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Structure and thermal stability of pyruvated carrageenans from the red alga *Coccotylus truncatus*

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ABSTRACT

The composition, structure, and thermal stability of carrageenans from unattached *Coccotylus truncatus* (the Baltic Sea, Estonia) were investigated. The complex polysaccharide was characterized by ^{13}C NMR and FTIR spectroscopy, ICP-OES and gel permeation chromatography methods. The main components of *C. truncatus* galactan are 3,6-anhydro-α-D-galactose-2-sulfate (30 ± 1.5%) and β-D-galactose-4-sulfate (45.3%), indicating a ι-carrageenan backbone. As the minor components, α-D-galactose-2,6-disulfate (12 ± 2%) from ν-carrageenan and 4′,6′-pyruvated β-D-galactopyranosyl residues (1.4%) from pyruvated α-carrageenan are found to be present, latter being responsible for the undersulfated nature of the galactan. The native polysaccharide with the average molecular weight of about 1500 kDa is highly susceptible to thermal degradation. The high-temperature treatment of this galactan gives products with 3,6-anhydro-α-D-galactose units predominantly at the reducing end. The carrageenan extraction from *C. truncatus* gives characteristically low yields (12–17%); weak gelling ability of the polysaccharides from this seaweed species (gel strength 30–40 g/cm^2) does not depend significantly on extraction conditions.

1. Introduction

The red alga *Coccotylus truncatus* (Pall.) M.J. Wynne and J.M. Heine (former names *Phyllophora truncata* and *Phyllophora brodiaei*) is one of the few marine species that can inhabit the brackish water of the Baltic Sea, although in a deep-growing dwarf form.[1] This seaweed (as unattached vegetative form) with another red alga *Furcellaria lumbricalis* forms voluminous community (known as the Kassari algal stratum) in Estonian waters in the central Baltic Sea region. The proportion of the two dominant species differs slightly depending on locality, *C. truncatus* accounting usually 30–35% of the biomass.[2] The utilization of this hardly separable seaweed mixture has been made since 1966 for the production of 'furcellaran' (polysaccharide blend from *F. lumbricalis*), but it is obvious that the final product contains galactans from both the dominant species. It has been proposed that the polysaccharides originating from the red alga *C. truncatus* may have economic value.[3] Recently, some rheological and turbidimetrical characteristics concerning the gelling product from this raw material have been investigated.[4,5]

While the composition and structure–property regularities of furcellaran have been thoroughly studied,[6,7] very little research has been conducted on the polysaccharides from *C. truncatus*. By the general primary structure of its major component, polysaccharides from *C. truncatus* belong to the ι-carrageenan pattern.[3,6] As is common for Phyllophoraceae, the carrageenan chemistry of *C. truncatus* varies considerably with life history generations. Based on immunoprecipitation and FTIR studies, it has been found that the carrageenans from gametophytic phase of this seaweed species belong to ι-family, while the sporophytic galactans contain both ι- and λ-structures.[8,9] The detailed compositional and structural peculiarities of *C. truncatus* polysaccharides have still remained unresolved.

An important characteristic of food grade carrageenans is their structural stability derived from the substitution pattern of hydroxyl groups. The destructive effect of acidic medium on red algal galactans is widely investigated,[10] several reports have been published on the influence of ultrasound and radiation.[11,12] The thermal degradation of carrageenans has been elucidated for both the dry preparations and the water solutions.[13–15] The susceptibility to degradation of various seaweed galactans usually follows the order: agarose > κ-carrageenan > ι-carrageenan, and has been related to the chain rigidity and sulfation degree.[11] For κ-type carrageenans, the intense destruction of polymeric chains has been reported to begin at temperatures above 115 °C.[14] As degraded carrageenans with an average molecular weight (M_w) below 50 kDa (named as poligeenans) have been associated with negative health

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effects,[16] the processing parameters for isolation of dietary carrageenans should yield the products with the minimum poligeenan fraction.

The aim of this work is to determine the structural and compositional characteristics of the polysaccharides isolated from the vegetative form of *C. truncatus*. Special attention is given to the thermal stability and gelling properties in connection with the structural characteristics of these seaweed galactans.

2. Results and discussion

2.1. Yields of extraction

The yields of polysaccharide extracted from *C. truncatus* were relatively low (12–17%) depending notably on the extracting agent. Pure water is the most efficient medium to isolate carrageenans from the algal tissue, lowest yields were obtained by the process involving hot alkaline media. According to the literature, polysaccharide yields from *C. truncatus* usually remain around 11–20%; however, values as low as 1–2% have been noted for sporophytes collected in colder season.[6,17] Although the low extraction yields have been attributed to the morphological peculiarities (thick cortex) of this seaweed species,[6] this is not confirmed in the present study as the further extraction from the seaweed residue gave notably low yields (0.2–0.3%).

