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Effects of structural peculiarities of carrageenans on their immunomodulatory and anticoagulant activities

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ABSTRACT

Biological activity of five carrageenan types – kappa, kappa/beta, kappa/iota, lambda and new type – iks – isolated from the most abundant species belonging to Gigartinaceae and Tichocarpaceae collected from the Pacific coast was investigated. The ability of carrageenans to influence on the cytokine production by human cells is greatly dependent on concentration and structure of polysaccharides. At high concentrations all types of carrageenans increased the level of pro-inflammatory IL-6 and TNF-α, while at low concentration (1–10 ng/mL) their activity was insignificant. All types of carrageenans induced the secretion of anti-inflammatory IL-10 in dose-dependent manner. Hybrid kappa/beta-carrageenan showed fairly high activity independent on concentration. At low concentrations (10 ng/mL) its activity was more than that of LPS. The structural analysis of polysaccharides suggests that additional sulphate ester residue of lambda-carrageenan increases the concentration of calcium in macrophage cytoplasm and may have an important role in the activation process of the formation of active oxygen forms. Kappa/iota carrageenan possessed for the potential anticoagulant activity, which was extremely strong in low concentration.

These results suggest that the immunomodulation and anticoagulant activity of carrageenans depends on the monosaccharide composition of polysaccharides, number, position and distribution of sulphate groups along galactan chain.

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1. Introduction

Cell walls of red algae are mostly composed of sulphated galactans such as agars or carrageenans which are not found in land plants, and have a wide application in practice. In food industry, carrageenans are widely utilized due to their excellent physical functional properties, such as thickening, gelling and stabilizing abilities. These polysaccharides have been safely consumed as constituents of food products for many years. According to the JECFA, only degraded carrageenans were associated with adverse effects and should not be used as food additives (Joint FAO/WHO, 2008). Carrageenans were used to reduce the amount of polymorphic transformation in tabletting (Schmidt, Wartewig, & Picker, 2003), to produce controlled release delivery system (Keppeler, Ellis, & Jacquier, 2009; Thommes & Kleinebudde, 2006), and to achieve interactions with drugs for modified release systems (Hugerth, 2001). Several carrageenans have been found to exhibit various biological activities, including antiviral, antitumor (Carlucci, Ciancia, Matulewicz, Cerezo, & Damonte, 1999; Yermak & Khotimchenko, 2003), antihyperlipidemic (Panlasigui, Baello, Dimatangal, & Dumelod, 2003), and anticoagulant activities (Caceres, Carlucci, Damonte, Matsuhiro, & Zuniga, 2000; Pereira et al., 2005). Carrageenans also possess immunoadjuvant properties, and they can exert both immunopotentiative and immunosuppressive actions (Bhattacharyya et al., 2010; Yermak & Khotimchenko, 2003; Zhou et al., 2004). However, the relationship between chemical structure and biological activity of native carrageenans was still not being studied fully.

Carrageenans are complex families of water-soluble, linear, sulphated galactans. They are composed of alternating 3-linked β-D-galactopyranose and 4 linked α-D-galactopyranose or 4-linked 3,6-anhydro-α-D-galactopyranose, forming the disaccharide repeating unit of carrageenans. The sulphated galactans are classified according to the presence of the 3,6-anhydro-bridge on the 4-linked galactose residue, and position and number of sulphate groups. In the case of carrageenans at least 17 different types have been defined, some of which have no commercial importance so far (Knutsen, Myslabodsky, Larsen, & Usov, 1994). Three major types of carrageenans, designated as kappa, lambda and iota, are commercially available and their biological activities are being studied (Yermak & Khotimchenko, 2003; Zhou et al., 2004). Recently, it was reported that the low molecular weight fractions

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of carrageenan from *Solieria chordalis* posses great immunostimulating properties (Stephanie, Eric, Sophie, Christian, & Yu, 2010). Recently for better understanding how the specific chemical structure of carrageenans contributes to immune response three types of carrageenans – kappa, iota and lambda – have been treated with specific carrageenases (which hydrolyze β-1 → 4 galactosidic bond), and interleukin-8 (IL-8) stimulation by carrageenans was determined. It was shown that a basic pathway of immune activation of carrageenans is initiated by the α-Gal-(1 → 3) Gal epitope (Bhattacharyya et al., 2010).

Native carrageenans always represent complex hybrid structures and are generally a mixture of galactans composed of different carrabiose types, the proportions and structures of which vary with species, ecophysiological and development conditions (Falshaw & Furneaux, 1994; Knutsen et al., 1994; Yermak & Khotimchenko, 2003). The structural diversity of these macromolecules, however, has given a major hindrance in establishment of their structure–activity relationship. Previously the most abundant algal species family Gigartinaceae and Tichocarpaceae collected from the Peter Great Bay of the Japan Sea were studied, and chemical structures of isolated carrageenans were established. We have isolated carrageenans from *Chondrus armatus*, *C. yendoi*, *C. pinnulatus* and *Tichocarpus crinitus* and shown that variations in carrageenan yield and composition depended on the environment conditions and algae life stages (Barabanova et al., 2008, 2005; Yermak, Kim, Titlyanov, Isakov, & Solov'eva, 1999). Gelling sulphated galactans from vegetative form of *C. pinnulatus* and *T. crinitus* were observed to have hybrid structure (kappa/iota and kappa/beta) (Barabanova et al., 2005; Yermak et al., 1999) and non-gelling fraction extracted from *T. crinitus* has unusually for carrageenans structure, designated as iks-carrageenan (Barabanova et al., 2008).

