Biochemistry (Moscow), Vol. 70, No. 3, 2005, pp. 350-356. Translated from Biokhimiya, Vol. 70, No. 3, 2005, pp. 430-437.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-109, October 10, 2004.

Comparative Study of Carrageenans from Reproductive and Sterile Forms of *Tichocarpus crinitus* (Gmel.) Rupr (Rhodophyta, Tichocarpaceae)

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Received May 6, 2004
Revision received June 3, 2004

Abstract—A comparative study of the structure and properties of the sulfated polysaccharides (carrageenans) isolated from the vegetative and reproductive forms of the red alga *Tichocarpus crinitus* was performed. The polysaccharides were separated into the gelling (KCl-insoluble) and non-gelling (KCl-soluble) fractions by precipitation with 4% KCl. The total content of polysaccharides extracted from the reproductive form of the alga was 1.8-fold more than that extracted from the vegetative form, and in the first case, the gelling polysaccharides mostly accumulated. The gelling polysaccharides from the vegetative form have the highest molecular weight (354 kD). According to the results of FT-IR and ^{13}C-NMR spectroscopy, the gelling polysaccharide fractions from both forms are κ/β carrageenans. The differences concern the content of the κ- and β-disaccharide units and the presence of a small content of the sulfated disaccharide segments (precursors of the κ-carrageenans) in the polysaccharide from the reproductive form of the alga. The non-gelling polysaccharide fractions from both forms of the plant are mixtures of sulfated galactans with a low content of 3,6-anhydrogalactose.

Key words: *T. crinitus*, carrageenan, life history stage of algae, ^{13}C-NMR spectroscopy, FT-IR spectroscopy

Carrageenans are sulfated polysaccharides of the red algae composed of repeated disaccharide residues of D-galactose and its derivatives connected with the alternating β-1,4 and α-1,3 glycoside bonds. The structure of carrageenans is variable due to the fact that the 4-O-substituted monosaccharide residue can be D-galactose or its 3,6-anhydroderivative, and different hydroxyl groups can be sulfated [1]. Scheme presents idealized structures of some types of carrageenan. Natural polysaccharides are often irregular structures and contain repeating units of several types, this being explained by the multistage biosynthesis of polysaccharides [2]. At the same time, the regular polysaccharides with a high content of 3,6-anhydrogalactose (κ- and ι-carrageenans) are of especial practical significance as gel-forming substances. Polysaccharides of a less regular structure with a low content of 3,6-anhydrogalactose (λ-carrageenans) do not form gels, but yield very viscous solutions and are used as stiffeners and stabilizers of suspensions and emulsions [3].

The structure of carrageenans depends on the species of the alga, its environment, and its life history stage [2-8]. The latter is especially important, since the red algae have a complex life cycle including the alternation of vegetative, sexual, and asexual reproduction. The first works on the carrageenan structure were performed mainly on samples of polysaccharides isolated from the mixture of different forms of the alga, this hampering greatly the interpretation of the results. The detailed analysis of the carrageenans from the algae *Iridaea undulosa* and *Gigartina skottsbergii* demonstrated that the cystocarps produce the gel-forming κ/ι-carrageenans and μ/ν-carrageenans, while tetrasporophytes form λ-carrageenans forming gels under high KCl concentration [9, 10]. Such distribution of carrageenans on the life history stage of the alga is characteristic for most species of Gigartinaceae and Phyllophoraceae [2, 11, 12], while the representatives of Solieriaceae and Hypneaceae produce only κ-

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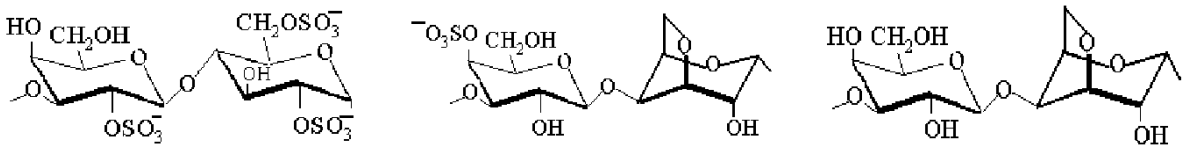

Idealized structures of some types of carrageenans
Scheme

and θ-carrageenans independently of the form of the alga [2].

