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Polysaccharide structure of tetrasporic red seaweed *Tichocarpus crinitus*

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ABSTRACT

Sulfated polysaccharide isolated from tetrasporic plants of *Tichocarpus crinitus* was investigated. The polysaccharide was isolated by two methods: with water extraction at 80 °C (HT) and with a mild alkaline extraction (AE). The extracted polysaccharides were presented by non-gelling ones only, while galactose and 3,6-AG were the main monosaccharides, at the same time amount of 3,6-AG in AE polysaccharides was the similar to that of HT. According to methods of spectroscopy and mass spectrometry, the polysaccharide from tetrasporic *T. crinitus* contains main blocks of 1,3-linked β-D-galactopyranosyl-2,4-disulfates and 1,4-linked 3,6-anhydro-α-D-galactopyranosyl while 6-sulfated 4-linked galactopyranosyl resudies are randomly distributed along the polysaccharide chain. The alkaline treatment of HT polysaccharide results in obtaining polysaccharide with regular structure that composed of alternating 1,3-linked β-D-galactopyranosyl-2,4-disulfates and 1,4-linked 3,6-anhydro-α-D-galactopyranosyl residues. Native polysaccharide (HT) possessed both high anticoagulant and antiplatelet activity measured by fibrin clotting and platelet aggregation induced by collagen. This activity could be connected with peculiar chemical structure of HT polysaccharide which has high sulfation degree and contains also 3,6-anhydrogalactose in the polymer chain.

1. Introduction

Carrageenans are sulfated linear galactans, whose basic structural units are disaccharide, carrabiose, consisting of alternating β-1,3-linked (Unit G) and α-1,4-linked (Unit D/DA) galactose residues. Variation on this basic structure results from the content of 3,6-anhydrogalactose, location, and number of sulfate groups (Anderson, Dolan, Lawson, & Penman, 1968; Dolan & Rees, 1965; Penman & Rees, 1973; Rees, 1963). The 1,3-linked D-galactose residues occur as the 2-,4- and 6-sulfate, or are occasionally unsulfated, while the 1,4-linked residues occur as the 2-sulfate, the 6-sulfate, the 2,6-disulfate, the 3,6-anhydrogalactose and 3,6-anhydrogalactose 2-sulfate. The hydroxyl groups on the carrabioses can be further substituted by pyruvate ketal, branched glycosides or occasionally be methyl ethers (Chiovitti et al., 1997; Chiovitti, Kraft, Bacic, Craik, & Liao, 2001). There are about 20 different carrageenan recognized, and referred to by Greek letters, that are grouped into families: κ, β, and λ (Stortz & Cerezo, 1992). However, carrageenan molecules occur as hybrids rather than pure idealized structures. Carrageenan biosynthesis depends on many factors that can be divided into exogenous, determined by environmental conditions in the seaweed habitats, such as solar illumination, temperature, salinity and etc., and into endogenous, which are related to the seaweed physiology, in particular the species affiliation and the stage of their development (Yermak & Khotimchenko, 2003). The latter is particularly important because red seaweeds have a complex life cycle involving the alternation of gametic and tetrasporic stages (Pereira, 2012). Moreover, the reproductive stage can be represented by cystocarpic plant (Lahaye & Kaeffer, 1997). It is shown, that gametophytes of *Gigartina pistillata* (Gigartinaceae) synthesize complex of κ/ι-carrageenan type with varying proportions of *ν*-carrabiose units, which are precursors of ι-type (Amimi, Mouradi, Givernaud, Chiadmi, & Lahaye, 2001). Pereira, Amado, Critchley, Van de Velde, and Ribeiro-Claro (2009) also showed that carrageenan from tetrasporophyte of Chondrocanthus teedei var. lusitanicus had a xi/tetha hybrid structure according to FRTIR-ATR and FT-RAMAN spectra. The detailed analysis of the carrageenans from *Gigartina skottsbergii* and *Gymnogongrus torulosus* demonstrates that their cystocarpic representatives produce κ/ι-carrageenan (Estevez, Ciancia, & Cerezo, 2002). The tetrasporic plants of the Gigartinaceae contain carrageenan types far different from those reported for cystocarpic

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plants. For example, the polysaccharide extracts obtained from the tetrasporic plants of *G. atropurpurea* appear to be a complex mixture or hybrid containing, not only λ-, ξ-, and π-carrageenans but also some carrageenan structures composed of unsulfated 3,6-anhydrogalactose units (Falshaw, Bixler, & Johndro, 2003). Such a distribution of carrageenans at the life history stages of the seaweed is a common characteristic for most species of Gigartinaceae and Phyllophoraceae (Craigie, 1990; Mccandless, West, & Guiry, 1982), while the representatives of Solieriaceae and Hypneaceae produce only κ- and θ-carrageenans independently on their life cycle phase (Craigie, 1990).

In contrast to other carrageenophytes, only very few data on cell wall polysaccharides of the family Tichocarpaceae are available. The red alga *Tichocarpus crinitus* is the unique member of this family, which is widely distributed in Far East seas including Sea of Japan and Ohotskoe Sea, as well as along the Pacific coast of Honshu and Hokaido, Japan (Perestenko, 1994). Previous investigations (Barabanova et al., 2005) of the structure and properties of carrageenans isolated from sterile and cystocarpic forms of this endemic red seaweed clearly demonstrate that gelling polysaccharide fractions of both forms are κ/β-carrageenans with a dominant content of κ-type disaccharide units in the sterile form (Barabanova et al., 2005). The application of the modern analytical tools-negative-ion ESI and tandem MALDI mass spectrometry revealed that mixture of oligosaccharides, released with enzymatic digestion from κ/β-carrageenan of *T. crinitus*, contain κ-carrabiose, κ-carratetraose as main components, and a set of hybrid oligosaccharides as less abundant components: β→κ, κ→β→κ, β→κ→κ (Anastyuk et al., 2011). Furthermore, it is shown that the non-gelling polysaccharide from sterile *T. crinitus* is represented by carrageenan unrelated to any previously observed structure (Barabanova et al., 2008). According to ^{13}C and H NMR data, the backbone of this polysaccharide following alkaline treatment is composed of alternating 1,3-linked β-D-galactopyranosyl-2,4-disulfates and 1,4-linked 3,6-anhydro-α-D-galactopyranosyl residues.

In the present study we investigated polysaccharides from tetrasporic plants of *T. crinitus* to complete elucidation of the life history stage effects on polysaccharide content of the seaweed.

2. Experimental

2.1. Materials

Tichocarpus crinitus (Gmel.) Rupr. was collected at a depth of 1.5–2 m in Sobol Bay (the Sea of Japan). Morphological and anatomic characteristics of the seaweed were determined according to Perestenko by light microscopy (Perestenko, 1994). Tetrasporic specimens containing mature tetraspores, were selected for further analyses. The seaweeds were washed with tape water in order to remove excess of salt. Bleaching of the seaweed was achieved by maintaining the specimen in pure acetone for 3 days prior being dried in air.