In spite of comprehensive knowledge concerning chemical structure and properties of these polysaccharides and their practical use in form of medical preparations, biological properties of these native carrageenans have not been investigated. Therefore, current research goals also focused on identifying more potent and specific structural types of carrageenans with immunomodulation and anticoagulant activity. For this purpose the anticoagulant effect of five natural sulphated carrageenans isolated from red seaweeds Gigartinaceae and Tichocarpaceae family, influence on the macrophage lysosomal activity, ROS formation and the concentration of calcium in macrophage cytoplasm and also ability of polysaccharides to induce the synthesis interleukins by human blood immune cells were determined by using *in vitro* and *ex vivo* models.

2. Materials and methods

2.1. Algae

The following representative species of red algae were collected at the Peter Great Bay (Japan Sea), which is near the border between the boreal and tropical zones: Order Gigartinales, Family Gigartinaceae – *C. armatus* (Harv.) Okam., *C. pinnulatus* (Harv.) Okam, family Tichocarpaceae – *T. crinitus* (Gmel) Rupr. All algae were harvested at the end of August and identified by Dr. T.V. Titlyanova. The selected seaweeds were in the vegetative form with lacking of any reproductive organs. Morphological and anatomic characteristics of the algae were determined according to Perestenko by light microscopy (Perestenko, 1980).

The algae were washed with tape water in order to remove some excess of salts. Bleaching of the seaweed was achieved by maintaining the specimen in pure acetone for 3 days prior being dried in the air.

2.2. Extraction of carrageenans

Dried and milled algae (50 g) were suspended in hot water (1.5 L) and the polysaccharides were extracted at 90 °C for 2 h in a water bath. The polysaccharides were separated into gelling – KCl-insoluble (a) and non-gelling KC1-soluble fractions (b) as described previously (Yermak et al., 1999) and their structures were established according to a published protocol (Barabanova et al., 2008, 2005; Yermak et al., 2006).

2.3. The chemical analysis of polysaccharides

The total amount of carbohydrates was estimated by the phenol–sulphuric acid method, using D-galactose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Monosaccharides as alditol acetate derivatives (Englyst & Cumming, 1984) were identified by GLC using Agilent 6850 gas chromatograph equipped with HP-5MS capillary column (30 m × 0.25 mm) with 5% phenyl methyl siloxane and flame-ionization detector (FID). The analyses were carried out at temperature programming from 175 to 220 °C with 3 °C/min. The content of 3,6-anhydrogalactose was determined according to the method of Usov and Elashvili (1991). The sulphate ester content of polysaccharide was determined according to the method Lahaye and Axelos (1993) by HPLC equipped (conductivity detector Waters 431) with an IC-Park A Anion column (50 mm × 4.6 mm; 10 μm, Waters), eluted by 2 mM borate/gluconate eluent (flow rate: 1.0 mL/min). The protein content of samples was determined according to the method of Lowry, Rosebrough, Farr, and Randall (1951) using crystalline bovine serum albumin as the standard.

The cationic composition was determined by atomic absorption spectroscopy. Sodium and potassium contents were determined after mineralisation of the carrageenan samples using a microwave digestor (MLS-1200 MEGA, Italy) and analyzed by ICP_AAS System (Leeman Labs Inc., PS 100, USA) with the following conditions: power – 10 kW, coolant gas – 13 L/min, nebulizer gas pressure – 40 Pa, pump rate – 1.0 mL/min.

2.4. Sedimentation and viscosity

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 0.1 M NaCl as the solvent. The apparent molecular weights of lambda- and iks-carrageenans in solution (0.1% (w/v) in 0.1 M NaCl) were calculated by the method of Archibald (Elias, 1961). The viscosity of carrageenan solutions (0.1–1.0 mg/mL in 0.1 M NaCl) was measured in a modified Ubellode Viscometer (Design Bureau Pushchino, Russia, 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 kappa-, kappa/beta- and kappa/iota-carrageenans 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 kappa-carrageenans, respectively, according to the literature data for this polymer–solvent system (Rochas, Rinaudo, & Landry, 1990).

2.5. Anticoagulant activity

The anticoagulant effects of carrageenan samples were assessed using APTT (activated partial thromboplastin time) assay with citrated plasma sample (1:10 (v/v), 3.8% sodium citrate) according to Fox et al. (1993). Coagulation time assays were performed

semi-automatically with a blood coagulation analyzer (BC2210, Kyoto-Daiichi Science, Japan). APTT assays were performed with activated Cephaloplastin Reagent (Dade®, Actin®, Dade Co. Ltd., USA).

