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High-field NMR spectroscopy of cystocarpic and tetrasporic carrageenans from *Iridaea undulosa*

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Abstract

High-field ^{1}H and ^{13}C NMR spectroscopy has been used to characterize the fine structure of the cystocarpic and tetrasporic carrageenans from *Iridaea undulosa*. The proportion of different carrageenans from the κ-family in cystocarpic samples were calculated. In the ^{13}C NMR spectra of the tetrasporic samples, twelve signals of approximately equal intensity were observed, indicating a nearly "perfect" λ-structure.

1. Introduction

Members of the family Gigartinaceae elaborate different carrageenans from karyotypically different generations [1,2]. Studies of the carrageenans from cystocarpic and tetrasporic stages from *Iridaea undulosa* [3] and *Gigartina skottsbergii* [4] showed that the cystocarpic samples elaborate two major products separable by potassium chloride precipitation: one of the products is a soluble, partly cyclized μ/ν-carrageenan, and the other product is a gelling κ/ι-carrageenan. The tetrasporic stages produce sets of λ-carrageenans gelling at high concentrations of potassium chloride.

The carrageenans isolated in similar amounts from cystocarpic *Iridaea undulosa* are a gelling fraction (C_1) and a nongelling fraction (C_4), and traces of two other gelling fractions (C_2 and C_3) [3]. The characteristics of the fractions varied from

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those of a κ/ι-carrageenan masked by many μ/ν-units for C_1 to those of a partially cyclized μ/ν-carrageenan in C_4 [3]. The tetrasporic stage from *Iridaea undulosa* is mainly made up of a λ-carrageenan. The major fraction (T_1, 72.3%) precipitates at 1.0–1.2 M concentrations of potassium chloride. Much less of a second λ-carrageenan (T_2, 3.6%) and a distinct galactan sulfate, containing L-galactose, were also isolated [3].

^{13}C NMR spectra of cystocarpic carrageenans have been routinely used; diads of κ-, ι-, and other carrageenans are fully assigned, and the anomeric resonances of μ- and ν-carrageenans are recognized [5]. On the other hand, ^1H NMR spectroscopy of carrageenans has received less attention since the pioneering work of Welti [6]. In this decade, Knutsen and co-workers confirmed previous assignments and made new ones, thus renewing its applications [7–9].

Herein, we report the use of high-field ^{13}C and ^1H NMR spectroscopy for the elucidation of the carrageenan structures of the main fractions obtained from the cystocarpic and tetrasporic stages of *Iridaea undulosa*.

2. Experimental

The carrageenans isolated from cystocarpic and tetrasporic samples from *Iridaea undulosa* were obtained and fractionated as previously reported [3].

NMR spectra of D_2O solutions were recorded at 70°C using a Varian VXR-400 NMR spectrometer. The C_1 sample contained 3-(trimethylsilyl)-1-propanesulfonic acid (DSS), while the other samples contained 99.9% acetone-d_6 as internal references. Prior to NMR analysis, the molecular weight of the tetrasporic samples (T_1 and T_2) had been reduced by ultrasonic irradiation [10].

The 400-MHz ^1H NMR spectra were recorded using either a 5-mm $^1H/^{19}F$ probe or a 5-mm switchable probe. Water suppression of the C_4, T_1, and T_2 samples was achieved by using an unoptimized WEFT pulse sequence [11]. The parameters were as follows: observe pulse angle, 45°; acquisition time, 2.048 s; spectral width, 4000 Hz; relaxation delay, 0.5–5.3 s; scans, 64 (C_4, T_1, and T_2), 1000 (C_1). Chemical shifts are referenced to Me_4Si by assigning to the residual line of acetone-d_6 a value of 2.20 ppm.

The 100-MHz ^{13}C NMR spectra were recorded using either a 5-mm switchable probe (T_1 and T_2) or a 10-mm broadband probe (C_1 and C_4). The parameters were as follows: pulse angle, 45° (C_1 and C_4), 60° (T_1 and T_2); acquisition time, 0.6 s; spectral width, 25 kHz; relaxation delay, 1.0 s (C_1 and C_4), 0.2 s (T_1 and T_2), and scans, 26 820–110 179. Chemical shifts are referred to Me_4Si by assigning to the central resonance of the methyl carbon of acetone-d_6 a value of 30.4 ppm.