2.2. Instruments

ESIMS spectra were recorded with 6510 LC Q-TOF mass spectrometer (Agilent, USA) with a dual electrospray-ionization source.

^{13}C NMR spectra of polysaccharide were recorded with a Bruker DPX-500 spectrometer operating at 60 °C and 62.9 MHz.

IR spectra were recorded on a Equinox 55 Fourier transform spectrophotometer (Bruker) with resolution of 4 cm^{-1}.

2.3. General methods

Total content of carbohydrate was determined by the phenol-sulfuric acid method using D-galactose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 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 mm × 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 (Englyst & Cummings, 1984). The content of the 3,6-ahydrogalactose was determined by complete reducing hydrolysis (Usov & Elashvili, 1991). The sulfate ester content of polysaccharide was determined according to Dodgson and Price (Dodgson & Price, 1962).

2.3.1. Polysaccharide extraction

Dried seaweed samples (10 g) were cut in small pieces and covered with distilled water in the ratio of 1:30 and polysaccharide extracted at room temperature for 24 h. The seaweed residues were removed by centrifugation and the supernatant was concentrated by rotary evaporation and poured into 3 vol. of ethanol. The precipitated polysaccharides (CT) were lyophilized. The seaweed residues were re-extracted twice with water (1:60) for 2 h at 80 °C. The suspensions were centrifuged (3000 rpm, 20 min, 20 °C) and supernatants were pooled and concentrated by rotary evaporation. The half of obtained solution was poured into 3 vol. of ethanol, yielding polysaccharide the fraction extracted with water at 80 °C (HT).

For alkali extraction, the dried seaweed samples (2 g) were placed in 120 mL of 0.6% solution of $NaHCO_3$ and heated at 80 °C (pH 7) for 4 h. The obtained extract was centrifuged (3000 rpm, 20 min, 20 °C) to remove algal residues, dialyzed against water (3 days) and than concentrated by rotary evaporation. Obtained solution was poured into 3 vol. of ethanol, yielding the alkali-extracted polysaccharide fraction (AE).

2.3.2. Polysaccharide fractionation with potassium chloride and calcium chloride

HT polysaccharides (100 mg) were dissolved in water (10 mL) and fractionated with KCl as described previously (Yermak et al., 1999). The precipitations were not formed. The supernatant obtained after KCl precipitation was dialyzed against water (3 days), concentrated and freeze-dried. Then the freeze-dried supernatant was dissolved in water to prepare 1% solution and solid ground KCl or $CaCl_2$ was added in small portions to hot polysaccharide solution (80 °C) with constant agitation to rich concentration of 0.2, 0.6, 1.0% for KCl and 1.0 and 4.0% for $CaCl_2$. The obtained solutions were left at 4 °C for 12 h and then centrifuged (10,000 rpm, 30 min, 4 °C). The precipitations were not formed. The supernatants were dialyzed against water for 4 days at 4 °C, concentrated and precipitated by 3 vol. of 95% ethanol and freeze-dried.

The AE fraction (100 mg) was dissolved in water (10 mL) and fractionated with potassium chloride and calcium chloride as described for HT polysaccharide.

2.3.3. Anion-exchange chromatography

A solution of HT polysaccharide (1%) in distilled water (25 ml) was added to a column (60 cm × 2.5 cm) of DEAE-Sepharose CL-6B. The polysaccharides were first eluted with water (HT1 fraction), and then sequentially with increasing concentration of NaCl – 0.5, 1.0 (HT2 fraction), 1.5 (HT3 fraction) and 2.0 M (HT4 fraction). The collected fractions (2.5–5 mL) were analyzed for carrageenan presence by a color reaction with 1,9-dimethyl methylene blue (DMMB) (Richardson et al., 2004).

2.3.4. Alkaline modification

HT polysaccharide (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 cooled down to room temperature and was neutralized to pH 5.5 with acetic acid. The alkali-modified carrageenan fraction (AM) was dialyzed against distilled water (3 day) and lyophilized.

2.3.5. Mild acid hydrolysis

HT polysaccharide (5 mg) was dissolved in 0.1 N hydrochloric acid (0.5 mL) and kept for 1, 2, 4, and 24 h at 37 °C. The depolymerization reaction was terminated by neutralization by NH_4OH. Then obtained oligosaccharides were precipitated with 5 volumes of methanol/water mixture (5:1, v:v) and centrifuged at 15,000 rpm for 12 min. The precipitates and supernatants were carefully separated, collected, and lyophilized. Supernatants were analyzed by mass spectrometry.

2.3.6. Determination of molecular weight

The viscosity of carrageenan solutions (0.1–1.0 mg/mL in 0.15 M NaCl) was measured in a modified Ubellode viscometer (Design Bureau Pushchino, Russia) with capillary diameter 0.3 mm at 25 °C, the time accuracy being with in ±0.1 s. The intrinsic viscosity of the carrageenan samples was calculated by the extrapolation of the dependence $\ln(\eta rel)/C$ to infinite dilution using the least square method. Viscosimetric molecular weights of carrageenan samples were calculated using the Mark–Houwink equation: $[\eta] = KM^{\alpha}$, where $[\eta]$ is the intrinsic viscosity and K and α are empirical constants constituting 3×10^{-3} and 0.95 at 25 °C in 0.1 M NaCl for carrageenans, respectively, according to the literature data for this polymer–solvent system (Rochas, Rinaudo, & Landry, 1990).

The molecular weight of AM was determined by the reducing sugars method with ferricyanide (Park & Johnson, 1949).

2.3.7. Fourier Transform Infrared spectroscopy (FT-IR)

Films of studied polysaccharides were obtained by drying 1 mL of 0.25–0.4% (w/v) their aqueous solution containing 8 mg of polysaccharide in polyethylene molds (about 0.5 cm deep, 2.5 cm diameter) at 37 °C. The films were clamped between NaCl windows. The spectra were normalized by the absorption of the monosaccharide ring at ~1070 cm^{-1} relatively the local basic line at ~1500–900 cm^{-1}.

2.3.8. NMR spectroscopy

Samples of polysaccharides were deuterium-exchanged twice from D_2O by freeze-drying prior being examined in a solution of 99.95% D_2O. The number of scans was 80 000. Chemical shifts are reported related to internal methanol (δ_C 50.15). Standard Bruker software (XWINNMR 1.2) was used to acquire the NMR data.

2.3.9. Mass spectrometry

Spectra were acquired in a negative-ion mode, with precalibration with a standard "HP-mix" for negative-ion mode. The capillary voltage was set to −4000 V, and the drying gas temperature was 325 °C. The fragmentor voltage was set to −160 V. The isolation window for MS/MS experiments was set to 1.3 mass units for singly-charged ions and 4 mass units for multiply-charged ions. The dried sample was dissolved in 1:1 acetonitrile–water (concentration of the sample was approx. 0.01 mg/mL) and introduced into the mass spectrometer at flow rate of 5 μL/min using a syringe pump, manufactured by KD Scientific (USA).

