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Structural peculiarities of polysaccharide from sterile form of Far Eastern red alga *Ahnfeltiopsis flabelliformis*

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ABSTRACT

KCl-insoluble sulfated polysaccharide from sterile alga *Ahnfeltiopsis flabelliformis* was investigated. Partial reductive hydrolysis and NMR spectroscopy showed that the polysaccharide comprises disaccharide units of carrabiose only. According to FT-IR-, 1D, 2D NMR spectroscopies and mass-spectrometry this polysaccharide is kappa/beta-carrageenan with ratio of kappa- and beta-types units 3:1 and contains minor amounts of iota- and gamma-carrageenans (precursor of beta-carrageenan). In addition, ESIMS/MS data suggested that xylose (minor amount) is present in the polysaccharide as a substituent one of hydroxyl group of galactose. According to aPTT and PT assays the studied carrageenan affected mostly intrinsic pathway of coagulation, while it effect on the extrinsic pathway is absent.

1. Introduction

Red algae (Rhodophyta) produce sulfated galactans, agars and carrageenans, which are the main components of their cell walls (Craigie, 1990). Sulfated galactans are widely used in food industry as thickeners, stabilizers and emulsifiers (Pomin, 2010), biotechnology (Stanley, 1990) and cosmetology (Yermak & Khotimchenko, 2003). Furthermore, they have a broad spectrum of biological activities: antiviral, anticancer, immunomodulating, anti-oxidant, anti-coagulant (Campo, Kawano, Silva, & Carvalho, 2009; Cardozo et al., 2007).

This large family of hydrocolloids is made up of linear chains of galactose, with alternating α-(1→3) and β-(1→4) linkages. In agarans, the α-linked galactose units are in the L configuration (L unit), whereas they are in D configuration (D unit) in carrageenans (Rees, 1969). Carrageenans are classified according to the number and the position of sulfated ester (S) and by the occurrence of 3,6 anhydro-bridges in the α-linked residues (DA unit) found in gelling galactans. The polysaccharide molecules containing 3,6-anhydrogalactose are able to form gels in the presence of specific ions (K^+, Ca^{2+} and others) (Knutsen, Myslabodski, Larsen, & Usov, 1994; Rees, 1969). The three most industrially exploited carrageenans, namely, kappa- (κ, DA-G4S), iota- (ι, DA2S-G4S) (gelling), and lambda- (λ, D2S6S-G2S) (non-gelling) carrageenans, are distinguished by the presence of one, two, and three ester-sulfate groups per repeating disaccharide unit, respectively and by the occurrence of 3,6-anhydrogalactose.

The chemical structures of carrageenans are very heterogeneous and are correlated to the algal sources, its environment, stage of its life cycle (i.e., gametophyte, tetrasporophyte), and the extraction procedures of the polysaccharides (Bulboa & Macchiavello, 2001; Craigie, 1990; Falshaw & Furneaux, 1994; Reis, Yoneshigue-Valentin, & Cesar, 2008; Yermak & Khotimchenko, 2003). This structural complexity is attributed to the occurrence of a mixture of carrageenans in algal extracts as well as the combination of different ideal carrabioses distributed along the polysaccharide chains giving rise to hybrid carrageenan (Bixler, 1996; Greer & Yaphe, 1984; Knutsen et al., 1994; Van de Velde, Peppelman, Rollema, & Tromp, 2001).

Structure of polysaccharides from the members of the family Phyllophoraceae presented by different generations was studied by a number of research groups. It was found that the alga *Mastocarpus stellatus* produced a hybrid kappa/iota-carrageenan with minor amounts of biosynthetic precursors – mu- and nu-carrageenans (Hilliou et al., 2012). Gelling polysaccharide from cystocarps *Stenogramme interrupta* was found to be iota/alpha-carrageenan, whereas non-gelling polysaccharide from tetrasporophyte consisted of zeta- and lambda-carrageenans

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(Cáceres, Carlucci, Damonte, Matsuhiro, & Zuñiga, 2000). At the same time alga *Phyllophora pseudoceranoides* synthesized iota-carrageenan irrespective of the life cycle stage (McCandless, West, & Guiry, 1982). *Phyllophora nervosa* had an unusual carrageenan of beta-family, constituting of omega- or hybrid omega/kappa-carrageenans (Usov & Arkhipova, 1981). It was shown that the polysaccharide from the gametophytes *Gymnogongrus torulosus* was a D/L-hybrid essentially consisting of kappa- and iota-carrageenan units and few amounts of agar-like structures (Estevez, Ciancia, & Cerezo, 2001). Attached and unattached red alga *Ahnfeltiopsis flabelliformis* (family Phyllophoraceae) was found to form an extensive population on the Pacific coast (Perestenko, 1994). Due to the fact that this alga can be a source of unusual polysaccharide, establishing its structure is interesting. Previously, a comparative chemical analysis of pigment and polysaccharides isolated from sterile and reproductive forms *A. flabelliformis* was carried out (Kravchenko, Belous, Glazunov, & Ermak, 2013). However, polysaccharide structure was not studied in details.

The aim of the current study was to investigate the structure of KCl-insoluble polysaccharides from sterile form of Far Eastern red alga *A. flabelliformis*.

2. Experimental

2.1. Algal material

The attached form of the sterile plants *A. flabelliformis* was collected in Troitsa Bay (Sea of Japan) in at 0.5 m depth in July 2012. Seaweeds were washed with running water for cleaning of epiphytes and removing soluble salts and material was frozen in airproof plastic bags. Morphological and anatomic characteristics of the seaweed were determined according to Perestenko by light microscopy (Perestenko, 1994).

2.2. Isolation and fractionation of polysaccharides

Phycobiliproteins extraction was carried out with 1.5% solution of sodium nitrate before isolation of polysaccharides (Kravchenko et al., 2013). After isolation of pigments, 5 g algal residues were filled with 150 mL distilled water. Polysaccharides were extracted in water bath at 80 °C for 3 h under constant stirring. The seaweed residues were removed by centrifugation and re-extracted twice with water. Obtained extracts were combined and centrifuged at 4000 rpm min^{-1} at 4 °C for 30 min to remove residues of the cell walls, concentrated by rotary evaporation and the polysaccharides were precipitated with 3 volumes of 96% ethanol. The precipitate was dissolved in distilled water, concentrated on a rotary evaporator and lyophilized. The polysaccharides were separated into KCl-insoluble and KCl-soluble fractions with 4% KCl as described previously (Yermak, Kim, Titlynov, Isakov, & Solov'eva, 1999).