2.2. Chemical composition

The composition of *C. truncatus* galactan is notably dependent on the conditions of extraction. A slight increase in 3,6-anhydrogalactose (3,6-AG) content from 19.1% to 21.2% during the alkaline extraction (compared to the water-extracted preparation) indicates the presence of galactose-6-sulfate or galactose-2,6-disulfate residues (the presence of the latter was confirmed by ^{13}C NMR studies). The observed results are among the highest values for this seaweed species. According to the literature, sporophytic and gametophytic polysaccharides from *C. truncatus* have been found to contain 9% and 16% of 3,6-AG, respectively.[9] Somewhat higher 3,6-AG contents (13–24.5%) have been reported for galactans isolated from the Baltic vegetative form.[4,6]

In order to determine the percentage of galactose-2,6-disulfate units, the native preparation was submitted to alkaline modification procedure. The analysis of this modified polysaccharide showed an increase in the 3,6-AG content by 4.8–6.7% (depending on the batch), thus the galactose-2,6-disulfate content in the native preparation was estimated to be 10–14%. Even after the chemical modification, the content of 3,6-AG in *C. truncatus* galactan was somewhat lower than that in the commercial preparation from Fluka (28.3% 3,6-AG).

The galactan from C. *truncatus* was found to contain 32.5% galactose. A pyruvate level of 0.5% makes it a moderately pyruvated carrageenan; the concentration of 4′,6′-pyruvated D-galactopyranosyl residues (confirmed by ^{13}C NMR studies) was estimated to be 1.4%. Taking into account the amount of pyruvated residues, the content of galactose-4-sulfate is about 45.3%.

The sulfur content of *C. truncatus* polysaccharide is relatively low for a ι-type carrageenan, and varies to a slight degree by the extraction medium, having a mean value of 8.6%. In ι-carrageenan, the sulfur content is usually 9.3–11.7%.[18] Hence, the galactan mixture from this seaweed species has more structural irregularities in addition to the alkali-labile units, and is farther from the idealized structure type of ι-carrageenan. Previously, somewhat lower sulfur contents (6.3–7.4%) for the Baltic *C. truncatus* (unattached vegetative form) galactan have been described.[4,6] Low levels of this element (about 6%) have also been reported for the gametophytic polysaccharides from *C. truncatus*.[9] On the other hand, carrageenans from the sporophyte form of this seaweed species have been characterized as more highly sulfated galactans with sulfur content over 10%.[9]

Considering the amount of negatively charged groups in the structure, the extracting media containing KOH at a concentration as low as 0.02 mol/L already saturate the galactan matrix with K^+-ions up to 60%, having notable effect to the mineral part of the polysaccharide preparation (Table 1). Although water-extraction results in more abundant Mg^{2+} and Ca^{2+} contents, the hot alkaline extraction yields products with higher overall mineral heterogeneity and substantially better solubility properties.

Concerning the typical toxic components, the water-extracted preparation was characterized by low cadmium content; arsenic concentration was under the detection limit (<0.06 mg/kg) in both the carrageenan and algae samples. The high iron content in both the native and alkali-extracted galactan preparations was attributable to the high concentration of this element in the algal raw material. The similar values for the galactan and seaweed samples indicate that iron does not selectively bind to the polysaccharide matrix of *C. truncatus* as is common for Mg^{2+} and Ca^{2+}.

Nitrogen content in the galactan is relatively high (0.73–0.82%), indicating notable amount of protein substances remaining in the preparation. This is obviously caused by the high protein content of *C. truncatus*, usually remaining in the range of 16–20%.[17] As noted previously for the other Baltic seaweed species *F. lumbricalis*, the proteinic pigments can be hardly separable from the polymer matrix by ordinary techniques.[19] It has been established that the amount of galactan-bound nitrogen does not depend significantly on the extraction conditions.

2.3. Structural characteristics

The FTIR spectra of the native and alkali-extracted polysaccharide preparations revealed absorption bands at 1375 and 1250 cm^{-1} (Fig. 1), which is indicative of the sulfate ester substitution. The intense signal at 932 cm^{-1} is attributable to 3,6-AG residues.[20] As expected for ι-carrageenan, the sharp adsorption bands at 850 and 805 cm^{-1} are indicative of the axial sulfate ester substitutions at the *O*-4 of a 3-linked galactose and at the *O*-2 of a 4-linked 3,6-AG, respectively.[21]

Table 1
Content of some elements in *C. truncatus* and galactan samples

Preparation	Element content (%)								Element content (mg/kg)							
	Na	K	Mg	Ca	N	P	S	Fe	Mn	Co	Ni	Cu	Zn	Mo	Cd	Ba
C. truncatus[a]	0.020	0.09	0.55	3.44	3.23	0.09	4.99	0.17	493	2.29	9.43	25.2	28.4	0.64	0.21	50.0
Galactan[b]	0.021	0.11	0.87	4.33	0.73	0.11	8.44	0.15	577	1.45	1.30	4.3	9.2	1.52	<0.05	73.3
Galactan[c]	0.041	6.19	0.22	2.86	0.82	0.09	8.66	0.16	162	2.35	6.05	29.2	16.8	2.07	0.37	88.1

[a] Algae washed thoroughly with tap water and distilled water.
[b] Extracted 4 h in water.
[c] Extracted 4 h in 0.02 M KOH solution.

