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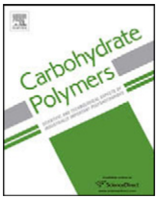

Analysis of structural heterogeneity of κ/β-carrageenan oligosaccharides from *Tichocarpus crinitus* by negative-ion ESI and tandem MALDI mass spectrometry

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ABSTRACT

A mixture of oligosaccharides, released with enzymatic digestion from κ/β-carrageenan of the red seaweed *Tichocarpus crinitus* was investigated. The composition, sequence and sulfation pattern of the oligosaccharide mixture were elucidated by negative-ion electrospray ionization and MALDI potential lift tandem TOF mass spectrometry. It was shown, that oligosaccharide mixture (DP up to 20), produced by κ-carrageenase, contained κ-carrabiose, κ-carratetraose as main components, and set of hybrid oligosaccharides as less abundant components: β → κ, κ → β → κ, β → κ → κ, and minor β → ι → κ, β → β → κ → κ, β → κ → β → κ, κ → β → β → κ, κ → β → κ → κ κ → β → β → β → κ, β → β → κ → β → κ, κ → β → κ → β → κ, κ → β → β → κ → κ. The application of arabinoosazone as a MALDI matrix for negative-ion mode with the addition of L-fucose allowed decreasing the in-source fragmentation. The oligosaccharides of κ/β-carrageenan were shown to maintain an ability to induce of TNF-α and IL-6 secretion of immune cells.

1. Introduction

Carrageenans are widely utilized in food industry due to their unique physico-chemical properties, such as thickening, gelling and stabilizing abilities (Güven, Guvener, & Guler, 1990). Carrageenans are composed of alternating α-(1-3) and β-(1-4) linked D-galactosyl residues (D- and G-units) and several types of these polysaccharides are identified on the basis of the structure of the disaccharide repeating unit, by the pattern of sulfation and the presence of 3,6-anhydrogalactose (A-unit) as 4-linked residue. Structural analysis of carrageenan is important for both ingredient suppliers and food industries. In addition, several potential pharmaceutical uses of carrageenans, including antitumor, antiviral, anticoagulant and immunomodulation activities, have been recently described. The degree of manifestation of biological activity of carrageenans seems to depend on the polysaccharide structure (Güven et al., 1990; Yermak & Khotimchenko, 2003). Over the last decade, the depolymerization of carrageenans had become a subject of interest due to medical effects. However, studies on oligosaccharides derived of carrageenan, especially studies on their immunomodulatory activities are comparatively few.

Natural carrageenans are mixtures of different sulfated polysaccharides and their composition may differ significantly (Craigie, 1990; Knutsen, Myslabodski, Larsen, & Usov, 1994). It is often difficult to determine whether the various carrageenans occur as a separate chemical entities in a mixture of cell wall polysaccharides or whether they are glycosidically linked in a single hybrid macromolecule. To investigate structural features of such polysaccharides precisely, enzymatic digestion with known mode of action and specificity, coupled with chromatographic methods of separation and further analysis with NMR-spectroscopy is often performed (Usov & Ivanova, 1987; Van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002). Carrabiose combinations have been demonstrated such as the κ/ι (A → G4S/A2S → G4S) carrageenan hybrid (or κ2-carrageenan) in several species of the *Gigartinaceae* family (Van de Velde, Peppelman, Rollema, & Tromp, 2001) and the κ/β-(A → G4S/A → G) carrageenan copolymer found in *Furcellaria* sp. and *Euchema gelatinae* (Greer & Yaphe, 1984; Knutsen & Grasdalen, 1987)

Previously, we have shown, that the gelling polysaccharides, isolated from the vegetative and reproductive forms of *T. crinitus* were κ/β-carrageenans type (A → G4S/A → G) (Barabanova et al., 2005). NMR-analyses of the enzymatic degradation products require purification of the oligosaccharides at mg scale which is time-consuming. However, the modern mass-spectrometric techniques, matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOFMS) and especially collisionally induced

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dissociation tandem electrospray ionization mass-spectrometry (CID ESIMS/MS), offering high sensitivity in the negative-ion mode and providing direct sequence information on the sulfated oligosaccharides (Zaia, 2004) had become an important complementary tool to other analytical methods for the study of sulfation moieties and heterogeneity in carrageenans. In this way, negative-ion tandem MALDI-TOFMS and ESIMS had been successfully employed to analyze heterogeneous carrageenan oligosaccharides, prepared using specific carrageenases (Antonopoulos, Favetta, Helbert, & Lafosse, 2005; Antonopoulos, Favetta, Helbert, & Lafosse, 2007; Antonopoulos, Hardouin, et al., 2005; Ekeberg, Knutsen, & Sletmoen, 2001; Yu et al., 2006). It was recently shown by our group, that the application of arabinoosazone as a matrix (Chen, Baker, & Novotny, 1997) in MALDI-TOFMS of singly charged multisulfated oligosaccharides (including disulfated monosaccharide), from fucoidan allowed to reduce in-source fragmentation and losses of the labile sulfate groups. Moreover, the fragmentation pattern, observed in a MALDI potential lift tandem TOF mass spectrometry (MALDI-LIFT-TOF/TOFMS) was similar to that observed with CID ESIMS/MS (Anastyuk et al., 2010).

In present work, ESIMS and tandem MALDI-TOFMS were applied to analyze structural heterogeneity of the oligosaccharides, obtained from κ/β-carrageenan of *T. crinitus* by enzymatic digestion with κ-carrageenase. Cytokine-induced ability of oligosaccharides was investigated using *in vitro* models.

2. Experimental

2.1. Materials

κ/β-carrageenan was extracted from red algae *T. crinitus*. Dried algae (10 g) were suspended in distilled water (300 mL) and the polysaccharides were extracted at 90 °C for 3 h in a boiling water bath. The suspensions were centrifuged (2500 × *g*, 20 min, 20 °C) and the algal residues were re-extracted twice with water for 2 h in a boiling water bath. The supernatants were pooled and concentrated in vacuum to about 100 mL. The polysaccharides were separated into the gelling – KCl-insoluble and non-gelling the KCl-soluble fractions according to Yermak and Khotimchenko (2003). KCl-insoluble fraction contained κ/β-carrageenan which was used for enzymatic degradation. The sample of κ-carrageenan standard was purchased from Sigma (USA).

Arabinoosazone (phenylosazone of D-arabinose) matrix for negative-ion mode MALDI-TOFMS was synthesized as described (Chen et al., 1997).

2.2. General methods

2.2.1. Preparation of oligosaccharides by enzymatic digestion

Oligosaccharides of κ/β-carrageenan were produced according to Rochas and Heyraud (1981) using recombinant *Alteromonas carrageenovora* κ-carrageenase. Prior to freeze-drying, degradation products were filtered through Amicon Centriprep (YM-30 Millipore, USA) to obtain preparation named **Tc-enz-OS.**

2.2.2. Size-exclusion chromatography

Oligosaccharides contained in the **Tc-enz-OS** preparation were purified by gel filtration on a Biogel P2 column (1.5 cm × 100 cm) equilibrated with 0.5 M ammonium hydrogenocarbonate buffer, pH 8.3. Elution was performed using the same buffer. Fractions were collected with fraction collector (BioRad, USA) by 2.5 mL. 44 subfractions were collected. Sugar-containing fractions, detected by phenol-sulfuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) were subjected to the mass-spectrometry analysis. Using mass-spectral data on the sample purity, the fractions were joined as follows: **SEC-1** (44-40), **SEC-2** (39-37), **SEC-3** (34-33), **SEC-4** (30-29), **SEC-5** (28-26). The high-molecular-weight fraction **SEC-6** (25-20) was joined by the data of phenol-sulfuric method. It contained whole peak, eluted just after the void volume. Oligosaccharides containing fractions (**SEC-1** through **SEC-6**) were freeze-dried.