2.6. Macrophage-phosphatase enhancing activity

Animal: male ICR mice, weighing 30–35 g were purchased from Samyuk Co. (Korea), and were housed and maintained at 24 h constant humidity (55%). They had free access to food (SAM 31, Samyuk Co., Korea) and water from the beginning of the experiment.

Preparation of the macrophage monolayer: the thioglycollate-elicited peritoneal macrophages were obtained from the mice, and the macrophage monolayer was prepared by the method of Matsumoto, Tanaka, Yamada, and Cyong (1990). More than 95% of the adherent cells showed typical macrophage morphology with the characteristics of staining.

Determination of phosphatase activity in macrophages was performed according to the method of Suzuki et al. (1990). Briefly, macrophages were cultured in the absence or presence of test samples for 15 h at 37 °C. Thereafter, the macrophage monolayer was solubilized by addition of 25 μl of 0.1% Triton X-100; then 150 μl of 10 nM *p*-nitrophenol phosphate and 50 μl of 0.1 M citrate buffer (pH 5.0) were added to each well and incubated for 1 h at 37 °C and then 50 μl of 0.2 M borate buffer (pH 9.8) was added. After 10 min extinction due to phosphatase activity of macrophages was measured at 405 nm.

2.7. IL-6, IL-10 and TNF inducing activity

Blood processing was performed using procedure of Bienvenu, Doche, Gutowski, Lenoble, and Pedrix (1995). Peripheral blood was collected by venapuncture into sterile siliconized tubes containing 30 IU of lithium heparinate per 5 mL tube diluted 1:5 in sterile Medium 199 (Sigma, USA) containing 300 mg/L of glutamine (Gibco, Life Technology, Germany) and 50 μg/mL of gentamicin. Diluted blood (0.1 mL) was transferred into sterile polypropylene plates and then incubated with the corresponding type of carrageenans (37 °C, 5% CO_2). A control incubation with LPS from *E. coli* (strain O55:B5) was performed for each experiment. Tubes were placed in ice for 6 h, centrifuged at 600 × *g* for 5 min, supernatants were collected and frozen followed by cytokine determination using specific ELISA (DuoSet Developing System, Genzyme, Boston, USA). The data of three independent experiments are presented.

2.8. Determination of reactive oxygen species (ROS) formation ("oxidative burst") in mouse macrophages

100 μL of suspension of peritoneal macrophages isolated from a BALB/C mice peritoneal fluid was placed in wells of 96-well microplate, which included 10 μL of solutions of tested matters. The incubation was conducted within 1 h at 37 °C. Then 10 μL of aqueous solution of dihydrorhodamine 123 (final concentration 1.0 μg/mL) was added to each well and microplate was incubated additionally 10 min at 37 °C. Intensity of a Rhodamine 123 fluorescence was measured with fluorescent plate reader Fluoroscan Ascent (ThermoLabsystems, Finland) at λ_{ex} = 485 nm and λ_{em} = 518 nm.

2.9. Lysosomal activity determination

100 μl of suspension of peritoneal macrophages isolated from a BALB/C mice peritoneal fluid was placed in wells of 96-well microplate, which included 10 μL of solutions of tested matters. The incubation was conducted within 1 h at 37 °C. Then 10 μL of aqueous solution of Acridine Orange Solution (final concentration 1.0 μg/mL) was added to each well and microplate was incubated additionally 30 min at 37 °C. Intensity of Acridine Orange fluorescence was measured with fluorescent plate reader Fluoroscan Ascent (ThermoLabsystems, Finland) at λ_{ex} = 485 nm and λ_{em} = 538 nm.

2.10. Determination of Ca^{2+} influx in mouse macrophages

The BALB/C mouse peritoneal macrophage liquid (20 μL) was transferred to glass cover slips and stored for 1 h in an incubator at 37 °C for cell adhesion. Then the cover slips were washed (3×) with phosphate buffered saline (PBS, pH 7.5) and transferred to 10 μM of Calcium Green-1/AM ("Molecular probes") in saline solution to load cells with fluorescent dye for 40 min at 37 °C. The components of saline solution were NaCl – 140 μM, KCl – 5 μM, $CaCl_2$ – 1 μM, HEPES – 10 μM, pH 7.4. Then, cells were washed with the same solution but without fluorescent dye and incubated 15–20 min in serum-containing medium to let cells recover. After that, cover slips were mounted on a cell chamber of fluorescent imaging system based on inverted microscope Axiovert 200 (Zeiss, Germany). The 75 W Optosource xenon arc lamp and DAC-controlled Optoscan monochromator (Cairn Research Ltd., UK) were used as a light source to excite fluorescence at λ = 488 nm; HQ FITC Filter-block (Chroma Technology Corp., USA) and A-Plan 40×/0.65 Ph2 Objective (Zeiss, Germany) were set for visualisation of Green-1 fluorescence in cells. A micropipette perfusion system with a flow rate of 100 μL/min was used to apply the test solution with compounds to macrophages plated on the glass cover slips. The images of green fluorescent cells were acquired using digital CCD video camera Hamamatsu Orca-ER C4742-95 (Hamamatsu Photonics K.K., Japan), captured and transferred to a IBM-compatible computer P-IV with Firewire data interface card. The fluorescence intensity of randomly selected 100 cell images was measured with AQM Advance 6 Software (Kinetic Imaging Ltd., UK) and expressed as an average pixel intensity of grey level for each cell determined. Effective concentrations were analyzed in triplicate. The means and standard errors for each treatment were calculated and plotted using Sigma Plot 3.02 Software (Jandel Scientific, San Rafeal, CA).