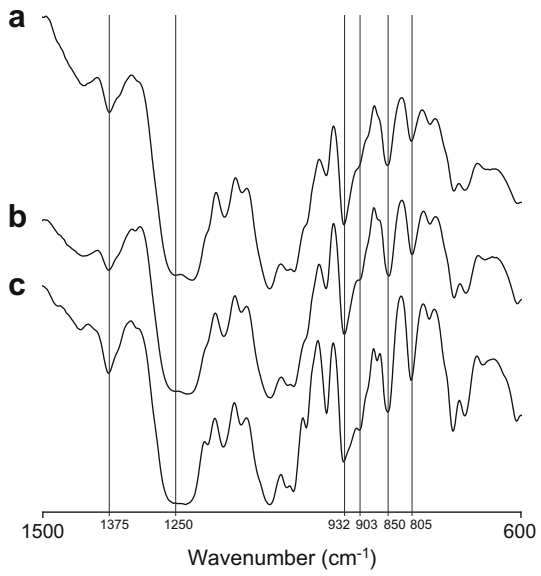

Figure 1. FTIR spectra of galactan from *C. truncatus* extracted 4 h in (a) water, (b) 0.02 M KOH, (c) ι-carrageenan (Fluka).

As seen from the FTIR spectra, the alkaline extraction only slightly modified *C. truncatus* galactan. The spectrum of the water-extracted polysaccharide exhibited a shoulder near 867 cm^{-1} that can be attributed to a sulfate group at the *O*-6 of a 4-linked galactose.[22] In this region, a small difference in spectra between the native and alkali-extracted preparations suggests the presence of ν-carrageenan, a biological precursor to ι-carrageenan.[20] Although the characteristic absorptions for galactose-2,6-disulfate (830 and 820 cm^{-1} for equatorial sulfate esters at *O*-2 and *O*-6, respectively) were not resolved, this is common for the preparations of low ν-carrageenan content.[23]

The FTIR spectra of *C. truncatus* galactan, particularly those of the alkali-extracted preparation, exhibited a broad shoulder with enhanced absorption at 903 cm^{-1}. This signal is normally associated with unsulfated 3-linked galactose residues[24,25] and/or unsulfated 3-linked residues bearing pyruvate acetal at *O*-4 and *O*-6,[26] being thus typical absorption band for agarose, some carrageenans (e.g. α-, β-, γ-, δ-, and π-type) and for many hybrid galactan blends. However, the FTIR spectra of ι-carrageenan preparations often show a weak absorption in this region.

The ^{13}C NMR spectra of the galactans investigated are shown in Figure 2a and b, and the values of chemical shifts of carbon signals are summarized in Table 2. The main components of the polysaccharide from *C. truncatus* were β-D-galactose-4-sulfate and 3,6-anhydro-α-D-galactose-2-sulfate, indicating the ι-carrageenan backbone. As a minor component, alkali-labile α-D-galactose-2,6-disulfate from ν-carrageenan[27] was also present in the galactan. Weak signals at 101.72, 67.40, 66.56, and 65.56 ppm were assigned to G-1, G-4, G-5, and G-6 of the 4′,6′-pyruvated β-D-galactopyranosyl unit; detectable band at 91.28 ppm confirmed the presence of pyruvated α-carrageenan (4′,6′-pyruvated carrabiose 2-sulfate) residues.[26] Diagnostic resonances included those for the acetal and methyl carbons of pyruvate acetal substituent at 101.72 and 25.43 ppm, respectively, which were clearly resolved from the noise. Although the signal for the carboxyl carbon of the pyruvic group was not observed in the ^{13}C NMR spectra, this is common for the unfractionated samples of red algal galactans, because the pyruvate substituent occurs usually at concentrations too low to be detected.[28] The presence of 4′,6′-pyruvated β-D-galactopyranosyl residues is commonly associated with agar-type polysaccharides, although many complex carrageenans can contain those structures in low amounts.[29] Nevertheless, significant proportion of pyruvate groups has been found in carrageenans obtained from some sporophytic Gigartinacean species.[26]

The ^{13}C NMR spectra of the native and alkali-extracted preparations differ only slightly. The spectrum of the alkali-extracted galactan demonstrates higher 3,6-AG content as seen from more intense bands at 77.04, 74.95, and 69.80 ppm, which correspond to A-5, A-2, and A-6, respectively. The pyruvate content appears to be not affected by the alkaline extraction, however the resonances for G-5 and G-6 of 4′,6′-pyruvated β-D-galactopyranosyl residues in alkali-extracted preparation were poorly resolved. This is apparently caused by the higher viscosity of the potassium-rich preparation. The spectrum of the preparation treated with strong alkali (not shown) indicated complete cyclization of α-D-galactose-2,6-disulfate residues.

The principal storage glucan of red algae, floridean starch, has often been reported to remain in the crude galactan preparations in relatively high quantities and could be readily detected by ^{13}C NMR investigations.[25] Both the alkali-treated and native preparations, particularly the latter, exhibited weak bands at 100.10, 73.75, and 71.83 ppm, which correspond to C-1, C-3, and C-5 of glucose units from floridean starch, respectively. Also traces of unsulfated forms of β-D-galactose and 3,6-anhydro-α-D-galactose from β- and κ-carrageenan, respectively, were detected as revealed by the characteristic signals at 94.58 and 95.24 ppm for A-1. The β- and κ-structures originate presumably from the minute amounts of *F. lumbricalis*, another dominant seaweed species present in the Kassari algal stratum that forms hardly separable mixtures with *C. truncatus*. The presence of λ-carrageenan is not confirmed by the current ^{13}C NMR study, as characteristic signals around 103.4 and 64.2 ppm for G-1 and G-4 of β-D-galactose-2-sulfate[30] were not detected.