2.2.3. Molecular weight determination

The κ/β-carrageenan was analyzed by analytical centrifugation. The sample formed a single sedimentation boundary, this indicating the homogeneity of the preparations.

The viscosity of carrageenan solutions (0.1–1.0 mg/mL in 0.1 M NaC1) 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 $\ln(\eta_{\mathrm{rel}})/C$ to infinite dilution using the least square method. The molecular weights of polysaccharides were calculated using the Mark–Houwink–Coon equation: $[\eta] = KM^{\alpha}$, where $[\eta]$ is the intrinsic viscosity and K and α are empirical constants constituting 3×10^{-3} and 0.95, respectively, according to the literature data for this polymer-solvent system (Rochas, Rinaudo, & Landry, 1990).

The molecular weight of oligosaccharides was calculated by the determination of the reducing end-groups using the colorimetric method of Park and Johnson (Park & Johnson, 1949).

2.3. Mass spectrometric analysis

ESIMS spectra were recorded with 6510 LC Q-TOF mass spectrometer (Agilent, USA) with a dual electrospray-ionization source. All spectra were acquired in the negative-ion mode, with precalibration with a standard "HP-mix" for negative-ion mode. The capillary voltage was set to −4000 V, and the drying gas temperature was 325 °C. The fragmentor voltage was set to −160 V. The isolation window for MS/MS experiments was set to 1.3 mass units for singly charged ions and 4 mass units for multiply charged ions. The dried sample was dissolved in 1:1 acetonitrile–water (concentration of the sample was approx. 0.01 mg/mL) and introduced into the mass spectrometer at flow rate of 5 μL/min using a syringe pump, manufactured by KD Scientific (USA).

MALDI-TOFMS spectra were recorded with UltraFlex III MALDI-TOF/TOF mass-spectrometer (Bruker, Germany) with nitrogen laser (337 nm), reflector and LIFT modes of operation. Instrument settings for the negative-ion mode: accelerating voltage, −25 kV; laser power, 19%; number of shots, 200; laser shot rate, 66 Hz. Sample preparation: 2 μL of the mixture, containing 0.5 μL of the sample (0.1 mg/mL for 0–2 kDa mass range) and 0.5 μL of L-Fuc (10 mg/mL) solutions in water, 1 μL of arabinoosazone matrix (10 mg/mL) solution in acetone was introduced onto the sample plate and air dried. The dehydroxybenzoic acid (DHB) solution (50 mg/mL), dissolved in 1:1 acetonitrile–water was used instead of arabinoosazone for the analysis of **SEC-6** fraction. Concentration of the sample in that case was 1 mg/mL.

2.4. ^{1}H NMR spectroscopy analysis

The ^{1}H NMR spectrum was recorded with a Bruker DRX-300 spectrometer at 30 °C using internal acetone (δ_{H} 2.225, δ_{C} 31.45) as reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and process the NMR data.

2.5. IL-6 and TNF inducing activity

Blood processing was performed using procedure of Bienvenu, Doche, Gutowski, Lenoble and Pedrix (1995). Human 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. The diluted blood (0.1 mL) was transferred into sterile polypropylene plates and then incubated with the carrageenans (37 °C, 5% CO_2). A control incubation with 1 or 100 ng/mL of LPS from *Escherichia coli* (strain 055:B5) was performed for each experiment. After 24 h the supernatants were collected and frozen, followed by cytokine determination using specific ELISA (DuoSet developing system, Genzyme, Boston, USA).

2.6. Statistical analyses

All results were expressed as mean ± the standard error of the mean (SEM) compared by ANOVA and the Newman–Keuls post test. Paired Students' *t*-tests were used to assess individual differences between samples and control. Probability values < 0.05 were considered statistically significant. Data were analyzed using the software Statistica 6.0 (StastSoft, USA).

3. Results and discussion

Recently, as already mentioned, we have investigated the structure and properties of gelling polysaccharides isolated from red algae *T. crinitus*. The results of FT-IR and ^{13}C NMR-spectroscopy showed that those polysaccharides had κ/β-carrageenan structure with composed mainly of the κ-carrabiose moiety predomination. However, it was impossible to determine precisely, if these polysaccharides possessed block κ/β-hybrid structure or there was a mixture of κ- and β-carrageenans. Nevertheless, the results of analytical centrifugation and gel filtration, as well as the identity of the IR spectra of polysaccharide fractions obtained by the fractionation using different concentrations of KCl, suggested the block structure (Barabanova et al., 2005). A detailed study on the structural features of the oligosaccharides from κ/β-carrageenan of *T. crinitus*, obtained by enzymatic hydrolysis using negative-ion tandem mass-spectrometry was performed.

3.1. Preliminary analysis

Segments of κ- and β-carrabiose units occurring in the *T. crinitus* carrageenan sample were estimated by ^1H NMR by integrating the α-anomeric signals resonating at 5.12 ppm (κ-carrabiose) and 5.10 ppm (β-carrabiose) (Fig. 1)a. Integration of the signals has allowed an estimation of 70/30 κ/β-carrabiose ratio. A less intense signal at 5.3 ppm, pointed out the occurrence of 3,6-anhydrogalactose-2-sulfate residue attributed to ι-carrabiose moieties. After enzymatic degradation, the ^1H NMR spectrum was more complex and presented an additional signal corresponding to the reducing and non-reducing ends of oligosaccharides produced after enzymatic degradation (Fig. 1b). The signals were ascribed according to previous NMR analysis of oligosaccharides. As expected, signals, corresponding to non-reducing and reducing κ-carrabiose moieties were observed at 5.10 (Anr-H1) and 5.32 (G4Srα-H1), respectively. As previously observed by Knutsen and Grasdalen (1992), the κ-carrageenase can accommodate β-carrabiose moieties in its active site and, as a consequence, β-carrabiose can be found at the nonreducing end but not at the reducing end.

The analysis of the oligosaccharide mixture **Tc-enz-OS** by negative-ion MALDI-TOFMS (Fig. 2a) and ESIMS (insert) has confirmed the occurrence of κ-oligosaccharides and hybrid κ/β-oligosaccharides as $[M-Na]^-$ ions (where M represents the sodium salt of oligosaccharides). The absence of abundant ions corresponding to, for example, κ-carrahexaose (*m*/*z* 1219.12) or κ-carraoctaose (*m*/*z* 1627.16), suggested that only end-products of the κ-carrageenase were occurring in the oligosaccharides mixture. This fact was confirmed by the MALDI-TOFMS of the oligosaccharides, derived from κ−carrageenan in the same way as **Tc-enz-OS** was obtained (Fig. 2b). The arabinoosazone (Chen et al., 1997) which was employed as a MALDI matrix for the analysis of carrageenan-derived oligosaccharides for the first time, allowed to reduce the in-source fragmentation and desulfation in comparision to the results, obtained with *nor*-harmane as a matrix (Antonopoulos, Hardouin, et al., 2005; Ciancia et al., 2005; Fukuyama et al., 2002). Moreover, it was found that addition of L-fucose in the matrix further decreased the intensity of the fragment ions (Fig. 2c without L-fucose versus Fig. 2b). It must be noted, that oligosaccharide mixture from κ−carrageenan contained small signal of tetrasulfated hexasaccharide at *m*/*z* 1321.25, having κ–ι–κ structure, that was suggested by MALDI-TOFMS/MS (data not shown). The addition of L-fucose thus increased the intensity of multiply sulfated oligosaccharides. Tetrasulfated oligosaccharides (for example, $(A\rightarrow G)_{10}(SO_3Na)_4$ was detected by ESIMS at *m*/*z* 848.699^{4-} and not detected by MALDIMS, see below) in **Tc-enz-OS** sample were not observed by MALDI-TOFMS, though.

