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ORIGINAL PAPER

Commercially available carrageenans show broad variation in their structure, composition, and functionality

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Abstract

Carrageenans are polysaccharides from red algae which are widely used as food additives and in other applications. Their structure is often described by different disaccharide repeating units, although it was already demonstrated that reality is more complex. In many studies, commercial carrageenans were used to establish structure function relationships, but a structural and compositional analysis was rarely conducted. Therefore, the aim of our study was to systematically and comprehensively characterize a broad collection of commercial carrageenans with different specifications from different manufacturers. For a more detailed characterization, an analytical approach based on partial enzymatic hydrolysis in combination with HPLC–MS and HPSEC-RI was developed and applied. Furthermore, rheology was used to gain detailed insights into the functionality of selected samples. Our results demonstrate that significant structural variation can be observed for commercial carrageenans. The samples contained different cations and the carrageenan type specified by the manufacturer did not always represent the structure of the corresponding polysaccharides. This was especially true for λ-carrageenans: Of the six commercial samples analyzed, none contained structural elements from the λ-type. Instead, the corresponding carrageenans contained κ-, ι- and ν-units. The application of the developed enzymatic-chromatographic approach showed that different hybrid carrageenans are present. In addition, the rheological analysis of the commercial carrageenan samples showed clear differences in the gelling properties upon calcium addition which could influence their behavior in different applications. Our results demonstrate that before an investigation of structure–function relationships, commercial carrageenan samples should be analyzed for their structure and composition. We also showed that the enzymatic-chromatographic approach described in this study is well suited for this purpose.

Keywords Carrageenans · Structural composition · Enzymatic hydrolysis · Cations · Sulfation · Mass spectrometry

Abbreviations

ICP-OES	Inductively coupled plasma atomic emission spectrometry
HPSEC	High-performance size exclusion chromatography
RI	Refractive index
MALLS	Multi angle laser light scattering

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Introduction

Carrageenans are polysaccharides extracted from red algae which are commonly used as food additives, e.g. as thickening, stabilizing or gelling agent [1]. They are linear polysaccharides and often described by using repeating disaccharide units. These units are composed of a 1,3-linked β-D-galactopyranose (G-unit) and a 1,4-linked α-D-galactopyranose (D-unit) or 3,6-anhydro-α-D-galactopyranose (DA-unit). The individual monosaccharide units can be substituted with sulfate groups [2, 3]. The substitution position depends on the type of carrageenan and is usually annotated by adding the position and "S" to the monosaccharide descriptor (e.g. G4S for a 1,3-linked galactose unit which is sulfated at position C4) [4].

Carrageenans are commonly classified into different types based on the disaccharide repeating units. These types are abbreviated using Greek letters, with κ-, ι- and

λ-carrageenan being the most commercially important carrageenans [2, 3, 5, 6]. The κ-carrageenan repeating unit is composed of a G4S and a DA-unit, while the ι-carrageenan repeating unit contains a G4S-unit and a DA2S-unit. In contrast, λ-carrageenan is composed of two galactose units (G2S and D2S,6S) [1, 4]. Carrageenans with a D-unit instead of a DA-unit are commonly referred to as natural precursors. For example, μ-carrageenan (G4S, D6S) is the precursor of κ-carrageenan, while ν-carrageenan is the precursor of ι-carrageenan. The sulfated D-units of precursor carrageenans can be converted into DA-units by the use of enzymes or under alkaline conditions. The latter usually occur during the extraction of carrageenans from red seaweed [2]. Due to the use of different bases, different cations can be associated with the carrageenans. The most abundant cations are potassium, sodium and calcium ions [7]. The cations are an important factor for the aggregation of carrageenan helices during gel formation [8]. Thus, they are important for the functional properties but possibly also for the physiological properties.

The usually applied classification of carrageenans assumes that only the disaccharide unit characteristic of one type of carrageenan is present in the polymer. However, some carrageenans contain different disaccharide repeating units within one polymer chain and are also known as hybrid carrageenans or kappa-2 carrageenans [5, 6, 9]. Hybrid/kappa-2 carrageenans gained relevance for selected applications and several types have been described and structurally characterized in the literature, such as κ/ι- [10, 11], ν/ι- [12] and μ/κ-hybrid carrageenans [13]. In addition, mixtures of different carrageenan types may occur, in which the individual polymers are not covalently attached to each other [9].

The physicochemical properties of carrageenans are closely linked to their chemical structure. For example, carrageenans without 3,6-anhydrogalactose units, such as λ-carrageenan, lack gel-forming properties. On the other hand, κ- and ι-carrageenans containing DA-units are able to form gels [1, 2]. In addition to the rheological properties [14–16], other physiological and technological effects of carrageenans have been investigated in many studies, such as the ice recrystallization inhibition activity [17–21], inflammatory effects in the gut [22–27] as well as anti-viral effects [28–30]. However, the structure and composition of the carrageenans used in these studies were either not characterized or only roughly characterized. In many cases, researchers rely on the specifications from the manufacturers or literature data. However, using well-characterized samples is crucial which has also been pointed out by the European Food Safety Authority [31]. The aim of our study was to comprehensively and systematically characterize different commercial carrageenans in detail to document possible structural and compositional differences, to point out influences on functionality and in general to raise awareness for this issue. Furthermore, an enzymatic-chromatographic approach was developed for a detailed structural characterization of the carrageenan samples.

Experimental

Carrageenans

Carrageenan samples were obtained from a variety of manufacturers, including samples from both the food industry (Eurogum (Denmark), CP Kelco (USA), Tate & Lyle (United Kingdom)) and chemical suppliers [Merck (Germany), Alfa Aesar (USA), Biosynth (Switzerland), Dextra Laboratories (United Kingdom)]. A total of 16 samples was included into the initial characterization, before selected samples were further characterized by partial enzymatic hydrolysis and rheology. The individual samples were named by using a descriptor for the carrageenan type (KC for κ-carrageenan, IC for ι-carrageenans, LC for λ-carrageenans and C for unspecified carrageenans) and consecutive numbers which correspond to different manufacturers. According to the manufacturer's specifications, 3 κ-carrageenans (KC1-3), 5 ι-carrageenans (IC1-5), 6 λ-carrageenans (LC1-6), and 2 unspecified carrageenans (C1 & C2) were present.

Other materials

Deuterium oxide (≥ 99.9% D) was purchased from Deutero (Germany), dimethyl sulfoxide (≥ 99.5%) from Carl Roth (Germany), and 35% (v/v) hydrogen peroxide from J.T. Baker (USA). Sodium dihydrogen phosphate dihydrate, Isopropyl β-D-1-thiogalactopyranoside (ultrapure), ammonium formate (≥ 99%) and acetonitrile (MS-grade) were obtained from VWR International (Germany). Disodium hydrogen phosphate (≥ 99%) was purchased from Honeywell (USA), and ampicillin from AppliChem (Germany). Neocarrabiose-4-sulfat sodium salt (≥ 95%) and Neocarratetraose-4,4-disulfate disodium salt (≥ 95%) were purchased from Biosynth (Switzerland). Other chemicals were obtained from Merck (Germany). For all experiments, chemical solvents with suitable purity and Milli-Q water were used.

NMR spectroscopy

NMR experiments were conducted by using a Bruker Avance Neo 400 MHz spectrometer, which was equipped with a temperature-controlled 5 mm-probe head. The spectra were recorded at a temperature of 65 °C and a standard ^{1}H pulse program from Bruker ('zg90') was used. The relaxation delay (d_1) was set to 25 s, the acquisition time was 2 s, and a total of 16 (or 32) scans were performed. For the analysis, carrageenan solutions with a concentration of

5 mg/mL were prepared in deuterium oxide and treated in a water bath at 70 °C until complete dissolution of the sample. As an internal reference, 0.5 µL of dimethyl sulfoxide was added (2.696 ppm, according to van de Velde et al. [32]). The signals of the anomeric protons of the D/DA-unit can be used to identify the different types of carrageenan [32].