In the experiments of carrageenan effect on lysosomal activity and its effect upon ROS formation in macrophage cytoplasm all the carrageenans were studied at concentration of 100 μg/mL first. Then, most active compound was selected and dose-dependent investigation was done; dose-dependent curves were plotted and its half maximal effective concentration (EC_{50}) or maximal stimulatory concentrations were calculated in dependence of curve shape.

Only figures demonstrating the carrageenan's effects at 100 μg/mL are included in the paper. The dose-dependent curves are not shown.

2.11. Statistical analysis

All measurements were done in three replicates. All results were expressed as mean ± standard deviation (SD) compared by ANOVA and the Newman–Keuls post test or paired Students' *t*-test used to assess individual differences between samples and control. *P* values < 0.05 were considered statistically significant. Data were analyzed using the software (Statistica 6.0; StastSoft, USA).

3. Results

3.1. Extraction and chemical analysis of carrageenans

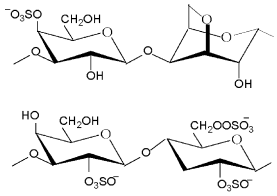
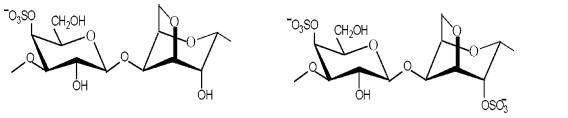
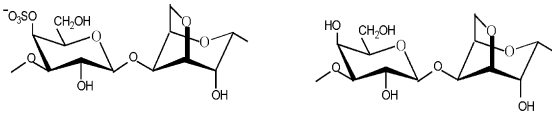
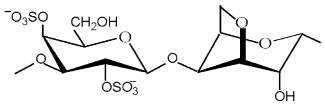
The polysaccharides were extracted from vegetative forms of seaweed and separated by 4% KCl into KCl-insoluble (a) and

Table 1
Characterization of carrageenan fractions from red algae.

Algae species	Fraction	Yield on dried alga (%)	Content, on dry weight of polysaccharide (%)									
			Sugar	Pr	Gal	3,6-AnGa	SO_3^{2-}	K	Na	Ca	Zn	Cd
C. armatus	a	30	47	1.9	37.0	33.0	22.0	0.4	0.6	2.9	0.11	ND
	b	10	49	2.1		–	28.0	0.2	0.5	1.5	0.10	ND
C. pinnu-latus	a	40	43	3.5	32.9	23.9	24.0	0.6	0.8	2.6	0.11	ND
T. crinitus	a	16	50	2.9	33.1	30.0	20.0	0.6	0.5	2.2	0.09	ND
	b	5	56	3.4	37.1	5.6	27.0	0.5	0.7	1.0	0.08	

Sugar, total sugar content; Pr, protein; Gal, galactose; 3,6-AnGal, 3,6-anhydrogalactose; So_3^{2-}, sulphate group; a, gelling fraction of polysaccharide; b, non-gelling fraction of polysaccharide.

Table 2
Chemical structures of the repeating units of carrageenans from algae of family Gigartinaceae and Tichocarpaceae.

Source of carrageenans	Type of carrageenan	The disaccharide repeating units	Reference
C. armatus	Kappa Lambda		Yermak et al. (1999)
C. pinnulatus	Kappa/iota		Yermak et al. (1999)
T. crinitus	Kappa/beta iks-type	 	Barabanova et al. (2005, 2008)

KCl-soluble (b) fractions. The yields and the chemical composition of these fractions are listed in Table 1. According to data obtained previously KCl-insoluble polysaccharides from *C. pinulatus* were identified as kappa/iota-carrageenan, from *C. armatus* as kappa-carrageenan, from *T. crinitus* as kappa/beta-carrageenan (Barabanova et al., 2005; Yermak et al., 1999). KCl-soluble (b) fractions from *C. armatus* were designed as lambda-carrageenan (Yermak et al., 1999) and from *T. crinitus* as new iks-carrageenan (Barabanova et al., 2008). The chemical structure of the disaccharide repeating units of carrageenans is listed in Table 2.