3.2. Purification of the oligosaccharide mixture

In order to enhance the sensitivity for the minor components and fractionate oligosaccharide mixture **Tc-enz-OS**, size-exclusion chromatography (SEC) was performed. Subfractions were joined according to ESI and MALDI-TOFMS data to obtain six main fractions (**SEC-1** to **SEC-6**). ESIMS was applied for the analysis of fractions to assist MALDI-TOFMS data.

3.3. MS/MS analysis of the oligosaccharide fractions

The elucidation of the structural features of sulfated oligosaccharides, obtained enzymatically from κ/β-carrageenan, by a negative-ion tandem mass-spectrometry was carried out, using the following observations, estimated in the former studies on carrageenan-derived oligosaccharides. It was shown, that the product ion spectra of $[M-Na]^-$ featured an extensive series of B- and C-type (according to nomenclature, suggested by Domon and Costello (Domon & Costello, 1988)) glycosidic cleavages, whereas the Y-type cleavage occurred mainly at the sulfated residues (Yu et al., 2006). Fragment ions from cross-ring cleavage were not observed, though during CID MS/MS of sulfated oligosaccharides from fucoidans (Anastyuk, Shevchenko, Nazarenko, Dmitrenok, & Zvyagintseva, 2009; Anastyuk et al., 2010; Daniel et al., 2007) and glycosaminoglicans (Zaia, 2004), ions of the $^{0,2}X/^{0,2}A$ – type were detected. As recently shown by ESIMS/MS and computational chemistry methods on 2-O- and 4-O-sulfated fucose residues (Saad & Leary, 2004), the mechanism of formation of the $^{0,2}X/^{0,2}A$ fragment ions required unsubstituted proton on the C-3 hydroxyl group. Hence, in case of enzymatically obtained oligosaccharides from carrageenan, when all protons of the C-3 hydroxyl groups are substituted, no $^{0,2}X/^{0,2}A$ – type ions could be produced during CID MS/MS (Antonopoulos, Favetta, et al., 2005; Ekeberg et al., 2001; Yu et al., 2006).

3.3.1. Fragmentation of the oligosaccharides from SEC-1 and SEC-2 fractions

Compositional analysis (data not shown) of **SEC-1** fraction by MALDI-TOF and ESIMS revealed that it was mainly composed of the κ-neocarrabiose (*m*/*z* 403.05, negative-ion mode). The negative-ion MS-analyses (data not shown) of the fraction **SEC-2** revealed, that it contained κ-neocarratetraose ion at *m*/*z* 811.09. Negative-ion tandem MALDI-TOF spectra (data not shown) were recorded for κ-neocarrabiose $[A\rightarrow G4SNa\text{-}Na]^-$ at *m*/*z* 403.05, κ-neocarratetraose

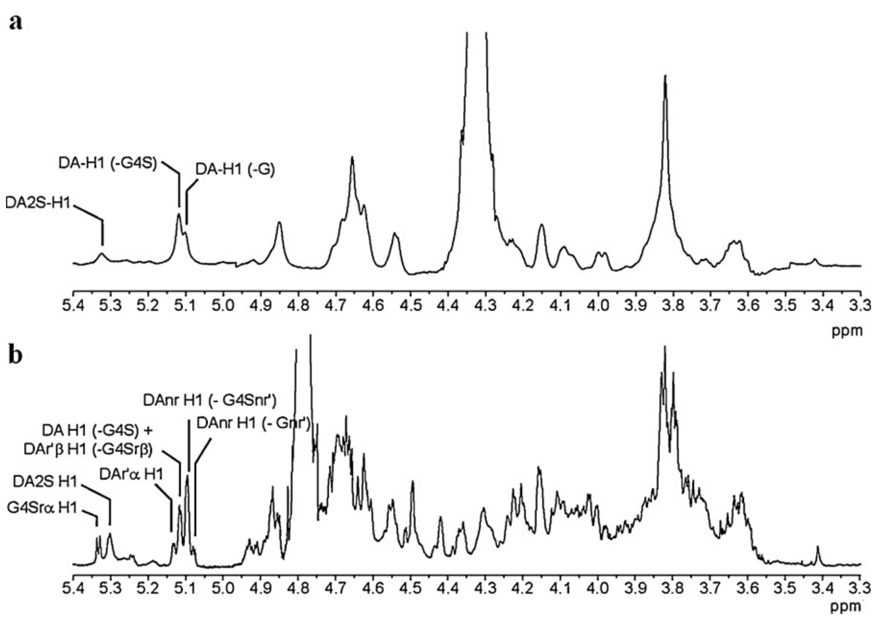

Fig. 1. Proton spectra of native κ/β-carrageenan from *T. crinitus* (a), after an enzymatic (b) hydrolysis.

$[(A{\rightarrow}G4SNa)_2\text{-}Na]^-$ ion at *m*/*z* 811.09. The fragmentation was very similar (except for B_2- and C_2-type ions, which exhibited low intensities) to that reported previously by Yu and co-workers using CID ESIMS/MS of the sodiated oligosaccharides from κ-carrageenan, obtained by enzymatic digestion (Yu et al., 2006).

3.3.2. Fragmentation of the oligosaccharides from SEC-3 fraction

The results of mass-spectral investigation of the fraction **SEC-3** by negative-ion MALDI-TOFMS (Fig. 3) and ESIMS are collected in Table 1. The ion with *m*/*z* 1015.24 (Table 1, position 1) was identified as a fragment from desulfation of the doubly sulfated ion at *m*/*z* 1117.18 (Table 1, position 2). This ion was also observed in ESIMS at *m*/*z* 1015.232, having low intensity.

A tandem MALDI-TOFMS of the most abundant ion $[M2Na{-}Na]^-$ at *m*/*z* 1117.18 (Fig. 4), unlike spectra of κ-oligosaccharides, contained intense signals from glycosidic bonds cleavage near the reducing end. The most intensive signals from the B_5 at *m*/*z* 835.2, C_5 at *m*/*z* 853.2 suggested that the main component was the A → G → A → G4SNa → A → G4SNa fragment (β–κ–κ). The presence of Y'_5 ion at *m*/*z* 973.2, along with ions of series of C_2, $B_{2\text{-}3}$ indicated the occurrence of a second structural variant: A → G4SNa → A → G → A → G4SNa (κ–β–κ). Thus, MS analysis