2.4. Biological activity

2.4.1. Ethical approval

The study protocol was approved by the medical ethical committee of the local hospital (Vladivostok, Russian Federation). Informed consent was obtained from all subjects who participated in the study. All donors were free of salicylic acid administration for 14 days before blood taking. Blood was drawn from the antecubital vein of normal healthy human volunteers and anticoagulated in plastic tubes Vacuette (Greiner Bio-One International AG, Kremsmuenster, Austria) with ACD (85 mM trisodium citrate; 71 mM citric acid; 111 mM dextrose) in a 6:1 (blood:ACD) as an anticoagulant or with 3.8% trisodium citrate in 1:9, citrate/blood (the exact anticoagulant for each assay was indicated below).

2.4.2. Thrombin time

Thrombin time was performed according to the method described earlier (Zhu, Di, Zhang, Zhang, & Chen, 2009). Sample aliquot (20 μL, 100 μg mL^{-1} in PBS) was added to 200 μL citric poor platelet plasma (PPP). Heparin used as a positive control, and PBS–as negative control. Mixtures were incubated at 37 °C for 2 min, then thrombin solution (200 μL, 0.5 U mL^{-1}) was added, and registration of thrombin time was performed. The influence on thrombin time was calculated according to the following equation: $\% = ((T_{sample} - T_{PBS})/T_{PBS}) \times 100$.

2.4.3. Washed platelet preparation

Blood was drawn into tubes with ACD with the ratio 6:1 (blood:ACD). Suspension of human platelets was prepared according to the modified method of double washing described earlier (Cazenave et al., 2004). To prepare platelet rich plasma (PRP), supernatants obtained after centrifugation at 120 × *g* for 12 min were collected. PRP was centrifuged 2200 × *g* for 15 min to prepare platelet pellet. The platelet pellet were washed by suspending in Tyrode's albumin buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.5 mM Na_2HPO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 6 mM glucose, and 0.35% bovine serum albumin) containing 10 U mL^{-1} heparin and 0.02 U mL^{-1} apyrase at pH 6.5, 37 °C. After 10 min incubation at 37 °C platelets were centrifuged again at 1900 × *g* for 8 min. Then platelet pellet was again resuspended with the same buffer but without heparin and after 10 min of incubation at 37 °C, the platelets were again washed using Tyrode's albumin buffer. Finally, the platelets were resuspended in the Tyrode's albumin buffer, pH 7.4 containing 0.02 U mL^{-1} apyrase. Platelets were counted using a Biola aggregometer (Moscow, Russia) provided with counter 230LA and adjusted to required number of platelets.

2.4.4. Platelet aggregation

Platelet aggregation speed was measured using a dual channel laser analyzer of platelet aggregometer Biola (Moscow, Russia) based on the turbidimetric method of Born and Cross (Born & Cross, 1963). Platelet suspension (300 μL, 300×10^3 μL^{-1}) was incubated with sample solution (30 μL, 100 μg mL^{-1} in Tyrode's albumin buffer) of saline solution (a negative control) for 2 min at 37 °C in a siliconized glass cuvette. Aggregation was initiated by addition of such agonist as collagen and thrombin. Thrombin (1 U mL^{-1}) and collagen (2 mg/mL) were used independently for induction of aggregation of washed platelets. Aggregation was then followed for 5 min with constant stirring at the speed of 800 rpm. In each case, aggregation induced by an agonist alone was considered as 100% aggregation speed. The sample aggregation speed changing was calculated according to the following formula: $\% = ((A_{sample} - A_{PBS})/A_{PBS}) \times 100$.

Table 1
Analysis of polysaccharide fractions from tetrasporic form of alga *T. crinitus*.

Fraction	Yield (%) on dried alga	Content, dry weight (%)					
		An	Gal	Glu	SO_4	Protein	An:Gal:SO_4
CT	2.6	3.5	13.8	1.1	nd	22.6	
HT	21.0	8.5	25.7	1.9	25.7	9.7	1.0:2.6:4.2
AM	52.8[a]	15.0	19.2	2.3	17.3	3.7	1.0:1.2:1.6
AE	13.5	9.7	30.4	4.9	30	9.9	1.0:3.0:4.8

[a] Percent from dry weight of HT polysaccharide.

2.4.5. Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was done by one-way Anova. Differences were considered to be statistically significant if $p < 0.05$.

3. Results

3.1. Extraction and chemical analysis of polysaccharides

The polysaccharides from tetrasporic plants of *T. crinitus* were isolated by two methods: (1) with sequential water extraction at 20 and 80 °C (CT and HT, respectively) and (2) with mild alkaline extraction. The yield of the HT polysaccharide fraction extracted with water at 80 °C was 21%, whereas the content of CT polysaccharide fraction extracted with water at 20 °C did not exceed 3%. According to the chemical analysis, the CT fraction consisted of galactose (Gal), glucose and 3,6-anhydrogalactose (3,6-AnGal) with a significant amount of protein (22.6%) (Table 1). HT polysaccharides were submitted to KCl and $CaCl_2$ precipitation. A non-soluble fraction (gelling) was not obtained by KCl or $CaCl_2$ at different concentrations of the used salts. Similar results were obtained for AE polysaccharides. Hence, HT and AE polysaccharides were presented only by soluble (non-gelling) polysaccharides in KCl or $CaCl_2$. Chemical characteristics of these polysaccharides are listed in Table 1. The ratios of 3,6-AnGal to galactose were about 1:3 for the HT and AE samples.

3.2. Molecular weight determination of polysaccharides

The viscosimetric molecular weight of HT and AE polysaccharides calculated by viscosity method and using the Mark–Houwink equation was 376 and 468 kDa, respectively. The molecular weight of AM determined by reducing sugars method was of 5.8 kDa.

3.3. NMR and FT-IR spectroscopy

NMR and FT-IR spectra were used to identify the polysaccharide structure. The IR spectrum of CT polysaccharide despite on the presence of absorbance at 1252 cm^{-1} of total sulfate groups but did not contain any absorbance at 935and 815 cm^{-1} (data not shown). The IR spectra of HT and AE polysaccharides had a strong absorbance at 1252 cm^{-1} corresponding to the significant amount of sulfate groups (Fig. 1a and b). There were also intense absorbencies at 932–935 cm^{-1} for 3,6-anhydrogalactose, 811–815 and 857 cm^{-1} that were attributed to sulfated ester groups at C-2 or at C-6 and C-4 positions, respectively (Chopin & Whalen, 1993; Pereira et al., 2009). It should be noted, that the IR spectra of AE and HT polysaccharides had some distinctions. The calculated the ratios of areas of absorbance bands in the IR spectra, which are characteristic for total sulfate (S_{1252}) content, polysaccharide concentration (S_{1074}), 3,6-AnGal ($S_{932–935}$) and sulfate positions ($S_{811–815}$, S_{857}) shown the changes in the sulfate content at C-4 and C-2 (or C-6) of galactose residues, while the total sulfate amount was change slightly. Moreover, analysis of the decomposed IR spectra on individual components showed an increase in the 3,6-AnGal and the non-sulfated galactose residues, and pronounced decrease in the amount of galactose residues with sulfate at C-4 in the AE polysaccharide spectrum (Fig. 1Ba and Bb).

