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Carbohydrate Research 325 (2000) 287–299

CARBOHYDRATE RESEARCH

The system of low-molecular-weight carrageenans and agaroids from the room-temperature-extracted fraction of *Kappaphycus alvarezii*

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Received 7 October 1999; accepted 20 December 1999

Abstract

The room-temperature-extracted fraction from the red seaweed *Kappaphycus alvarezii* consists mainly of low-molecular-weight carrageenans, with structural dispersion around a basic κ-pattern. This dispersion results from: (a) low percentages of 3,6-anhydrogalactose and the presence of precursor units; (b) important quantities of 6-*O*-methyl β-D-galactose (4-sulfate) residues; (c) significant amounts of ι-repeating structure, and (d) small amounts of non-sulfated and disulfated β-D-galactose residues. Significant quantities of α-L-galactose units suggest the presence of agaroids, as it has been reported in several other carrageenophytes.

Keywords: Seaweed polysaccharides; Carrageenans; Room-temperature extract; *Kappaphycus alvarezii*; Structure determination; Low molecular weight; L-Galactose

1. Introduction

Kappaphycus alvarezii is a red seaweed of great commercial value that is cultivated mainly in the Far East as raw material for the industrial production of κ-carrageenan (alternating 3-linked β-D-galactose 4-sulfate and 4-linked 3,6-anhydro-α-D-galactose units). Usually, the polysaccharide is extracted with hot, neutral or alkaline, water from the native or alkali-treated seaweed and precipitated with ethanol to produce the commercial carrageenans [1]. Their structure has been extensively studied [2–5]; it consists, predominantly, of a high-molecular-weight κ-carrageenan. When the extraction was carried out under conditions necessary to preclude the cyclization reaction, some precursor units were also detected. A low percentage of ι-structure (alternating 3-linked β-D-galactose 4-sulfate and 4-linked 3,6-anhydro-α-D-galactose 2-sulfate units) (~ 3%) was also present [3–5], possibly forming independent polysaccharide chains [4].

During the alkaline treatment of the seaweed or its further washing, alkali-soluble and cold-water-soluble materials were removed [5]. Also, in the ethanol precipitation step of the κ-carrageenan isolation, the ethanol-soluble material is lost.

Highly soluble low-molecular-weight carbohydrates have been found in some car-

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0008-6215/00/$ - see front matter
PII: S0008-6215(00)00006-9

rageenophyte red seaweeds [6–8] and contain not only unusual units, such as non-sulfated and disulfated D-galactose units and single stubs of xylose and galactose, but also L-galactose and/or 3,6-anhydro-L-galactose-containing agaroids [6–10].

We report the study of similar products extracted from *K. alvarezii* with water at room temperature.

2. Results

The seaweed was extracted twice with water at room temperature. Yields and analyses of the extracts (E1 and E2) are given in Table 1, while the monosaccharide compositions are shown in Table 2. The low yield of the second extraction shows that this procedure was exhaustive. Attempts to fractionate E1 by stepwise precipitation in solutions of potassium chloride of increasing concentration only yielded traces ($< 1\%$) of insoluble products at 0.3–0.4 M (E1F1) and 1.0–1.2 M KCl (E1F2), while most of the sample (78.3%, 99.0% of the recovered material) remained soluble in 2.0 M KCl (E1F3). Yields and analyses of the precipitating and soluble products are given in Table 1; the monosaccharide compositions are shown in Table 2.

Optical rotations of E1 and E2 ($[\alpha]_D$ 33.8 and 26.3°, respectively) are lower than those reported for κ/ι-carrageenans ($[\alpha]_D$ 56.1–66.5° [11], $[\alpha]_D$ 63.0–68.4° [12]) or partially cyclized μ/ν-carrageenans ($[\alpha]_D$ 55.1° [11], $[\alpha]_D$ 44.3° [12]). The same happens with the optical rotation of fraction E1F3 ($[\alpha]_D$ 34.1°) and for the alkali-treated derivatives, they are even lower ($[\alpha]_D$ 14.6, 12.1, and 12.0°; for E1T, E1F3T, and E1F3T3, respectively). These values are in agreement with the presence of L-galactose in these products (E1 7.9%, E1F3 9.1%, E1T 8.7% and E1F3T3 12.3%); no 3,6-anhydro-α-L-galactose was detected.