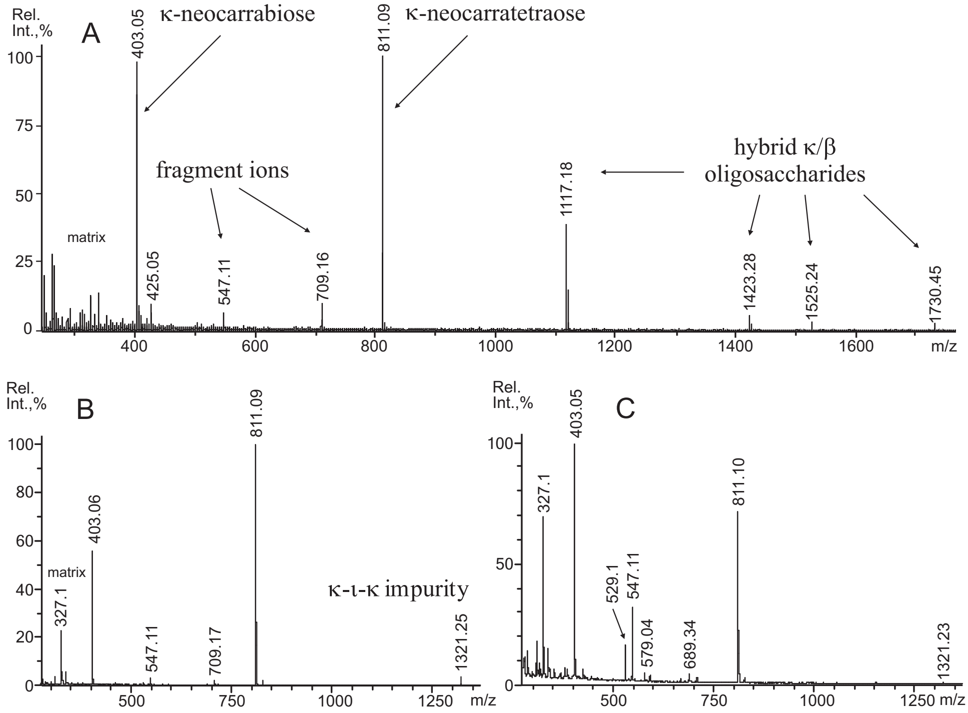

Fig. 2. (a) Negative-ion mode MALDI-TOFMS of enzymatic digestion products (oligosaccharides) of a carrageenan from the red seaweed *T. crinitus* using arabinoosazone as a matrix. (b) Negative-ion mode MALDI-TOFMS of the oligosaccharides from κ-carrageenan standard (Sigma), digested with the same enzyme, with addition of L-fucose in the matrix. (c) Negative-ion mode MALDI-TOFMS of the same oligosaccharide mixture without addition of L-fucose.

Table 1
Composition of the oligosaccharide fraction **SEC-3**, estimated by mass-spectrometry.

#	Observed *m*/*z*		Composition/structural characteristics of oligosaccharides
	MALDIMS [M*−Na]$^-$	ESIMS [M−*n*Na]$^{n-}$	
1	1015.24	1015.232$^-$	[A → G → (A → 4SNa)$_2$-NaSO$_3$+H-Na]$^-$
2	1117.18	547.108^{2-}	A → G4SNa → A → G → A → G4SNa A → G → (A → G4SNa)$_2$
3	1135.11	Not observed	Not established
4	1219.11	391.050^{3-}	Probably, A → G → A2SNa → G4SNa → A → G4SNa
5	1237.12	397.060^{3-}	(G → G)$_1$(A → G)$_2$(SO$_3$Na)$_3$
6	1423.28	700.152^{2-}	(A → G)$_2$(A → G4SNa)$_2$ impurity

* M represents the sodium salt of oligosaccharides.

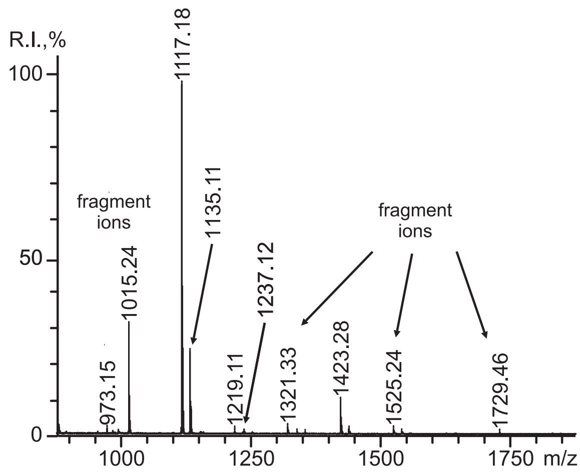

Fig. 3. Negative-ion mode MALDI-TOFMS of **SEC-3** fraction of oligosaccharides from enzymatic digestion products of carrageenan from the red seaweed *T. crinitus*.

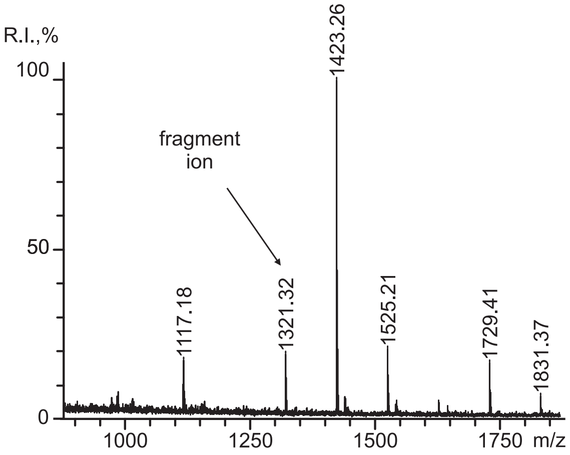

Fig. 5. Negative-ion mode MALDI-TOFMS of **SEC-4** fraction of oligosaccharides from enzymatic digestion products of carrageenan from the red seaweed *T. crinitus*.

revealed, that selected ion, most probably, had two isomeric forms (κ–β–κ and β–κ–κ), which are likely obtained from the degradation products of hybrid κ/β-carrageenan by the κ-carrageenase and are in agreement with detailed NMR analysis (Knutsen & Grasdalen, 1992).

The ion at *m*/*z* 1135.11 (Table 1, position 3), detected by MALDI-TOFMS and having mass difference of 18 Da from *m*/*z* 1117.18 could be a structural variant of above-mentioned A → G → (A → G4SNa)$_2$, where one of 3,6-anhydrogalactose was substituted by galactose, but, however, this presumption was not supported by ESIMS. The ion of triply sulfated carrahexaose at *m*/*z* 1219.11 (Table 1, position 4) was detected by ESIMS more effectively, than by MALDIMS. Despite the fact, that triply sulfated oligosaccharides with higher DP were detected in MALDI readily (see below), it was unable to record MALDI-TOF/TOFMS. Probably, this triply sulfated hexasaccharide had β–ι–κ structure that was suggested with the clear signal to ι-carrabiose in the NMR spectrum (Fig. 1b). The ion at *m*/*z* 1237.12 of the fraction **SEC-3** also had higher intensity in ESIMS (Table 1, position 5). This ion appeared to contain hexose (galactose) instead of 3,6-anhydrogalactose. Its structural properties were not determined due to the low intensity.

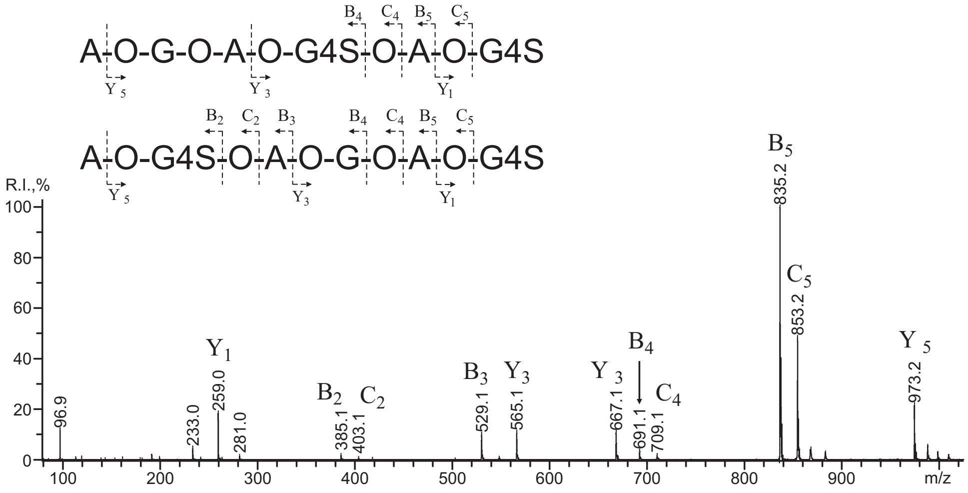

Fig. 4. Negative-ion mode tandem MALDI-TOFMS of [M2Na−Na]$^-$ ion at *m*/*z* 1117.18