It should be noted that it is difficult to evaluate the impact of all factors on the qualitative and quantitative characteristics of polysaccharides being synthesized in the alga.

The alga *Tichocarpus crinitus* belongs to the family Tichocarpaceae that is widely spread in the seas of the Far East. This seaweed exhibits reproductive and vegetative (sterile) stages and spends a long time in a sterile state [13]. Due to its rapid growth, large size, and characteristic features of its development, *T. crinitus* can be considered as a promising species for industrial production of polysaccharides and for introduction into mariculture. The first information concerning *T. crinitus* as the source of carrageenan polysaccharides was published in 1969 [14]. Chemical analysis [15, 16] and ^{13}C-NMR spectroscopy [17] revealed that the fraction of polysaccharide forming gels in the presence of potassium chloride corresponded to not completely sulfated κ-carrageenan. The fraction soluble in the presence of potassium chloride exhibited the usual for λ-carrageenan structure of the carbohydrate chain with alternating α-1,3 and β-1,4 bonds between the residues of D-galactopyranose [18]. Later, we investigated physicochemical properties of carrageenans from *T. crinitus* [19] and showed that the growth of biomass of the sterile alga *T. crinitus* as well as its qualitative and quantitative polysaccharide composition significantly depended on the environmental conditions, such as temperature of water and photon irradiance [20]. It has been unknown, however, whether polysaccharide composition of the alga *T. crinitus* changes while the transition from one stage of life history to the other.

The goal of the present work was to compare the structure and properties of carrageenans isolated from the vegetative and reproductive forms of *T. crinitus*.

MATERIALS AND METHODS

Algae. The vegetative and reproductive forms of the algae *T. crinitus* were collected in the same place in the Sea of Japan (Peter the Great Bay, Cape Fal'shivyi) in the end of October. The algae were washed with running water to remove soluble salts, treated with acetone to remove pigments, and dried.

Isolation of polysaccharides and their fractionation. Dried algae were ground and water was added (in the ratio of 1 : 30). Extraction of polysaccharides was performed for 3 h at 90°C under constant stirring. The resulting extract was filtered and then centrifuged at 4000 rpm for 20 min and the algal pellet re-extracted twice with water. The supernatants were pooled. Polysaccharides were separated into the gelling and non-gelling fractions as described previously [19].

Analytical methods. Total content of carbohydrates was determined by the phenol–sulfuric acid method using D-galactose as the standard [21]. Neutral monosaccharides were determined as alditol acetates derivatives by gas–liquid chromatography using an Agilent 6850 chromatograph (Germany) equipped with a capillary HP-5MS column (30 m × 0.25 mm) with 5% Phenyl Methyl Siloxane (Agilent) and a flame ionization detector at 175-225°C; the rate of temperature change was 3°C/min [22].

The content of 3,6-anhydrogalactose was determined by complete reducing hydrolysis [23]. The content of the sulfate groups was determined turbidimetrically [24].

Analytical centrifugation. High-speed sedimentation of carrageenan samples was performed in a MOM 3130 analytical centrifuge (Hungary) (schlieren optics, double chamber capillary cell) at 30,000 rpm using 0.15 M NaCl as the solvent.

Gel chromatography on CL-2B Sepharose. Carrageenan (15 mg) was dissolved in 3 ml of 0.15 M NaCl, applied on a column (24.5 × 1.5 cm), and then eluted with 0.15 M NaCl. Fractions (3.8 ml) were collected and assayed for the carbohydrate content. The column was calibrated using sulfated dextrans of 36-50, 400-600, and 1400 kD (Sigma, USA).