Alkaline treatment of E1 gave E1T with low yield (49%). Potassium chloride stepwise fractionation of E1T gave two fractions (E1T1 and E1T2) precipitating at low concentrations of potassium chloride (0–0.1 M, 16% and 0.1–0.3 M, 8%, relative to E1T), a third frac-

Table 1
Yields and analyses of carrageenans E1 and E2 of the fractions obtained by fractionation of E1 with potassium chloride, and of those obtained from E1 and E1F3 by alkaline treatment and further potassium chloride fractionation

Fraction	Range of precipitation (M) KCl	Yield [a,b] (%)	Sulfate (SO_3Na) (%)	Gal [c]:3,6-AnGal:sulfate molar ratio	Proteins (%)	Molecular weight
E1		6.0	26.1	1:0.39:1.20	7.8	15,500
E2		0.2	15.5	1:0.68:0.96	3.8	2500
E1F1	0.3–0.4	0.2	18.0	1:0.29:1.04	n.d. [d]	n.d.
E1F2	1.0–1.2	0.6	20.1	1:0.28:0.90	n.d.	n.d.
E1F3	2.0[e]	78.3 (99.0)	19.4	1:0.42:1.06	6.8	12,000
E1T		49.0	18.5	1:0.69:1.01	3.6	10,400
E1T1	0–0.1	16.0 (27.6)	20.3	1:0.29:1.36	6.7	20,900
E1T2	0.1–0.3	8.0 (13.8)	23.0	1:0.34:1.41	n.d.	21,200
E1T3	1.2–1.4	17.0 (29.3)	25.1	1:0.22:1.22	8.8	28,900
E1T4	2.0 [e]	17.0 (29.3)	19.9	1:0.24:0.52	5.3	16,300
E1F3T		60.2	24.7	1:0.78:1.32	1.5	18,800
E1F3T1	0–0.1	5.9 (10.3)	6.9	1:0.10:0.74	23.7	n.d.
E1F3T2	1.2–1.4	6.1 (10.5)	24.6	1:0.16:1.13	12.9	n.d.
E1F3T3	2.0 [e]	45.9 (79.3)	24.8	1:0.24:0.97	1.1	1900

[a] Yields of E1 and E2 are given for 100 g of seaweed; yields of E1F1–E1F3 and of E1T are given for 100 g of E1, and those of E1T1–E1T4 for 100 g of E1T. Yield of E1F3T is given for 100 g of E1F3 and those of E1F3T1-E1F3T3, for 100 g of E1F3T.

[b] In parentheses, percentage of the total recovered.

[c] Results of the phenol–sulfuric acid determination are expressed as galactose (previous discount of the amount of 3,6-anhydrogalactose).

[d] n.d. = not determined.

[e] Soluble in 2.0 M KCl.

Table 2
Monosaccharide composition (mol/%) of carrageenans E1 and E2, of the fractions obtained from E1 by fractionation with potassium chloride, and of those obtained from E1 and E1F3 by alkaline treatment and further potassium chloride fractionation

Fraction	Gal	6-*O*-Me-Gal [a]	2-*O*-Me-Gal	3,6-AnGal [b]	Xyl	Glc
E1	57.2 [c]	14.5	1.4	21.6	1.9	3.4
E2	47.7	2.1	tr.	46.7	2.0	1.5
E1F1	50.6	10.6		28.9	9.9	
E1F2	39.0	8.2	10.6	33.0	7.0	2.2
E1F3	48.0 [d]	14.9		27.5	8.1	1.5
E1T	38.5 [e]	15.0	1.5	32.6	4.7	7.7
E1T1	69.6	7.3		18.2	tr	4.9
E1T2	48.0	24.0		28.0	tr	tr
E1T3	24.6	40.0		29.8	tr	5.6
E1T4	46.5	15.4	3.5	11.1	10.6	12.9
E1F3T	33.7	14.1	2.0	39.9	8.3	2.0
E1F3T1	63.6	2.7	8.1	3.2	16.4	6.0
E1F3T2	87.1	1.1		10.4	1.4	
E1F3T3	61.8 [f]	11.9	2.2	15.6	5.7	2.8