2.3. Analytical methods

Monosaccharide composition was determined by total reductive hydrolysis (Englyst & Cummings, 1984; Usov & Elashvili, 1991). Neutral monosaccharides were analyzed as alditol (Englyst & Cummings, 1984) and aldononitrile acetate derivatives (Usov & Elashvili, 1991) by gas–liquid chromatography (GLC) with 6850 chromatograph (Agilent, USA) equipped with a capillary column HP-5MS (30 m × 0.25 mm, 5% Phenyl Methyl Siloxane) and a flame ionization detector. The analyses were carried out at a temperature gradient program from 175 to 225 °C; the rate of temperature change was 3 °C min^{-1}. The sulfate esters content of polysaccharide was determined by the turbidimetric method (Dodgson & Price, 1962).

2.4. Partial reductive hydrolysis

5 mg polysaccharide was placed in a test tube with 40 mg borane-4-methyl-morpholine (Sigma, USA) and 1 mL of 2 M aqueous trifluoracetic acid (TFA) containing 1 mg mL^{-1} mio-inositol (Acros Orcanics, USA) as internal standard. The solution was heated at 65 °C for 8 h. The solvent was evaporated and three portions of 3 mL of 96% ethanol were added to the hydrolysate and evaporated to remove traces of TFA. 1 mL of 6% hydroxylamine hydrochloride was added to the vacuum-dried hydrolysate residue. The solution was heated at 100 °C for 1 h. After cooling, 1 mL acetic anhydrate was added and the tube was heated at 100 °C for 1 h. 3 mL toluene was added three times and the solvent was evaporated to dryness. Then 5 mL CH_3Cl and 5 mL water were added. After shaking, the chloroform layer was separated and evaporated to almost dryness. Product of partial reductive hydrolysis was analyzed by GLC. The analyses were carried out at a temperature gradient from 175 to 290 °C; the rate of temperature change was 10 °C min^{-1}. Agarose (Sigma, USA) and kappa-carrageenan from *Euchemia cottonii* (Sigma, USA) were used as standards for agarobiose and carrabiose production.

2.5. Mild acid hydrolysis

Hydrolysis was carried out according to Yu et al. method (Yu et al., 2002). 1 mg polysaccharide sample was dissolved in 200 μL 0.1 N HCl and heated at 37 °C for 2 h and 24 h. Hydrolysis was stopped by the addition of some drops 0.1 N NH_4OH solution (pH 8–9). Then the sample was freeze dried.

2.6. Fourier transform-infrared spectroscopy (FT-IR)

IR spectra of the studied polysaccharides were recorded in films on a Vector 22 Fourier transform spectrophotometer (Bruker, Germany) with 4 cm^{-1} resolution. Sample preparation: 8 mg compound was dissolved in 1 mL H_2O and was heated at 37 °C on a polyethylene substrate until a dry film was produced. Then, the film was pressed between two NaCl plates and IR spectra were recorded. The spectra were normalized by the absorption of the monosaccharide ring skeleton at ~1074 cm^{-1} ($A_{1074} \approx 1.0$).

2.7. NMR spectroscopy

Sample of polysaccharide was deuterium-exchanged twice from D_2O by freeze-drying prior to being examined in a solution of 99.95% D_2O. One- (^1H, ^{13}C NMR) and two-dimensional (^1H–^1H COSY, ^1H–^1H ROESY and ^{13}C–^1H HSQC) spectra of polysaccharide were recorded with DPX-700 (176.05 MHz) spectrometer (Bruker, Germany) operating at 35 °C (for 1D) and 50 °C (for 2D). Chemical shifts were described relative to internal standard – methanol (δ_C 50.15, δ_H 3.337). XWINNMR 1.2 software (Bruker, Germany) was used to acquire and process the NMR data.

2.8. Mass spectrometry

A 2,5-dihydroxybenzoic acid (DHB) (Sigma, USA) was used as matrix for matrix-assisted laser desorption/ionization mass spectrometry (MALDIMS). Spectra were recorded with an Ultra Flex III MALDI-TOF/TOF mass spectrometer (Bruker, Germany) with nitrogen laser (355 nm), reflector and "potential LIFT" modes of operation. Instrument settings for the positive- and negative-ion modes: accelerating voltage, 25 kV; laser power, ~20–30 μJ; number of shots, 250; laser shot rate, 66 Hz. Sample preparation: 1 μL aqueous solution of sample in concentration 5 mg mL^{-1} and 1 μL of

1 M matrix solution with ratio ethanol:H_2O 1:1 were mixed. 1 μL of mixture was introduced on a stainless steel target and air dried.

2.9. Anticoagulant activity

2.9.1. Prothrombin time (PT)

Polysaccharides and unfractionated heparin in final concentrations of 10 and 1 μg mL^{-1}, respectively, were incubated for 1 min at 37 °C with 0.1 mL of human plasma followed by an addition of 0.2 mL of a solution of thromboplastin calcium mixture (Tekhnologiya-Standart, Russia) preheated at 37 °C. Then the time of clot formation was registered. The influence on PT was calculated according to the following equation:

$$\% = \frac{t_{sample} - t_{PBS}}{t_{PBS}} \times 100$$

2.9.2. Activated partial thromboplastin time (aPTT)

Polysaccharides and unfractionated heparin in final concentrations of 10 and 1 μg mL^{-1}, respectively, were incubated for 2 min at 37 °C with 90 μL of human plasma followed by the addition of 90 μL aPTT reagent (ellagic acid phospholipid reagent) (Tekhnologiya-Standart, Russia). After incubation at 37 °C for 3 min, 40 μL of $CaCl_2$ of 0.025 M was added, and the timer was started. The influence on aPTT was calculated according to the following equation:

$$\% = \frac{t_{sample} - t_{PBS}}{t_{PBS}} \times 100.$$

3. Results and discussion

Sterile form of red alga *A. flabelliformis* was collected in July at a depth of 0.5 m. After the preliminary extraction of pigments with 1.5% solution $NaNO_3$ (Kravchenko et al., 2013), polysaccharides were isolated by sequential extraction with distilled water at 80 °C. Obtained polysaccharides (Σ) were separated into a KCl-insoluble and KCl-soluble fractions. The yields and composition of the polysaccharide fractions are given in Table 1. As seen from Table 1, the total amount of KCl-insoluble polysaccharide was 6.2-fold more than KCl-soluble.