3.2. Molecular weight determination of carrageenans

The polysaccharide fractions were analyzed by analytical centrifugation. All samples formed a single sedimentation boundary.

The apparent molecular weights of the lambda- and iks-carrageenans, determined by sedimentation analysis, were 200,000 and 500,000 g/mol, respectively. Viscosimetric molecular weights of kappa-, kappa/beta- and kappa/iota-carrageenans, calculated by the Mark–Houwink were 200,000, 420,000, and 470,000 g/mol.

3.3. Anticoagulant activity of carrageenans

The anti-coagulant activity of native polysaccharide from seaweeds of families Gigartinaceae and Tichocarpaceae was studied by APTT assay. As could be seen lambda- and kappa/iota-carrageenans possess high anticoagulant activity at low concentration of 100 μg/mL (Table 3). Kappa/beta-carrageenan containing only one sulphate group on two disaccharide chain exhibits low anticoagulant activity.

3.4. Immunomodulatory properties

3.4.1. Macrophage-phosphatase activity

As is shown in Table 3, all types of carrageenan have insignificantly macrophage-phosphatase activity. Carrageenans with high degree of sulphating – lambda- and iks-types were the most active ones.

Table 3
Biological activities of carrageenans.

Type of carrageenan	Macrophage-phosphatase (%)	Anti-coagulant (APTT, s)
Kappa	159 ± 15	187.0 ± 8.3
Lambda	212 ± 32	580.0 ± 69.4
Kappa/beta	157 ± 22	81.3 ± 10.3
X (iks)	186 ± 11	343.0 ± 25.6
Kappa/iota	151 ± 12	≥600 ± 47.3
Control (saline)	100	58.7 ± 3.5

Macrophage-phosphatase activity was determined at carrageenans concentration equal to 1000 μg/mL; APPT, at 100 μg/mL. Each experiment was done in triplicate. Each value represents the mean ± SD ($n = 3$). Paired Students' t-tests were used to assess individual differences between samples and control. Differences between samples and control were significant, $p < 0.05$. Control, saline as a negative control.

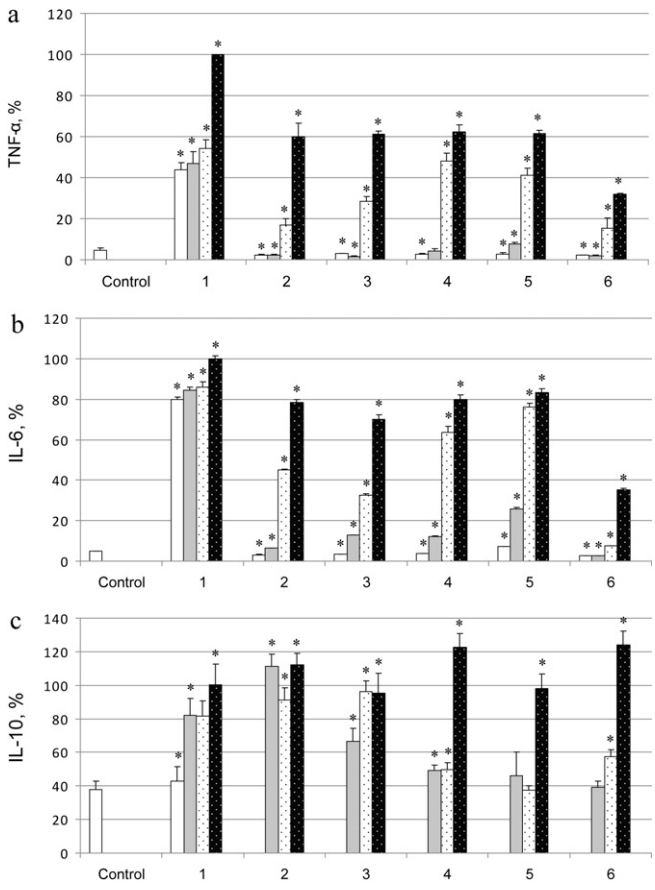


Fig. 1. The influence of carrageenans on the induction of TNF-α and interleukin-6 and interleukin-10: 1, LPS; 2, kappa/beta carrageenan; 3, kappa/iota carrageenan; 4, kappa-carrageenan; 5, lambda-carrageenan; 6, iks-carrageenan: □–C = 1 ng/mL; ▩ –C = 10 ng/mL; ⊡ –C = 100 ng/mL; ■ –C = 1 μg/mL. Mean ± SD contents cytokines (%) are presented. Whole blood was obtained from 5 healthy subjects and incubated with the samples (columns 1–6) in different concentration or saline solution (control column). *Differences between samples and control were significant; $p < 0.05$.