Molecular weight

The weight average molecular weight (M_W) of the carrageenans was determined by using a Hitachi system (Merck, Germany) with a refractive index detector (L-7490, Hitachi, Merck, Germany) and a multi angle laser light scattering (MALLS) detector (SLD7100, PSS Polymer Standards Service, Germany). The system was equipped with a TSKgel G6000PW_{XL} column (Tosoh Bioscience, Japan) and the column temperature was set at 60 °C. 0.1 M lithium chloride was used as the eluent to prevent aggregation of the carrageenans, as recommended by Lecacheux et al. [33], with an isocratic flow rate of 0.5 mL/min. The M_W of the samples was determined using a refractive index increment of 0.115 g/mL [33]. For the analysis, carrageenan solutions with a concentration of 2 mg/mL were used. The sample solutions were prepared in 0.1 M lithium chloride (eluent) and treated in a water bath at 60 °C until the polysaccharides were completely dissolved.

Inductively coupled plasma atomic emission spectrometry (ICP-OES)

For the ICP-OES analysis of associated cations as well as sulfur, 10 mg of carrageenan were dissolved in 10 mL of 1% (v/v) nitric acid. The solutions were analyzed on a Varian 715-ES (Agilent Technologies, USA) or an iCAP 7000 instrument (Thermo Fisher Scientific, USA). The instrument was calibrated with standard solutions of sodium, potassium, calcium and sulfur, covering a concentration range of 0.25—50 mg/L. Based on the sulfur content, the sulfate content of the carrageenans was calculated.

Sulfate analysis

To release the sulfate groups, the polysaccharide was subjected to acid hydrolysis. For this purpose, 25 mg of the carrageenan sample were mixed with 2.25 mL of 2.3 M trifluoroacetic acid and 0.25 mL of 35% (v/v) hydrogen peroxide. Hydrolysis was carried out for 16 h at 100 °C. Subsequently, the samples were cooled to room temperature (20 °C) and then centrifuged for 10 min at 220 × g. After centrifugation, 250 µL of the supernatant was dried by evaporation. The dried residue was dissolved in 3.25 mL of water and treated in a water bath at 70 °C for 15 min [34]. Sulfate analysis was carried out on a Dionex ICS-6000 system (Thermo Fisher Scientific, USA) equipped with a conductivity detector and a Dionex AERS 500e suppressor (4 mm, Thermo Fisher Scientific, USA) to suppress the conductivity of the eluent. 30 mM sodium hydroxide solution was used as the eluent with an isocratic flow rate of 0.38 mL/min. Separation of inorganic ions was carried out on a Dionex IonPac AS10 column (250 × 4 mm, particle size 8.5 µm, Thermo Fisher Scientific, USA) and the column temperature was set at 30 °C.

Production of recombinant enzymes

Two carrageenases from *Zobellia galactanivorans* (ZGAL_236 (κ-carrageenase) and ZGAL_4265 (ι-carrageenase), according to Ficko-Blean et al. [35]) were cloned and heterologously expressed in this study. Genes were amplified from genomic DNA (DSM 12802, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany) by PCR using the Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific, USA). The primers used are listed in Table S1. Specific overhangs were added to the genes, PCR products were purified by using the MicroElute Cycle-Pure Kit (Omega Bio-Tek, USA), and cloned into the pLIC-SGC1 vector (plasmid #39187, Addgene, USA) by ligation-independent cloning [36]. The vector was previously linearized by BseRI and the gene vector constructs were transformed into *E. coli* NovaBlue. The sequence of the plasmids containing the gene of interest was verified by Sanger sequencing (Eurofins, Germany).

For protein expression, *E. coli* (BL21) cells (Thermo Fisher Scientific, USA) were transformed with the plasmids and selected on an agar plate containing 100 µg ampicillin/mL. A single colony was used to inoculate 5 mL of LB medium (100 µg ampicillin/mL). The cells were grown over night at 37 °C with constant shaking (225 rpm). The suspension was transferred into 400 mL of LB medium (100 µg ampicillin /mL) and grown another 4 h (37 °C, 225 rpm). Overexpression was induced by adding 80 µL of 500 mM isopropyl β-D-1-thiogalactopyranoside solution. The culture was incubated over night at 20 °C with constant shaking (225 rpm). Subsequently, cells were removed by centrifugation (4.000 × g) and the cell pellet was resuspended in lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, pH 8.0). Bacterial cells were lysed by sonication (pulse 3 × 20 s, amplitude 50%, pause 59,9 s) using a Sonifier W-250 D (Branson Ultrasonics, USA) and the lysate was centrifuged at 4 °C and 14.000 × g for 30 min. The supernatant was then used for immobilized metal affinity chromatography to purify the overexpressed proteins.

For protein purification, 4 mL of a Ni–NTA resin (Thermo Fisher Scientific, USA) were used, pre-equilibrated with wash buffer (2 mM sodium phosphate, 12 mM sodium chloride, 10 mM imidazole, pH 8.0). The supernatant was

added to the Ni–NTA-resin and incubated at 4 °C for 30 min. After incubation the resin was washed twice with 4 mL of wash buffer. The proteins were eluted by adding 5 mL of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole, pH 8.0).

Partial enzymatic hydrolysis

1% (w/v) carrageenan suspensions were prepared and treated in a water bath at 60 °C until the polysaccharides were completely dissolved. For partial enzymatic hydrolysis, 250 µL of the carrageenan solution were mixed with 200 µL of water and 50 µL of enzyme solution. The samples were incubated for 24 h at 37 °C with constant agitation. After incubation, the enzymes were inactivated at 95 °C for 10 min. For comparison, carrageenan solutions without the addition of enzyme (250 µL of 1% (w/v) carrageenan solution, 250 µL of water) and enzyme solutions without the addition of polysaccharide (50 µL of enzyme solution, 450 µL of water) were prepared.

HPLC–MS

The analysis of enzymatically released oligosaccharides was carried out on a LCMS-2020 system (Shimadzu, Japan) equipped with an ACQUITY UPLC BEH amide column (2.1 mm × 150 mm, particle size 1.7 µm, Waters, USA). 20/80 (v/v) acetonitrile/water (A) and 80/20 (v/v) acetonitrile/water (B) were used as eluents. Both eluents contained 10 mM ammonium formate and 0.2% formic acid and the following gradient program was applied: 5 min at 100% B, in 26 min linear to 20% B, in 1 min back to 100% B and finally 8 min at 100% B. The analysis was carried out at a flow rate of 0.4 mL/min and a column temperature of 35 °C was used. MS detection with electrospray ionization was carried out in negative full scan mode (*m/z* 250–1000). For sample preparation, 50 µL of the hydrolysate were mixed with 150 µL acetonitrile to precipitate undigested high molecular weight material and to adjust the solvent composition. The samples were centrifuged for 4 min at 16.100 × g and the supernatant was used for HPLC–MS analysis.

HPSEC-RI

For HPSEC analysis, 100 µL of the hydrolysate were mixed with 150 µL of 0.1 M lithium chloride solution (eluent). The analysis was carried out on a Hitachi system (Merck, Germany) equipped with a TSKgel G6000PW$_{XL}$ column (Tosoh Bioscience, Japan). 0.1 M lithium chloride was used as the eluent with an isocratic flow rate of 0.5 mL/min. The analysis was performed at a temperature of 60 °C and a refractive index detector (L-7490, Hitachi, Merck, Germany) was used for detection.