3. Results

The ^{13}C NMR spectra of C_1, C_4, and T_1 are given in Fig. 1. The spectrum of T_2 is not shown because it is almost identical to that of T_1 (see later). The polysaccha-

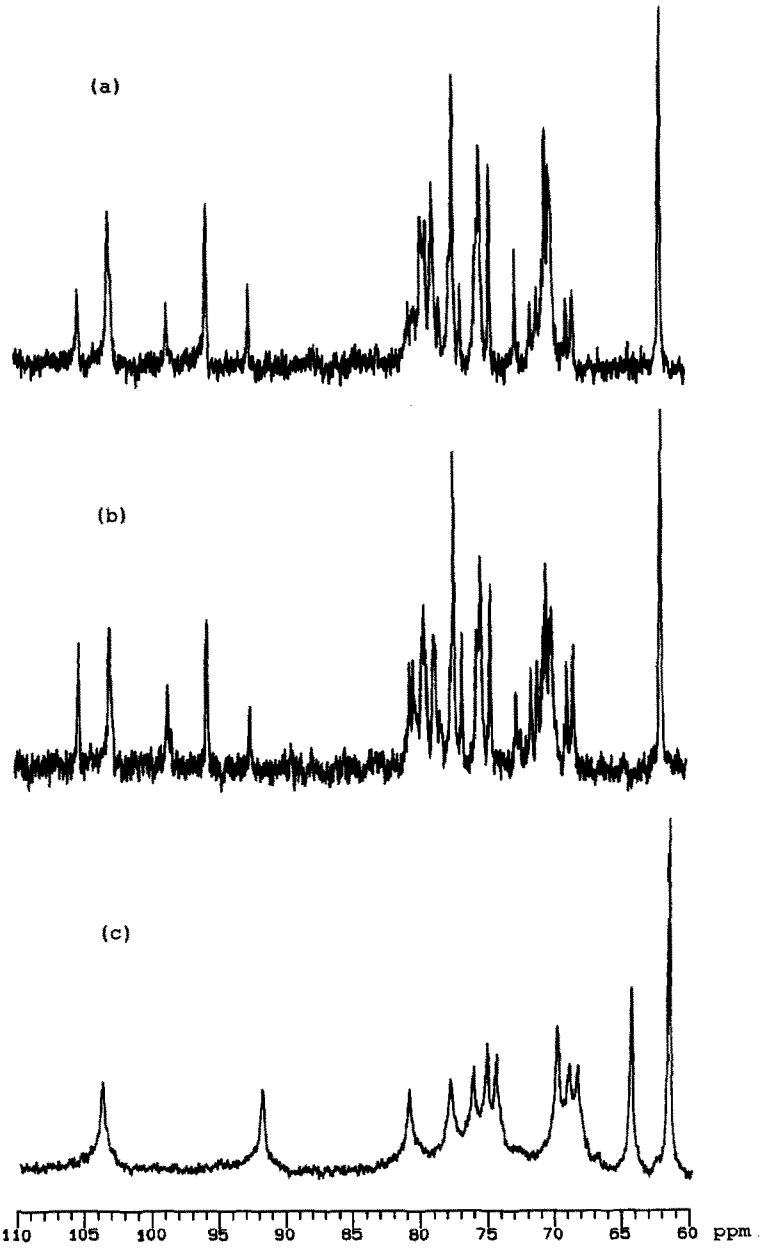

Fig. 1. ^{13}C NMR spectra of carrageenans from *Iridaea undulosa*: (a) C_1, (b) C_4, and (c) T_1.

rides from the tetrasporic plants were treated by ultrasonic irradiation to reduce the viscosity of their solutions prior to spectroscopic analysis [10]. The chemical shifts, putative assignments, and relative areas for the anomeric resonances for cystocarpic carrageenans C_1 and C_4 and tetrasporic carrageenans T_1 and T_2 are