The galactose and 3,6-anhydrogalactose are the main monosaccharides in KCl-insoluble fraction (Table 1). A 3,6-anhydrogalactose content was 9.5 times higher in the KCl-insoluble fraction than in KCl-soluble one. Both polysaccharides also contained glucose and xylose. Their amounts were negligible in KCl-insoluble polysaccharide. The presence of glucose in the studied samples is probably due to the occurrence of floridean starch in the algal cell wall (Craigie, 1990). The presence of xylose in the polysaccharide can be caused either xylan that co extracted with galactan (Craigie, 1990), either as single xylose residues that replace hydroxyl groups of galactan (Lahaye & Rochas, 1991; Usov & Bilan, 1998). Generally, xylose residues at C6 of 3-linked β-D-galactose or at C3 4-linked α-D-galactose are characteristic for red algae of family Corallinaceae (Navarro & Stortz, 2008; Usov & Bilan, 1998; Usov, Bilan, & Shashkov, 1997) and Solieriaceae (Chiovitti et al., 1997), respectively. High xylose content was determined in KCl-soluble polysaccharide from *Gymnogongrus griffithsiae* (Talarico et al., 2004) as well as in studied *A. flabelliformis*.

Partial reductive hydrolysis was carried out for identification of isolated polysaccharide. Obtained hydrolysate was analyzed by GLC. According to the results, a KCl-insoluble polysaccharide from *A. flabelliformis* contains only disaccharide units of 4-O-β-D-galactopyranosyl-3,6-anhydro-D-galactose (carrabiose) that allows it to include a group of carrageenan. The molar ratio of AnGal:Gal should be 1:1 for regular carrageenans structures. As seen from Table 1, polysaccharide from *A. flabelliformis* exhibit irregular structure.

FR-IR and NMR spectroscopic techniques were used for polysaccharides identification. Obtained spectrum was compared with that of carrageenans with known structures. The IR spectrum of KCl-insoluble polysaccharide showed a very intense absorption band at about 1229 cm^{-1} (see supplementary materials) characteristic of the sulfated ester groups (Pereira, Amado, Critchley, Van de Velde, & Ribeiro-Claro, 2009). This fact agrees well with chemical analysis results (Table 1). The absorption band at about 932 cm^{-1} (3,6-anhydrogalactose) and 847 cm^{-1} (sulfate group at C-4 of the galactose residue) indicated the presence of kappa-type of carrageenan (Rees et al., 1993). Weak band at 892 cm^{-1} indicated the presence of unsulfated galactose residue, which is typical for both beta- and alpha-types of carrageenan (Pereira et al., 2009). The absence of a clear absorption band at 805 cm^{-1}, characteristic of the sulfate group at C-2 3,6-anhydrogalactose of alpha-carrageenan, was evidence to the beta-type of polysaccharide.

^{13}C NMR spectrum of polysaccharide contains more than 12 signals. Four signals of different intensities at 95.2, 95.6, 103.2 and 103.3 ppm were observed in the anomeric carbons resonance area of the ^{13}C NMR spectrum of polysaccharide. It may indicate the presence of two types of disaccharide units (Fig. 1B). Double signals at 103.2 and 103.3 ppm were characteristic of the C-1 galactose residues, whereas signals at 95.6 and 95.2 ppm were assigned to C-1 3,6-anhydrogalactose residues of kappa- and beta-carrageenans, respectively (Liao, Kraft, Munro, Craik, & Bacic, 1993; Miller & Blunt, 2000; Usov & Shashkov, 1985). ^{1}H NMR spectrum also contained four anomeric signals of different intensities at 4.61 and 4.64 ppm (H-1 galactose), at 5.10 and 5.12 ppm (H-1 3,6-anhydrogalactose) corresponding to beta- and kappa-carrageenans, respectively (Fig. 1A) (Kolender & Matulewicz, 2004; Van Velde, Knutsen, Usov, Rollema, & Cerezo, 2002). Integration of the signals at 5.12, 5.10 and 4.87 ppm showed that kappa- and beta-units of carrageenan have a ratio of 3:1. In addition, weak signals at 5.32 and 5.20 ppm present in ^{1}H NMR spectrum (Fig. 1A), which can be assigned to the α-anomeric protons of 3,6-anhydrogalactose 2-sulfate of iota-carrageenan and galactose 6-sulfate of gamma-carrageenan, respectively (Van de Velde et al., 2002).

It was shown by DEPT experiment that there were two oxymethylene groups at 62.0 and 70.1 ppm, the latter of which was substituted as seen from the values of its chemical shift. Two-dimensional COSY (Fig. 2A), ROESY and HSQC (Fig. 2B) spectroscopy was used to assign signals of monosaccharide residues protons, to

Table 1
Yields and chemical analysis of polysaccharide fractions from sterile form of *A. flabelliformis*, collected in Troitsa Bay in July 2012.

Fraction	PS yield, % dried algal weight	Content, % sample weight					Molar ratio AnGal:Gal
		Gal	3,6-AnGal	Glc	Xyl	SO_3Na	
Σ	31.8	34.9	15.0	2.0	2.1	18.7	1:2.1
A	25.1	35.1	21.8	2.1	1.1	21.6	1:1.4
B	4.1	33.6	2.3	4.2	5.1	23.4	1:13.0

Note: A – KCl-insoluble fraction, B – KCl-soluble fraction, PS – polysaccharide, Σ – hot water extracted sum polysaccharide, Gal – D-galactose, 3,6-AnGal – 3,6-anhydro-D-galactose, Glc – glucose, Xyl – xylose.