2.4. Gelling ability

Viscous polysaccharides from *C. truncatus* show weak gelling ability that does not depend considerably on the extraction conditions. The gel strength values for the alkali-extracted and native preparations were 30 and 40 g/cm^2 (for 2% gels), respectively. The native galactan gelled in 0.05 M $CaCl_2$ solution gave the highest gel strength values (70 g/cm^2) observed for this polysaccharide backbone. For comparison, the commercial ι-carrageenan preparation from Fluka did not form gels with measurable strength at 2% concentration. Somewhat higher gelling ability of *C. truncatus* galactan preparations can be attributed to their abundant inorganic part, especially to the considerable amount of polysaccharide-bound Ca^{2+} ions. Small differences in gel strength values between the native and alkali-extracted preparations can also be attributed mainly to the variable counter-ion content in those samples, at that, the impairing effect of precursor structures on gelling properties[31] was not evidenced.

2.5. Molecular weight distribution and thermal destruction

Figures 3 and 4 show the molecular weight distribution of the galactans under study. Using the sample solvent of the same composition as that of the GPC eluent (0.1 M $NaNO_3$) enabled interferences (broad negative peaks) to be reduced in the low-molecular region of an internal ethylene glycol standard at 22.89 mL. Due to the small difference in concentration between the HPLC eluent and the solvent used to prepare the galactan solution, a characteristic peak at 22.14 ± 0.01 mL was observed in all chromatograms.

M_w of the native galactan from *C. truncatus* was estimated at about 1500 kDa. Somewhat lower M_w values (about 1400 kDa) were measured for the preparation obtained through the hot alkaline extraction procedure. The presence of a minor peak at