Determination of viscosity of carrageenan solutions. 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) (capillary

diameter 0.3 mm) at 26°C, the time accuracy being within ±0.1 sec. 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.

Fourier transform IR spectroscopy (FT-IR). IR spectra of the investigated samples were recorded using a Vector 22 Fourier transform spectrophotometer (Bruker, Germany) with resolution of 4 cm^{-1}. Eight milligrams of the sample was dissolved in 1 ml of water, placed in a polyethylene plate (1 cm in diameter), and dried at 37°C to obtain a dry film. Then the film was fixed between two NaCl windows and the IR spectra were recorded. The spectra were normalized by the absorption of the monosaccharide ring at ~1070 cm^{-1}.

^{13}C-NMR spectroscopy. ^{13}C-NMR spectra of polysaccharides were recorded on a DRX-500 spectrometer (Bruker) in a D_2O solution at 60°C using methanol as the internal standard (δ 50.15 ppm).

RESULTS AND DISCUSSION

All the algae *T. crinitus* were collected at a single place in the Sea of Japan (Peter the Great Bay, Cape Fal'shivyi) from the same depth, to avoid the influence of the accessory factors as the photon irradiance, water motion, and salinity on the composition of the investigated polysaccharide. The macrophytes were represented by two life history stage (forms)—vegetative and reproductive (containing cystocarps).

Polysaccharides from both forms were extracted with hot water and fractionated using precipitation with KCl to obtain the gelling (KCl-insoluble) and non-gelling (KCl-soluble) fractions. The yield and composition of the polysaccharide fractions are presented in Table 1. The total amount of polysaccharides isolated from the reproductive form of the alga was 1.8-fold more than that from the vegetative form. In the first case, the gelling polysaccharides were mostly accumulated. The galactose and 3,6-anhydrogalactose were main monosaccharides in all fractions. The molar ration of these monosaccharides for the regular structures must constitute 1 : 1. As seen from Table 1, all isolated polysaccharides except for the KCl-insoluble fraction from the vegetative form of the alga exhibit irregular structure.

The polysaccharide fractions were analyzed by analytical centrifugation and gel filtration. All samples formed a single sedimentation boundary, this indicating the homogeneity of the preparations. This is also supported by gel chromatography on CL-4B Sepharose. The unimodal distribution of the elution curves of the polysaccharide fractions was observed by gel filtration.

The viscosity of the diluted polysaccharide solutions was determined in 0.1 M NaCl. The values of the intrinsic viscosity for all polysaccharide fractions are presented in Table 2. The molecular weights of polysaccharides were calculated using the Mark–Houwink–Coon equation:

Table 1. Characteristic of the polysaccharide fractions isolated from two forms of the alga *T. crinitus*

Life history stage	Sample	Yield, %	Content, %			Molar ratio AnGal/Gal
			Gal	3,6-AnGal	sulfates	
Vegetative	A	16.0	33.1	30.0	20.0	1.0 : 1.1
	B	5.0	37.7	5.6	27.0	1.0 : 6.0
Reproductive	A	30.0	39.0	25.5	15.0	1.0 : 1.4
	B	7.0	33.2	7.2	26.8	1.0 : 4.0

Note: A stands for the KCl-insoluble polysaccharide fraction, and B is the KCl-soluble polysaccharide fraction.

Table 2. Values of intrinsic viscosity and molecular weight for carrageenans isolated from *T. crinitus*

Polysaccharide fraction	Vegetative form		Reproductive form	
	|η|, ml/g	M, daltons	|η|, ml/g	M, daltons
KCl-insoluble	$0.578 \cdot 10^3$	354 000	$0.271 \cdot 10^3$	159 000
KCl-soluble	$0.216 \cdot 10^3$	145 000	$0.345 \cdot 10^3$	205 000

$[\eta] = KM^{\alpha}$, where $[\eta]$ is the intrinsic viscosity and K and α are empirical constants constituting $3 \cdot 10^3$ and 0.95, respectively, according to the literature data for this polymer–solvent system [25]. The highest molecular weight (354 kD) was determined for the polysaccharides of the gelling fraction from the vegetative form of the alga, this corresponding to the results obtained by gel chromatography on CL-2B Sepharose (Table 2).