Table 1
Idealized repeating units of carrageenans

	Unit A 3-linked β-galactose	Unit B 4-linked α-galactose
κ	4-sulfate	3,6-anhydro
ι	4-sulfate	3,6-anhydro 2-sulfate
μ	4-sulfate	6-sulfate
ν	4-sulfate	2,6-disulfate
λ	2-sulfate [a]	2,6-disulfate

[a] 70% in the original definition.

listed in Tables 2 and 3, respectively. The spectra of C_1 and C_4 are almost identical, despite the marked difference in their solubility behavior. ^{1}H NMR spectra of the four carrageenan fractions give broad and sometimes overlapping

Table 2
^{13}C NMR spectral data of carrageenans C_1 and C_4

C_1 δ (ppm)	Area [a]	C_4 δ (ppm)	Area [a]	Tentative assignments [b]
105.2	14	105.2	17	Aμ-1(105.3), Aν-1(105.3)
103.0	42	102.9	34	Aι-1(102.7), Aκ-1(103.0)
98.7	7	98.7	12	Bν-1(98.8)
		98.3	4	Bμ-1(98.2)
95.7	29	95.7	24	Bκ-1(95.7)
92.6	9	92.5	10	Bι-1(92.7)
80.6–80.2	s	80.6–80.3	s	Aν-3(80.5)
79.7–79.3	m	79.5	m	Aκ-3(79.3), Bν-4(79.4)
78.9	m	78.8–78.6	m	Bκ-4(78.8), Bι-4(78.8)
78.4	s	78.3	s	Bι-3(78.4)
77.3	l	77.3	l	Bκ-5(77.3), Bι-5(77.6), Aι-3(77.4)
76.8	s	76.7	m	Bν-2(76.3)
75.5	m	75.6–75.5	m	Bι-2(75.5)
75.3	l	75.3	m	Aι-5(75.3), Aκ-5(75.2), Aν-5(75.3)
74.6	m	74.6	m	Aκ-4(74.5)
72.7	s	72.6	s	Aι-4(72.7)
71.6	s	71.6	s	Aν-4(73.9) [c]
71.1	s	71.0	s	Aν-2(70.8)
70.6	s	70.6	s	Bι-6(70.3)
70.4	l	70.4	l	Bι-6(70.3), Aκ-2(70.1), Bκ-2(70.3)
70.1–70.0	m	70.1–69.9	m	Aκ-2(70.1), Bκ-6(70.0), Aι-2(69.9)
68.8	s	68.8	s	Bν-3(68.6), Bν-5(68.6)
68.4	s	68.3	m	Bν-6(68.1)
61.8	l	61.9–61.8	l	Aι-6(61.8), Aκ-6(61.8), Aν-6(61.7)

[a] Area-% of the anomeric peaks, for nonanomeric peaks s, small; m, medium; l, large.
[b] Roman letter A or B refers to a 3-linked β- or a 4-linked α-unit, respectively (ref 12, Table 1); the Greek letter refers to the idealized repeating unit (Table 1). In parentheses, predicted chemical shift (ref 12).
[c] See Discussion.

Table 3
^{13}C NMR spectral data of carrageenans T_1 and T_2

T_1 δ (ppm)	T_2 δ (ppm)	Tentative assignments [a]
103.9(55) [b]	103.9(54)	Aλ-1(103.7)
92.0(45)	92.0(43) [c]	Bλ-1(94.7) [d]
81.0	81.0	Bλ-4(79.1), Aλ-2(79.3)
78.0	78.0	
76.2	76.3	Bλ-2(76.7), Aλ-3(78.4), Aλ-5(75.9)
75.3	75.3	
74.6	74.6	
70.0	70.0	Bλ-3(68.8), Bλ-5(68.6), Bλ-6(68.1)
69.1	69.1	
68.6	68.6	
64.5	64.6	Aλ-4(66.3) [d]
61.7	61.7	Aλ-6(61.6)

[a] For nomenclature, see Tables 1 and 2. In parentheses, predicted chemical shifts (ref 12).
[b] In parentheses, area percent of the anomeric peaks.
[c] In addition, 3% of a peak at 95.4 ppm appears.
[d] See Discussion.

signals (Fig. 2), thus precluding the complete assignment of diad structures. However, in the 5.00–5.60 ppm region, the anomeric protons for the α-linked units (unit B, Table 1) are resolved, which permitted the identification and quantification of diad structures (Table 4).