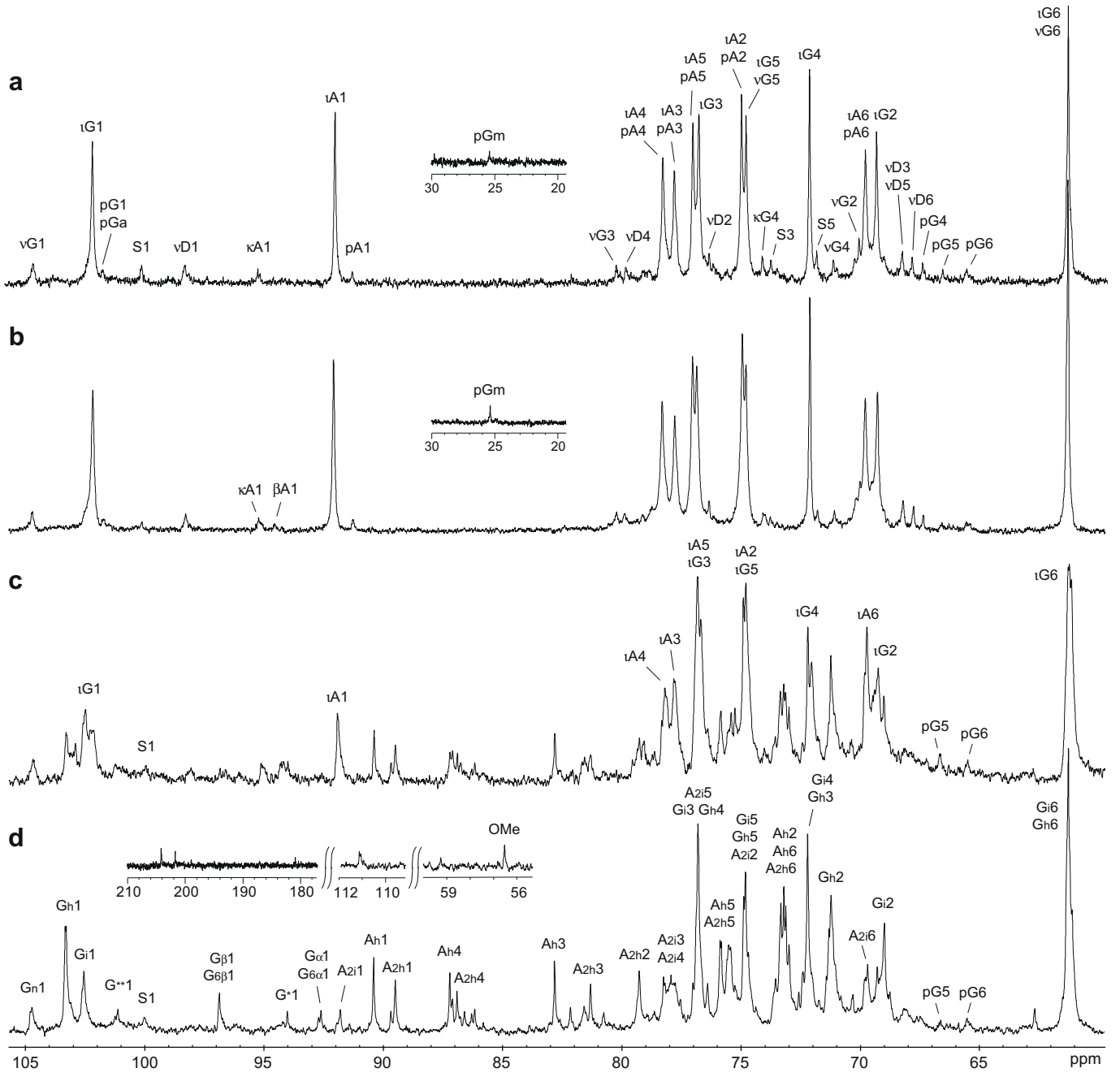

Figure 2. ^{13}C NMR spectra of *C. truncatus* galactan preparations recorded at 40 °C. (a) Water-extracted galactan, 38,000 transients; (b) alkali-extracted galactan, 100,000 transients; thermally degraded (7 days at 85 °C) water-extracted galactan preparation (c) before, 40,000 transients, and (d) after removal of high-molecular fraction, 26,000 transients collected. A_h—hydrated aldehyde of a 3,6-anhydro-α-D-galactose residue; A_{2h}—hydrated aldehyde of a 3,6-anhydro-α-D-galactose-2-sulfate residue; G_h—terminal β-D-galactose-4-sulfate linked to a 3,6-anhydro-α-D-galactose or 3,6-anhydro-α-D-galactose-2-sulfate; G_i—internal β-D-galactose-4-sulfate residue; G^*—β-D-galactose-2-sulfate linked to a α-D-galactose-2,6-disulfate or α-D-galactose-2-sulfate or α-D-galactose-6-sulfate; G^{**}—β-D-galactose-2-sulfate linked to a 3,6-anhydro-α-D-galactose-2-sulfate; G_α—reducing α-D-galactose; $G_{6\alpha}$—reducing α-D-galactose-6-sulfate; G_β—reducing β-D-galactose; $G_{6\beta}$—reducing β-D-galactose-6-sulfate; G_n—β-D-galactose-4-sulfate linked to a reducing form of α-D-galactose-6-sulfate or α-D-galactose-2,6-disulfate; pGm—methyl carbon of the pyruvated acetal substituent; S—glucose units in floridean starch. κ and β indicate corresponding units from κ- and β-carrageenans. For other signal marks, see Table 2.

20.33 mL in GPC elution profile of the alkali-extracted preparation revealed that the alkaline medium not only decreased the polymerization degree of the high-molecular fraction, but also induced a partial hydrolysis of the galactan matrix and the formation of low-molecular weight degradation products. As seen from chromatogram (Fig. 4a), the commercial ι-carrageenan preparation from Fluka also contained minor amounts of low-molecular constituents (with M_w values below 0.5 kDa) as hydrolysis products and/or standardization additives.

The polysaccharides from *C. truncatus* were found to be highly susceptible to thermal degradation; even temperatures as low as 60 °C resulted in a significant drop in M_w characteristic to 1100 kDa. At the degradation temperature of 80 °C, a small peak at 20.23 mL appeared in the chromatogram (Fig. 3), indicating the formation of low-molecular products similar to those observed in the alkali-extracted preparation. A sharp increase in this peak was evidenced in a narrow temperature region between 80 and 85 °C with a substantial cleavage of the high-molecular part (Figs.

Table 2
^{13}C NMR chemical shifts (ppm) for signals of carrageenan structures

Galactan	Unit	^{13}Carbon chemical shift					
		C-1	C-2	C-3	C-4	C-5	C-6
ι-Carrageenan[3]	G	102.2	69.3	76.8	72.2	74.8	61.3
	A	92.1	75.0	77.8	78.3	77.0	69.8
ν-Carrageenan[27]	G	105.3	70.8	80.5	71.6	75.3	61.7
	D	98.8	76.3	68.6	79.4	68.6	68.1
Pyruvated α-carrageenan*,[26]	pG	101.9	69.1	76.7	67.4	66.7	65.5
	pA	91.4	75.2	77.7	78.3	77.1	69.9
Native galactan from *C. truncatus***	ιG[a]	102.16	69.33	76.77	72.14	74.79	61.30
	ιA[a]	92.00	74.98	77.80	78.28	77.02	69.80
	νG[b]	104.65	70.06	80.22	71.14	74.79	61.30
	νD[b]	98.28	76.34	68.24	79.84	68.24	67.84
	pG[c]	101.72	nd.	nd.	67.40	66.56	65.56
	pA[d]	91.28	74.98	77.80	78.28	77.02	69.80

nd.—not determined due to overlapping signals.
[a] Corresponding units from ι-carrageenan.
[b] Corresponding units from ν-carrageenan.
[c] 4′,6′-Pyruvated β-D-galactose from pyruvated α-carrageenan.
[d] 3,6-Anhydro-α-D-galactose-2-sulfate from pyruvated α-carrageenan.
* 4′,6′-Pyruvated carrabiose 2-sulfate (pyruvate acetal: methyl 25.5 ppm, acetal 101.6 ppm, carboxyl 175.7 ppm).
** pyruvate acetal: methyl 25.43 ppm, acetal 101.73 ppm, carboxyl—not detected.