[a] Always in the D-configuration.
[b] The ratio 3,6-anhydro-D:L-galactose was measured for E1 and E1F3, and only the D-isomer was detected.
[c] 49.3% of D-galactose and 7.9% of L-galactose.
[d] 38.9% of D-galactose and 9.1% of L-galactose.
[e] 29.8% of D-galactose and 8.7% of L-galactose.
[f] 49.5% of D-galactose and 12.3% of L-galactose.

tion gelified at 1.2–1.4 M KCl, while a fourth one remained soluble in 2.0 M KCl (E1T3, 17% and E1T4, 17%, both relative to E1T).

The major fraction from the potassium chloride fractionation of E1 (E1F3) was also submitted to an alkaline treatment; three fractions were obtained at 0–0.1 M KCl (E1F3T1, 5.9%), 1.2–1.4 M KCl (E1F3T2, 6.1%) and 2.0 M KCl (E1F3T3, 45.9%). Although the ranges of precipitation are similar (no precipitation at 0.1–0.3 M KCl was found by fractionation of E1F3T), yields of the fractions, especially that of the soluble fractions (E1T4 and E1F3T3), were different. This has been previously observed [13], showing that the solubility of carrageenan molecules in potassium chloride solutions is influenced by their molecular surroundings [11]. Yields and analyses of the alkali-treated samples (E1T and E1F3T) and of the fractions obtained from them are given in Table 1, while their monosaccharide composition is given in Table 2.

FTIR spectra of E1 and of all the products obtained from it by fractionation or by alkaline treatment and further fractionation are very similar, showing absorptions at 932–934 cm^{-1}, corresponding to the 3,6-anhydro ring and at 851 and 807 cm^{-1}, due to the C-4-axial sulfate group in the 3-linked β-D-galactose 4-sulfate units and to the C-2-axial sulfate group in the 4-linked 3,6-anhydro-α-D-galactose 2-sulfate residues, respectively. No absorption around 820 cm^{-1} (primary sulfate) was observed. The spectrum of E2 is similar to those above-mentioned spectra of E1 and derivatives, but E2 lacks the peak at 807 cm^{-1}. A shoulder at 972 cm^{-1} is clear in the spectra of E1T and E2. This signal was previously attributed to the presence of a 'deviant' ι-carrageenan (alternating 3-linked β-D-galactose 4-sulfate and 4-linked α-D-galactose 2-sulfate) [14], but no 3,6-di-*O*-alkylgalactose was detected in the ethylation or methylation analyses of E1T and E2 (see below). No absorption was found at 900–905 cm^{-1} suggesting the absence of major amounts of the unsulfated 3-linked β-D-galactose and/or its 6-*O*-methyl derivative [15,16].

Carrageenan E1 and its cyclized derivative E1T; E1F3 and its derivatives E1F3T and E1F3T3 were submitted to methylation and ethylation analyses. Data in Tables 3 and 4 indicate that E1 is mainly a κ/ι-carrageenan

(ratio κ/ι 2.6:1) with small amounts of nonsulfated 3-linked β-D-galactose and its 2,4- and 4,6-disulfated derivatives, and nonsulfated 4-linked α-D-galactose. The absence of 2,3-di-*O*-methyl- and 3-*O*-methyl-galactose suggests that the 4-linked α-D-galactose 6-sulfate and 2,6-disulfate units shown in the ^{13}C NMR spectra (see below) were cyclized during the methylation procedure. Ethylation analysis of E1 (Table 4) indicated that 26.9% of the 3-linked β-D-galactose 4-sulfate units were naturally methylated on C-6.