^{13}C-NMR analysis and FT-IR spectroscopy were used to identify the type of carrageenans. All spectra of the polysaccharides were compared with the spectra of the known idealized structures of carrageenans. An intensive absorption band at 1240-1250 cm^{-1} was observed in the IR spectra of the investigated carrageenans indicated the presence of a significant amount of the sulfate groups [26]. The IR spectra of the gelling polysaccharides were similar for both forms of the alga (Fig. 1, a′ and b′). These spectra exhibited an intense absorption band at 932 cm^{-1} that is characteristic for 3,6-anhydrogalactose and an absorption band at 847 cm^{-1} of the sulfate group at the O-4 of galactose [27]. Based on these data, the polysaccharides of the gelling fractions can be identified as κ-carrageenans [28]. The weak absorption band at 893 cm^{-1} indicates the presence of non-sulfated galactose residue, this being characteristic for both β- and α-carrageenans [29, 30]. The absence of an absorption band at 805 cm^{-1} of the sulfate group at the O-2 of 3,6-anhydrogalactose of α-carrageenan suggests the presence of β-carrageenan. Thus, the results of IR spectroscopy indicate that the gelling polysaccharides from both forms of the alga are κ/β-carrageenans.

Analysis of the ^{13}C-NMR spectra of the polysaccharides of the KCl-insoluble fractions supports the results of FT-IR spectroscopy. The ^{13}C-NMR spectra of the gelling polysaccharide fractions from the reproductive (Fig. 2) and vegetative (Fig. 3) forms of the alga show four signals in the region of resonance of the anomeric carbon atoms. The signals at δ 95.3 ppm correspond to the C-1 atoms of the 3,6-anhydro-D-galactose residues of β-carrageenan, and the signals at δ 95.8 ppm (Figs. 2 and 3) correspond to the C-1 atoms of the 3,6-anhydro-D-galactose residues of κ-carrageenan. The poorly resolved signals of double integral intensity at δ 103.2 and 103.3 ppm (Figs. 2 and 3) are the result of the overlap of the signals from the C-1 atoms of the galactose residues of κ- and β-carrageenans [31, 32]. The high field in the ^{13}C-NMR spectra of the polysaccharides are also typical for κ- and β-carrageenans. Moreover in the spectra of the gelling polysaccharide from the reproductive alga the additional weak resonance at 105.4 ppm is observed. It can correspond to the C-1 atom of the galactose residues carrying the sulfate group at O-4, that may point to presence of μ- and ν-carrageenans, which are precursors of κ-carrageenans [33, 34].