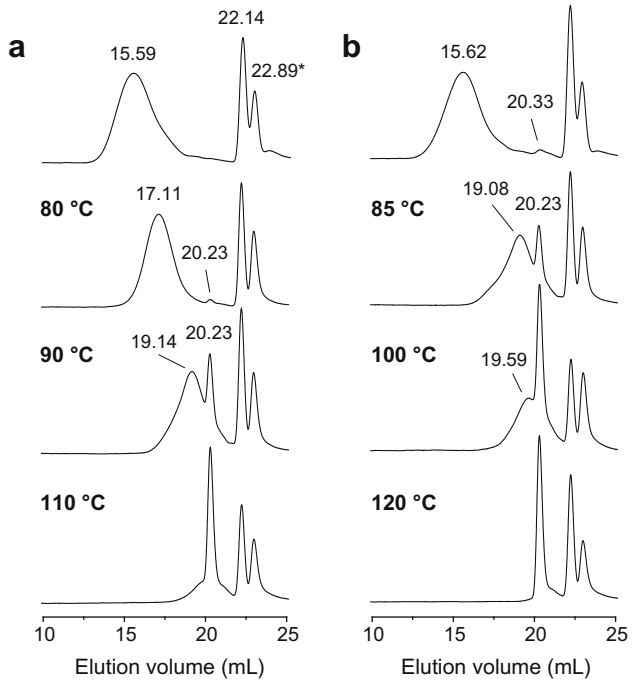

Figure 3. Gel permeation chromatography elution profiles for *C. truncatus* galactan extracted in (a) water, (b) 0.02 M KOH solution, and for water-extracted galactan preparations degraded 7 days at 80–120 °C. * Peak at 22.89 mL corresponds to internal marker of ethylene glycol.

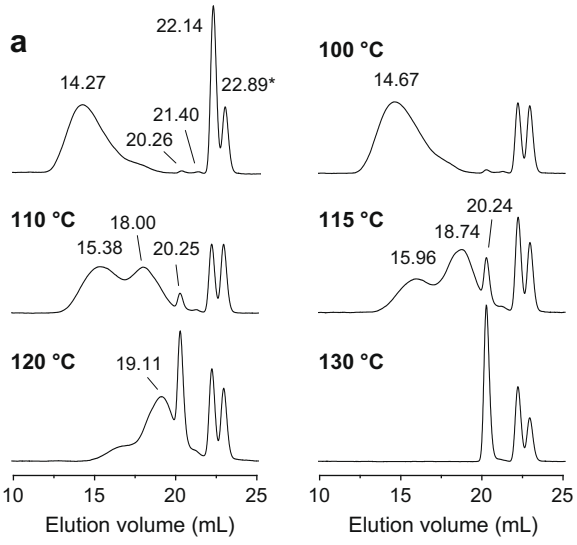

Figure 4. Gel permeation chromatography elution profiles for the commercial ι-carrageenan preparation from Fluka (a) before and after 7 days of thermal treatment at 100–130 °C. * Peak at 22.89 mL corresponds to internal marker of ethylene glycol.

3 and 5a). Almost all the highly polymerized components were decomposed in the preparation treated at 110 °C. In contrast, ι-carrageenan from Fluka was fairly stable at temperatures below 90 °C (Fig. 5a). The extensive destruction of the polymeric chains in this preparation took place in the region of 100–130 °C. At the degradation temperature of 110 °C, two macromolecular fractions with M_w values of 34 and 2100 kDa were clearly differentiated, and the formation of low-molecular component was evidenced as a small peak at 20.25 mL in chromatogram (Fig. 4). Although the sample treated at 120 °C contained a substantial amount of partly degraded polysaccharides, the high-molecular component was completely decomposed at the temperature of 130 °C.

The destruction of 3,6-AG in macromolecular assembly is evidently connected with the length of the polysaccharide chains, and is associated with the formation of low-molecular fraction. In all cases, severe decrease in 3,6-AG content occurred as the M_w of the galactan preparation dropped below 10 kDa. For thermally labile polysaccharides from *C. truncatus*, the decomposition of 3,6-AG began already at 80–85 °C, whereas the same characteristic for the ι-carrageenan preparation from Fluka was 115–120 °C (Fig. 5b). Higher susceptibility to thermal degradation of *C. truncatus* polysaccharides can be accounted to the specific structural irregularities and to a smaller polymerization degree of its native preparation.

The ^{13}C NMR spectrum of the thermally treated (7 days at 85 °C) *C. truncatus* galactan preparation indicated substantial changes in the polysaccharide structure (Fig. 2c). A concomitant alteration in the solubility properties of this partially degraded preparation