In addition, the cystocarpic carrageenans have five additional major resonances in the 4.60–5.00 ppm range: two of the signals (4.91–4.92 and 4.83–4.84 ppm) correspond to H-4 of the β-linked galactose (unit A, Table 1) of κ-, ι-, and ν-carrageenans [6,8], and to H-2 of the unit B of ι- (and possibly ν-) carrageenans [6]. The partially resolved signals at 4.67, 4.63–4.64, and 4.62 ppm include the β-anomeric protons, H-4 and H-5 of 3,6-anhydrogalactose residues [6]. It is not known whether ν-carrageenan gives signals in this region.

The tetrasporic carrageenans T_1 and T_2 give only three peaks in the region 4.50–5.00 ppm, at 4.71, 4.59, and 4.54 ppm. They are tentatively assigned to correspond to H-2 of unit B and to H-1 and H-2 of unit A.

Table 5 gives the putative diad composition of the cystocarpic carrageenans calculated by a combination of chemical method [3] and ^1H and ^{13}C NMR spectroscopy. Similar values were obtained by each analysis; nevertheless, it must be considered that the amount of μ-carrageenan computed by direct analysis is less reliable, as it is calculated by difference between the 6-sulfate present in the original polysaccharide and that determined after sodium periodate oxidation. On the other hand, ^1H NMR spectroscopy shows the virtual absence of the μ-carrageenan, suggesting that its proportion was overestimated by substraction of the amounts indicated by colorimetric analysis.