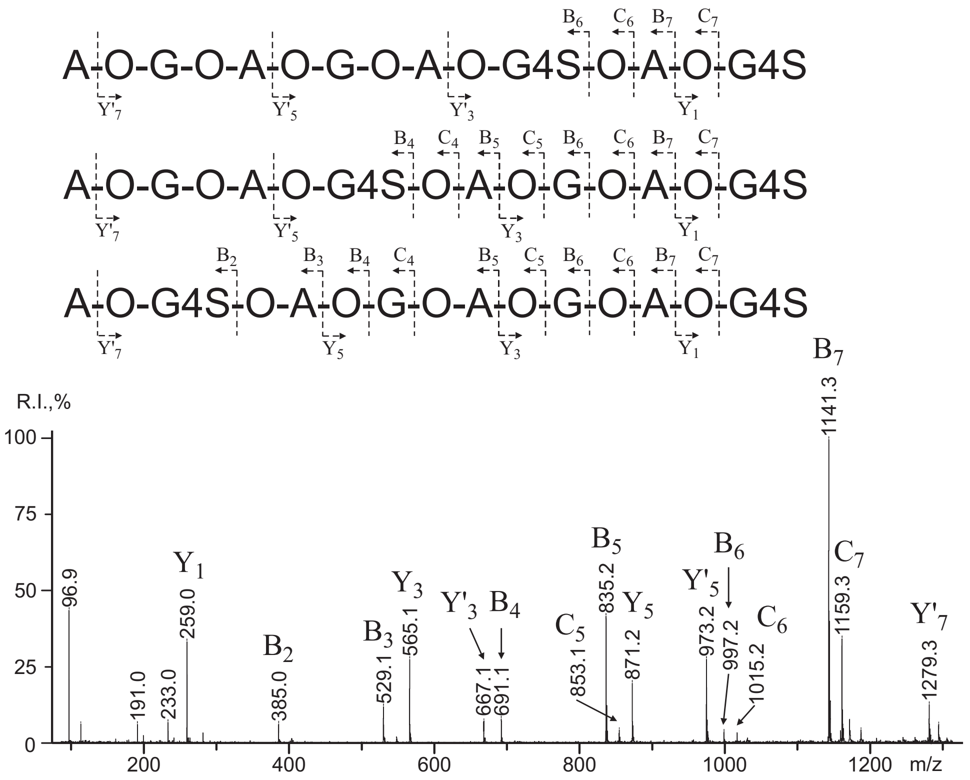

Fig. 6. Negative-ion mode tandem MALDI-TOFMS of [M2Na−Na]$^-$ ion at *m*/*z* 1423.26

3.3.3. Fragmentation of the oligosaccharides from SEC-4 fraction

The results of mass-spectral investigation of the fraction **SEC-4** by negative-ion MALDI-TOFMS (Fig. 5) and ESIMS are collected in Table 2. The ion with *m*/*z* 1321.32 (Table 2, position 2) was identified as a fragment from desulfation of the doubly sulfated ion at *m*/*z* 1423.26 (Table 2, position 3), since this ion was not detected by ESIMS.

A tandem MALDI-TOFMS of the most abundant ion from fraction **SEC-4**, [M2Na−Na]$^-$ at *m*/*z* 1423.26 (Table 2, position 3), shown at Fig. 6, was similar to the previous spectrum (Fig. 4). It also featured intense signals from glycosidic bonds cleavage (B_7 and C_7-type ions from near the reducing end). The Y-type cleavages in this tandem MALDI-TOFMS occurred mainly at the sulfated residues, as was observed by Yu and coworkers in CID ESIMS/MS spectra of singly charged (sodiated) multisulfated oligosaccharides (Yu et al., 2006). Hence, selected ion could have three structural variants: β–β–κ–κ, β–κ–β–κ, κ–β–β–κ.

The fragmentation pattern of triply sulfated ion at *m*/*z* 1525.21 (Table 2, position 4), shown at Fig. 7 was more complex. The most intensive fragment ions, as at the previous spectra, was B_7-type ion at *m*/*z* 1243.2, arising from the reducing end, and Y_5-type ion at *m*/*z* 973.2 from the cleavage of κ–β–κ fragment from the reducing end. Almost the same intensity had Y_3-type ion at m/z 667.1 from the cleavage of dehydrated κ–κ fragment from the reducing end. The Y_7-type fragment ion at *m*/*z* 1381.2, the series of the ions of B_{2-5}, C_{2-5} from the cleavage of singly sulfated κ–β fragment

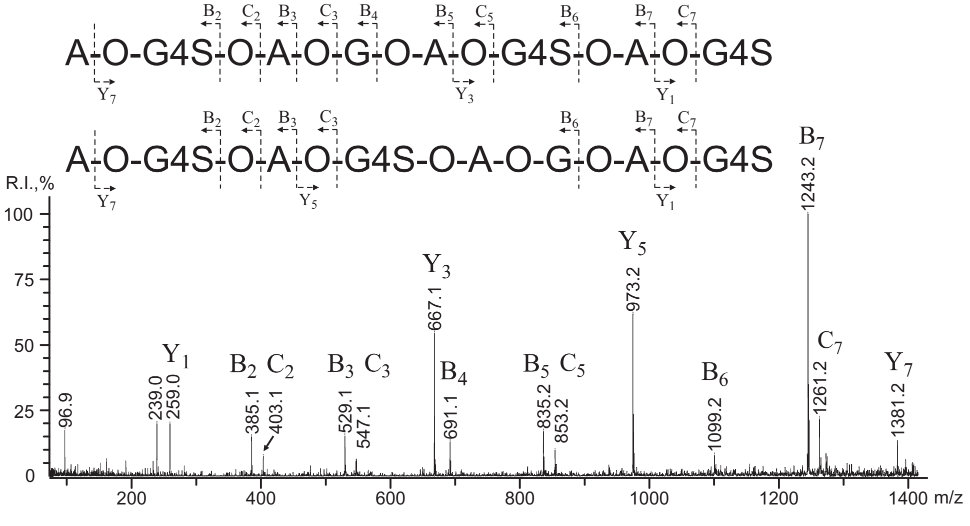

Fig. 7. Negative-ion mode tandem MALDI-TOFMS of [M3Na−Na]$^-$ ion at *m*/*z* 1525.21

Table 2
Composition of the oligosaccharide fraction **SEC-4**, estimated by mass-spectrometry.

#	Observed *m*/*z*		Composition/structural characteristics of oligosaccharides
	MALDIMS [M*−Na]$^-$	ESIMS [M−*n*Na]$^{n-}$	
1	1117.18	547.097^{2-}	(A → G)$(SO_3Na)_2$ impurity
2	1321.32	Not observed	[(A → G)$_4(SO_3Na)_2$ -$NaSO_3$+H−Na]$^-$
3	1423.26	700.158^{2-}	A → G → A → G → A → G4SNa → A → G4SNa A → G → A → G4SNa → A → G → A → G4SNa A → G4SNa → A → G → A → G → A → G4SNa
4	1525.21	493.090^{3-}	A → G4SNa → A → G4SNA → A → G → A → G4SNa A → G4SNa → A → G → A → G4SNa → A → G4SNa
5	1729.41	853.197^{2-}	(A → G)$_3(SO_3Na)_2$ impurity
6	1831.37	Not observed	(A → G)$_3(SO_3Na)_3$ impurity

* M represents the sodium salt of oligosaccharides.

from the non-reducing end, and the high intensity of Y_3-type ion suggested structural variant κ–β–κ–κ. Despite lack of doubly sulfated B_4-type ions, indicating the cleavage of κ–κ fragment from the non-reducing end, the high intensity of the above-mentioned Y_5 ion allowed to propose the other structural variant – κ–κ–β–κ.