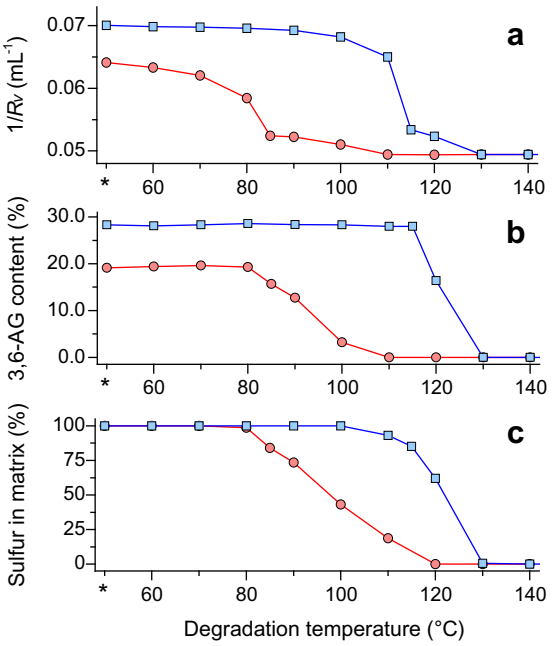

Figure 5. Degradation profiles for carrageenan preparations after 7 days of thermal treatment. Influence of degradation temperature on (a) M_w characteristic of the main component ($1/R_v$, mL^{-1}; R_v—elution volume), (b) 3,6-AG content, and (c) sulfur content in galactan matrix (relative to the total sulfur); (▫) ι-carrageenan from Fluka, (●) native galactan from *C. truncatus*; * indicates untreated preparation.

was also noted. Although the product was readily solubilized in water (as 2% solution, w/w), the high-molecular fraction (M_w over 10 kDa) precipitated completely within 10 h at 4 °C. The resulting clear, low viscous solution was submitted to further ^{13}C NMR analysis for more detailed characterization of the low-molecular component (Fig. 2d). The separation of the high-molecular fraction was clearly evidenced from the change in signal intensity for A-1 of 3,6-anhydro-α-D-galactose-2-sulfate.

The low-molecular degradation products formed during the thermal treatment of carrageenans in dry state were found (after solubilization) to be similar to those obtained previously by the acid hydrolysis[32–34] of these polysaccharides. The formation of the oligosaccharides with the reducing terminal 3,6-anhydro-α-D-galactose residues was evidenced from the well-resolved resonances at 90.38, 87.19, and 82.20 ppm, which correspond to A-1, A-4, and A-3, respectively. Signals at 89.48, 86.89, 81.30, and 79.30 ppm were assigned to A-1, A-4, A-3, and A-2 of the reducing 3,6-anhydro-α-D-galactose-2-sulfate units, and the ones at 103.24 and 71.23 ppm to G-1 and G-2 of the terminal D-galactose-4-sulfate residues, respectively. The two latter signals showed significantly higher intensities compared to the respective resonances for the internal D-galactose-4-sulfate residues (at 102.51 and 69.00 ppm), revealing the presence of a substantial amount of disaccharides.

The spectrum exhibited detectable bands at 204.2 and 201.7 ppm, indicating the presence of aldehydic residues; also a minor signal at 180.9 ppm was observed. A weak low-field signal at 111.13 ppm was presumably due to the occurrence of a small proportion of furanose forms derived from 3,6-AG. The resonance at 56.53 ppm could possibly be attributed to the methoxy group at the C-3 position of a D-galactose moiety.[35] The signals at 96.83 and 92.59 ppm were attributed to the anomeric carbon of unbound reducing form of galactose or galactose-6-sulfate and the resonance at 104.68 ppm to G-1 of the β-D-galactose-4-sulfate linked to the internal or reducing form of α-D-galactose-2,6-disulfate or α-D-galactose-6-sulfate. No substantial change in the intensity of this signal was observed during the fractionation, indicating a significant amount of ν-carrageenan units remaining in the high-molecular fraction. The weak signals at 101.08 and 94.00 ppm can arise from G-1 of the diads containing 3-linked D-galactose-2-sulfate residues. Thus, although not confirmed in the ^{13}C NMR studies of undegraded *C. truncatus* galactan, this polysaccharide may contain traces of λ-carrageenan units.

It becomes evident that during the thermal treatment the 2-sulfated glycosidic linkages are cleaved at a higher rate than the non-sulfated ones. The process gives products with 3,6-AG units at the reducing end. As seen from the ^{13}C NMR spectrum of the low-molecular fraction of the *C. truncatus* galactan preparation degraded at 85 °C, the reducing 3,6-AG residues already have more than half of their sulfate groups removed. At this, about 15% of the total sulfur is released from the galactan matrix (Fig. 5c) and converted into the acidic products (obviously, $NaHSO_4$ and $KHSO_4$) that further promote the polysaccharide cleavage. The acidic nature of the degradation products was evidenced by the low pH values of their water solutions (pH 2.7 for 2% solution of the galactan treated at 85 °C).

3. Conclusion

The vegetative unattached form of the red alga *C. truncatus* contains hybrid ι-/ν-carrageenans with small amount of pyruvated 3-linked D-galactose residues. The native polysaccharide with M_w of 1500 kDa is characterized by relatively low content of sulfur (8.4%) and 3,6-anhydro-α-D-galactose (19.1%), latter being subject to increase during the treatment with strong alkali. The yields of polysaccharide extraction from *C. truncatus* are relatively low (12–17%) resulting in products with weak gelling ability (gel strength 30–40 g/cm^2) and calcium-rich mineral part. Alkaline extraction in 0.02 M KOH solution results in observable drop in M_w value and induces the formation of low-molecular degradation products. Compared to the commercial ι-carrageenan preparation, the native galactan from *C. truncatus* is more susceptible to thermal degradation, with intensive cleavage of the polymeric chains beginning at 80 °C. The labile properties may reduce the usability of this polysaccharide in certain food applications.