In E1F3, the major fraction obtained from E1, the κ/ι ratio is lower (κ/ι 2.1:1), and the percentages of the above-mentioned unusual units are slightly increased. The presence of small amounts of 3-*O*-methylgalactose (3%) in the permethylated derivative of this fraction

Table 3
Composition of partially methylated monosaccharides produced by permethylation of E1, E1F3, E1F3T, E1F3T3, E1T, E2, and F1

Monosaccharide [a,b]	E1	E1F3	E1F3T	E1F3T3	E1T	E2	F1
2,3,4,6-Gal	tr [c]	tr	1.1	tr	tr	tr	tr
2,4,6-Gal	5.3	6.8	13.1	11.9	6.3	1.9	27.1
2,3,6-Gal	tr			5.7	2.6	2.6	8.6
2,6-Gal [d]	33.9	47.0	36.5	50.7	43.6	51.3	19.6
2,4-Gal							6.2
6-Gal	3.4	3.9	6.3	6.5	5.1	tr	16.2
2-Gal	1.1	2.4	5.5	4.0	3.6	1.9	1.8
3-Gal	tr	3.0	2.1	2.3	1.0	1.0	2.8
Gal	tr	1.0	1.3	1.0	1.0	tr	1.9
2-AnGal	40.7	24.3	27.3	10.0	23.8	38.7	3.9
AnGal	15.6	11.6	6.8	5.3	11.9	2.6	tr
2,3,4-Xyl	tr	tr	tr	2.6	1.1	tr	11.9

[a] Mol/% of monosaccharides having methyl groups at the positions indicated.
[b] Traces of glucose were detected in all the samples.
[c] Percentages lower than 1% are given as trace (tr).
[d] Hydrolysis of the permethylated polysaccharide and derivatization to the corresponding aldononitrile acetates showed the absence of 4,6-Gal.

Table 4
Composition of partially ethylated and methylated ethylated monosaccharides produced by perethylation of E1, E1F3, E1F3T, E1F3T3, E1T, and E2

Monosaccharide [a,b,c]	E1	E1F3	E1F3T	E1F3T3	E1T	E2
2,3,4,6-Gal	tr [d]	1.2	1.7	1.2	tr	tr
2,4,6-Gal	3.2	7.0	7.4	6.4	3.6	1.4
2,4,VI-Gal		4.6	1.9	1.3	1.5	
2,3,6-Gal	1.0			4.9	1.9	1.5
2,6-Gal	31.0	33.7	28.9	42.9	30.7	48.9
2,VI-Gal	11.4	8.3	8.6	9.3	11.8	1.0
2,3-Gal			3.1			tr
6-Gal	3.8	6.8	13.1	4.1	5.2	2.6
2-Gal	2.0	2.7	3.5	3.7	2.3	1.0
3-Gal	tr	1.0	1.1	2.6	tr	tr
2-AnGal	34.6	24.1	22.0	18.8	30.2	39.9
AnGal	11.6	9.4	8.7	3.0	12.8	3.7
2,3,4-Xyl	1.4	1.2	tr	1.8	tr	tr

[a] Mol/% of monosaccharides having ethyl groups at the positions indicated.
[b] The position of methyl groups is given in roman numeral, while that of the ethyl groups is given in arabic numerals.
[c] Traces of VI-Gal, Gal, and Glc were present in all the samples.
[d] Percentages lower than 1% are given as trace (tr).

(Table 3) would suggest remains of precursor units, but the fact that similar amounts of this sugar were found in alkali-treated fractions (E1F3T, E1F3T3 and E1T, Tables 3 and 4) indicates that it could arise from an unidentified 2,6-disubstituted, C-6 non-sulfated α-D-galactose residue (see ^{13}C NMR spectra). On the other hand, minor amounts of 2-*O*-methylgalactose may be interpreted as 4-linked α-galactose 6-sulfate units, in the D- or L-configuration, bearing terminal xylose at C-3, as in the case of the agaroid from *Laurencia nipponica* [17]. In E1F3 and its derivatives C-6 methyl groups were found not only in the 3-linked β-D-galactose 4-sulfate units, but also in their unsulfated counterpart (Table 4).

E2 shows the same characteristics as E1 and its derivatives, being more of the κ type (ratio κ/ι ~ 15:1, Table 3).

Fig. 1 shows the ^{13}C NMR spectra of E1, E1F3, E1F3T, and E1F3T3.