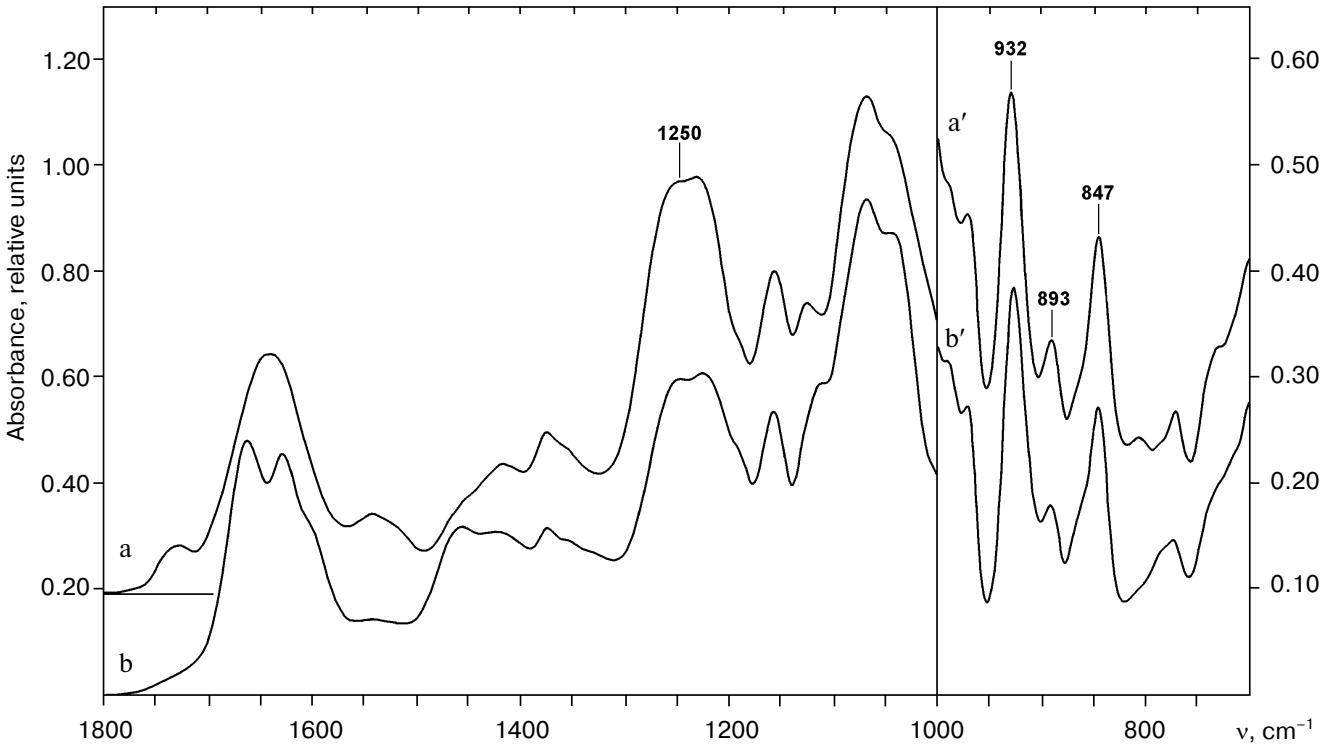

Fig. 1. IR spectra of KCl-insoluble fractions from the vegetative (a and a′) and reproductive form of *T. crinitus* (b and b′).