Rheology

To assess the rheological characteristics of the seven commercial carrageenan samples a Physica MCR 301 (Anton Paar GmbH, Austria) rheometer was used. The rheological measurements were conducted in pure polysaccharide solutions in Milli-Q water and upon addition of $CaCl_2$ (Carl Roth GmbH & Co. KG, Germany) to characterize the gelling behavior. Calcium chloride was chosen due to the typical use of carrageenan in dairy products, which are rich in calcium ions. Each carrageenan sample was weighed to obtain a suspension with a polysaccharide concentration of 5 mg/mL. The polysaccharides were completely dissolved by heating to a temperature of 60 °C. For the samples containing 0.1 M $CaCl_2$, the salt and carrageenan were weighed simultaneously and were dissolved subsequently. All samples were maintained at 60 °C until transferred onto the measurement geometry of the rheometer. The measurement geometries were preheated to 60 °C. Once the sample was loaded, the temperature was lowered to 25 °C within 7 min. To allow for sample relaxation or gelation, the temperature was kept constant for 5 min prior to initiating the frequency sweep. The frequency sweep to determine the storage modulus G' and the loss modulus G'' took 25 min. To prevent water evaporation during all measurements, the sample was overlaid with a low viscosity paraffin oil (Carl Roth GmbH & Co. KG, Germany). More details on this method can be found in [16]. Due to the absence of gelation of the samples without added salt, the measurements were carried out using a double gap geometry (DG26.7-SN97573, Anton Paar GmbH, Austria), which is suited for less viscous samples. The amplitude γ was set to 10% during the frequency sweep between 0.1 and 10 Hz to ensure that the strain applied remained within the linear viscoelastic region. In case of the gel-forming samples containing $CaCl_2$, a plate-plate geometry (PP25-SN19091, Anton Paar GmbH, Austria) with a diameter of 25 mm and a constant gap width of 1 mm was used. The amplitude γ during the frequency sweep had to be set to 2% to remain the strain within the linear viscoelastic region. It should be noted that gel formation can potentially lead to an inaccurate measurement due to volume changes during gelation [37]. Therefore, the normal force was carefully monitored during the cooling process and the measurement. No significant deviation was detected indicating that gelation did not impact the accuracy of the measurement. Triplicate measurements were conducted for each sample and the corresponding standard deviation was

calculated. The graphs of the storage and loss moduli for each sample are provided in the supplementary materials.

Results & discussion

To study the structural and functional heterogeneity of commercial carrageenans, 16 samples were purchased from different suppliers. The carrageenans were classified into different types (see Section "Carrageenans") and intended for use as laboratory chemicals or food additives. All samples were analyzed for their structural composition by using NMR spectroscopy, HPSEC-RI/MALLS, ICP-OES, and ion chromatography. Based on the results, selected carrageenans were subjected to further analysis by partial enzymatic hydrolysis and rheology.

Compositional analysis

The results of the NMR spectroscopic analyses of the structural units are shown in Table 1. The characteristic signals of the D-/DA-units were assigned according to van de Velde et al. [32] and signal integrals were used to calculate the portions of the individual structural elements. Only κ-units were detected in all κ-carrageenan samples, whereas the NMR spectrum of IC1, IC2, IC4, and IC5 solely contained signals which resulted from ι-units (exemplary spectra are shown in Fig. S1). However, IC3 contained 73% ι-units and 27% κ-units suggesting the presence of a carrageenan mixture or a hybrid carrageenan. Notably, none of the λ-carrageenans contained signals of λ-units. LC1, LC3, LC4, and LC5 were composed of comparable portions of κ- (42–47%), ι- (31–37%) and ν-units (21–30%), whereas LC2 contained 72% κ-units and 28% ι-units. The two unspecified carrageenans C1 and C2 also contained κ- and ι-units (C2) or κ-, ι-, and ν-units (C1). Therefore, our results show that the analyzed commercial λ-carrageenans as well as the unspecified carrageenans are structurally heterogeneous polysaccharides.

Table 1 Carrageenan composition of the commercial samples used in this study as determined by ^{1}H NMR spectroscopy

Sample	Structural unit (portion, mol%)	Sample	Structural unit (portion, mol%)
KC1	κ	LC1	κ (47), ι (31), ν (22)
KC2	κ	LC2	κ (72), ι (28)
KC3	κ	LC3	κ (42), ι (37), ν (21)
IC1	ι	LC4	κ (42), ι (33), ν (24)
IC2	ι	LC5	κ (44), ι (33), ν (23)
IC3	κ (27), ι (73)	LC6	κ (59), ι (41)
IC4	ι	C1	κ (37), ι (33), ν (30)
IC5	ι	C2	κ (59), ι (41)

The results also demonstrate that it should not be assumed that commercial λ-carrageenans actually contain λ-units. Furthermore, the samples KC2, IC4, and C2 contained a signal at 5.40 ppm, whereas IC3 contained a signal at 5.22 ppm. The corresponding signals could not be assigned to any carrageenan unit. By comparison with different standard compounds, these signals were assigned to sucrose (5.40 ppm) and glucose (5.22 ppm). These sugars were most likely added to standardize the viscosity of the individual carrageenans. Therefore, the presence of mono- and dimeric sugars must also be taken into account when commercial carrageenans are used to study physiological effects. The results of the molecular weight analyses by HPSEC-RI/MALLS are shown in Table S2. The M_W and the polydispersity index of the samples as well as the chromatograms obtained for the samples (Fig. S2) demonstrated that all carrageenans had a rather high molecular weight and a rather narrow molecular weight distribution. Both parameters were in a range typical for carrageenans [33, 38–40].

The contents of potassium, calcium, and sodium cations (determined by ICP-OES) are shown in Table 2. As expected, the three κ-carrageenan samples mainly contained potassium ions besides smaller amounts of sodium and calcium ions. The ι-carrageenan samples were more heterogeneous, although potassium was the predominant cation in all five ι-carrageenan samples. In addition, IC1, IC3, and IC4 showed significant amounts of calcium, whereas for samples IC2 and IC5 significant amounts of sodium were detected. The observations made for κ- and ι-carrageenan are in good agreement with the literature [7]. The λ-carrageenans and the unspecified samples showed even more variation.

Table 2 Contents of K^+, Na^+, and Ca^{2+} (determined by ICP-OES) of the carrageenans used in this study

Sample	K^+, g/100 g	Na^+, g/100 g	Ca^{2+}, g/100 g
KC1	6.84	0.10	0.82
KC2	7.44	0.08	0.64
KC3	6.81	0.37	0.30
IC1	4.45	0.07	4.00
IC2	4.26	3.15	1.34
IC3	5.71	0.09	2.65
IC4	3.84	1.37	2.96
IC5	5.43	4.46	0.04
LC1	0.98	0.25	5.48
LC2	5.73	0.30	2.33
LC3	1.77	6.12	0.29
LC4	5.36	5.79	0.13
LC5	2.46	6.25	0.26
LC6	1.72	6.59	0.16
C1	1.69	5.58	0.37
C2	4.18	2.39	0.11

Notably, the carrageenans LC3, LC5, and LC6 as well as C1 mainly contained sodium ions. Potassium was observed as the predominant cation in LC2 and C2, whereas sample LC1 showed high amounts of calcium. Furthermore, a 1:1 mixture of potassium and sodium was detected for sample LC4. The results demonstrate that commercial carrageenans show significant heterogeneity with regards to the associated cations. The contents of the three cations in combination with the sulfate contents also suggest that a slight excess of salt is present in all carrageenans. This is most likely caused by the inclusion of some of the salt present during the manufacturing (inclusion during precipitation or gel pressing). Because the cation type in the carrageenans is largely dependent on the conditions during extraction, it can be concluded that significantly different methods were used during the production of the analyzed samples. The results also demonstrate that the cation type must be taken into account when the functional properties of carrageenans are being analyzed because it may influence aggregation [8].

The sulfate contents of the carrageenans are shown in Table 3. Generally, comparable sulfate contents were obtained by using the two methods, but some differences were observed between the carrageenan types. For the κ-carrageenan and unspecified carrageenan samples, the sulfate contents calculated from ICP-OES were slightly lower than from the IC analysis, whereas no trend was observed for ι-carrageenans. In contrast, the sulfate contents determined by ICP-OES were in some cases higher than the ones determined by IC in case of the λ-carrageenan samples. Nevertheless, both methods are suitable to obtain information on the degree of sulfation. As expected, the ι-carrageenan samples contained higher sulfate contents than the κ-carrageenan samples which is caused by the presence of a second sulfate group at the DA-unit. However, IC3 and IC4 showed a clearly lower sulfation degree. This can be explained by the presence of κ-units (IC3) as well as the presence of glucose and sucrose (IC3 and IC4). Furthermore, the presence of sucrose also explains the sulfate content of C2 which was lower than expected for a carrageenan with 41% ι-units. Although the obtained values for κ- and ι-carrageenans were slightly lower than in the literature [34], the results confirm the carrageenan type determined by NMR spectroscopy. The sulfate contents obtained for the LC samples were all below the theoretical value for λ-carrageenan (calculated from the mass fraction of sulfate in a carrageenan with strictly repeating G2S-units and D2S,6S-units). This is in good agreement with the absence of λ-units in the NMR spectra. For LC2 and LC6, the sulfate contents were in good agreement with the contents which were expected based on the NMR spectroscopically determined carrageenan composition. The samples LC1, LC3, LC4, LC5, and C1 showed higher degrees of sulfation which is in good agreement with the occurrence of ν-units (trisulfated repeating unit) in these carrageenans. Therefore, the determination of the sulfate content is in some cases suited to confirm the carrageenan types and the purity of the carrageenans. However, it cannot provide unambiguous information on the structural composition of carrageenans without further analyses. This is for example illustrated by the mean sulfate content of the ι-carrageenans (IC1-5) and the λ-carrageenans (LC1-6) which is very similar between the two groups.