3.3.4. Fragmentation of the oligosaccharides from SEC-5 fraction

The results of mass-spectral investigation of the fraction **SEC-5** by negative-ion MALDI-TOFMS (Fig. 8) and ESIMS are collected in Table 3. The ions with *m*/*z* 1423.31, 1627.44, 1933.54, 2239.69, 2341.59, detected by MALDI-TOFMS were assumed to be fragment ions from desulfation (Table 3, positions 1, 3, 7, 9, 10), since they were not visible under ESIMS. It is worth mentioning, that four times sulfated oligosaccharides (Table 3, positions 4, 13) were detected by ESIMS as multiply charged ions (having low intensities, though), while their singly charged variants in MALDI-TOFMS were not observed.

A tandem mass-spectrum (not shown) of the doubly sulfated ion at *m*/*z* 1729.37 (Table 3, position 5), demonstrated a number of fragment ions, most intensive of which (B_5 at *m*/*z* 835.2, Y_1 at *m*/*z* 259.0) were singly sulfated. Though the intensity of the spectrum peaks were low and some peaks were missing, the doubly sulfated Y'_n-type ions with odd indexes ($n = 3, 5, 7$) suggested, that at least four structural variants of the selected ion could exist in the mixture. Using similar approach for the interpretation of this spectrum as for already described above, the four possible structural variants were suggested: κ–β–β–β–κ, β–κ–β–β–κ, β–β–κ–β–κ, β–β–β–κ–κ.

The interpretation of the tandem mass-spectrum (not shown) of the triply sulfated ion at *m*/*z* 1831.30 was similar to already described spectra. It suggested four structural variants (Table 3, position 6).

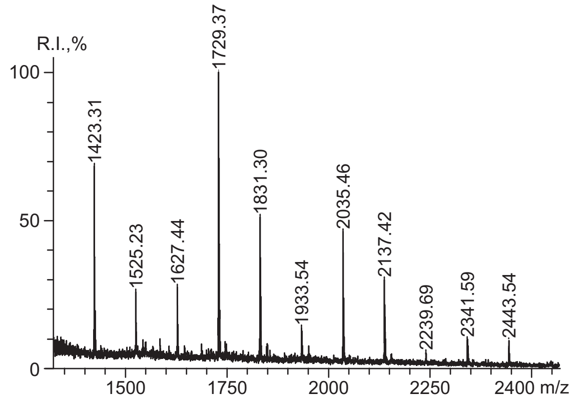

Fig. 8. Negative-ion mode MALDI-TOFMS of **SEC-5** fraction of oligosaccharides from enzymatic digestion products of carrageenan from the red seaweed *T. crinitus*.

3.4. Mass-spectrometric analysis of the high-molecular fraction SEC-6

The results of mass-spectral investigation of the fraction **SEC-6** by negative-ion MALDI-TOFMS and ESIMS collected in Table 4. The oligosaccharides were poorly visible by both ESIMS and MALDIMS (data not shown) with arabinoosazone as a MALDI matrix. Dehydroxybenzoic acid (DHB) as a matrix gave better results when sample concentration increased up to 1 mg/ml. Unfortunately, this led to increased fragmentation and desulfation of the analyte molecules. The addition of L-fucose gave unsuccessful results in this case. The *m*/*z* values, shown at the Table 4 on positions 1, 2, 4, 5, 7, 8 corresponded to the fragment ions from successive desulfation. Also, MALDI-TOFMS (data not shown) of the fraction **SEC-6** contained intensive fragment ions from the glycosidic bonds cleavage in the low-molecular mass-range. However, the ions of triply sulfated oligosaccharides with *m*/*z* 2750.61, 3056.71, likely occurring natively in the oligosaccharide mixture (Table 4, positions 3, 6) were detected by both ESIMS and MALDI-TOFMS.

4. Cytokin-inducing activities

Although the action of carrageenans on the immune response has been studied by many investigators *in vitro* and *in vivo* experiments, little is known on the effect of the molecular weigh of carrageenan on the immunomodulatory effects. Immunological tests carried on oligosaccharides from carrageenans are not yet fully documented and, only of lambda- or kappa-carrageenan have been investigated through *in vivo* (Yuan, Song, Li, Li, & Dai, 2006; Zhou et al., 2004). Carrageenans are well known to stimulate a production of cytokines by cells. These cytokines play a critical role in regulating inflammatory and immunological processes of the host. Recently the carrageenans have been treated by enzymes and interleukin-8 (IL-8) stimulation by modified polysaccharides was determined for better understanding how the specific chemical structure of carrageenans contributes to immune response (Brattacharyya et al., 2010). It was shown that a basic pathway of immune activation of carrageenans is initiated by the α-Gal-(1 → 3) Gal epitope. In our case the relationship between immunostimulate activity and molecular weight of carrageenan is illustrated through their ability of producing cytokine- TNF-α and interleukin IL-6. The average molecular mass of the native carrageenan polysaccharide was 200 kDa and that of the oligosaccharide mixture, obtained from κ/β-carrageenans by enzymatic digestion with κ-carrageenase, was 4.3 kDa respectively. It must be noted that maximum observed molecular weight of oligosaccharides, estimated by mass-spectrometry was 3.5 kDa (see Table 4).

Experiments were performed to determine in human blood cell the effect of polysaccharide (κ/β-carrageenans) and oligosaccharides on the production of TNF-α and interleukin IL-6. Ability of carrageenans to activate cells and to cause in them synthesis of cytokines has been correlated to activity of LPS, cytokine-induction

Table 3
Composition of the oligosaccharide fraction **SEC-5**, estimated by mass-spectrometry.

#	Observed m/z		Composition/structural characteristics of oligosaccharides
	MALDIMS $[M^*-Na]^-$	ESIMS $[M-nNa]^{n-}$	
1	1423.31	Not observed	$[(A\rightarrow G)(A\rightarrow G4SNa)_3\text{-}NaSO_3+H\text{–}Na]^-$
2	1525.23	493.080^{3-}	$(A\rightarrow G)(A\rightarrow G4SNa)_3$ impurity
3	1627.44	Not observed	$[(A\rightarrow G)_3(A\rightarrow G4SNa)_2\text{-}NaSO_3+H\text{–}Na]^-$
4	Not observed	389.548^{4-}	$(A\rightarrow G)_4(SO_3Na)_4$
5	1729.37	853.192^{2-}	A → G4SNa → A → G → A → G → A → G → A → G4SNa A → G → A → G4SNa → A → G → A → G → A → G4SNa A → G → A → G → A → G4SNa → A → G → A → G4SNa A→G → A → G → A → G → A → G4SNa → A → G4SNa
6	1831.30	595.123^{3-}	A → G4SNa → A → G → A → G4SNa → A → G → A → G4SNa A → G4SNa → A→G → A → G → A → G4SNa → A → G4SNa A → G4SNa → A → G → A → G4SNa → A → G → A → G4SNa A → G → A → G4SNa → A → G → A → G4SNa → A → G4SNa
7	1933.54	Not observed	$[(A\rightarrow G)_6(SO_3Na)_2\text{-}NaSO_3+H\text{–}Na]^-$
8	2035.46	1006.256^{2-}	$(A\rightarrow G)_6(SO_3Na)_2$
9	2137.42	697.158^{3-}	$(A\rightarrow G)_6(SO_3Na)_3$
10	2239.69	Not observed	$[(A\rightarrow G)_7(SO_3Na)_2\text{-}NaSO_3+H\text{–}Na]^-$
11	2341.59	Not observed	$[(A\rightarrow G)_7(SO_3Na)_3\text{-}NaSO_3+H\text{–}Na]^-$
12	2443.54	799.192^{3-}	$(A\rightarrow G)_7(SO_3Na)_3$
13	Not observed	619.147^{4-}	$(A\rightarrow G)_7(SO_3Na)_4$

[*] M represents the sodium salt of oligosaccharides.