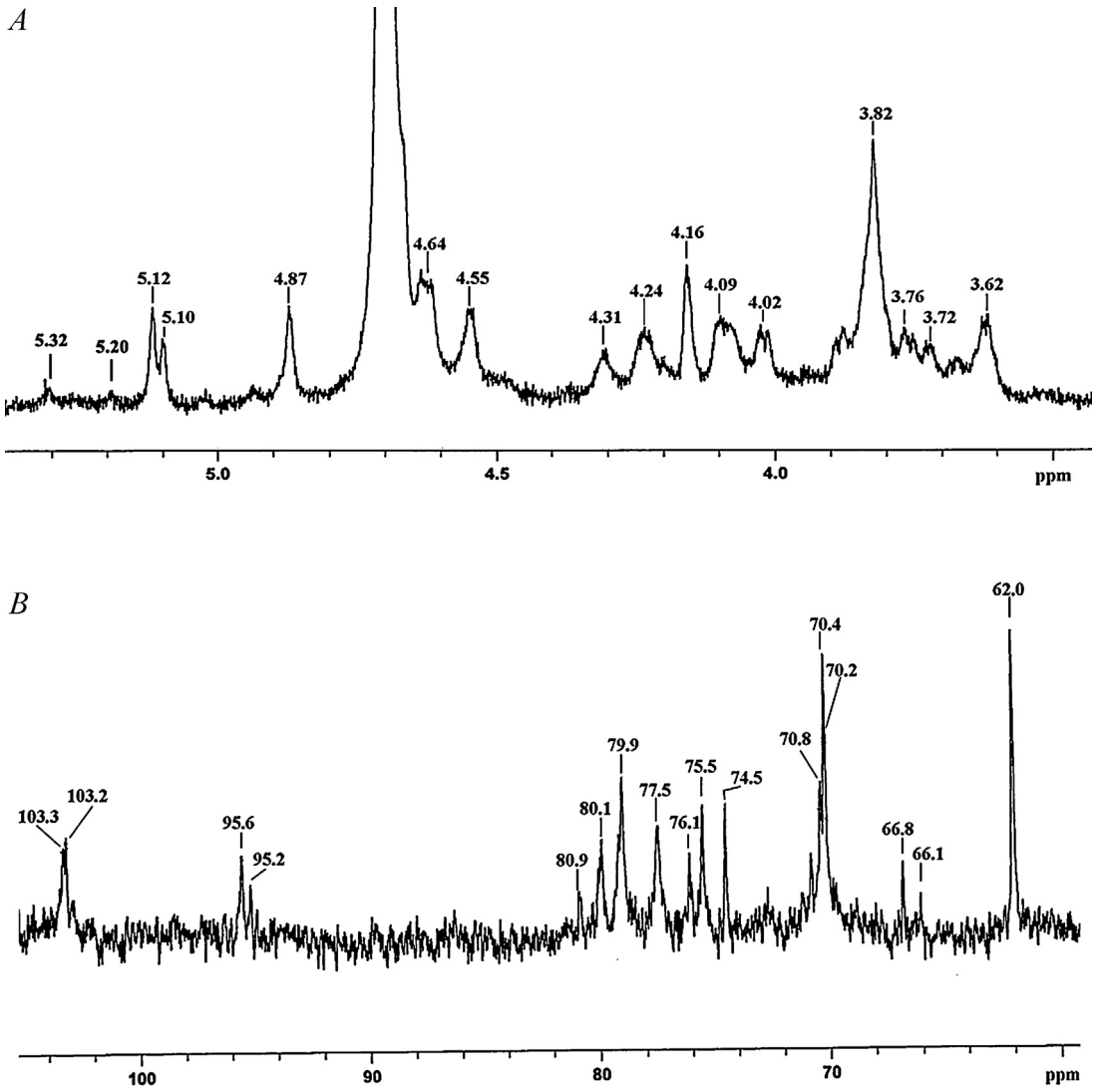

Fig. 1. ^{1}H (A) and ^{13}C (B) NMR spectra of KCl-insoluble polysaccharide from sterile form of *A. flabelliformis*.

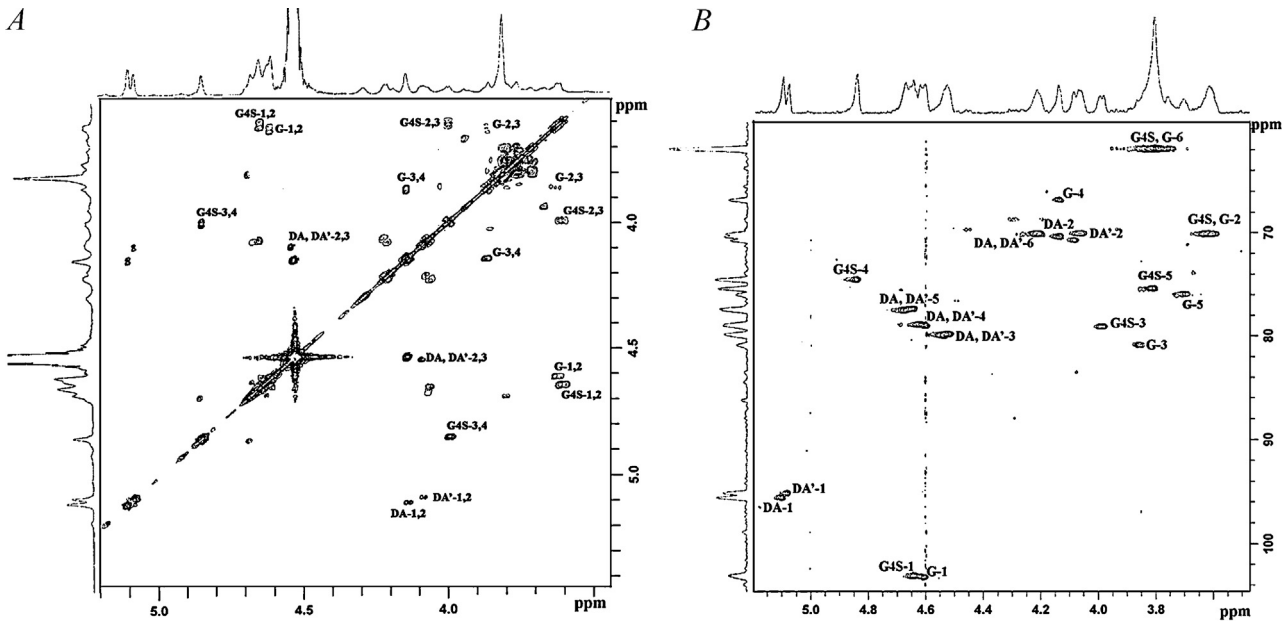

Fig. 2. ^{1}H–^{1}H COSY (A) and ^{13}C–^{1}H HSQC (B) spectra of KCl-insoluble polysaccharide fraction from *A. flabelliformis*.

Table 2
Chemical shifts values of protons and carbon (δ ppm) in ^{1}H and ^{13}C NMR spectra of KCl-insoluble polysaccharide from *A. flabelliformis*.

Carrageenan type	MS residue	^{1}H chemical shifts (ppm)					
		H-1	H-2	H-3	H-4	H-5	H-6
Kappa	G4S	4.64	3.60	3.99	4.87	3.80	3.80–3.70
	DA	5.12	4.14	4.52	4.62	4.66	4.20–3.62
Beta	G	4.61	3.62	3.86	4.14	3.70	3.80–3.70
	DA′	5.10	4.08	4.53	4.62	4.66	4.20–3.62
Carrageenan type	MS residue	^{13}C chemical shifts (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
Kappa	G4S	103.2	70.0	79.0	74.3	75.5	62.0
	DA	95.6	70.2	79.7	78.7	77.3	70.1
Beta	G	103.3	70.1	80.6	66.7	76.1	62.0
	DA′	95.2	70.6	79.7	78.7	77.3	70.1

Note: MS – monosaccharide.

carry out C/H correlation shown in Table 2 and to determine the type of glycosidic linkages between monosaccharide units.