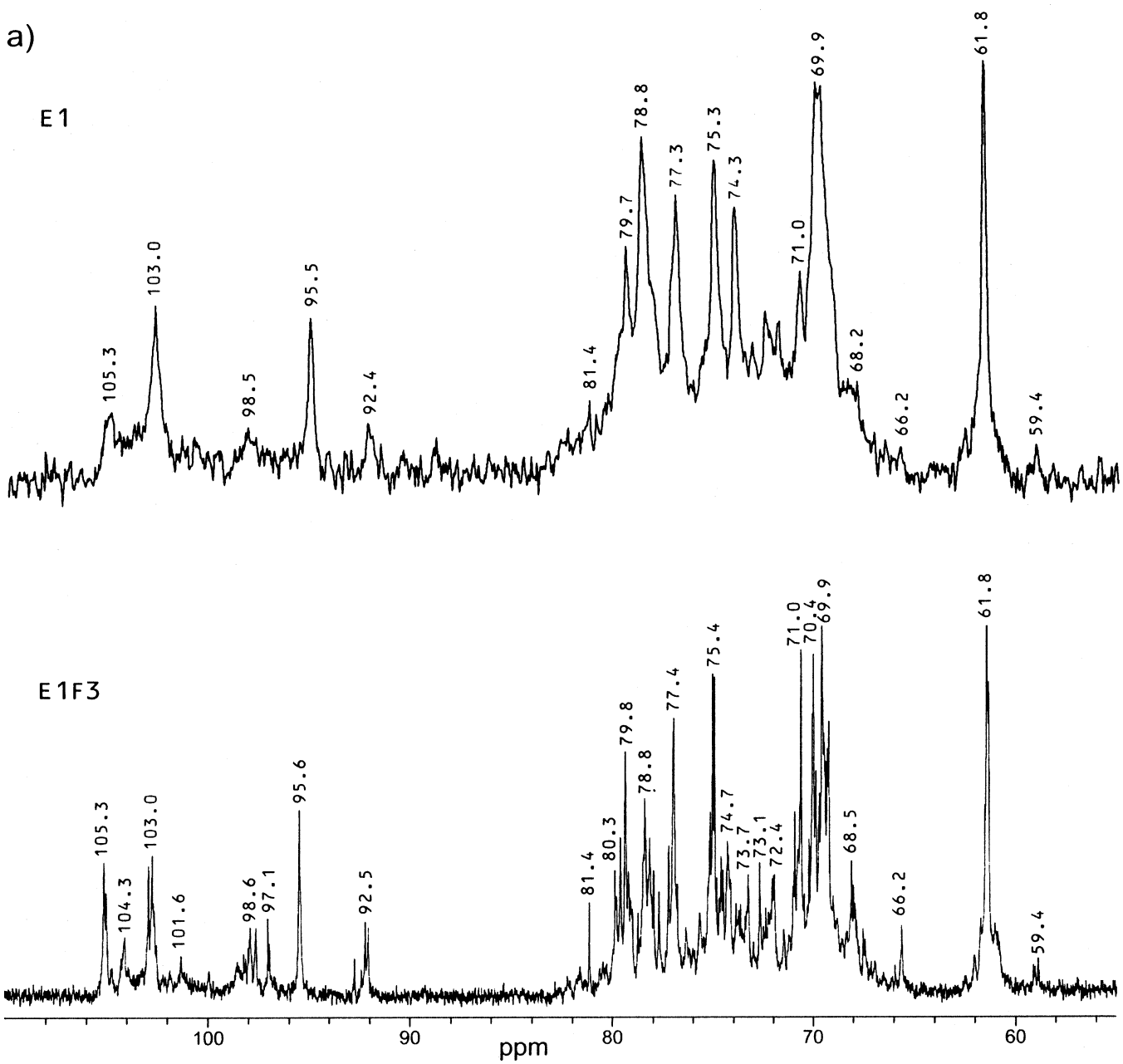

Fig. 1. ^{13}C NMR spectra of (a) E1 and E1F3, and (b) E1F3T and E1F3T3, showing the increasing complexity of the fractions after fractionation.