Table 4
Composition of the high-molecular oligosaccharide fraction **SEC-6**, estimated by mass-spectrometry.

#	Observed m/z		Composition
	MALDIMS $[M^*-Na]^-$	ESIMS $[M-nNa]^{n-}$	
1	2545.68	Not observed	$[(A\rightarrow G)_8(SO_3Na)_2\text{-}NaSO_3+H\text{–}Na]^-$
2	2647.67	Not observed	$[(A\rightarrow G)_8(SO_3Na)_3\text{-}NaSO_3+H\text{–}Na]^-$
3	2750.61	901.221^{3-}	$(A\rightarrow G)_8(SO_3Na)_3$
4	2852.81	Not observed	$[(A\rightarrow G)_9(SO_3Na)_2\text{-}NaSO_3+H\text{–}Na]^-$
5	2954.82	Not observed	$[(A\rightarrow G)_9(SO_3Na)_3\text{-}NaSO_3+H\text{–}Na]^-$
6	3056.71	1003.586^{3-}	$(A\rightarrow G)_9(SO_3Na)_3$
7	3158.90	Not observed	$[(A\rightarrow G)_{10}(SO_3Na)_2\text{-}NaSO_3+H\text{–}Na]^-$
8	3261.02	Not observed	$(A\rightarrow G)_{10}(SO_3Na)_2$
9	Not observed	848.699^{4-}	$(A\rightarrow G)_{10}(SO_3Na)_4$

[*] M represents the sodium salt of oligosaccharides.

activity of which has been accepted for 100%. The effect of carrageenans to influence on the cytokine production is largely dependent on the concentration (Table 5). The oligosaccharides of carrageenan were shown to maintain an ability to induce of TNF-α and IL-6. At high concentration (100 ng/mL) of parent carrageenan (polysaccharide) and oligosaccharides possessed strongly marked activity in the synthesis of IL-6 and TNF-α and their activities were comparable. At low concentration (1 ng/mL) their activity was insignificant comparable with the activity of LPS and only oligosaccharide stimulated induction of TNF-α, compared to control. Biosynthesis of TNF-α, by oligosaccharide was aloud higher than that by the parent carrageenan (more than 1.6 times). According to Brattacharyya et al. (2010) oligosaccharides of carrageenan with molecular weights 1550, 400, 8000 Da, obtained by hydrolysis of carrageenan by κ-carrageenases, induced secretion of IL-8 by cells in culture. The shorter degraded oligosaccharides induced a higher IL-8 response, consistent with increased exposure of the immunogenic α-1-3 galactosidic epitope.

Table 5
Biological activity of κ/β-carrageenan and oligosaccharides, obtained enzymatically.

Sample		TNF (pg/ml)	IL-6 (ng/ml)
LPS*	1 ng	76.2 ± 7.4	6.9 ± 0.04
	100 ng	196.4 ± 8.2	8.1 ± 0.9
κ/β, polysaccharide	1 ng	11.78 ± 0.23	0.8 ± 0.01
	100 ng	108.2 ± 2.8	5.7 ± 0.01
κ/β oligosaccharides	1 ng	18.3 ± 5.4	0.5 ± 0.03
	100 ng	110.2 ± 9.4	4.6 ± 0.1
Control	13.5 ± 1.4	1.1 ± 0.005	

The mean ± SED ($n = 5$) contents cytokines (%) are presented. Whole blood was obtained from 5 healthy subjects and incubated with the LPS and carrageenans samples in different concentration or saline solution (control). Paired Student's *t*-tests were used to assess individual differences between samples and control. $P < 0.05$ were considered statistically significant.

[*] LPS-lipopolysaccharide.

5. Conclusion

The mass-spectrometric investigation of the multisulfated oligosaccharides, obtained by κ–carrageenase digestion of κ/β-carrageenan from *T. crinitus* has confirmed their hybrid structure. It was found, that the enzyme was able to cleave glycosidic bonds between –κ–κ–, but unable to digest tetrasaccharide κ–κ. Oligosaccharides having –κ–κ–**x** or –**x**–κ–κ motifs on the reducing- and non-reducing ends were cleaved at slow rate (**x** = β or ι). Due to this fact, hybrid κ/β and κ/β/ι were detected: β → κ, κ → β → κ, β → κ → κ, including minor β → ι → κ, β → β → κ → κ, β → κ → β → κ, κ → β → β → κ, κ → β → κ → κ, κ → β → β → β → κ, β → β → κ → β → κ, κ → β → κ → β → κ, κ → β → β → κ → κ. The digestion of the κ-carrageenan standard gave only κ and κ → κ oligosaccharides with the minor κ → ι → κ. The results shown using DHB as a MALDI matrix were similar to the results, obtained with *nor*-harmane as matrix, when excess desulfation was observed, as well as the cleavages of the glycosidic bonds (Antonopoulos, Hardouin, et al., 2005), when high-molecular (DP up to 20) oligosaccharides were analyzed. In opposite, the application of arabinoosazone as a matrix allowed minimizing desulfation and effectively analyzing multisultated (up to 3) oligosaccharides (DP 2-10) in a tandem (LIFT-TOF/TOF) mode. It was shown, that the MALDI LIFT-TOF/TOFMS fragmentation pattern of the multisulfated oligosaccharides was similar to CID ESIMS/MS of singly charged (sodiated) ions: mass-spectra showed the Y-type ions from glycosidic bonds cleavage at the sulfated residues, while B- and C-type cleavages of glycosidic bonds were intensive if they were located between the sulfated residues. The Y-ions with even-indexes, (Yu et al., 2006) as long as the signals from cross-ring cleavages were not observed, suggesting that the observed fragment ions were not the products of secondary cleavages. The successful application of the MALDI potential lift tandem TOF mass spectrometry using arabinoosazone as a matrix for sequencing of carrageenan-derived oligosaccharides, to our knowledge, was performed for the first time. The technique for "cooling" the matrix and thus reducing the in-source fragmentation by addition of L-Fuc into the matrix/sample (originally developed for peptides (Harvey, 2011)) was successfully employed for multisulfated oligosaccharides. The oligosaccharides of κ/β-carrageenan were shown to maintain an ability to induce of TNF-α and IL-6 secretion of immune cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.04.081.

References

Anastyuk, S. D., Shevchenko, N. M., Nazarenko, E. L., Dmitrenok, P. S., & Zvyagintseva, T. N. (2009). Structural analysis of a fucoidan from the brown alga *Fucus evanescens* by MALDI-TOF and tandem ESI mass spectrometry. *Carbohydrate Research*, *344*(6), 779–787.

Anastyuk, S. D., Shevchenko, N. M., Nazarenko, E. L., Imbs, T. I., Gorbach, V. I., Dmitrenok, P. S., et al. (2010). Structural analysis of a highly sulfated fucan from the brown alga *Laminaria cichorioides* by tandem MALDI and ESI mass spectrometry. *Carbohydrate Research*, *15*(345), 2206–2212.

Antonopoulos, A., Favetta, P., Helbert, W., & Lafosse, M. (2005). On-line liquid chromatography electrospray ionization mass spectrometry for the characterization of kappa- and iota-carrageenans. Application to the hybrid iota-/nu-carrageenans. *Analytical Chemistry*, *77*(13), 4125–4136.

Antonopoulos, A., Favetta, P., Helbert, W., & Lafosse, M. (2007). On-line liquid chromatography–electrospray ionisation mass spectrometry for kappa-carrageenan oligosaccharides with a porous graphitic carbon column. *Journal of Chromatography A*, *1147*(1), 37–41.