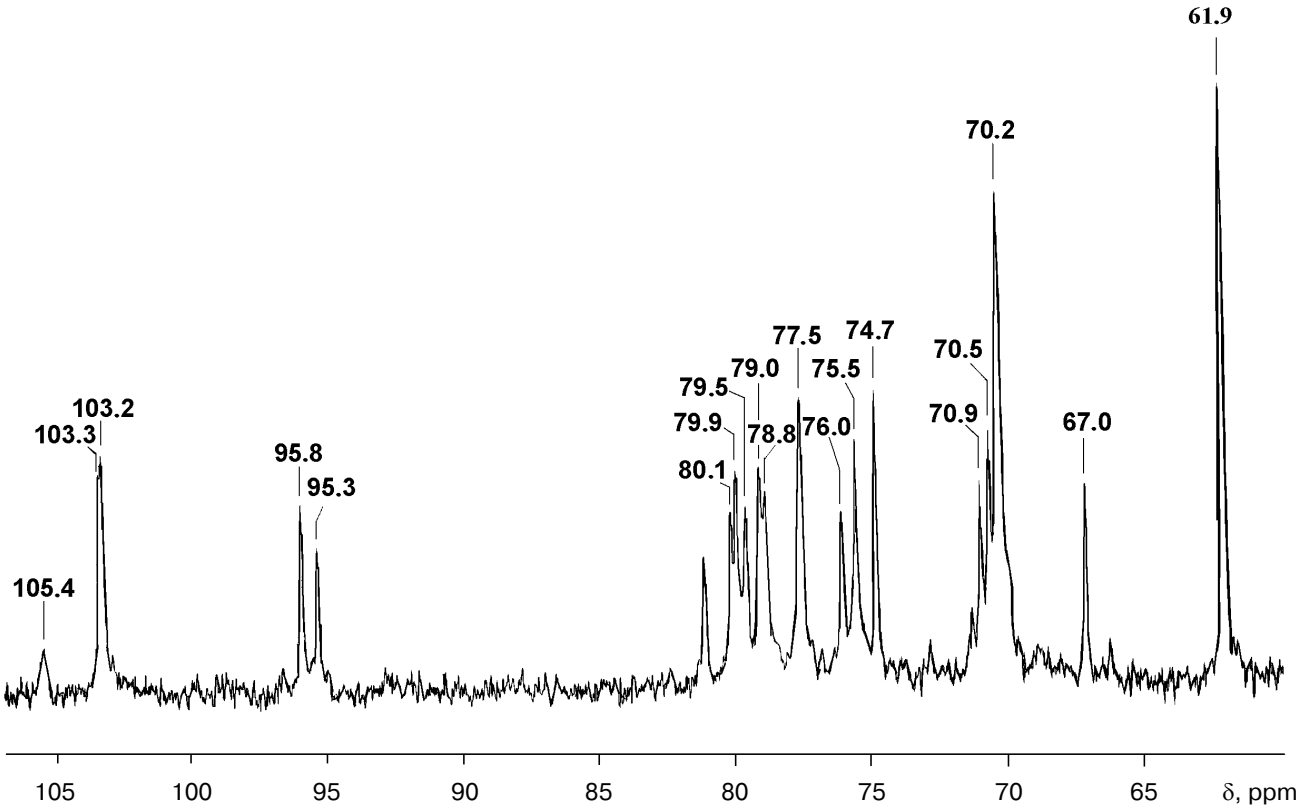

Fig. 2. ^{13}C-NMR spectrum of the KCl-insoluble polysaccharide fraction from the reproductive form of *T. crinitus*.