There were the correlation signals C-1/H-1 at 95.2/5.10, 95.6/5.12, 103.2/4.64 and 103.3/4.61 ppm, corresponding to the four types of galactose residues in the anomeric area of heteronuclear 2D HSQC spectrum (Fig. 2B, Table 2). It was shown from the spectra that two types of disaccharide units were in the polysaccharide structure, which differed from each other only by the presence or absence of sulfate groups at C-4 of galactopyranose residue. The correlation signals at 74.3/4.87 and 66.7/4.14 ppm corresponded to C-4/H-4 sulfated (G4S) and unsulfated (G) 3-linked β-galactose. The presence of the sulfate group at C-4 β-galactose indicated that the signal in the ^{13}C NMR spectrum was shifted to 7.6 ppm in the weak field compared to the signal at C-4 unsulfated β-galactose. This fact is in good agreement with literature data (Liao et al., 1993; Miller & Blunt, 2000). Correlation signal at 62.0/3.8 ppm was assigned to the proton at C-6 both 3-linked G4S and G (Fig. 2B). Distinct cross-peaks at 79.7/4.53 and 78.7/4.62 ppm were in the spectrum as the result of the overlapping of the signals C-3 and C-4 DA and DA′ residues, respectively. According to HSQC spectrum, triplet in the region 70.0–70.8 ppm was a result of multiple carbon signals overlapping: G4S-2 and G-2, DA-2 and DA′-2, as well as DA-6 and DA′-6 (Fig. 2B, Table 2). Glycosylation effect of galactopyranose residue at the C-3 indicates that the monosaccharide residues in the both disaccharide units have D-configuration that is in good agreement with the results of the partial reductive hydrolysis indicating the presence of carrabiose units only.

The ROESY experiment allowed the verification of bonding between monosaccharide residues. Correlation peaks between H-1 DA and H-3, H-4 G4S (5.12/3.99 and 5.12/4.87, respectively) and H-1 DA′ and H-3, H-4 G (5.10/3.86 and 5.10/4.14, respectively), as well as H-1 G4S and H-4,5 DA (4.64/4.62 and 4.64/4.66, respectively) and H-1 G and H-4,5 DA′ (4.61/4.62 and 4.61/4.66, respectively) were compatible with alternating α-(1→3) and β-(1→4) linkages in the disaccharide repeating units of the polysaccharide.

Thus, according to chemical analysis, FT-IR, 1D and 2D NMR spectroscopy investigated KCl-insoluble polysaccharide from sterile form *A. flabelliformis* is kappa/beta-carrageenan with ratio of kappa- and beta-types units 3:1 and contains minor amounts of iota- and gamma-carrageenan. It should be noted that algae *G. torulosus* and *G. griffithsiae* belonging to family Phyllophoraceae as studied alga *A. flabelliformis*, produce kappa/iota-carrageenan with few content of agar-like structures (Estevez, Ciancia, & Cerezo, 2008; Talarico et al., 2004).

Matrix-assisted laser desorption/ionization mass spectrometry (MALDIMS) was applied for the elucidation of structural peculiarities of the polysaccharide including information on the minor inclusions (gamma- and iota-chains). The sample was depolymerized by mild acid hydrolysis to oligosaccharides, suitable for MALDIMS.

According to ^{13}C and ^{1}H NMR data the polysaccharide under study contains chains of beta-carrageenan. By assuming that the speed of hydrolysis of unsulfated polysaccharide is higher than highly sulfated one, an analysis of oligosaccharides, obtained by 2 h and 24 h (0.1 N HCl at 37 °C) was performed. MALDIMS of hydrolysis products (2 h, not shown) using negative-ion mode showed the presence of both short and longer (up to 1.5 kDa) sulfated oligosaccharides. It is worthy to mention that positive-ion mode (data not shown) revealed the presence of unsulfated fragments. The fact of detection of initially unsulfated fragments (beta-chains) at the early stage of acid hydrolysis (2 h) suggests that polysaccharide under study contains relatively large amounts of that chains.

MALDIMS of hydrolysis products (24 h) showed that oligosaccharide composition was similar excepting intensities of some fragments were higher. In addition, mass spectrum was almost cleared of a satellite signals, corresponding to the cleavage of water molecules (M-18). Probably, M-18 satellite signals were detected due to the excess fragmentation of oversulfated fragments with higher degree of polymerization (DP), still retained in the mixture on 2 h of hydrolysis. Thus, fragments obtained at 24 h of hydrolysis were assumed to be stable enough for the analysis and their structural features could enlighten the structure of the initial polysaccharide.

The composition and sequence of the key fragments at 2 and 24 h of mild acid hydrolysis (both sulfated and neutral) that was elucidated by tandem MALDIMS using a potential LIFT technique is depicted at Table 3. It must be noted that according to half-quantitative evaluation of the mixture contents, the main component was a disaccharide chain of kappa-carrageenan (Fig. 3A and B; *m/z* 449.01 and 403.05; Table 3, No. 1) and a "hybrid" tetrasaccharide that contains fragments of both kappa- and beta-chains (Fig. 3A and B; *m/z* 755.13 and 709.16; Table 3, No. 6). The presence of these fragments in the main chain of the polysaccharide is consistent with spectroscopy data (Fig. 1B).

A tandem MALDIMS of the ion at *m/z* 667.04 (Fig. 4A) was interesting because of the presence of the rare $^{0,2}A_3$ signal at *m/z* 607.0, corresponding to the cross-ring cleavage of hexapyranose ring of 6-sulfated Gal residue (D6S) at the non-reducing end. The rest of the signals, allowing the elucidation of the sequence of the saccharides were typical (Goncalves, Ducatti, Grindley, Duarte, & Noseda, 2010; Yu et al., 2006) for the tandem mass spectra obtained by collisionally-induced dissociation electrospray ionization mass spectrometry (CID ESIMS/MS). Thus, the minor ion under study was assumed to contain inclusion of gamma-chain (the precursor of beta-chain) that in accord with the ^{1}H NMR data (Table 3, No. 6, Fig. 1A).

Table 3
Structural characteristics of key fragments detected by MALDI mass spectrometry in a mixture of oligosaccharides obtained by the acid hydrolysis of the KCl-insoluble polysaccharide from *A. flabelliformis* for 2 and 24 h.