Antonopoulos, A., Hardouin, J., Favetta, P., Helbert, W., Delmas, A. F., & Lafosse, M. (2005). Matrix-assisted laser desorption/ionisation mass spectrometry for the direct analysis of enzymatically digested kappa- iota- and hybrid iota/nu-carrageenans. *Rapid Communication in Mass Spectrometry.*, *19*(16), 2217–2226.

Barabanova, A. O., Yermak, I. M., Glazunov, V. P., Isakov, V. V., Titlyanov, E. A., & Solov'eva, T. F. (2005). Comparative study of carrageenan from reproductive and sterile forms of *Tichocarpus crinitus*. (Gmel.) Rupr. (Rhodophyta, Tichocarpaceae). *Biochemistry (Moscow)*, *70*, 430–437.

Bienvenu, J., Doche, C., Gutowski, M., Lenoble, M., & Pedrix, J. (1995). Production of proinflammatory cytokines and cytokines involved in the TH1/TH2 balance is modulated by pentoxifylline. *Journal of Cardiovascular Pharmacology*, *25*(Suppl. 2), 80–84.

Brattacharyya, S., Liu, H., Zhang, Z., Jam, M., Dudeja, P. K., Michel, G., et al. (2010). Carrageenan-induced innate immune response is modified by enzymes that hydrolyze distinct galactosidic bonds. *Journal of Nutritional Biochemistry*, *10*, 906–913.

Chen, P., Baker, A. G., & Novotny, M. V. (1997). The use of osazones as matrices for the matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates. *Analytical Biochemistry*, *244*(1), 144–151.

Ciancia, M., Sato, Y., Nonami, H., Cerezo, A. S., Erra-Balsells, R., & Matulewicz, M. C. (2005). Autohydrolysis of a partially cyclized mu/nu-carrageenan and structural elucidation of the oligosaccharides by chemical analysis, NMR spectroscopy and UV-MALDI mass spectrometry. *Arkivoc*, *12*, 319–331.

Craigie, J. S. (1990). Cell walls. In K. M. Cole, & R. Y. Sheath (Eds.), *Biology of the red algae* (pp. 221–257). Cambridge: Cambridge University Press.

Daniel, R., Chevolot, L., Carrascal, M., Tissot, B., Mourao, P. A., & Abian, J. (2007). Electrospray ionization mass spectrometry of oligosaccharides derived from fucoidan of *Ascophyllum nodosum*. *Carbohydrate Research*, *342*(6), 826–834.

Domon, B., & Costello, C. E. (1988). A systematic nomenclature for carbohydrate fragmentations in Fab-Ms Ms spectra of glycoconjugates. *Glycoconjugate Journal*, *5*(4), 397–409.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugar and related substances. *Analytical Chemistry*, *28*, 350–356.

Ekeberg, D., Knutsen, S. H., & Sletmoen, M. (2001). Negative-ion electrospray ionisation–mass spectrometry (ESI–MS) as a tool for analysing structural heterogeneity in kappa-carrageenan oligosaccharides. *Carbohydrate Research*, *334*(1), 49–59.

Fukuyama, Y., Ciancia, M., Nonami, H., Cerezo, A. S., Erra-Balsells, R., & Matulewicz, M. C. (2002). Matrix-assisted ultraviolet laser-desorption ionization and electrospray-ionization time-of-flight mass spectrometry of sulfated neocarrabiose oligosaccharides. *Carbohydrate Research*, *337*(17), 1553–1562.

Güven, K. C., Guvener, B., & Guler, E. (1990). Pharmocological activities of marine algae. In I. Akatsuka (Ed.), *Introduction to applied psycology* (pp. 67–92). The Hague, The Netherlands: SPB Academic Publishing.

Greer, C. W., & Yaphe, W. (1984). Characterization of hybride (beta-kappa-gamma) carrageenan from *Eucheuma gelatinae* J. Agardh (Rhodophyta, Solieriaceae). Using carrageenans, infrared and ^{13}C-nuclear magnetic resonance spectroscopy. *Botanica Marina*, *27*(3), 473–478.

Harvey, D. J. (2011). Analysis of carbohydrates and glycoconjugates by matrix-assisted laser desorption/ionization mass spectrometry: An update for the period 2005–2006. *Mass Spectrometry Reviews*, *30*(1), 1–100.

Knutsen, S. H., & Grasdalen, H. (1987). Characterization of water-extractable polysaccharides from Norwegian *Furcellaria lumbricalis* (Huds.) Lamour. (Gigartinales, Rhodophyceae) by IR and NMR spectroscopy. *Botanica Marina*, *30*, 497–505.

Knutsen, S. H., & Grasdalen, H. (1992). The use of neocarrabiose oligosaccharides with different length and sulphate substitution as model compounds for ^{1}H-NMR spectroscopy. *Carbohydrate Research*, *229*, 233–244.

Knutsen, S. H., Myslabodski, D. E., Larsen, B., & Usov, A. (1994). A modified system of nomenclature for red algal galactans. *Botanica Marina*, *37*(2), 163–169.

Park, J. T., & Johnson, M. J. (1949). A submicrodetermination of glucose. *The Journal of Biological Chemistry*, *181*, 149–151.

Rochas, C., & Heyraud, A. (1981). Acid and enzymic hydrolysis of kappa carrageenan. *Polymer Bulletin*, *5*, 81–86.

Rochas, C., Rinaudo, M., & Landry, S. (1990). Role of the molecular weight on the mechanical properties of kappa carrageenan gels. *Carbohydrate Polymers*, *12*, 255–266.

Saad, O. M., & Leary, J. A. (2004). Delineating mechanisms of dissociation for isomeric heparin disaccharides using isotope labeling and ion trap tandem mass spectrometry. *Journal of The American Society for Mass Spectrometry*, *15*(9), 1274–1286.

Usov, A. I., & Ivanova, E. G. (1987). Characterization of hybrid structure of substituted agarose from *Polysiphonia morrowii* (Rhodophyta, Rhodomelaceae) using β-agarase and ^{13}C-NMR spectroscopy. *Botanica Marina*, *30*(5), 365–370.

Van de Velde, F., Knutsen, S. H., Usov, A. I., Rollema, H. S., & Cerezo, A. S. (2002). ^{1}H and ^{13}C high resolution NMR spectroscopy of carrageenans: Application in research and industry. *Trends in Food & Technology*, *13*, 73–92.

Van de Velde, F., Peppelman, H. A., Rollema, H. S., & Tromp, R. H. (2001). On the structure of κ/ι-hybrid Carrageenans. *Carbohydrate Research*, *331*, 271–283.

Yermak, I. M., & Khotimchenko, Y. S. (2003). Chemical properties, biological activities and applications of carrageenan from red algae. In M. Fingerman, & R. Nagabhushanam (Eds.), *Recent advances in marine biotechnology* (pp. 207–257). Enfield, Plymouth: Science Publishers Inc.

Yu, G., Zhao, X., Yang, B., Ren, S., Guan, H., Zhang, Y., et al. (2006). Sequence determination of sulfated carrageenan-derived oligosaccharides by high-sensitivity negative-ion electrospray tandem mass spectrometry. *Analytical Chemistry*, *78*(24), 8499–8505.

Yuan, H., Song, J., Li, X., Li, N., & Dai, J. (2006). Immunomodulation and antitumor activity of kappa-carrageenan oligosaccharides. *Cancer Letters*, *243*, 228–234.

Zaia, J. (2004). Mass spectrometry of oligosaccharides. *Mass Spectrometry Reviews*, *23*(3), 161–227.

Zhou, G., Sun, Y., Xin, H., Zhang, Y., Li, Z., & Xu, Z. (2004). In vivo antitumor and immunomodulation activities of different molecular weight lambda-carrageenans from *Chondrus ocellatus*. *Pharmacological Research*, *50*, 47–53.