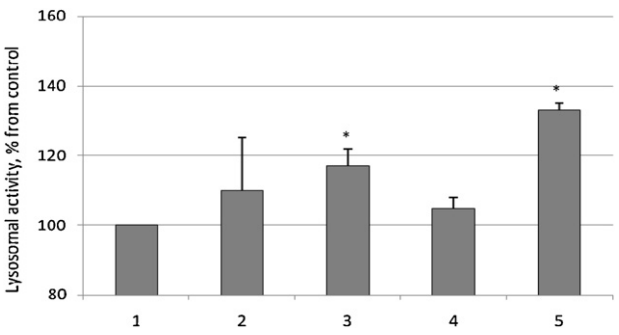


Fig. 2. Influence of different compounds applied at concentration of 100 μg/mL upon lysosomal activity of mouse macrophages: 1, control; 2, kappa/iota-carrageenan; 3, lambda-carrageenan; 4, kappa-carrageenan; 5, kappa/beta-carrageenan. Suspension of peritoneal macrophages was incubated with addition of solutions of tested samples (columns 2–5) or saline solution as control (column 1). Data shown as a mean ± SD ($n = 3$); *$p < 0.05$.

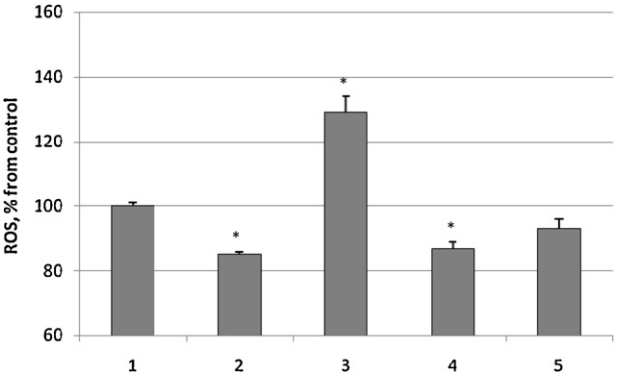


Fig. 3. Influence of different compounds applied at concentration of 100 μg/mL upon ROS formation in mouse macrophages: 1, Control; 2, kappa/iota-carrageenan; 3, lambda-carrageenan; 4, kappa-carrageenan; 5, kappa/beta-carrageenan. Suspension of peritoneal macrophages was incubated with addition of solutions of tested samples (columns 2–5) or saline solution as control (column 1). Data shown as a mean ± SD ($n = 3$); *$p < 0.05$.

3.4.2. *Effect of carrageenans on the production of cytokines by human cells*

Carrageenans are well known to stimulate a production of cytokines by cells (Bhattacharyya et al., 2010). Experiments were performed to determine the effect of different types of carrageenans on the production of TNF-α, proinflammatory interleukin (IL) IL-6 and antiinflammatory IL-10 in human blood cells (Fig. 1).

Ability of carrageenans to activate cells and to cause synthesis of cytokines has been correlated to activity of LPS, cytokine-induction activity of which has been accepted for 100%. Fig. 1 shows that all carrageenans stimulate induction of TNF-α and IL. However, activity of polysaccharides was found to stimulate formation of the cytokines in the dose-dependent manner and to depend on the structure of carrageenans. At high concentration all the types of carrageenans caused strong increase in the level of cytokines, in comparison with a control, while at low concentration (1–10 ng/mL) their activity was insignificant. Lambda- and kappa-carrageenans at the concentration of 100 ng/mL possessed appreciable activity during secretion of IL-6 and TNF-α. All the types of carrageenans possessed the ability to induce the synthesis of anti-inflammatory IL-10, which increased with concentration. At concentration of 1 μg/mL kappa- and iks–carrageenans possessed the most activity than other carrageenans. Kappa/beta-carrageenan showed fairly high activity independent on the concentration and its activity was some 30% more, than that of LPS at concentrations of 10 ng/mL.

3.4.3. *Effect of carrageenans on lysosomal activity*

Using the technique of lysosome staining with fluorochromes followed by fluorescence analysis of cells, it was found that all the carrageenans at concentration of 100 μg/mL authentically stimulated lysosomal activity of mouse peritoneal macrophages. Among researched substances kappa/beta-carrageenan has shown the greatest efficiency (Fig. 2). The kappa/beta-carrageenan action was dose-dependent and accompanied by increasing in the order of 30% stimulation of lysosomal activity compared to the control level expressing in increase of lysosomal amount, size and acidity. The maximal stimulatory concentration was found to be 37 μg/mL.

3.4.4. *Influence of researched polysaccharides on formation of reactive oxygen species (ROS) in mouse macrophages*

Influence of polysaccharides on formation of reactive oxygen species (ROS) in mouse macrophages has been investigated. As shown in results, lambda-carrageenan possessed the influence on the macrophage, activating the process of ROS formation on 30% in comparison with the control (Fig. 3). The half maximal effective concentration (EC_{50}), was equaled to 61 μg/mL.