The ^{13}C NMR spectra of the HT and AE fractions were similar, poorly resolved and difficult to assign, but a few signals could be detected in the anomeric region. These spectra contained the broad and overlapping signals at 101.8, 103.3, 100.6 and 93.6 ppm that pointed out the presence of some structural types of carrageenans (Fig. 2a) and according to the literature data, the resonance at

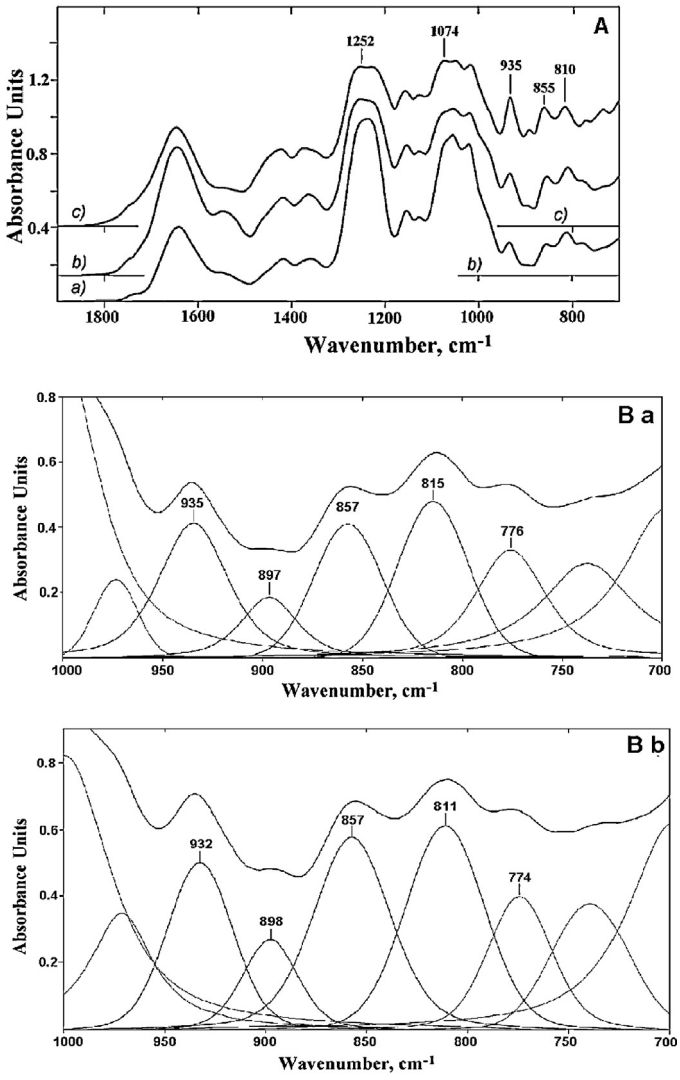


Fig. 1. IR spectra of polysaccharides from tetrasporic form of alga *T. crinitus*: Aa – alkali-extracted polysaccharide (AE); Ab – water extracted polysaccharide at 80 °C (HT); **Ac** – alkali-modified polysaccharides (AM). IR spectrum of polysaccharides from tetrasporic form of alga *T. crinitus* were decomposed on individual components: Ba – alkali-extracted polysaccharide (AE); Bb – water extracted polysaccharide at 80 °C (HT).

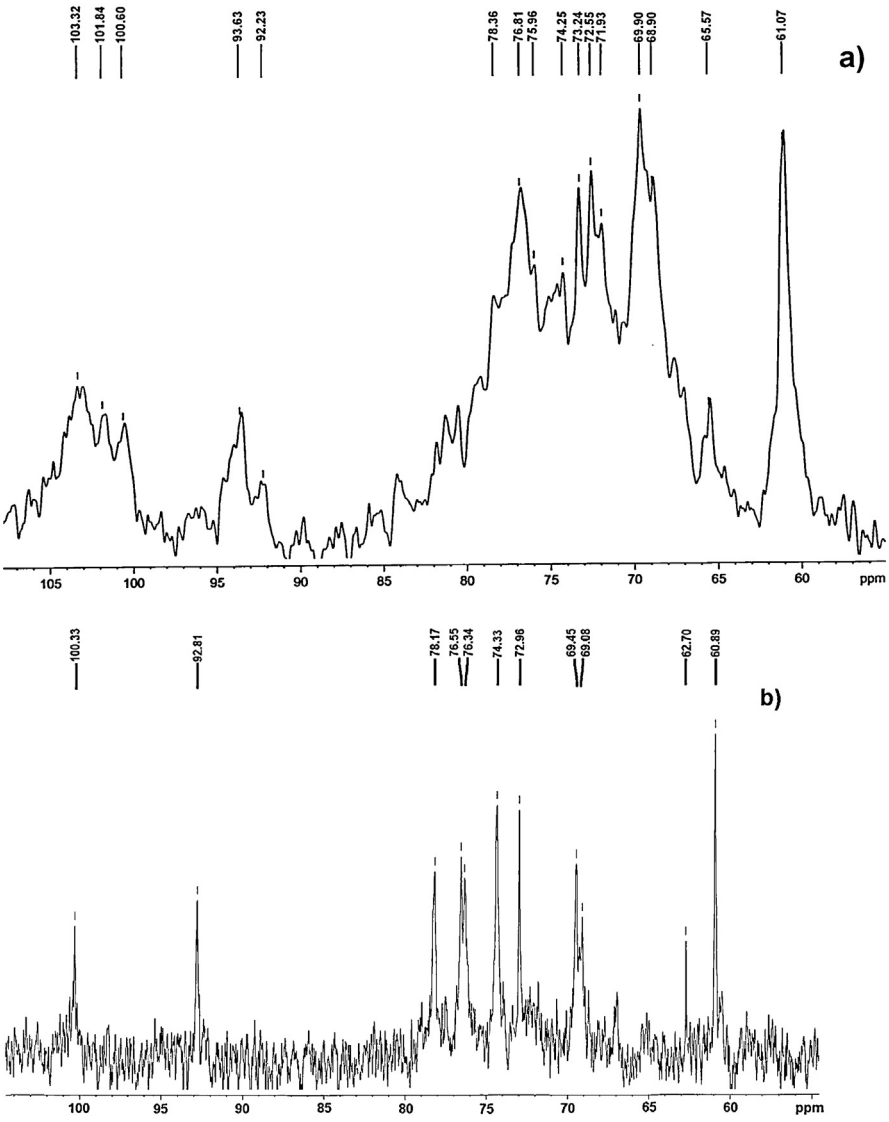


Fig. 2. a – ^{13}C NMR-spectrum of water extracted polysaccharide at 80 °C (HT) from tetrasporic form of alga *T. crinitus*; b – ^{13}C NMR-spectrum of alkali-modified polysaccharides (AM) from tetrasporic form of alga *T. crinitus*.