Table 3 Sulfate content of the commercial carrageenans used in this study

Sample	Sulfate content, % (w/w)	
	IC	ICP-OES
KC1	19.4 ± 0.3	18.1
KC2	17.1 ± 0.1	16.2
KC3	17.8 ± 0.1	16.4
IC1	29.6 ± 0.3	28.2
IC2	29.3 ± 0.4	28.8
IC3	22.7 ± 0.3	21.7
IC4	23.3 ± 0.9	24.7
IC5	29.5 ± 0.3	30.1
LC1	29.3 ± 0.7	29.4
LC2	22.6 ± 0.1	21.9
LC3	26.9 ± 0.4	29.7
LC4	31.3 ± 0.4	35.1
LC5	27.7 ± 0.4	32.5
LC6	24.0 ± 0.3	26.7
C1	29.6 ± 0.1	28.9
C2	17.9 ± 0.4	17.4

The contents were determined by ion chromatography (IC) and by using the sulfur content obtained from the ICP-OES measurements

Detailed characterization of selected carrageenans

The compositional analysis of the carrageenans showed significant heterogeneity with regards to the structural elements and cations present. However, the methods used cannot differentiate between mixtures of individual carrageenans and hybrid carrageenans. Therefore, an enzymatic chromatographic approach was developed to gain further insights into the structural composition and five potential hybrid carrageenans were selected for a more detailed characterization: IC3, LC1, LC2, C1, C2. These samples were selected because they showed varying ratios of κ- and ι-units (LC2: 72:28, C2: 59:41, IC3: 27:73) and contained ν-units as well as different portions of κ- and ι-units (LC1 & C1). To gain insights into their functionality, the rheological properties of the selected carrageenans were also analyzed and compared to KC1 and IC1.

Characterization after partial enzymatic hydrolysis

To obtain more detailed information about the presence of carrageenan mixtures or hybrid carrageenans, an analytical approach based on partial enzymatic hydrolysis followed by chromatographic analysis was developed. To selectively cleave κ- and ι-units, a κ-carrageenase and a ι-carrageenase from *Zobellia galactanivorans* [35] were heterologously expressed in *E. coli*. The activity as well as the specificity of the two enzymes were tested by hydrolyzing a 0.5% solution of KC1 and IC1 at 37 °C for 24 h. To gain insights into the extent of carrageenan hydrolysis, the hydrolysates were analyzed by HPSEC-RI (Fig. 1). This approach allows for the reliable detection of general hydrolytic activity as well as the detection of high molecular weight blocks which are resistant to hydrolysis by the individual enzymes. To analyze the oligosaccharides liberated by κ- or ι-carrageenase, the hydrolysates were also analyzed by HPLC–MS (Fig. 1). A HILIC column was selected for chromatographic separation of the carrageenan oligosaccharides and MS detection was carried out after electrospray ionization in negative mode. By using this setup, dimeric repeating units as well as oligosaccharides with a higher molecular weight could be detected.

The HPSEC-RI chromatograms clearly demonstrated that the κ-carrageenase completely hydrolyzed KC1, because the high molecular weight peak disappeared and later eluting low molecular weight compounds were formed. In contrast, a peak shift was observed after the incubation of IC1 with κ-carrageenase. This is most likely the result of small

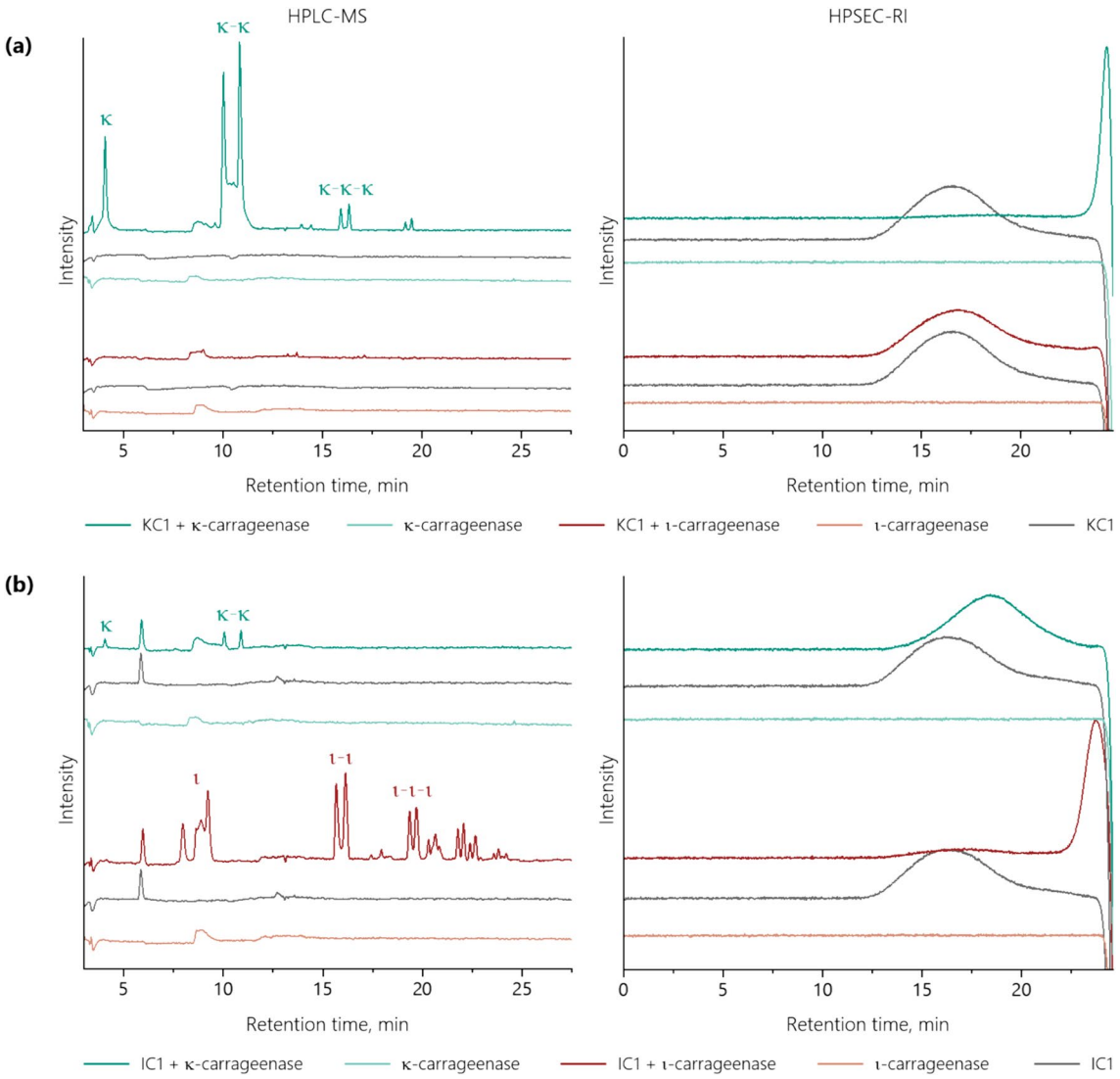

Fig. 1 HPLC–MS and HPSEC-RI chromatograms of KC1 (**a**) and IC1 (**b**) after partial enzymatic hydrolysis with κ- and ι-carrageenase. The chromatograms of the untreated carrageenans and the enzyme solutions are shown for comparison. The letters κ and ι are used as descriptors for the corresponding disaccharide repeating unit