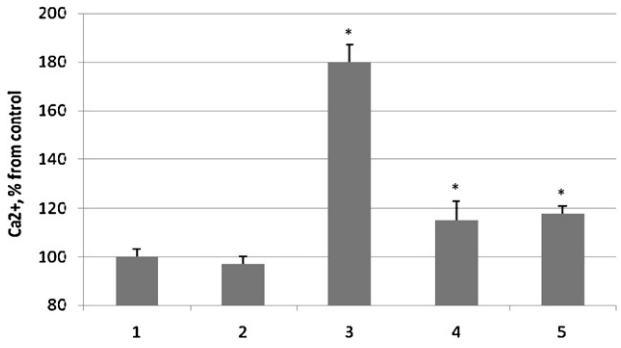


Fig. 4. Influence of different compounds applied at concentration of 100 μg/mL upon concentration of Ca^{2+} in cytoplasm of mouse macrophages: 1, Control; 2, kappa/iota-carrageenan; 3, lambda-carrageenan; 4, kappa-carrageenan; 5, kappa/beta-carrageenan. Suspension of peritoneal macrophages was incubated with addition of solutions of tested samples (columns 2–6) or saline solution as control (column 1). Data shown as a mean ± SD ($n = 3$); $^*p < 0.05$.

3.4.5. *Influence of carrageenans on concentration of calcium ions in cytoplasm of cells*

Ability of carrageenans (at concentration of 100 μg/mL) to influence on intracellular concentration Ca^{2+} in cytoplasm of mouse macrophages has been investigated. The result showed that the presence of lambda-carrageenan in the incubation medium within 5 min leads to almost two fold increase in concentration of calcium in the cytoplasm (Fig. 4).

4. Discussion

Carrageenans are very diverse by composition and chemical structure as well as their physico-chemical properties and biological effects. The wide range of potential pharmacological uses especially as anticoagulants and immunomodulators is the main reason for the increased interest in these polysaccharides. Since the structures of carrageenans are complex and heterogeneneous ones, and as a lot of studies on biological activity were carried out using commercial samples of carrageenans or relatively crude polysaccharide preparations it was not easy to determine the overall relationship between their activity and structure. For the purpose of searching the relationships of various preparations of these polysaccharides (pure or hybrid type) with different degree of sulphation and position of sulphate groups and also with presence of 3,6-anhydrocalactose residue carrageenans were examined by *in vitro* and *ex vivo* models. This relationship is illustrated through their immunomodulatory and anticoagulant properties.

Anticoagulant activity is among the most widely studied property of sulphated polysaccharides. Liner sulphated galactans were shown to express anticoagulant activity not merely as function of charge density. The structural basis for this activity certainly depends on the monosaccharide composition, sites of sulphation and/or the glycosidic linkage (Groth, Grunewald, & Sunsanne, 2009).

Carrageenans are different from each other by the amount of 3,6-anhydro-D-galactose they contain as well as the numbers and position of ester sulphate groups. As was shown earlier (Farias, Valente, Pereira, & Mouráo, 2000) lambda-carrageenan, which contained 2-sulphated and 6-sulphated galactose units, but had not 3,6-anhydro-D-galactose groups, possessed greater antithrombotic activity than the other types of carrageenans, probably, to its higher sulphate content. At the same time Pereira et al. (2005) have shown that the presence of 2,3-disulphates galactose units in the sulphated galactan from marine alga *Botryocladia occidentalis* had an amplifying effect on anticoagulant activity, but the proportion and/or the distribution of these units along a polysaccharide chain may be a critical motif to posses its activity. In our case kappa/beta-carrageenan with low sulphate content and a hybrid structure showed low activity, while kappa/iota-, lambda- and iks-carrageenan types possessed high anticoagulant activity. In the last types of polysaccharides kappa/iota-one had greater anticoagulant activity than other carrageenan types at the low concentration of the substance. According to our current data, kappa/iota-carrageenan (disaccharide chain of iota-carrageenan β-galactose is partially sulphated at C4 position and additionally C2 position in α-3,6-anhydrogalactose) contains three sulphate ester residue per two disaccharide chain and 3,6-anhydrogalactose unit, iks-type of carrageenan contains repeating 3-linked β-D-galactopyranosyl-2,4-disulphate residues, but also 4-linked 3,6-anhydro-α-D-galactopyranosyl residues (Table 2). Lambda-carrageenan, where the β-galactose is partially sulphated at C2 position and α-galactose at C2 and C6 position, contains 3 sulphate ester residue per disaccharide chain, but does not contain 3,6-anhydrogalactose units (Table 2). According to the chemical analysis (Table 1) lambda-carrageenan contains the highest amount of sulphate groups (28% on dry weight of polysaccharide). However, lambda-carrageenan possessed lesser activity than iota. So, we have not found a correlation between anticoagulation activity of carrageenans and amount of sulphates. This indicated that existence of these functional groups is required but not sufficient to confer anticoagulant activity, and that the position of those groups and distribution of sulphate groups along the galactan chain may thus be a determining factor that was remarked earlier (Farias et al., 2000).