101.3 ppm is described for C-1 in diad G2S,4S-DA of polysaccharide fraction from cystocarpic *Gigartina skottsbergii* (Ciancia, Matulewicz, Finch, & Cerezo, 1993). We used the anion-exchange chromatography on DEAE Sepharose CL-6B for separation of the observed carrageenan mixture. The polysaccharides were sequentially eluted with distilled water and a gradient of NaCl in water solutions (Fig. 3). The main fraction (HT1) was eluted by water with yield of 51.1%. The elution of polysaccharides by the NaCl gradient lead to 3 fractions: HT2 (3.2%), HT3 (2.2%) and HT4 (6.8%). The NMR and IR spectra of the HT1 fraction were identical to those obtained for original polysaccharide.

To obtain the well resolved NMR spectra of HT polysaccharide, it was subjected to alkaline treatment. The ^{13}C NMR spectrum of AM revealed twelve signals with strong intensity due to the 12 carbon atoms (Fig. 2b) and showed two signals at 101.4 and 93.8 ppm in the resonance region of the anomeric carbon atoms indicating the presence of one disaccharide repeating unit in this fraction. This spectrum corresponded to the spectrum of x-carrageenan with alternating 1,3-linked β-D-galactopyranosyl-2,4-disulfates and 1,4-linked 3,6-anhydro-α-D-galactopyranosyl residues (Barabanova et al., 2008). According to the chemical analysis the alkali treatment resulted in the increase in 3,6-anhydrogalactose and the decrease in sulfate content of investigated polysaccharides (Table 1). The increase of the absorption at 935 cm^{-1} was also observed in the IR spectrum of modified polysaccharides and pointed out to the presence of a precursor, 1,4-linked galactose-6-sulfate, in the native polysaccharide (Fig. 1Ac). The

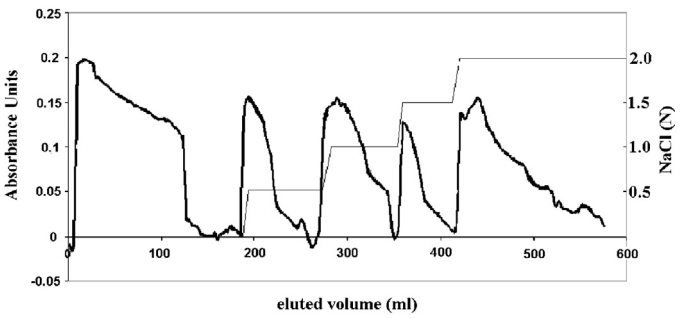
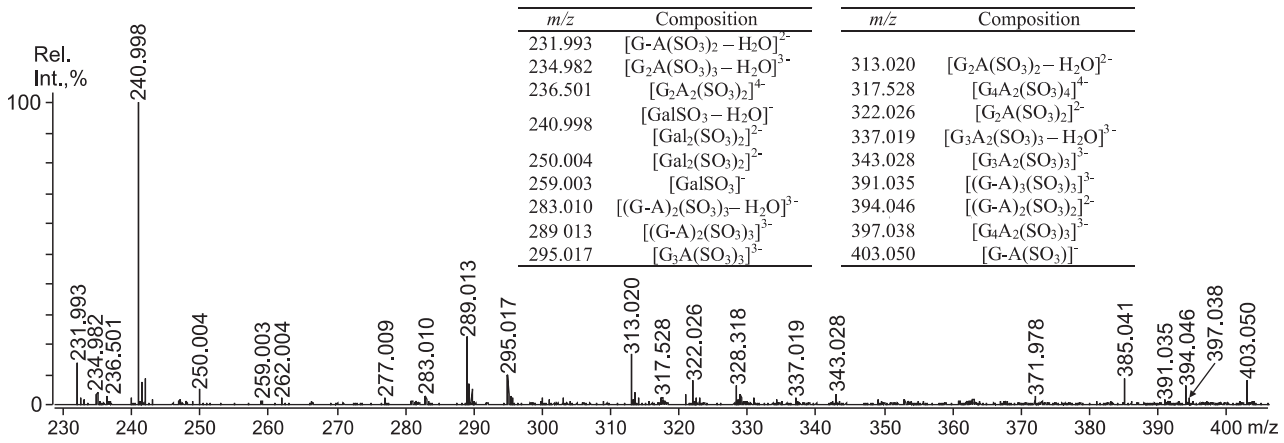


Fig. 3. Anion-exchange chromatography of the water extracted polysaccharide at 80 °C (HT) of alga *T. crinitus* on DEAE Sepharose CL-6B. The polysaccharides were sequentially eluted with distilled water and gradient of NaCl in water solutions.



m/z	Composition
231.993	$[G\text{-}A(SO_3)_2 - H_2O]^{2-}$
234.982	$[G_2A(SO_3)_3 - H_2O]^{3-}$
236.501	$[G_2A_2(SO_3)_2]^{4-}$
240.998	$[GalSO_3 - H_2O]^-$ $[Gal_2(SO_3)_2]^{2-}$
250.004	$[Gal_2(SO_3)_2]^{2-}$
259.003	$[GalSO_3]^-$
283.010	$[(G\text{-}A)_2(SO_3)_3 - H_2O]^{3-}$
289 013	$[(G\text{-}A)_2(SO_3)_3]^{3-}$
295.017	$[G_3A(SO_3)_3]^{3-}$

m/z	Composition
313.020	$[G_2A(SO_3)_2 - H_2O]^{2-}$
317.528	$[G_4A_2(SO_3)_4]^{4-}$
322.026	$[G_2A(SO_3)_2]^{2-}$
337.019	$[G_3A_2(SO_3)_3 - H_2O]^{3-}$
343.028	$[G_3A_2(SO_3)_3]^{3-}$
391.035	$[(G\text{-}A)_3(SO_3)_3]^{3-}$
394.046	$[(G\text{-}A)_2(SO_3)_2]^{2-}$
397.038	$[G_4A_2(SO_3)_3]^{3-}$
403.050	$[G\text{-}A(SO_3)]^-$

Fig. 4. ESIMS of oligosaccharide mixture, obtained by acid hydrolysis (HCL, 2 h, 1 M at 37 °C) of HT polysaccharide.

ratio of 3,6-AnGal to galactose in AM polysaccharides also differed from that of native polysaccharide and was of 1:1.2 (Table 1).