No.	Negative-ion mode of registration		No.	Positive-ion mode of registration	
	m/z	Composition/structural characteristics of the ion $[M^a-Na]^-$		*m/z*	Composition/structural characteristics of the ion $[M+Na]^+$
1	403.04	$[DA\text{-}G4S]^-$ $[G4S\text{-}DA]^-$	17	347.09	$[G\text{-}DA+Na]^+$ $[DA\text{-}G+Na]^+$
2	547.10	$[DA\text{-}G4S\text{-}DA]^-$	18	365.13	$[G\text{-}D+Na]^+$ $[D\text{-}G+Na]^+$
3	565.12	$[DA\text{-}G\text{-}D6S]^-$ $[D6S\text{-}G\text{-}DA]^-$ $[G\text{-}G4S\text{-}DA]^-$ overlapping of matrix associates	19	491.14	$[DA\text{-}G\text{-}DA+Na]^+$
4	649.03	$[DA\text{-}G4S\text{-}DA2S]^-$ $[DA2S\text{-}G4S\text{-}DA]^-$ only in the spectrum for 2 h hydrolysis	20	509.14	$[G\text{-}DA\text{-}G+Na]^+$ $[DA\text{-}G\text{-}D+Na]^+$
5	667.04	$[DA\text{-}G4S\text{-}D6S]^-$ $[D6S\text{-}G4S\text{-}DA]^-$	21	611.09	$[D\text{-}DA\text{-}G4S+Na]^+$ $[G4S\text{-}DA\text{-}D+Na]^+$ $[DA\text{-}G4S\text{-}D+Na]^+$
6	709.16	$[G4S\text{-}DA\text{-}G\text{-}DA]^-$ $[G\text{-}DA\text{-}G4S\text{-}DA]^-$ $[DA\text{-}G4S\text{-}DA\text{-}G]^-$	22	653.18	$[G\text{-}DA\text{-}G\text{-}DA+Na]^+$ $[DA\text{-}G\text{-}DA\text{-}G+Na]^+$
7	727.16	$[G\text{-}DA\text{-}G4S\text{-}D]^-$ $[G\text{-}D\text{-}G4S\text{-}DA]^-$	23	671.17	$[DA\text{-}G\text{-}D\text{-}G+Na]^+$ $[G\text{-}D\text{-}G\text{-}DA+Na]^+$
8	811.09	$[(DA\text{-}G4S)_2]^-$ $[(G4S\text{-}DA)_2]^-$ [G-DA-G4S-DA2S] [G4S-DA2S-G-DA]	24	**797.23**	$[Ac(AnGal)_2Gal_2(SO_3Na)+Na]^+$
9	829.11	$[G\text{-}DA\text{-}G4S\text{-}D6S]^-$ $[G\text{-}D6S\text{-}G4S\text{-}DA]^-$	25	899.16	$[(AnGal)_3Gal_2(SO_3Na)+Na]^+$
10	853.21	$[(AnGal)_3Gal(SO_3Na)]^-$	26	917.18	$[(AnGal)_2Gal_3(SO_3Na)+Na]^+$
11	871.21	$[(AnGal)_2Gal_3(SO_3Na)]^-$	27	**959.29**	$[Ac(AnGal)_2Gal_3(SO_3Na)+Na]^+$
12	955.14	$[(AnGal)_3Gal_2(SO_3Na)_2]^-$	28	1061.23	$[(AnGal)_3Gal_3(SO_3Na)+Na]^+$
13	1015.27	$[(AnGal)_3Gal_3(SO_3Na)_2]^-$	29	1079.24	$[(AnGal)_2Gal_4(SO_3Na)+Na]^+$
14	1033.27	$[(AnGal)_2Gal_4(SO_3Na)]^-$	30	**1103.33**	$[Ac(AnGal)_3Gal_3(SO_3Na)+Na]^+$
15	1117.22	$[(AnGal)_3Gal_3(SO_3Na)_2]^-$	31	1163.18	$[(AnGal)_3Gal_3(SO_3Na)_2+Na]^+$
16	1219.18	$[(AnGal)_3Gal_3(SO_3Na)_3]^-$	32	1265.43	$[(AnGal)_3Gal_3(SO_3Na)_3+Na]^+$

[a] M is sodium salt of mono/oligosaccharides if it is sulfated.

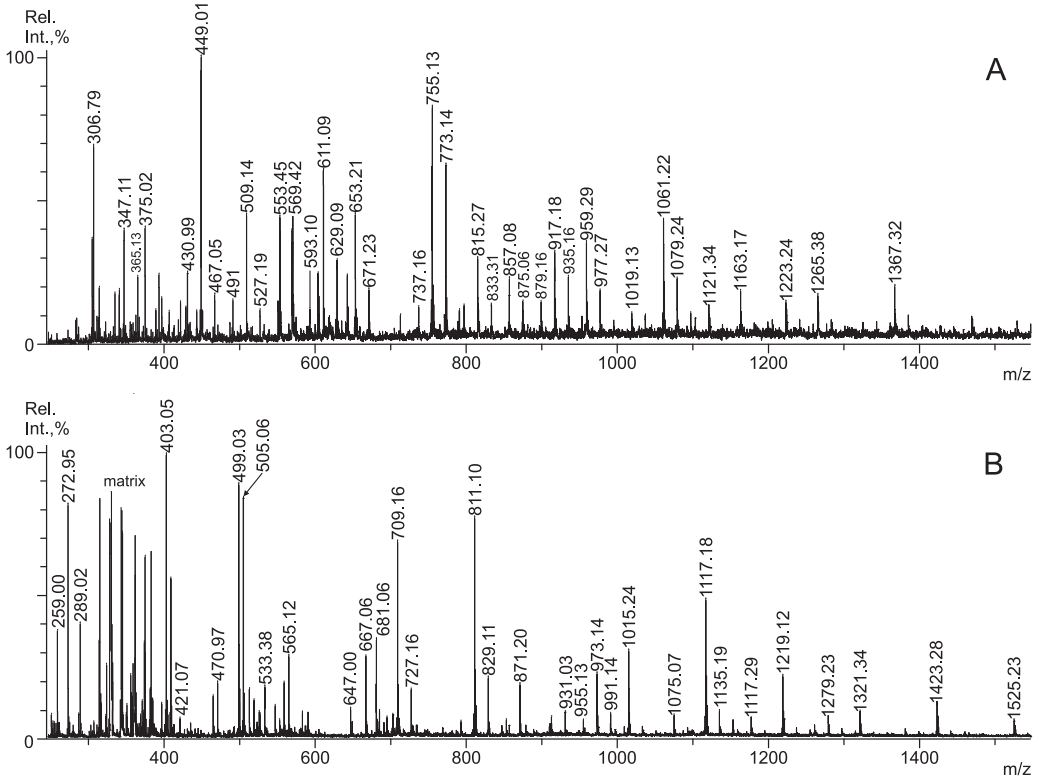

Fig. 3. MALDI mass spectra of oligosaccharides obtained by acid hydrolysis of KCl-insoluble polysaccharide (HCl, 37 °C, 24 h) from sterile form *A. flabelliformis* recorded in positive-ion (A) and negative-ion (B) modes.