Although the action of carrageenans on the immune response has been studied by a lot of investigators by *in vitro* and *in vivo* experiments, there is a little information about the effect of the different types of carrageenans on the immunomodulatory properties. It is known that carrageenan exposure predictably induces an increase in interleukin secretion by cells, in tissue culture or in tissues from human or animal colon (Bhattacharyya et al., 2010; Zhou et al., 2004). The induction of the formation of gamma-interferon, an important nonspecific factor protecting the organism from infections, is one of the early events of the systemic effect of carrageenan (Quan, Kolb, & Lspinats, 1980). It was reported that carrageenan enhanced resistance to intraperitoneal *Salmonella typhimurium* infection (Vijayakumar, Palanivel, & Muthukkaruppan, 1989), protected mice from a lethal challenge with *Klebsiella pneumoniae* DT-S infection (Tateda et al., 1995). Ogata, Matsui, Kita, and Shigematsu (1999) found that pretreatment of mice with carrageenan had increased serum levels of tumor necrosis factor (TNF-α) after intravenous administration of lipopolysaccharide (LPS) to mice. Since these cytokines play a critical role in regulating inflammatory and immunological processes of a host, *in vivo* administration of carrageenan may influence antibacterial host-defense systems in the case of infection, not only through macrophage blockade but also by modulation of cytokine production.

Our previous results demonstrated that pretreatment of mice with kappa- and lambda-carrageenans from *C. armatus* increased mouse resistance to the toxic effect of LPS from *Y. pseudotuberculosis* (Ermak et al., 2006) and improved non-specific resistance to impact of LPS *E. coli* inducing endotoxinemia in mice (Khasina, Sgrebneva, Yermak, & Maleev, 2007). The degree of protection depended on the type of carrageenan. It has been assumed, that the protective effect of these types of carrageenans is connected with immunomodulatory action.

For precise interpretation of the results obtained from the experiments using carrageenan *in vivo*, it is necessary that all the possible effects of this polysaccharide *in vitro* should be considered. Our results show that carrageenans have immunostimulatory activity, but structural difference of carrageenans obviously plays a determinant role in the immunomodulatory properties of polysaccharides.

Determination of peritoneal macrophage lysosomal activity and ROS formation and effect of the compounds upon Ca^{2+} influx is one of the aspects of current study on carrageenan immunomodulatory activity. The present study demonstrates the ability of carrageenans to activate prime macrophages, resulting in modulating of ROS and cytokine production. Thus, the findings that kappa/beta- and lambda-carrageenans can effectively stimulate lysosomal activity and ROS formation, correspondingly, in immune cells, reflect the potency of these compounds in induction of cellular immune response and may correlate with increase in antimicrobial resistance of experimental animals after some carrageenan applications.

In the present study, the involvement of intracellular Ca^{2+} as a possible messenger in the carrageenan's effects was investigated. Since the macrophage ROS formation stimulation occurred at the same concentration of lambda-carrageenan that increased $[Ca^{2+}]$, it was quite possible that the stimulatory effect was Ca^{2+}-dependent and the ROS synthesis stimulation was implemented via the regulation of cellular membrane permeability for calcium ions. Considering the structure of carrageenans (Table 1) the additional sulphate ester residue seems to have an important role in the activation process of the formation of active forms of oxygen and increase in concentration of calcium in cell cytoplasm.

The ability of carrageenans to influence on the cytokine production is largely dependent on the concentration and structure of polysaccharides. Lambda-carrageenan possessed the high activity in the synthesis of IL-6 at concentrations of 100 ng/mL, which was in agreement with Bhattacharyya et al. (2010). As was shown by those authors that lambda-carrageenan produced an IL-8 response higher than that of iota-carrageenan, perhaps due to the more favorable structure with less internal bonding between C3 and C6 of nonsulphated saccharide, producing the pyranose epimer. It is noteworthy, that kappa/beta-carrageenan in contrast to the other types possessed low activity to induce the secretion of IL-6 and TNF-α at concentration of 100 ng/mL; however its ability to induce the production of anti-inflammatory IL-10 at low concentrations (10–100 ng/mL) was more than that of LPS. This difference between activities of carrageenans may be attributed to the less number of ester sulphate groups on disaccharide chain of kappa/beta-carrageenan than the other type of carrageenans and its hybrid structure.

Therefore, the peculiar differences in carrageenan structure may attribute seriously the influence on the level of immunomodulatory effects. There are three main dissimilarities in primary structure (Tables 1 and 2): molar ratio of galactose to 3,6-anhydrogalactose, the number and the position of sulphate group in some β-D-galactose residues and regular or irregular (hybrid) structure of carbohydrate chain of polysaccharides.

In conclusion, our data demonstrated immunomodulatory and anticoagulant properties of carrageenans. The differences in structure of individual or hybrid types of carrageenans observed effect on the investigated properties. These results suggest that the immunomodulatory and anticoagulant activity of carrageenans should depend on the basic polysaccharide structure, monosaccharide composition, number, position and distribution of sulphate groups along the galactan chain.

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