3.4. Mass spectrometry

To elucidate detailed structural features of HT polysaccharide, it was hydrolyzed in mild conditions (Section 2.3.5) to oligosaccharides. The composition of the oligosaccharide mixture was characterized with a negative-ion ESIMS (Fig. 4). The structural features of the most interesting polysaccharide fragments (2 h hydrolysis) were elucidated using a tandem CID ESIMS. Structural features of the following components were suggested: the main component of the hydrolysate (due to ESIMS data, *m/z* 240.998, spectrum available as the supplementary material) was G(2,4)S-(1,4)-A; the component at *m/z* 250.004 was found to have three structural variants (Fig. 5): G(2,4)S-(1,4)-G, G6S-(1,3)-G2(4)S, G-(1,3)-G(2,4)S; the component at *m/z* 295.017 was found to have two structural variants (Fig. 6): G2S-(1,4)-A-(1,3)-G(2,4)S-(1,4)-G and G-(1,3)-G2S-(1,4)-A-(1,3)-G(2,4)S.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.04.063.

3.5. Biological activity of HT polysaccharide

Biological activity of HT polysaccharide was investigated by means of thrombin time assay and platelet aggregation induced by thrombin and collagen. HT polysaccharide possessed anticoagulant activity measured by thrombin time assay increasing coagulation time by 40% in comparison with the control (PBS) but it was still less than that of heparin (Table 2). Moreover, being effectless in regard to platelet aggregation induced by thrombin, HT polysaccharide also showed an antiplatelet effect by decreasing collagen-induced platelet aggregation by about 55% (Table 2).

4. Discussion

The endemic seaweed *Tichocarpus crinitus* of the far eastern region is a potential source for industrial production of polysaccharides and a promising species for introduction into mariculture. In the paper we continued our investigation of the polysaccharide composition of this seaweed in relation to the phase of its life cycle that remains an important research objective.

The chemical analysis of polysaccharides extracted from tetrasporic *T. crinitus* showed that galactose and

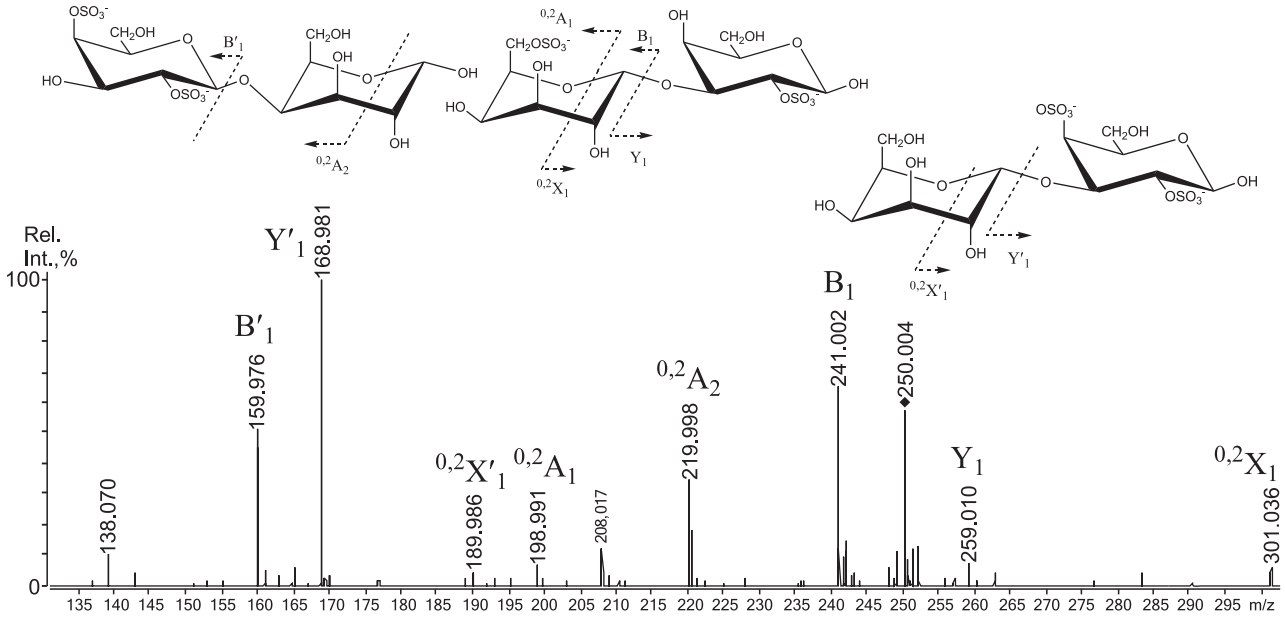


Fig. 5. Tandem CID ESIMS of the ion at *m/z* 250.004.