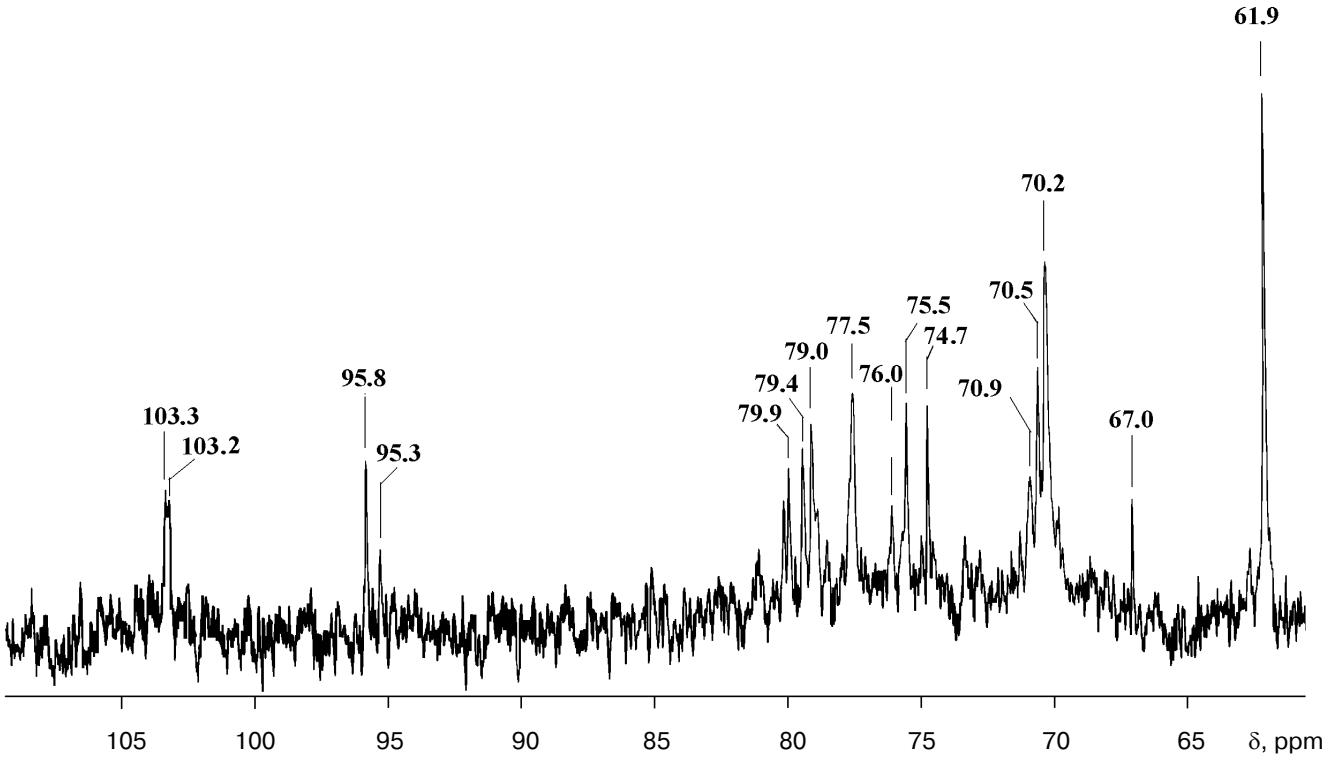

Fig. 3. ^{13}C-NMR spectrum of the KCl-insoluble polysaccharide fraction from the vegetative form of *T. crinitus*.