portions of κ structural elements within IC1 which cannot be detected by NMR spectroscopy but are hydrolyzed by the enzyme and lead to a molecular weight reduction. The HPSEC results are also supported by the HPLC–MS oligosaccharide analysis. The HPLC–MS chromatogram of the hydrolysate obtained from the incubation of KC1 with κ-carrageenase contained several peaks which were in part present as double peaks (result of mutarotation of the reducing oligosaccharides). The two early eluting peaks were identified as κ-carrabiose and κ-carratetraose by using standard compounds. The identification of all other oligosaccharides was carried out by analyzing the mass spectra of the corresponding peaks (see Figs. S3 and S4). However, especially the spectra of larger oligosaccharides were characterized by multiple signals (double / triple charge quasimolecular and fragment ions) and significant source fragmentation (cleavage of sulfate). Therefore, only the later eluting small double peak at 16 min was identified as κ-carrahexaose, whereas the other peak could not be assigned to a specific structure. In the κ-carrageenase hydrolysate of IC1, trace amounts of κ-carrabiose and κ-carratetraose were present. This confirms the occurrence of small amounts of κ-units in IC1 as well as the specificity of the κ-carrageenase. ι-carrageenase completely degraded IC1 to low molecular weight products, while no change was observed for KC1. In the HPLC–MS chromatogram obtained from the hydrolysis of IC1 with ι-carrageenase, three peaks could be assigned to ι-carrabiose, ι-carratetraose, and ι-carrahexaose. The mass spectra of the other later eluting peaks contained to many fragments which prevented an unambiguous assignment. However, the two methodological approaches demonstrated the specificity of the enzymes and allowed for the detection of κ and ι structural elements and longer κ- and ι-blocks. Thus, the analytical approach is well suited to analyze potential hybrid carrageenans.

The chromatograms obtained from the enzymatic hydrolysis of IC3 (NMR: 27% κ-units, 73% ι-units) confirm that a high portion of ι-carrageenan structural elements were present (Fig. 2a). This can be seen from the detection of ι-carrageenan oligosaccharides by HPLC–MS and the detection of high portions of low molecular weight compounds by HPSEC-RI after ι-carrageenase hydrolysis. However, the HPSEC-RI chromatograms revealed that IC3 is not completely digested and small portions of polymeric compounds with varying molecular weight are present. κ-carrageenase also released significant amounts of κ-carrageenan-derived oligosaccharides and low molecular weight compounds. Furthermore, the κ-carrageenase digestion resulted in a slight shift of the IC3 peak to higher retention times in the HPSEC-RI chromatogram. The underlying, relatively minor reduction in molecular weight suggested that consecutive κ-units are present in rather small blocks. The broad molecular weight distribution after ι-carrageenase hydrolysis could be caused by some long κ-chains, small alternating blocks of κ- and ι-units or some longer carrageenan chains with alternating κ- and ι-units. Nevertheless, the combination of the results indicated that IC3 is largely composed of a ι/κ hybrid carrageenan which mainly contains long blocks of consecutive ι-units and comparably small blocks of consecutive κ-units.

Comparable elution profiles were obtained for sample C2 (NMR: 59% κ-units, 41% ι-units, Fig. 2b). However, the intensities of the peaks corresponding to oligomeric ι-units, low molecular weight products after ι-carrageenase digestion, and polymeric compounds after κ-carrageenase digestion were lower than for IC3. In contrast, higher intensities were observed for the peaks corresponding to oligo- and polymeric products derived from κ-units. This is most likely a result of the higher portion of κ-units in C2 compared to IC3. Therefore, the results from the enzymatic hydrolysis showed that C2 is largely composed of a κ/ι hybrid carrageenan with rather long blocks of ι-units and comparably short blocks of κ-units.

For sample LC2 (NMR: 72% of κ-units, 28% of ι-units), significant amounts of κ-oligosaccharides and a clear molecular weight reduction were detected after incubation with κ-carrageenase (Fig. 3). In contrast, ι-carrageenase did not have a significant impact on the HPSEC-RI chromatogram (only traces of low molecular weight compounds) and only small amounts of ι-carrageenan oligosaccharides were liberated. This indicates that LC2 is composed of long blocks of κ-units, whereas a major part of the ι-units is most likely present in smaller sections or as single residues. This is also supported by additional oligosaccharides present in the chromatogram of the κ-carrageenase hydrolysate (eluting between 17.9 and 22.3 min) which could be derived from κ-oligosaccharides with single ι-units (mass spectra are shown in Fig. S5). Although the presence of small portions of isolated κ or ι chains cannot be completely excluded, LC2 is mainly composed of a κ-/ι-hybrid carrageenan with long blocks of consecutive κ-units and small sections with ι-units.

The carrageenan samples LC1 (NMR: 47% κ-units, 31% ι-units, 22% ν-units) and C1 (NMR: 37% κ-units, 33% ι-units, 30% ν-units) showed comparable HPLC–MS and HPSEC-RI elution profiles (Fig. 4): Only κ-oligosaccharides were released and only κ-carrageenase resulted in a formation of low molecular weight compounds, while ι-carrageenase only resulted in a slight change of the HPSEC-RI elution profile. The variation in the intensity of oligosaccharides and low molecular weight compounds after κ-carrageenase digestion can be well explained by the higher portion of κ-units in LC1. The undigested residue after κ-carrageenase hydrolysis may result from some complex carrageenan chains, which contain small blocks of κ-units and/or longer blocks of ι-, and ν-units. However, the results suggested that LC1 and