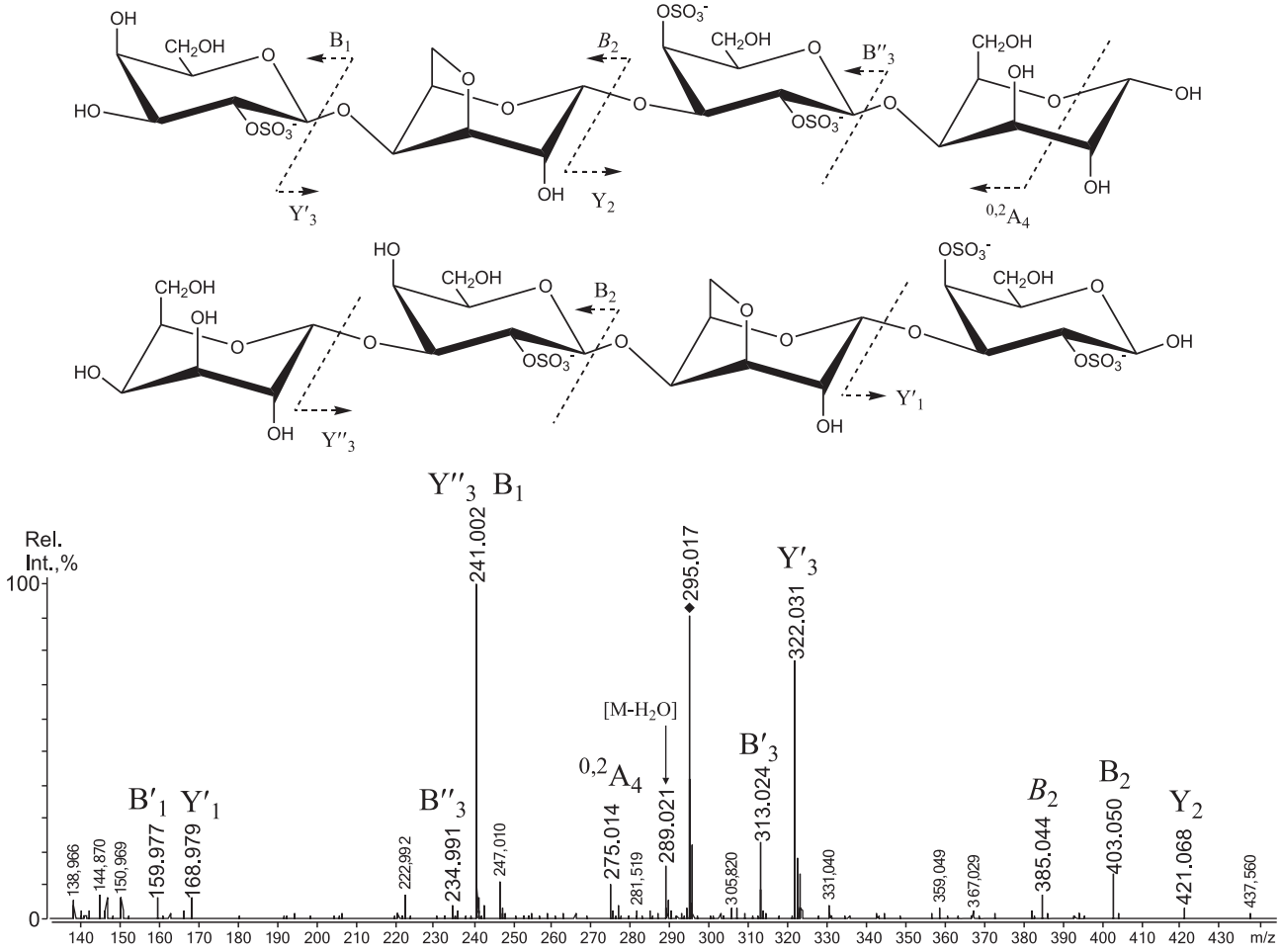


Fig. 6. Tandem CID ESIMS of the ion at *m*/*z* 295.017.

3,6-anhydrogalctose were the predominant sugars with a significant amount of protein after the cold extraction (Table 1). Usually, polysaccharide fraction obtained by "cold water" extraction is not commonly reported in the literature due to the presence of low molecular-weight components (salts and pigments) in the seaweed tissue. We showed that cold extracts obtained from tetrasporic plants of *T. crinitus* contained a track amount of carrageenan-like sample with molecular weight 4.6 kDa.

Total polysaccharide yield of tetrasporic *T. crinitus* extracted at 80 °C averaged 21% which was similar to the polysaccharide yield to sterile form of *T.crinitus* (Barabanova et al., 2005). In contrast to this, the polysaccharides extracted from tetrasporic plants contained only non-gelling fractions (Table 1). Similar results were reported for tetrasporic plants of *Iridaea undolosa*, where the yield of biosynthesized non-gelling lambda carrageenans was predominant (Stortz & Cerezo, 1993).

According to NMR and IR spectra, the polysaccharide from tetrasporic *T. crinitus* was a mixture of several types of carrageenan. The alkaline treatment of this polysaccharide resulted in a polysaccharide fraction with regular structure, which corresponded to structure of modified polysaccharide from sterile *T. crinitus*. The non-gelling polysaccharide extracted from vegetative *T. crinitus* had unusual structure and viscoelastic behavior (Barabanova et al., 2008). Following alkaline treatment, the backbone of this polysaccharide was composed of alternating 1,3-linked β-D-galactopyranosyl-2,4-disulfates and 1,4-linked 3,6-anhydro-α-D-galactopyranosyl residues. The exceptional viscoelastic behavior of non-gelling polysaccharide from vegetative *T. crinitus* might be due to the presence of 3,6-DA. The presence of 3,6-anhydrogalctose is not typical for non-gelling carrageenan types. A low quantity of 3,6-DA (1–5 mol%) was also reported in carrageenan from tetrasporic material of *Psilophycus alveatus*, *Gigartina lanceata*

Table 2
Antithrombotic activity of HT polysaccharide.

Sample	Thrombin time (%)[a]	Thrombin-induced platelet aggregation (%)[b]	Collagen-induced platelet aggregation (%)[b]
Control (PBS)	100.0 ± 2.5	100.0 ± 0.1	100.0 ± 12.1
Heparin	234.8 ± 18.9*	80.5 ± 2.6*	23.0 ± 6.3*
HT polysaccharide	142.8 ± 17.7*	88.7 ± 10.6	45.0 ± 2.2*

[a] Thrombin time change in the presence of HT polysaccharide and heparin ($C = 10\ \mu g \times mL^{-1}$, final value). The results are expressed as % change in thrombin time relative to the negative control (100%).

[b] Platelet aggregation speed change under the influence of thrombin and collagen in the presence of HT polysaccharide and heparin ($C = 10\ \mu g \times mL^{-1}$, final value). The results are expressed as % of change in aggregation speed relative to the negative control (100%).

* $P < 0.05$ in comparison with control (PBS).

and *Chondracanthus chauvinii* (Falshaw & Furneaux, 1995, 1998). In tetrasporic plants of *Sarcothalia atropurpurea*, this monosaccharide was defined as a component of native carrageenan according to the results of the direct constituent sugar analysis (Falshaw et al., 2003).

Another distinctive feature of non-gelling polysaccharides from *T. crinitus* is the presence of two sulfate groups at C-2 and C-4 in 3-linked galactose residues. Usually, non-gelling carrageenans have one sulfate group, which is attached at C-4 (μ- and ν-types) or C-2 (λ-type) in 3-linked galactose residues. However, the sulfation in the 2- and 4-position of the 3-linked unit was also reported for polysaccharide from *Psaromenia berggrenii* (Miller & Furneaux, 1996). A novel carrageenan structure composed of 3-linked β-D-galactopyranosyl 2-sulfate units alternating with 3,6-anhydro-α-D-galactopyranosyl units in *Callophyllis lambertii* has been suggested (Falshaw, Furneaux, & Stevenson, 2005; Miller, 2003). Falshaw, Bixler, and Johndro, 2001 described carrageenan samples that contained some miscellaneous 3-linked or 4-linked monosaccharide units which do not correspond to the idealized carrageenan structure. There were units with ambiguous linkage/substitution patterns. For example, 2,3,4-Gal is derived from a galactopyranosyl unit that is either linked or substituted at the 2-, 3- and 4-positions. It could correspond to 2,4-disulfated 3-linked galactopyranosyl unit as it was shown for *T.crinitus* (Barabanova et al., 2008) or to 2,3-disulfated 4-linked galactopyranosyl unit as it was observed in the polysaccharide from the red seaweed *Champia novae-zelandiae* (Miller & Furneaux, 1996). Since seaweeds are natural organisms, there is always potential for structures that do not fit in a rigid naming system. These unusual monomers may not be contained in homogeneous polymers but scattered along a chain of other carrageenan structures and act as spacers where they may interrupt double helix formation, and thus prevent, or weaken gelation (Lawson, Rees, Stancioff, & Stanley, 1973; Penman & Rees, 1973)