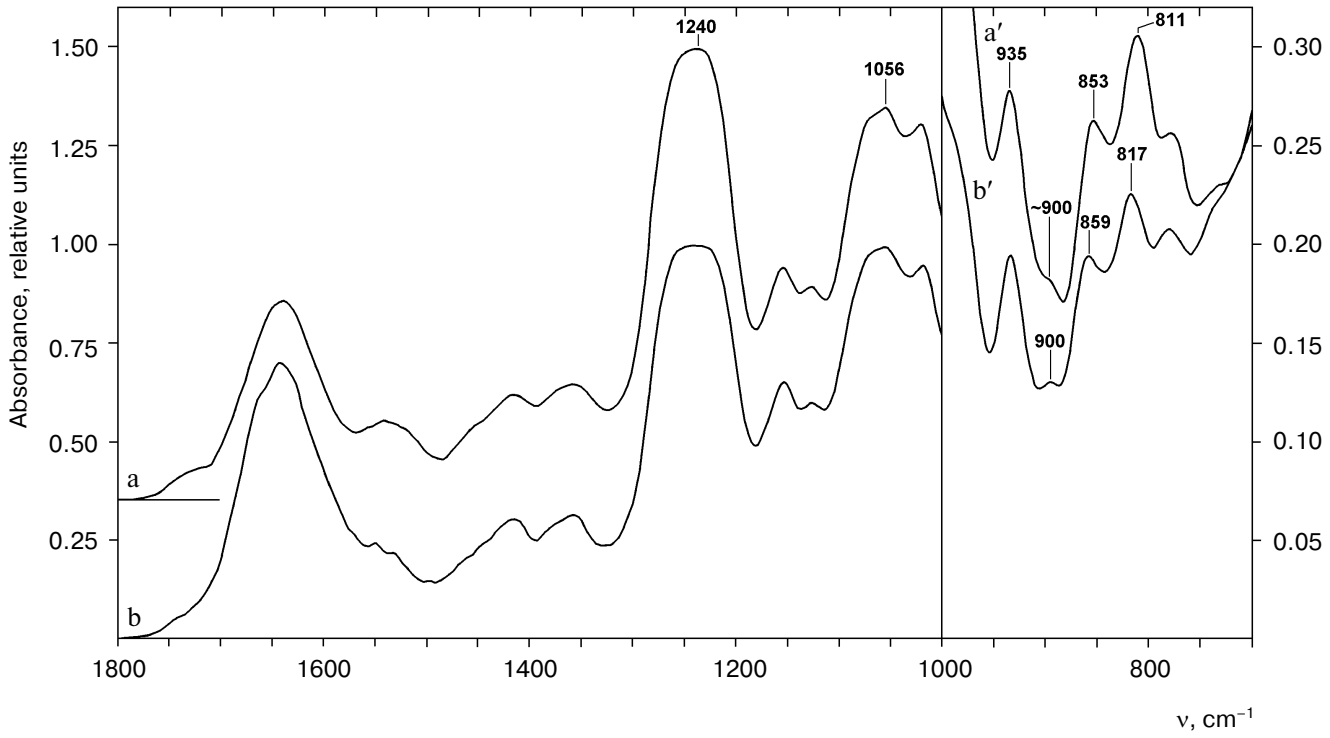

Fig. 4. IR spectra of the KCl-soluble fractions from the vegetative (a and a′) and reproductive form of *T. crinitus* (b and b′).

Thus, according to the data of ^{13}C-NMR and IR spectroscopy, the polysaccharides of the gelling fractions isolated from the two forms of the alga *T. crinitus* are κ/β-carrageenans. These fractions differ in the ratio of the disaccharide residues of types of carrageenans, which results from the intensity of the corresponding signals in ^{13}C-NMR spectra. In the reproductive form (Fig. 2), their content is close (60 : 40), while in the vegetative form (Fig. 3) the disaccharide units of the κ-type predominate (80%). Currently, it is impossible to determine precisely whether these polysaccharides have block κ/β structure or there is a mixture of κ- and β-carrageenans. However, the results of analytical centrifugation and gel filtration, as well as the identity of the IR spectra of different polysaccharide fractions obtained by the fractional KCl precipitation (data not shown), suggest the block structure.

One more difference between the gelling fractions from the different forms of the alga is that polysaccharides from the reproductive form contain a small amount of disaccharide residues corresponding to the precursors of κ-carrageenans. It is accordance with the data of ^{13}C-NMR studies and IR spectroscopy and the results of chemical analysis (Table 1) indicating the irregular structure of the investigated polysaccharides.

The IR spectra of the non-gelling polysaccharides from the different forms of the alga (vegetative and reproductive) exhibit a identical picture in the characteristic region (950-800 cm^{-1}). The IR spectra of the polysaccharides from both forms of the alga in both fractions have a weak absorption band at 935 cm^{-1} that is characteristic of 3,6-anhydrogalactose (Fig. 4, a′ and b′), this being supported by the data of chemical analysis. The IR spectra of polysaccharides isolated from the vegetative form of *T. crinitus* (Fig. 4a′) show intense absorption bands at 811 and 853 cm^{-1}. The corresponding bands in the IR spectra of the non-gelling polysaccharides isolated from the reproductive form of the alga (Fig. 4b′) are shifted to 817 and 859 cm^{-1}, respectively.

^{13}C-NMR spectra of polysaccharides of the non-gelling fractions are rather complicated and difficult to assign. Currently, the results suggest that the non-gelling polysaccharides from the two forms of the alga are mixtures of sulfated galactans containing a small amount of 3,6-anhydrogalactose. The alga *Gigartina skottsbergii* with cystocarps produces polysaccharide of a complex structure containing together with the main κ-, ι-, μ-, and ν-carrageenans a hybrid structure of carrageenans of κ- (41%), ι- (18%), and λ- (29%) types and galactose (4.5%) [35]. The precise structure of the polysaccharides from the non-gelling fractions will be determinate more correctly after chemical modification.

The authors express their gratitude to Prof. A. I. Usov and Prof. V. E. Vaskovsky for their help in discussing of the results.

This work was supported by the Programs for Fundamental Research of the Presidium of Russian Academy of Sciences "Physico-Chemical Biology" and "Fundamental Science for Medicine".

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