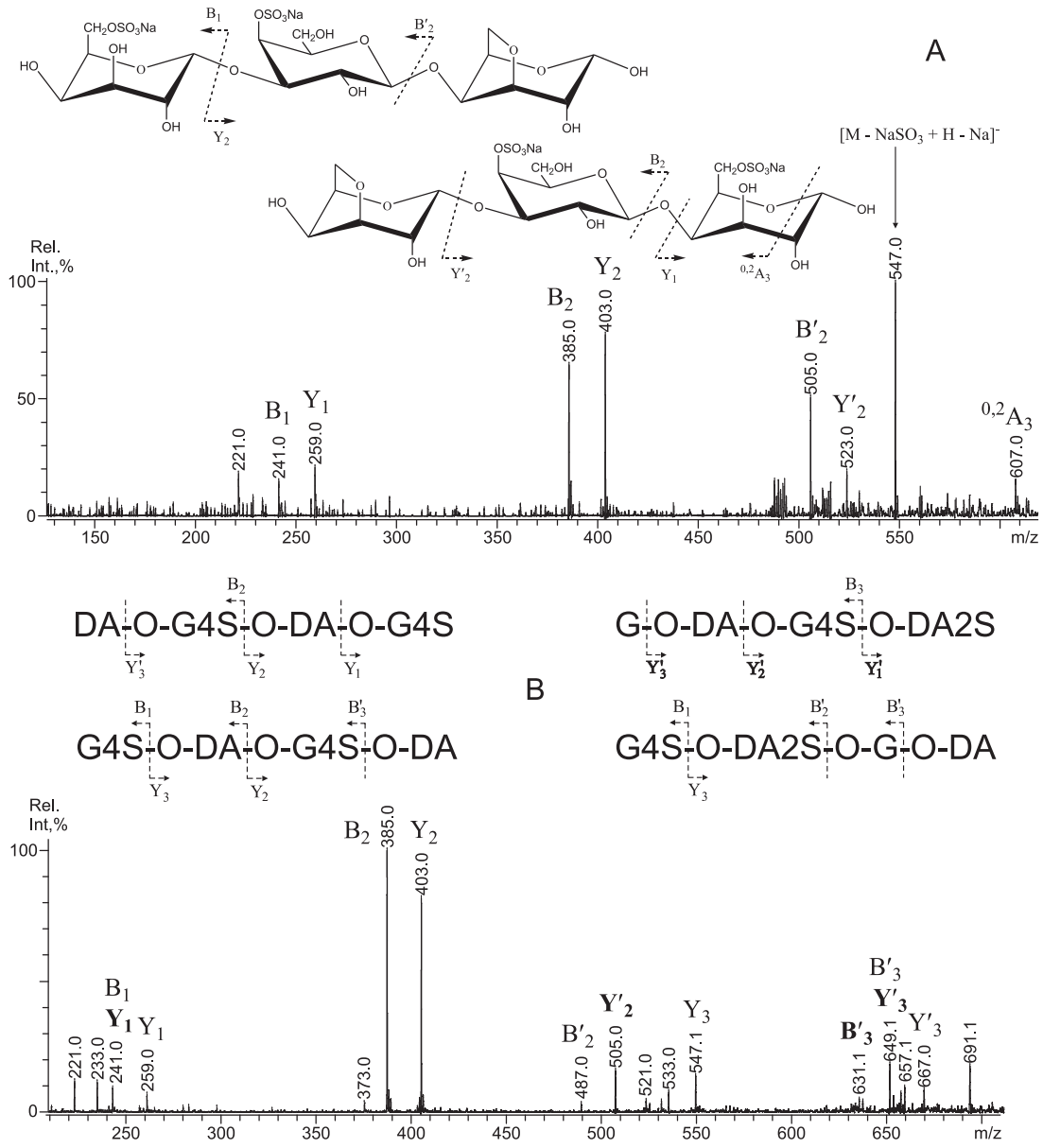

Fig. 4. Tandem MALDI mass spectra of anions at *m/z* 667.04 (A) and *m/z* 811.09 (B).

A tandem MALDIMS of the ion at *m/z* 811.09 (Fig. 4B) is interesting due to the presence of the fragment ion $\mathbf{Y_2}'$ at *m/z* 505.0, corresponding to the breaking of glycosidic linkage and cleavage of the iota-chain fragment. It must be noted that a tandem MS of the ion at the same *m/z*, produced with recombinant *Alteromonas carrageenovora* kappa-carrageenase from purchased kappa-carrageenan did not exhibit a fragment ion at *m/z* 505.0 (Anastyuk et al., 2011). Thus, it was shown that oligosaccharide, matching by *m/z* with fragments, built up of kappa-blocks in our case could contain residues of beta- and iota-chains (Table 3, No. 8).

A tandem MALDIMS of the ion at *m/z* 671.17 (Fig. 5B), acquired in a positive-ion mode, had +18 Da mass difference with an "ideal" fragment, built up of beta-chains showing the fact of desulfation. This fragment contains the residues of unsulfated Gal residue. The excess loss of sulfate from C-6 of Gal residue could occur either in the ion source of mass spectrometer or during hydrolysis that was observed recently for the hybrid iks-carrageenan sample from tetrasporic red seaweed *Tichocarpus crinitus* (Byankina (Barabanova) et al., 2013). The presence of 4-linked Gal residue was additionally confirmed by the $^{0,2}A_3$ fragment ion at *m/z* 449.1, corresponding to the cross-ring cleavage of one of the 4-linked Gal residues (Fig. 5, upper structure).

It is worthy to mention that positive-ion mode ESIMS (see supplementary materials) of oligosaccharides, obtained under 0.1 N HCl at 24 h revealed signals, corresponding to the ions of $[Xyl + Na]^+$ at *m/z* 173.053 and $[XylHex + Na]^+$ at *m/z* 335.113. Despite of the low intensity of the ion at *m/z* 335.113, some structural information was extracted in tandem CID MS/MS mode. The analysis confirmed that the disaccharide was built up of the xylose and hexose (probably, galactose) residues. The Gal residue was located at the non-reducing end. The fact that xylose was linked to galactose residues was observed by MALDIMS in oligosaccharide from the red seaweed *Gracilaria corticata* (Chattopadhyay et al., 2008). In addition, for Corallinaceae (Navarro & Stortz, 2008; Usov et al., 1997) and Solieriaceae (Chiovitti et al., 1997) families it was shown that β-D-Xyl residues were the substitute of hydroxyl group at C-6 of 3-linked β-D-Gal residues or at C-3 of 4-linked α-D-Gal residues,