Common industrial procedure for extraction of carrageenan to convert precursor elements into 3,6-anhydrogalactose is alkaline extraction. This procedure followed by fractionation with potassium chloride also provides an alternative method to separate different types of carrageenans for further analysis (Rodriguez et al., 2005). Falshaw et al. (2001) showed that non-gelling fractions of the mixed life phase material of *Chondrus crispus*, *Gigartina skottsbergii* and *Sarcothalia crispata* extracted by low concentration of alkali may contain a significant amount of κ, ι, μ and ν carrageenans. The most plausible explanation for this behavior may be a less 'blocky' character of the hybrid polymers found in this fraction, i.e. these hybrid polymers are more randomly distributed and, thereby more soluble in KCl (Falshaw et al., 2001). To define more accurately structural features of HT polysaccharide, the modern negative-ion tandem CID ESIMS/MS technique was used. It was recently shown that the negative-ion CID ESIMS spectra of carrageenan-derived oligosaccharides featured extensive series of B- and C-type glycosidic cleavages, whereas the Y-type cleavage occurred mainly at the sulfated residues (Yu et al., 2006) thus providing an information on the sulfation pattern. Linkage type information could be found upon observation of the cross-ring cleavages ($^{0,2}X/^{0,2}A$), like those observed in CID MS/MS of sulfated oligosaccharides from 3-/4-linked oligosaccharides from fucoidan (Anastyuk, Shevchenko, Nazarenko, Dmitrenok, & Zvyagintseva, 2009). However, none were detected in our previous work on *T. crinitus* (Anastyuk et al., 2011). As shown recently by ESIMS^n on hexoses (Minamisawa & Hirabayashi, 2005) and computational chemistry methods on 2-, 3- and 4-sulfated fucose residues (Tissot, Salpin, Martinez, Gaigeot, & Daniel, 2006), the mechanism of formation of the $^{0,2}X/^{0,2}A$ fragment ions required unsubstituted proton on the C-3 hydroxyl group. Hence, in case of enzymatically obtained oligosaccharides from carrageenan (Anastyuk et al., 2011), when all protons of the C-3 hydroxyl groups were substituted, no cross-ring cleavages could occur. Nevertheless, tandem CID ESIMS spectra of the polysaccharide fragments, obtained from HT polysaccharide by mild acid hydrolysis contained information on cross-ring cleavages allowing us to deduce the location of the 6-sulfated Gal residues in the oligosaccharide chain. Tandem CID ESIMS of the ion at *m*/*z* 250.004 shown at Fig. 5 contained abundant signal from the ion of $^{0,2}A_2$-type of cleavage, suggesting that 4-linked galactose was located at the reducing end. The abundant ions from the cleavages glycosidic bonds cleavages of Y- and B-type suggested that 4-linked Gal residues also could be, probably, unsulfated. 3-linked Gal residues were doubly-sulfated, in except of desulfation in the ion source of the mass spectrometer with loss of the water molecules (Fig. 4). High lability of the sulfate groups at C-4 (C-6) of Gal residues in mild acid hydrolysis conditions was also observed for fucoidans (Anastyuk, Imbs, Shevchenko, Dmitrenok, & Zvyagintseva, 2012). The tandem CID ESIMS of the triply-sulfated tetrasaccharide ion at *m*/*z* 259.017 (Fig. 5) suggested two structural variants, one with the unsulfated reducing 4-linked Gal residue ($^{0,2}A_4$-type ion) and on the same residue on non-reducing end. 3-linked Gal residues were 2,4-disulfated, while non-reducing one could be monosulfated at C-6. The fact of presence of 3,6-anhydrogalactose residues in the structure of oligosaccharides confirms the proposition that HT polysaccharide contains them. The low amount of detected 4-linked 6-sulfated Gal residues in the hydrolysate could be explained by the following. Inserts of 6-sulfated 4-linked Gal residues were randomly distributed along the polysaccharide chain, and thus they did not accumulate as G(2,4)S-(1,4)-DA repeating unit in hydrolysis products.

It is well known that sulfated polysaccharides possess anticoagulant activity (Pomin, 2010). The major factor for the anticoagulant action of heparin-like compound is believed to be not so much the polyanion nature of these compounds as their configuration, molecular mass, and specific fine structural features (Becker, Guimaraes, Mourao, & Verli, 2007; Melo, Pereira, Foguel, & Mourao, 2004). It was showed that algal sulfated polysaccharides and extracts enriched with them exert a strongly marked dual effect on hemostasis: anticoagulant and proaggregating activity increase simultaneously (de Azevedo et al., 2009). Hence, we investigated antithrombotic properties of HT polysaccharide. According to our data HT polysaccharide possessed both high anticoagulant and antiplatelet activity measured by fibrin clotting and platelet aggregation induced by collagen (Table 2). Notwithstanding, HT polysaccharide action was not as high as for heparin, it also possessed an interesting antithrombotic effect. This activity could be connected with peculiar chemical structure of HT polysaccharide which has high sulfation degree and contains also 3,6-anhydrogalactose in the polymer chain. HT polysaccharide possessed both high anticoagulant and antiplatelet activity measured by fibrin clotting and platelet aggregation induced by collagen. This activity could be connected with peculiar chemical structure of HT polysaccharide which has high sulfation degree and contains also 3,6-anhydrogalactose in the polymer chain.

5. Conclusion

Sulfated polysaccharide isolated from tetrasporic plants of *Tichocarpus crinitus* was investigated. The polysaccharide was isolated by two methods: with water extraction at 80 °C (HT) and with a mild alkaline extraction (AE). The extracted polysaccharides were presented by non-gelling fraction only, while galactose and 3,6-DA were the main monosaccharides, at the same time amount of 3,6- DA in AE polysaccharides was the same as in HT. The alkaline treatment of HT polysaccharide resulted in polysaccharide with regular structure that composed of alternating

1,3-linked β-D-galactopyranosyl-2,4-disulfates and 1,4-linked 3,6-anhydro-α-D-galactopyranosyl residues. According to spectroscopy methods the polysaccharide from tetrasporic *T. crinitus* contained that main block. The application of the mass spectrometry for the analysis of oligosaccharides obtained by mild acid hydrolysis of the HT polysaccharide allowed us to confirm that native polysaccharide indeed contained blocks of 1,3-linked β-D-G-2,4-disulfates and 1,4-linked 3,6-anhydro-α-D-galactopyranosyl while 6-sulfated 4-linked galactopyranosyl resudies were randomly distributed along the polysaccharide chain. Polysaccharide possessed both high anticoagulant and antiplatelet activity measured by fibrin clotting and platelet aggregation induced by collagen. This activity could be connected with peculiar chemical structure of HT polysaccharide which has high sulfation degree and also 3,6-anhydrogalactose in the polymer chain.

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