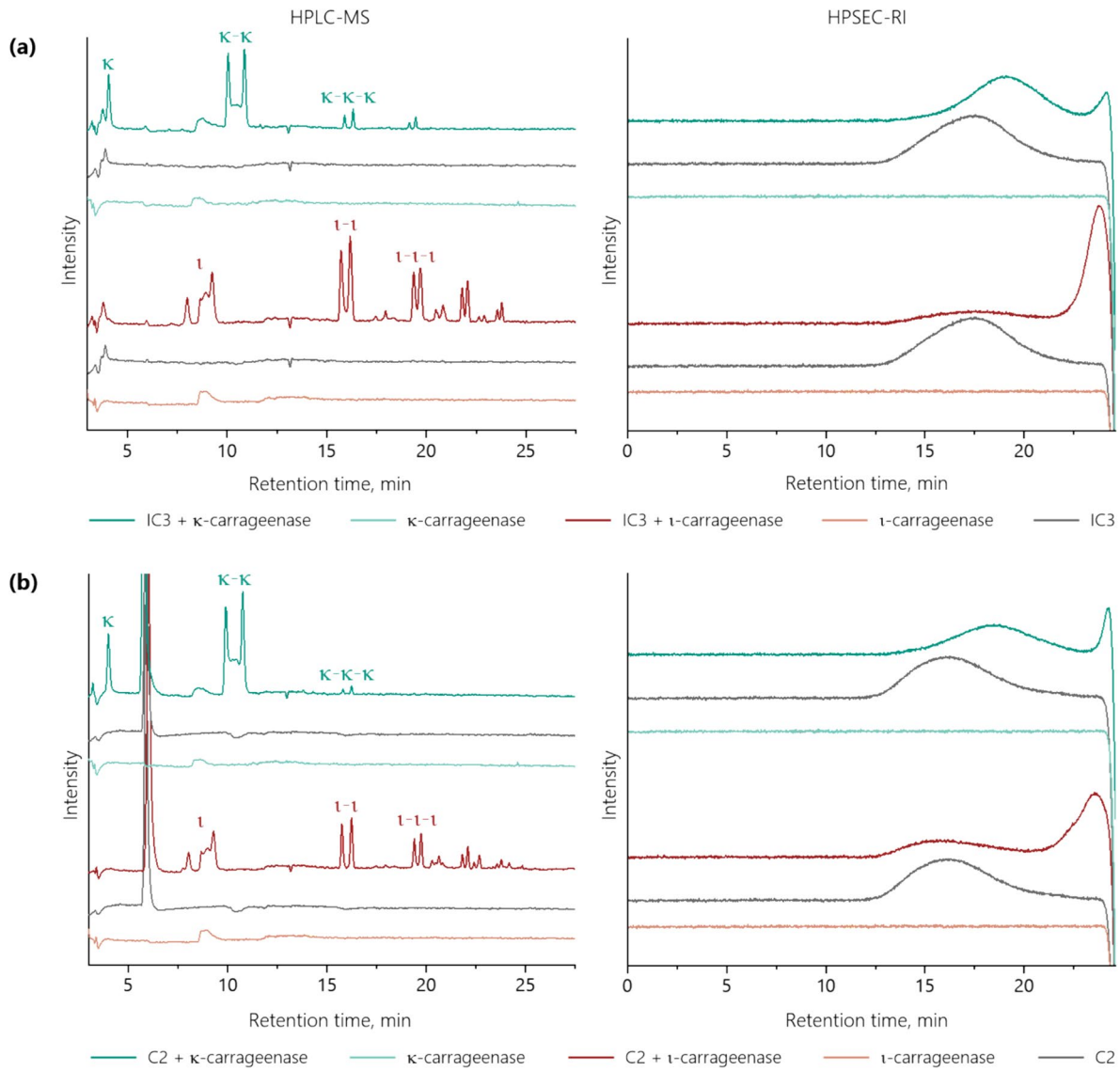

Fig. 2 Chromatograms from HPLC–MS and HPSEC-RI analysis of IC3 (**a**) and C2 (**b**) after partial enzymatic hydrolysis with κ- and ι-carrageenase. The chromatograms of the untreated carrageenans and the enzyme solutions are shown for comparison. The letters κ and ι are used as descriptors for the corresponding disaccharide repeating unit

C1 are hybrid carrageenans with long blocks of κ-units, which are intercepted by short blocks of ι- and ν-units. This observation is also supported by the presence of an additional oligosaccharide in the κ-carrageenase hydrolysate (eluting at 17.9 min, for mass spectra see Fig. S6 and S7).

Altogether, the chromatographic analysis after hydrolysis with selective carrageenases confirmed the structural heterogeneity of commercial carrageenans and revealed differences and similarities between the investigated samples. The results demonstrate that the developed approach can be used to obtain detailed information on the structural composition of carrageenans and is thus well-suited to complement existing methods. To gain further insights into potential variations in the functional properties of the individual polysaccharides, the rheological properties of the five carrageenans were analyzed and compared to KC1 and IC1.

Rheological characterization

The rheological characterization aims to establish a connection between the structural composition of the commercial carrageenan samples and their functional properties. Given that gel formation is the most relevant functional property for industrial applications, this aspect was investigated. Unfortunately, the definition of a gel is not uniform across different scientific fields. As a result, there is no universally accepted gel definition. According to

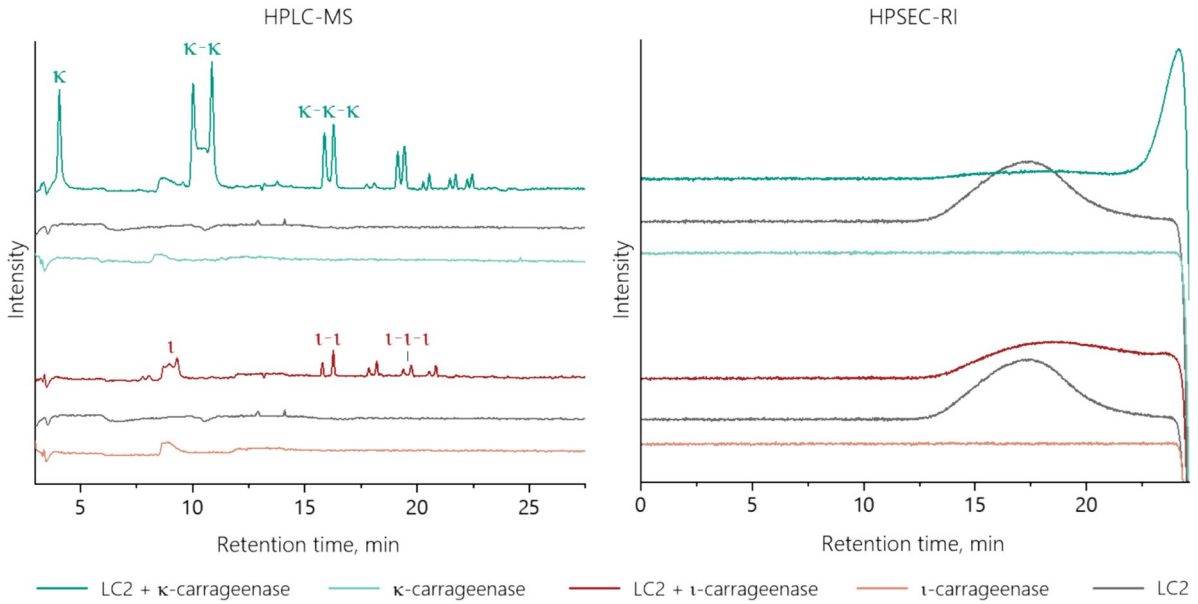

Fig. 3 Chromatograms from HPLC–MS and HPSEC-RI analysis of LC2 after partial enzymatic hydrolysis with κ- and ι-carrageenase. The chromatograms of the untreated carrageenans and the enzyme solutions are shown for comparison. The letters κ and ι are used as descriptors for the corresponding disaccharide repeating unit

Guenet [41], a gel must fulfill two key characteristics: It must consist of a continuous network of connected fibers or other structures and the formation or melting of a thermoreversible gel must occur through a first-order transition. Picout and Ross-Murphy proposed another definition of a gel focusing on the rheological properties [42, 43]. Following their definition, a substance can be stated as a gel if the storage modulus G' is consistently greater than the loss modulus G''. Furthermore, both moduli G' and G'' exhibit near-parallel behavior across a broad range of frequencies. The storage modulus G' defines the elastic properties of the material. It reflects the mechanical energy stored inside the sample during deformation. In contrast, the loss modulus G'' characterizes the viscous behavior of the material and represents the energy dissipated during flow under shear stress. For characterizing elastic materials, the loss factor tanδ is a commonly used parameter. It is defined as the ratio of the loss modulus G'' to the storage modulus G' (Eq. 1).

Equation 1: Definition of the loss factor tanδ

$$\tan\delta = \frac{\text{Loss Modulus} G''}{\text{Storage Modulus } G'}$$

Due to the well-known ability of κ- and ι-carrageenan to form gels when specific ions such as calcium ions are present, a simplified gel definition is used in this study. When the storage modulus G' is clearly greater than the loss modulus G'' over the respective frequency range, a gel is considered to be present.

To characterize the rheological behavior of carrageenan in water, the seven selected commercial carrageenans (KC1, IC1, IC3, C1, C2, LC1, LC2) were first subjected to rheological measurements without $CaCl_2$ addition. Following sample preparation, it was evident that no gelation occurred, and that the samples remained highly fluid. Consistent with this observation, the measured storage moduli G' were correspondingly low remaining below 1 Pa (Fig. S8). The lack of data points at higher frequencies, particularly for sample LC2 and IC3, can be attributed to the inability to measure such an excessively low storage modulus. All values of the loss modulus G'' exhibit a linear increase with increasing frequency, consistently falling in a range of 0.01 to 1 Pa. In addition, all G'' values exceed their corresponding values of the storage modulus. Consequently, the loss factors tanδ for all samples exceeded 1 in all cases (Fig. 5). Therefore, it can be concluded that gel formation in the absence of salt does not take place, the different samples show predominant viscous behavior, and the rheological properties of the various solutions exhibit no significant differences despite their varying structural composition. These observations are valid for the applied test conditions. However, gelation might occur at lower temperatures or higher carrageenan concentrations.

The addition of $CaCl_2$ to a final concentration of 0.1 M alters the rheological properties of the carrageenan solutions. This is evident in the results of the rheological measurements shown in Fig. 6. The storage modulus G' showed a tremendous increase for all samples and remained at a constant level, except for samples C1 and LC1 (Fig. 6c).