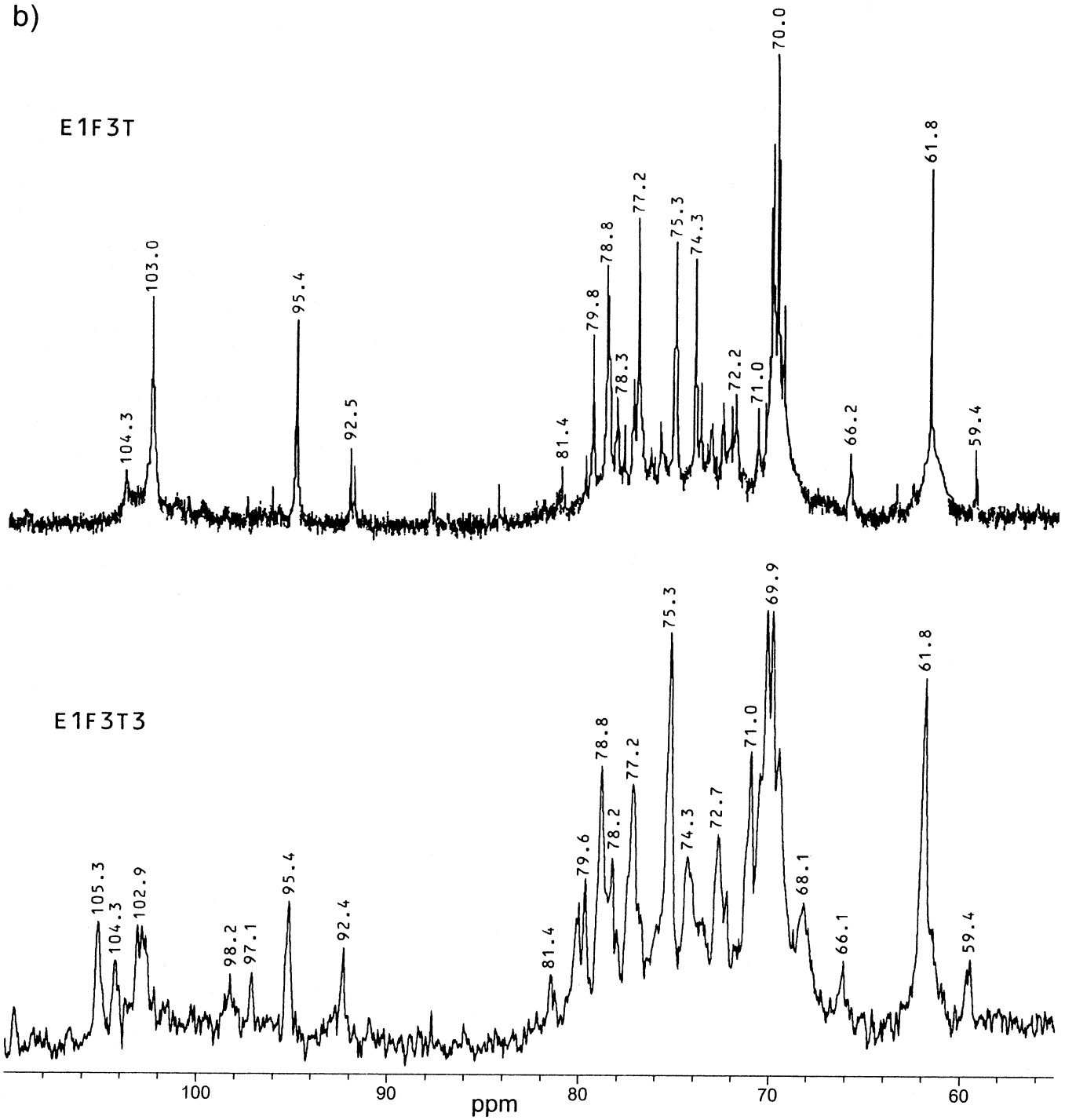

Fig. 1. (*Continued*)

The major signals present in the ^{13}C NMR spectra of E1 and E2 (not shown) were readily assigned to the repeating units of a κ-carrageenan [18,19]. Some minor absorptions reflect the heterogeneity of the samples; in E1 these signals correspond to low percentages of ι-carrageenans [18,20] and precursor structures [3,21]. These μ/ν-structures (105.1–105.2 ppm and 98.2–98.6 ppm, for the anomeric signals) were also found with different intensity in most of the fractions. It must be taken into account that these peaks, as expected, are not present in the spectra of E1T and E1F3T, but they appear in the spectrum of the alkali-treated fraction E1F3T3. E2 lacks the signals corresponding to ι-repeating units, in agree-

ment with its methylation analysis (Table 3), but shows terminal reducing α- and β-galactose absorptions (93.0 and 97.1 ppm, respectively [22]), corresponding to its low molecular weight (Table 1).