4. Experimental

4.1. Materials

The samples of algae (the mixture of unattached forms of *C. truncatus* and *F. lumbricalis*) were collected from Kassari Bay (N 58° 41.08′; E 22° 50.16′, the Baltic Sea, Estonia) in the middle of August 2007, using SCUBA diving down to the depth of 8 m. The specimens of unattached vegetative form of *C. truncatus* (accounting 38% of the total wet biomass) were separated carefully from the algal mixture, washed thoroughly with tap water, then with distilled water, and dried at room temperature. Dextran markers for gel permeation chromatography (GPC) analyses and the commercial ι-carrageenan preparation were from Fluka.

4.2. Extraction, modification, and degradation

The air-dry algal sample was refluxed in a 33-fold mass of the extracting medium (distilled water or 0.02 M KOH solution); the time of extraction was counted in the boiling state. The hot extract was filtered through a porous glass filter (porosity no. 2) into cold (7 °C) isopropanol (99.9% v/v, 3-fold volume per extract), leading to the precipitation of polysaccharides. The galactans precipitated were separated from the alcohol–water mixture by filtration through a porous glass filter (porosity no. 3) and washed with cold (7 °C) isopropanol. The isolated polysaccharide mixture was dried to a constant weight in a drying oven (60 °C, 2 days), and then milled.

For alkaline modification, the 1 M NaOH solution containing 0.5% of $NaBH_4$ and 0.25% of polysaccharide was heated in a water bath at 80 °C for 3 h. After cooling, the solution was dialyzed (molecular weight cutoff 12 kDa) against bidistilled water, concentrated in vacuo, and freeze dried.

For thermal stability analysis, air-dry polysaccharide preparations were held in an air thermostat at a constant temperature (60–160 °C) for 7 days. The extent of polymeric chain cleavage was investigated by GPC.

4.3. Chemical analysis

3,6-AG content was determined colorimetrically using a resorcinol–acetal method and fructose as a standard sugar.[36] Galactose content was estimated by anthrone assay.[37] Pyruvate acetal substitution was detected as the 2,4-dinitrophenylhydrazone derivative of pyruvic acid[38], the value corrected according to Duckworth and Yaphe.[39] Nitrogen content was measured by the Kjeldahl procedure. The other elements were determined using the ICP-OES method. Sulfur released from the galactan matrix was estimated chromatographically.[40]

4.4. FTIR spectroscopy

The FTIR spectra of carrageenan samples were scanned using a PerkinElmer FTIR System Spectrum BX spectrometer (12 scans per spectrum; nominal resolution: 4 cm^{-1}) from thin (0.015 mm) films obtained by a slow evaporation of 1% solutions in polystyrene Petri dishes at room temperature. The spectra were recorded in the 4000–370 cm^{-1} region.

4.5. NMR spectroscopy

Proton-decoupled ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopic analyses were carried out using a Bruker AVANCE III spectrometer operating at 800 MHz. The spectra from a 2% carrageenan solution in D_2O (w/w) were obtained at 40 °C, and maximum 100 thousand transients were collected before the Fourier transform. The chemical shifts were converted to a tetramethyl silane scale on the basis of the C-6 signal from the galactose subunit having a constant value of 61.3 ppm for these carrageenans.[3]

4.6. Gel testing

For gel strength assessments, a suitable gel tester equipped with a hemispherically tipped plunger (an effective cross-section area of 1 cm^2) was constructed. The gel strength measurements were made in triplicate for 2.0% gels (w/w) formed by dissolving the dry galactan in hot water after gelling in an air thermostat at 20 °C for 4 h. The cylindrical samples were 35 mm in diameter and 35 mm in height. The force needed to rupture the gel by the plunger was expressed in g/cm^2; the constant increase in the stress to the gel surface by addition of mass 350 g/min was achieved.

4.7. Gel permeation chromatography

GPC of carrageenans was performed on a chromatograph equipped with a PerkinElmer Series 200 pump, a Knauer Smartline 2300 refractive index detector, a Knauer Smartline column thermostat, and two Shodex OHpak SB-806MHQ columns in series. Elution was carried out using a 0.1 M $NaNO_3$ solution as the mobile phase at a flow rate of 0.8 mL/min. The temperature of the columns was maintained at 60.0 °C. A calibration curve was constructed using 10 dextran standards (668, 410, 273, 148, 80.9, 48.6, 23.8, 11.6, 5.2, and 1.3 kDa), the elution volume was corrected to the internal marker of ethylene glycol (0.01% in sample) at 22.89 mL. The equation of the curve was as follows: $\log M_w = 0.02x^2 - 1.3503x + 22.361$ (M_w, average molecular weight; x, elution volume; $R^2 = 0.9987$). The carrageenan concentration used was 0.07%, and the sampling volume 100 μL.

To obtain more reliable results, the galactan samples were dissolved in the same solvent used as an eluent in the GPC system. For better solubilization, the sols were kept overnight under a constant shaking at 35 °C. The final solubilization was assured by heating the polymeric solutions in a boiling water bath under a vigorous stirring for 10 min. The hot (60 °C) sol was filtered through a 0.45 μm membrane (Spartan 30/0.45RC), allowed to cool down, and then injected into the HPLC system.

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