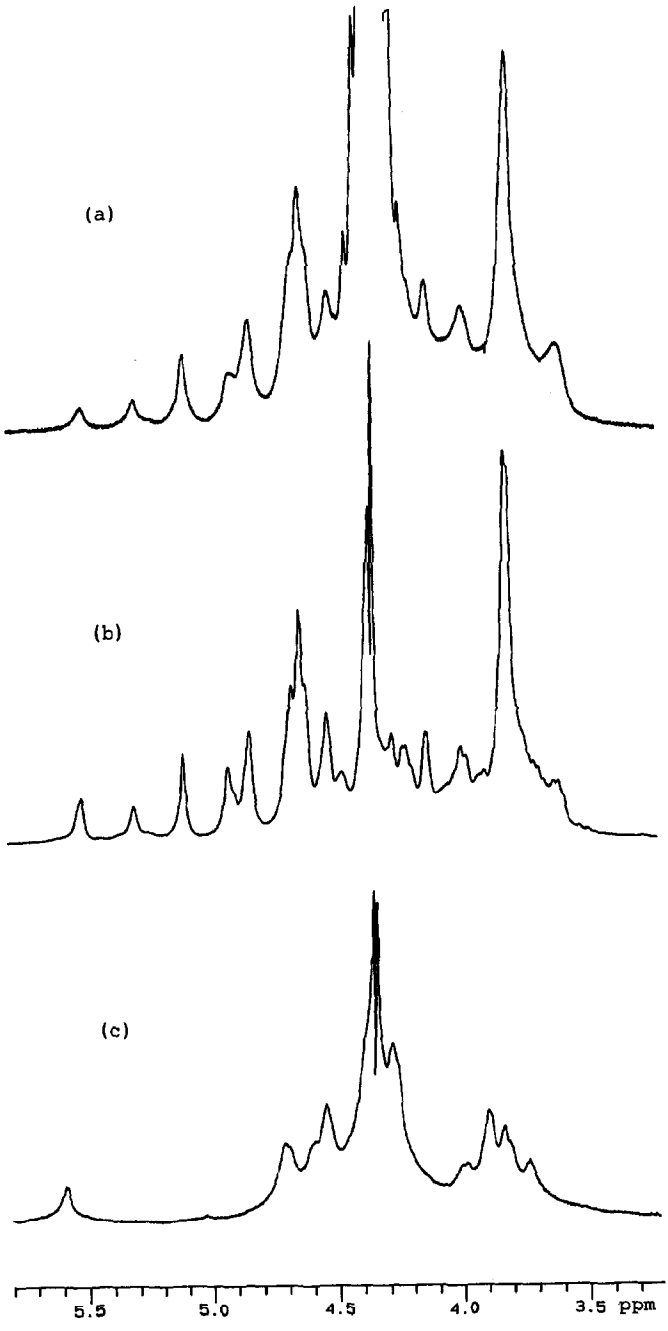

Fig. 2. ^{1}H NMR spectra of carrageenans from *Iridaea undulosa*: (a) C_1, (b) C_4, and (c) T_1.

Table 4
Chemical shifts (ppm) of signals in the ^{1}H NMR of carrageenans in the 5.00–5.60 ppm region

Fraction				Assignment [a]
C_1	C_4	T_1	T_2	
		5.59(100)	5.59(100)	Bλ-1
5.51(20) [b]	5.51(26)			Bν-1
5.29(22)	5.30(21)			Bι-1
5.24(2)	5.24(2)			Bμ-1
5.09(56)	5.10(51)			Bκ-1

[a] For nomenclature, see Tables 1 and 2.
[b] In parentheses, area percent.

Table 5
Composition of the cystocarpic carrageenans in diads

	κ-Diads	ι-Diads	μ-Diads	ν-Diads
C_1				
Analysis [a]	——	69 ——	9	22
^{13}C NMR	62	18		20
^{1}H NMR	56	22	2	20
C_4				
Analysis [a]	——	50 ——	19	31
^{13}C NMR	48	19	8	24
^{1}H NMR	51	21	2	16

[a] Analysis: methylation and determination of 3,6-AnGal, 6-sulfate, and 2,6-disulfate content.

4. Discussion

^{13}C NMR spectroscopy has been used to determine the proportion of κ-, ι-, μ-, and ν-carrageenans in fractions obtained from cystocarpic plants [13]. The most useful data are obtained from the resonances that correspond to the α-anomeric carbons, as the β-anomeric carbons give overlapping signals. ^{1}H NMR spectroscopy has also been used to quantify the proportion of each carrageenan of the κ-family [13], provided that the α-anomeric protons give four resolved signals. Again, the signals for the β-anomeric protons cannot be used to either identify or quantify diads.

To the best of our knowledge, there are no reports of NMR spectroscopy of nonhydrolyzed λ-carrageenans [10,14]. Bremond et al. [15] reported the ^{13}C NMR spectrum of a polysaccharide purported to be a λ-carrageenan; however, there is an overabundance of anomeric signals of variable intensities in the spectrum to permit the identification of the λ-carrageenan structure. Rochas and Lahaye [10] also reported the spectrum of a λ-type carrageenan isolated from *Gigartina canaliculata* depolymerized by ultrasonication, but the spectrum indicated at least six anomeric signals and thus a structural diversity not expected for a classical λ-structure.