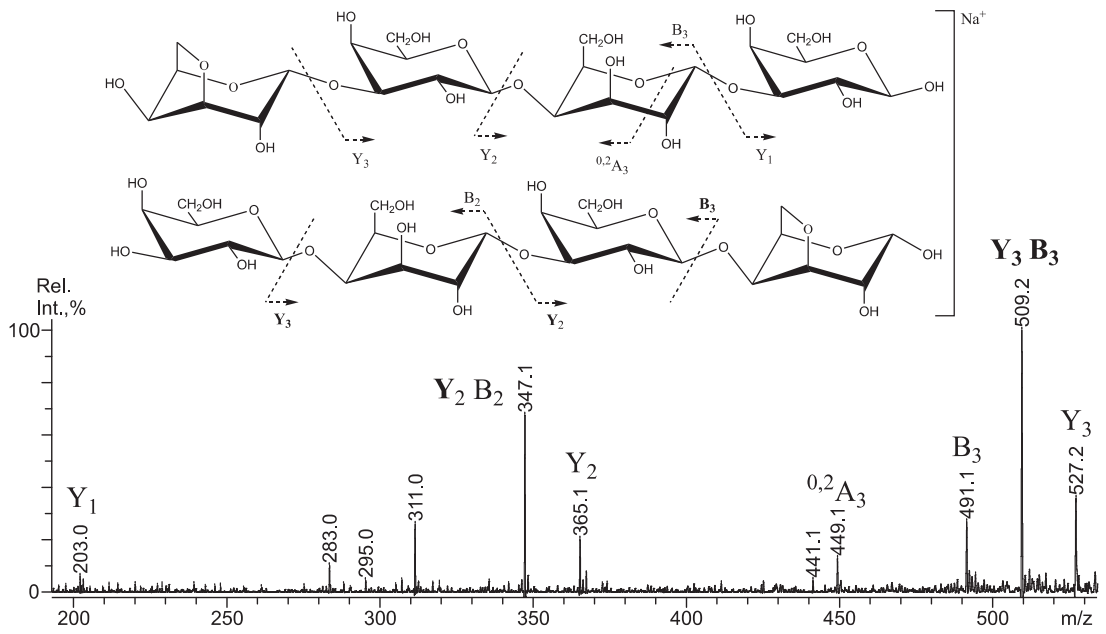

Fig. 5. Tandem MALDI mass spectrum of cation with *m/z* 671.17.

respectively. Thus, the acquired data allowed us to conclude that xylose residues, detected by monosaccharide composition analysis (Table 1) are incorporated into the structure of the polysaccharide as substitute of one of hydroxyl groups of galactose residues.

Anticoagulant activity is among the most widely studied property of sulfated polysaccharides. As known marine sulfated polysaccharides are capable of interacting with certain cationic proteins, such as (co)-factors of the coagulation cascade during clotting-inhibition processes (Pomin, 2009). The anticoagulant effect of studied KCl-insoluble polysaccharide in comparison with KCl-soluble and Σ (sum) polysaccharide substances in this study was investigated by their capacity to inhibit the clotting process in prothrombin time and activated partial thromboplastin time.

The effect on the extrinsic pathway of blood coagulation was estimated by the prothrombin time (PT). This assay showed that none of the investigated polysaccharides had any effect toward extrinsic pathway of blood coagulation (Fig. 6). However, the results of the activated partial thromboplastin time (aPTT) used to estimate the effect of the samples on the intrinsic pathway of blood coagulation represented the following data. The most active of the investigated polysaccharides was KCl-soluble type (increase in clotting time by 67%) whereas the sum and KCl-insoluble polysaccharides retarded coagulation by 25 and 50%, respectively (Fig. 6). It can be connected with the sulfation level and monosaccharides contents as well (Table 1). 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 (Pomin & de Souza Mourão, 2012; Yermak et al., 2012).

Hence, results of the aPTT and PT assays suppose that carrageenans affected mostly intrinsic pathway of coagulation, while their effect on the extrinsic pathway is absent. Our results practically correspond to conclusions made by Silva and coauthors who also observed that carrageenans affected intrinsic coagulation pathway (Silva et al., 2010).

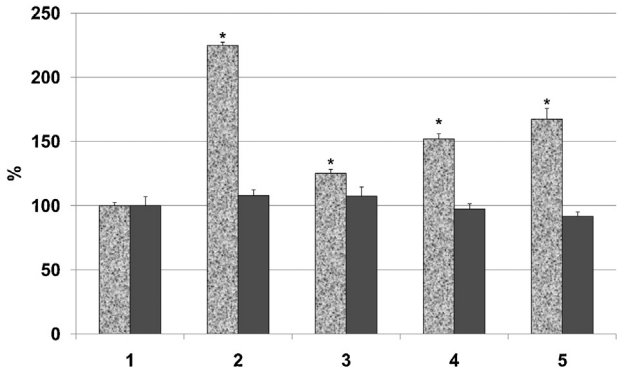

Fig. 6. Coagulation time change in the presence of red algal polysaccharides ($C = 10\ \mu g\ mL^{-1}$, final value): 1, control (PBS); 2, heparin ($C = 1\ \mu g\ mL^{-1}$, final value); 3, $\sum$ polysaccharide; 4, KCl-insoluble polysaccharide; 5, KCl-soluble polysaccharide. The results are expressed as % change in thrombin time relative to the negative control (100%). ▨ – activated partial thromboplastin time; ■ – prothrombin time.

4. Conclusions

Sulfated polysaccharide from sterile plant of *A. flabelliformis* extracted after preliminary pigments isolation was investigated. Obtained polysaccharide was generally presented by KCl-insoluble fraction with galactose and 3,6-anhydrogalactose as the main monosaccharides. According to FT-IR-, 1D and 2D NMR spectroscopies KCl-insoluble polysaccharide is kappa/beta-carrageenan with ratio of kappa- and beta-types units 3:1. Partial reductive hydrolysis data is in a good agreement with NMR spectroscopy, indicating that the polysaccharide comprises disaccharide units of 4-O-β-D-galactopyranosyl-3,6-anhydro-D-galactose (carrabiose) only. Mass spectrometry method used for analysis of oligosaccharides obtained by mild acid hydrolysis of KCl-insoluble polysaccharide allowed us to confirm that polymer chain consisted of kappa- and beta-types units and contained minor amounts of iota- and gamma-carrageenans (precursor of beta-carrageenan). In addition, ESIMS/MS data suggested that xylose (minor amount) determined by chemical analysis is present in the polysaccharide as a substituent one of hydroxyl group of galactose. Anticoagulant activity of studied polysaccharides was investigated. According to aPTT and PT assays the studied carrageenan affected mostly intrinsic pathway of coagulation, while it effect on the extrinsic pathway is absent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.04.022.

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