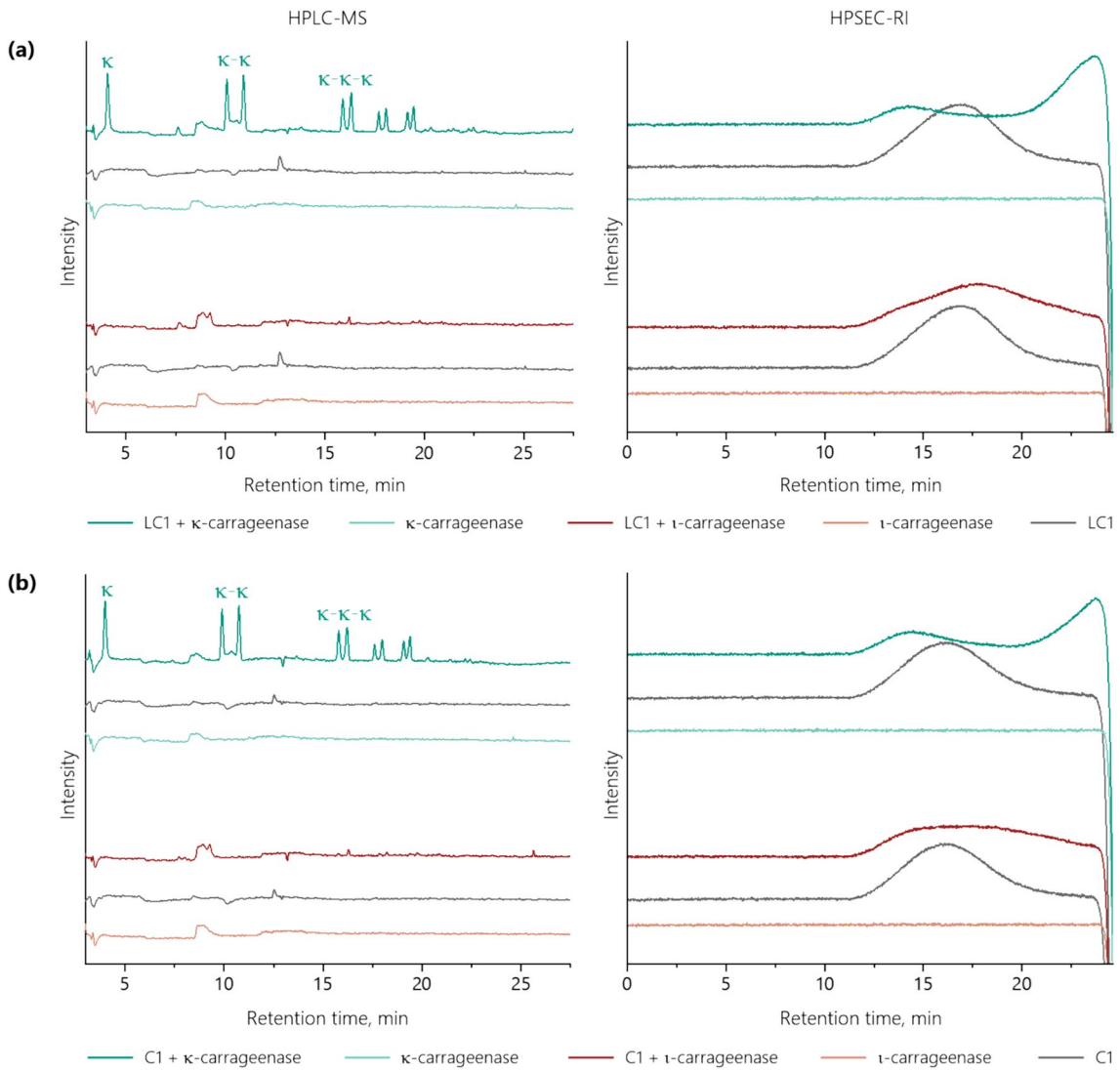

Fig. 4 Chromatograms from HPLC–MS and HPSEC-RI analysis of LC1 (**a**) and C1 (**b**) after partial enzymatic hydrolysis with κ- and ι-carrageenase. The chromatograms of the untreated carrageenans and the enzyme solutions are shown for comparison. The letters κ and ι are used as descriptors for the corresponding disaccharide repeating unit

The pure κ-carrageenan (KC1) exhibited the highest storage modulus and maintained a constant value above 100 Pa, while samples C1 and LC1 retained values of G' below 1 Pa over the frequency range of 0.1 to 10 Hz. Even with the addition of $CaCl_2$, those samples remained highly fluid, which made it difficult to obtain precise measurements with the plate-plate measurement geometry. Therefore, the data of samples C1 and LC1 exhibit high standard deviations and occasionally, measurements with a storage modulus of 0 Pa were observed. The values of the storage modulus G' for the remaining samples IC1, IC3 and C2 were approximately around 10 Pa.

In addition to the storage modulus G′, Fig. 6 also illustrates the values of the corresponding loss modulus G″. Samples KC1 and LC2 exhibited a constant loss modulus between 1 and 10 Pa over the measured frequency range from 0.1 to 10 Hz. Furthermore, the values for samples IC1, IC3 and C2 were slightly lower than those of KC1 and LC2. The loss modulus exhibited a constant behavior up to a frequency of 1 Hz. Beyond this frequency, the values of the loss modulus G'' experienced a serious increase for all three samples. The samples LC1 and C1 showed even lower loss modulus values, starting around 0.2 Pa, which then increased tremendously during the measurement. In comparison to the storage modulus G', the loss modulus values were generally lower, except for samples LC1 and C1.

Consequently, the loss factor tanδ for KC1, LC2, IC1, IC3 and C2 were generally below 1. This observation holds true only up to a frequency of 1 Hz for IC1, IC3 and C2 (Fig. S10). Due to the inaccurate measurements of the storage

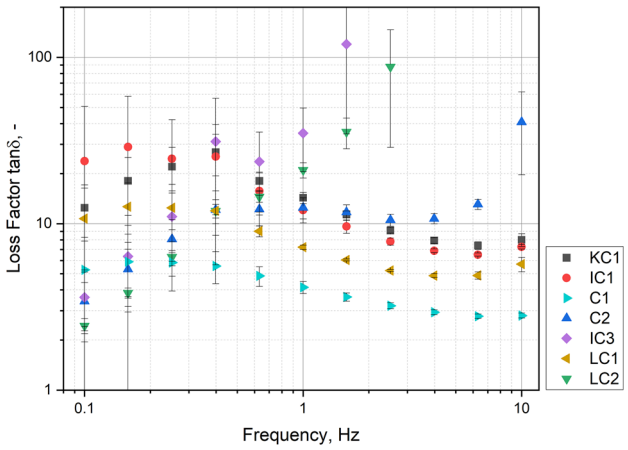

Fig. 5 Loss factors tanδ of the selected commercial carrageenans measured in water. The frequency sweep ranged from 0.1 to 10 Hz with a strain amplitude γ of 2%

modulus for sample C1, it was not feasible to derive reliable loss factors for this sample. Therefore, C1 is not shown in Fig. S10.

Based on these findings, the samples KC1, LC2, IC1, IC3 and C2 formed gels in presence of 0.1 M $CaCl_2$. Unlike the other samples, LC1 and C1 retained their liquid state and did not form gels. Both samples contained ν-units and $CaCl_2$ addition only had a minor influence on their rheological properties. These results indicate that the presence of ν-units in κ/ι hybrid carrageenans, and thus the partial substitution of 3,6-anhydrogalactose with disulfated galactose, has a negative effect on gelation and the development of a gel-like character upon $CaCl_2$ addition. This is in good agreement with data on ι/ν hybrid carrageenans and the observation that D-units do not adopt a helical conformation which is crucial for gel formation [3, 41]. Van de Velde demonstrated that ι-carrageenan loses its ability to form gels when the content of ν-units exceeds 20 mol% [7]. This observation seems to apply also to κ/ι/ν hybrid carrageenans, as a ν-units content of 22 mol% was already sufficient to prevent gel formation. Due to the absence of samples with lower content of ν-units, it remains unclear whether κ/ι hybrid carrageenans exhibit even greater sensitivity to the presence of ν-units than ι-carrageenan.