In both of the above-mentioned spectra (E1F3T3 and E2), signals at 81.4, 70.6, 77.9, and 69.6 ppm were assigned to C-3, C-4, C-5, and C-6, respectively, of nonreducing terminal 3,6-anhydro-α-D-galactose units; the signal at 71.0 ppm was attributed to C-2 of a reducing β-galactose terminal unit; and that at 67.9 ppm to C-2 of reducing α-galactose terminal residues, indicating that oligosaccharides of the type α-D-3,6-AnGal→(β-D-Gal4S→α-D-3,6-AnGal)$_n$→Gal are present [19]. The signal at 81.4 ppm was observed in most of the spectra.

It has been reported that the anomeric signals of a sulfated galactan with a nearly perfect ι-disaccharide repeating structure occur at essentially the same position as those of its 6′-O-methylated counterpart [23]. The same happens with the κ-disaccharide repeating units and their 6′-O-methylated derivative; only the values of the signals corresponding to C-4, C-5, and C-6 are modified. A weak absorption at 59.4 ppm is clearly seen in the spectra of E1 and its derivatives, which corresponds to the methoxyl group on C-6 of the β-D-galactose (4-sulfate) units.

The presence of small amounts of the diad β-D-Gal 4,6S→α-D-3,6-AnGal is consistent with the spectra of E1F3T and E1F3T3 (signals at 74.2, 73.0, and 68.4 ppm correspond to C-4–C-6, respectively, of the 3-linked unit; the δ values of the other carbon atoms coincide with signals corresponding to the major diads present in the samples) [24], in agreement with the low, but significant amounts of 2-*O*-methylgalactose detected by methylation analysis (see above).

The presence of a β-carrageenan repeating structure (alternating 3-linked β-D-galactose and 4-linked 3,6-anhydro-α-D-galactose units) was discarded due to the absence of signals at 80.9 and 66.8 ppm [25]. Unsulfated 3-linked β-D-galactose residues of an α-carrageenan (alternating 3-linked β-D-galactose and 4-linked 3,6-anhydro-α-D-galactose 2-sulfate units) were also discarded due to the lack of peaks at 82.4 and 67.4 ppm, corresponding to the C-3 and C-4 of the unsulfated 3-linked unit [26].

The signals at 104.3, 74.4, 76.8, 70.5, and 66.2 ppm were assigned to C-1–C-5, respectively, of a terminal β-D-xylose, probably linked to C-6 of some of the β-D-galactose units; these signals are especially clear in the spectrum of E1F3 [27–29].

In some of the spectra small peaks at 104.3 (shoulder) and 101.0–101.6 ppm were observed, and they were tentatively assigned to the anomeric signals of linked β-D-galactose and α-L-galactose (6-sulfate) units [30].

Fraction E1F3 was submitted to ion-exchange chromatography on a DEAE Sephadex A-25/Sephadex G-50 (23:27) column [31], and the elution was carried out by a stepwise increase of the concentration of sodium chloride. The elution pattern is given in Fig. 2, while Table 5 shows yields, sulfate content, and monosaccharide composition of the fractions. Nine fractions were obtained; most of them (F1–F4 and F8 and F9) contained small amounts of glucose, as in the starting sample, but in fractions F5–F7 glucose was the major monosaccharide. The yield of glucan, considering only fractions F5–F7, was much higher than that expected from the starting sample. The yield of glucan in the other fractions was of the order of that of the starting material (1.5%) (Table 5). The presence of a linear glucan with α-(1→6) linkages and trace amounts of α-(1→4) linkages was evident from the methylation analyses (major quantities of 2,3,4-tri-*O*-methylglucose) and ^{13}C NMR spectra (signals at 100.4, 76.1, 74.2, 72.9, 72.3, and 68.3 ppm, corresponding to C-1–C-6, respectively, of the α-D-glucose repeating units [32]) of fractions F5–F7 (not shown).