In a previous publication [12] we calculated the expected chemical shifts for a λ-carrageenan; the anomeric resonances were 103.7 and 94.7 ppm. These values are close to those reported by Noseda and Cerezo [14] for a room temperature NMR spectral analysis of a partially autohydrolyzed λ-carrageenan. The chemical shifts obtained in the present study (at 70°C) are 103.9 and 92.0 ppm (Fig. 1 and Table 3). The discrepancy in chemical shift values between the anomeric resonance of unit B in the native carrageenan molecule and an autohydrolyzed derivative might be due to differences in the conformation of the two samples. The lower-than-expected chemical shift for the anomeric carbon of unit B suggests that the strong shielding effect of the 2-sulfate of unit A on the anomeric carbon of the α-galactose 2,6-disulfate unit of λ-carrageenan is caused by a modification of the preferred torsional angles around the glycosidic bond (conformational map) [12,16]. The upfield shift of the other glycosidically linked carbon Aλ-3 (Table 3) confirms this suggestion. Other discrepancies appear in nearly all the predicted values for other chemical shifts [12], thus indicating conformational changes induced by sulfation. Most notable is the signal at 64.5–64.6 ppm. This is the region in which the carbon of the hydroxymethyl group usually resonates, but in this case it is assigned to C-4 of unit A. As discussed in a previous publication [12], sulfation of C-2 of unit B produces a notorious upfield shift on the neighboring C-4 of unit A. When C-4 is not sulfated (as in a diad like β-Gal-3,6-AnGal 2-sulfate), its calculated chemical shift is 64.8–65.1 ppm, which is close to the value found for λ-carrageenan.

The upfield shift of C-2 sulfation of unit B on the C-4 of unit A is also observed in the signal appearing at 71.6 ppm in the spectra of the cystocarpic samples. This signal is now assigned to the C-4 of unit A of a ν-carrageenan, appearing 2.3 ppm upfield from that calculated [12]. This resonance was reassigned by comparison of its intensity in the spectra of the cystocarpic samples of *Gigartina skottsbergii* [13] (M. Ciancia, personal communication) and *Iridaea undulosa* (present work) of known composition.

Insofar as we know, these are the first reported ^{1}H NMR spectra of λ-carrageenans. The signal for the anomeric α-proton appears at 5.59 ppm, close to, but separated from, a similar unit in ν-carrageenans (5.51 ppm). The β-anomeric proton resonance is close to the signals of other secondary sulfated protons, which results in the appearance of only three signals downfield of the HOD signal.

The system of cystocarpic carrageenans from *Iridaea undulosa* is composed of two main fractions, C_1 and C_4 [3]. The results of this study indicate that the proportions of various structural diads in these fractions are similar. The samples are composed of κ-diads, with lesser amounts of ι- and ν-diads (Table 5). Little to no μ-carrageenan is present in fractions C_1 and C_4. A small increase in the proportion of ν-diads, with a concomitant decrease in the κ-diads, is observed between the gelling and nongelling fractions. The difference in gelling behavior between the cystocarpic fractions depends on the varied distribution of these diads, since the variation in diad density between the gelling and nongelling fractions is not significant. Therefore, this discontinuity in the structure–solubility relationship disallows the prediction of the gelling properties of carrageenans of

the κ-family based only on the presence of certain diad structures. If the small increase in κ-units at the expense of ν-units occurs by the same biosynthetic pathway, only a cyclization–desulfation sequence may be postulated [17] for the cystocarpic system from *Iridaea undulosa*. As no evidence exists for the enzymic conversion of ι- to κ-carrageenans, it may be suggested that this stage of the seaweed shows two different pathways, one leading to the precursor richer in μ-units, and the other leading to the one richer in ν-units. This scenario is different from the mechanism observed for *Gigartina skottsbergii* [13]. NMR spectral data suggest that the previous analytical data [3] of sulfate and 3,6-anhydrogalactose content were underestimated and that the periodate-labile 6-sulfated units were overestimated.

The ^{13}C NMR spectra of sonicated tetrasporic carrageenans show twelve signals of approximately equal intensity (Fig. 1) for both fractions T_1 and T_2, indicating a nearly "perfect" λ-structure. This means that in our previous paper the sulfate content was underestimated [3]. The spectrum of T_2 is identical to that of T_1, except in the spectrum of T_2 (the minor fraction), an additional signal of low intensity is present at ~95.4 ppm (Table 3), together with other signals of the same intensity in the nonanomeric region. This indicates the presence of an additional structural unit. Methylation analysis, in conjunction with the fact that T_2 contains more sulfate than T_1, suggests that the additional unit arises from extra sulfation of the β-galactose unit in position 4 and/or 6.

Acknowledgments

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