In addition, more information upon the rheological behavior of the formed gel can be drawn from Fig. 6. The magnitude of the storage modulus G' serves as indicator for the strength of the gel network. Sample KC1 containing pure κ-carrageenan exhibited the highest storage modulus values and consequently formed the most stable gel. The proportion of κ-units appears to influence the magnitude of the storage modulus G'. With increasing content of structural components of the κ-type of carrageenan (IC3 < C2 < LC2 < KC1), the storage modulus increased (Fig. 7). This finding aligns

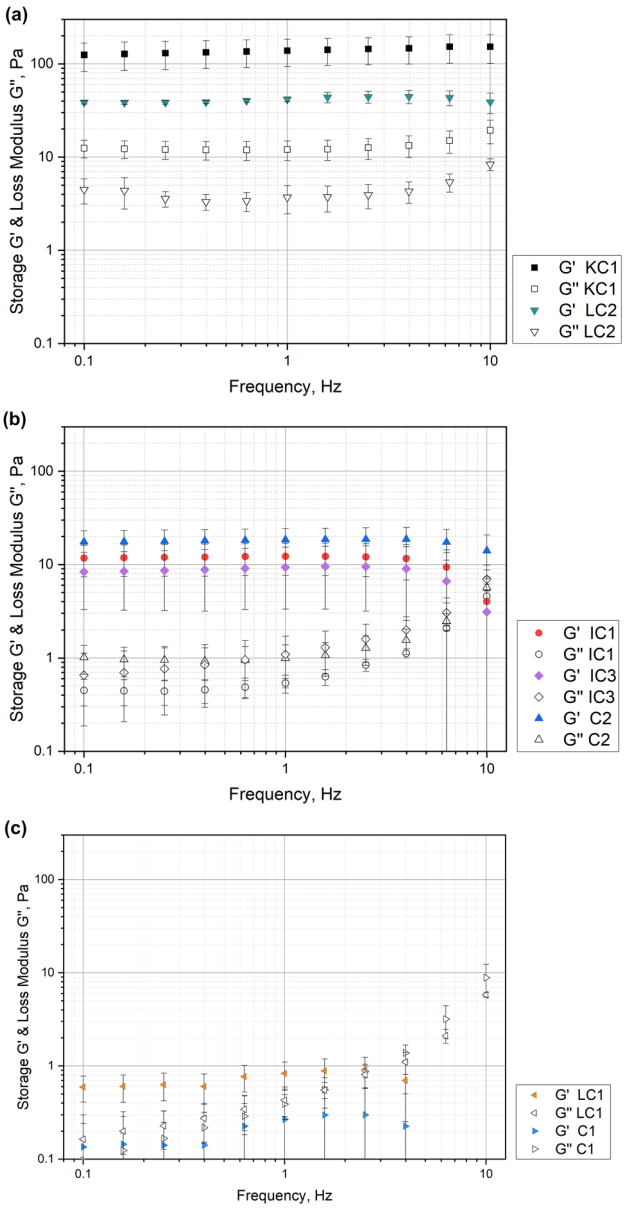

Fig. 6 Storage and loss moduli G' and G'' of the seven commercial carrageenan samples in 0.1 M $CaCl_2$ solution measured at a frequency range between 0.1 and 10 Hz and an amplitude γ of 2%. Graph (**a**) displays the results for the samples KC1 and LC2, graph (b) presents the finding for samples IC1, IC3 and C2, and graph (**c**) shows the data for samples LC1 and C1

with the data reported by Bui et al. [44], where the storage modulus of mixed κ-ι-carrageenan gels is mainly dependent on the portion of κ-carrageenan. Our data demonstrate that commercial hybrid carrageenans exhibit a similar behavior.

Visual examination of the samples confirmed that KC1 produced the most stable gel upon $CaCl_2$ addition. None of the other samples formed a cohesive and self-supporting gel. Additionally, the combined plot of the storage modulus G' and loss modulus G'' in Fig. 6 allows an estimation of the

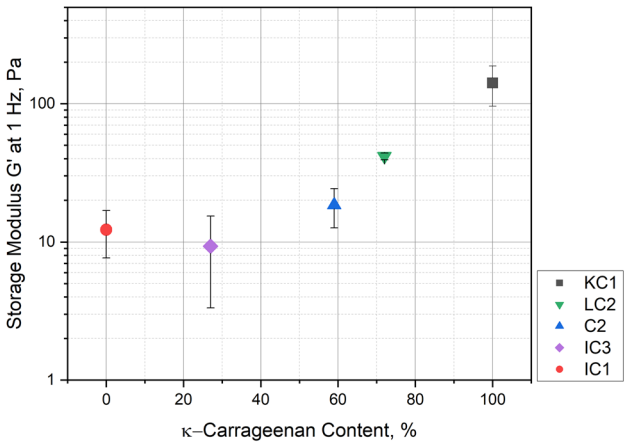

Fig. 7 Relationship between the storage modulus G' measured at a frequency of 1 Hz and the κ-carrageen content

gel point. The point where the storage modulus G' and the loss modulus G'' intersect defines the gel point and marks the transition of the sample's behavior from solid-like to liquid-like. The gel structure can no longer withstand the energy input from the measurement at this critical frequency resulting in the rupture of the gel. This intersection of G' and G'' for samples KC1 and LC2 did not fall within the measurement range of 0.1 to 10 Hz. This further confirms the formation of stable gels in these samples. Furthermore, the gel points for the samples IC1, IC3 and C2 in Fig. 6b were clustered around a frequency of 10 Hz. While the gel points of IC1 and IC3 were indistinguishable, sample C2, with a slightly higher content of κ-units of 59%, exhibited a gel point slightly above 10 Hz. The absence of gel formation in the samples LC1 and C1 eliminates the possibility of a gel point for these samples. Therefore, the gel point's position also supports the correlation between the κ-unit content and the gel properties.

Altogether, the results from the rheological characterization clearly demonstrate that the selected commercial carrageenans not only show heterogeneity in their structural, but also their functional properties. All analyzed hybrid carrageenans showed gelling behavior, except for samples LC1 and C1, which both contain ν-units. Therefore, the gel forming capability of hybrid carrageenans depended on the portion of κ- and the presence of ν-units.

Conclusion

Our results demonstrate that commercially available carrageenans show significant variation with regard to the structure of the polysaccharides as well as with regard to the cations in the carrageenan samples. Furthermore, the carrageenan type specified by the manufacturers is not always in agreement with the actual molecular structure. Furthermore, our results suggested that the investigated λ-carrageenans, as well as one ι-carrageenan and two unspecified carrageenans, were actually hybrid carrageenans with κ-, ι-, and (in part) ν-units. Therefore, a detailed structural characterization is essential when carrageenans are purchased to investigate structure function relationships. Otherwise, the properties cannot be related to specific structural elements and false conclusion might be drawn about the physiological and technological effects of carrageenans. This was further supported by the rheological characterization. In absence of calcium ions, no gelation was observed. In contrast, the addition of $CaCl_2$ at a final concentration of 0.1 M induced gel formation in all samples, except of samples LC1 and C1. The formed gels displayed considerable variations in their characteristics. It was shown that the proportion of κ-units in the hybrid carrageenans corresponds to the value of the storage modulus G' and the location of the gel point, suggesting that the content of κ-units is a key factor for gel formation. Moreover, the presence of ν-units seems to hinder gelation of κ/ι hybrid carrageenans upon $CaCl_2$ addition. The findings of this study emphasize that future studies should focus on an analysis of the properties of well-defined carrageenans. The samples characterized in this study as well as the characterization of carrageenans after partial enzymatic hydrolysis are well suited for this purpose.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-024-04605-w.

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Data availability Data are included as electronic supplementary material. Further data can be made available on request.

Declarations

Ethical approval This article does not contain any studies with human or animal subjects.

Conflict of interest The authors have no conficts of interest to declare.

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