The percentages of glucose were discounted and the 'carrageenan' monosaccharide compositions of the nine fractions are given in Table 6. The qualitative pattern of composition is similar in all fractions and also similar to that of the starting product, the differences between the composition of the fractions being only quantitative. The sequence of percentages of sulfate in each fraction is more regular than in Table 5, but lower percentages of sulfate

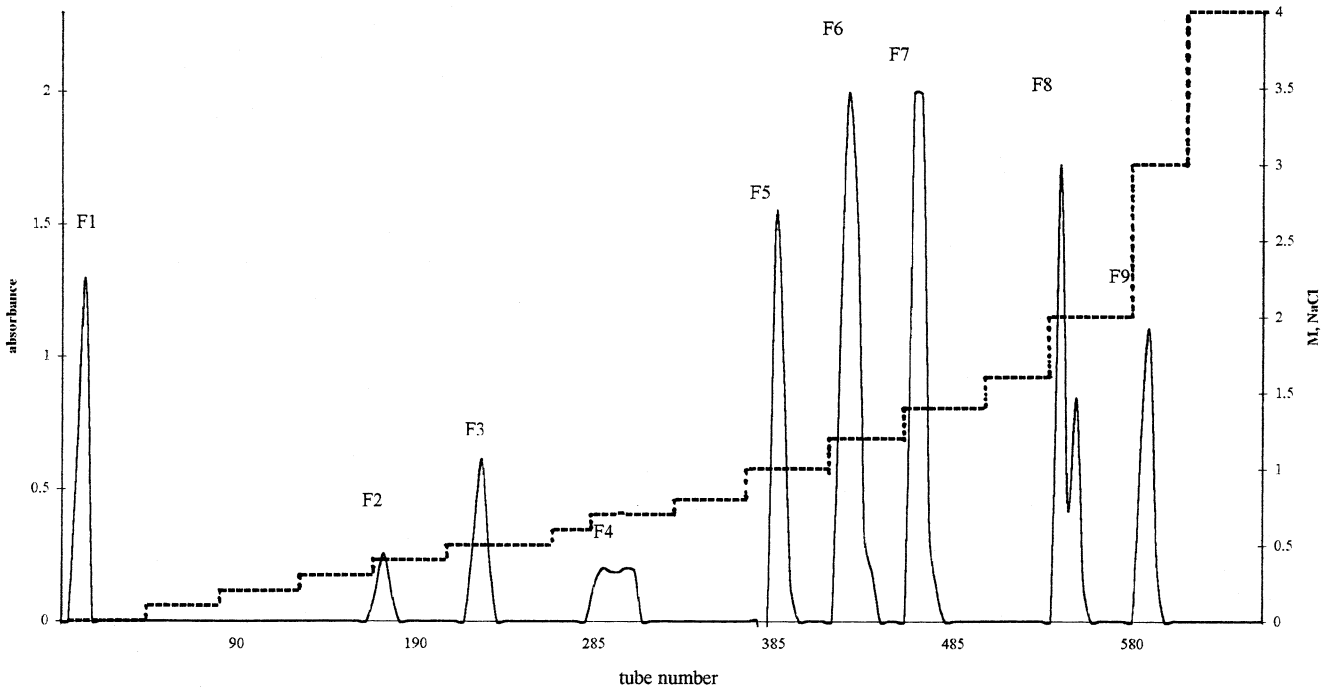

Fig. 2. Elution pattern of the ion-exchange chromatography of E1F3.

Table 5
Yields, sulfate content, and monosaccharide compositions (mol%) of the fractions obtained by ion-exchange chromatography of E1F3 on DEAE A-25/Sephadex G-50 (46:54)

Fraction	M, NaCl	Yield [a] (%)	Sulfate (SO_3Na) (%)	Gal	6-*O*-Me-Gal	2-*O*-Me-Gal	3,6-AnGal	Xyl	Glc
E1F3 [b]		78.3 (99.0)	19.4	48.0	14.9		27.5	8.1	1.5
F1	0.0	16.5 (20.5)	3.6	61.8	tr [c]	3.7	2.2	26.9	5.4
F2	0.4	1.4 (1.7)	7.5	56.7	3.8	6.9	9.4	19.9	3.3
F3	0.5	4.3 (5.3)	17.2	51.4	2.2	1.9	29.9	10.1	4.5
F4	0.6	1.5 (1.9)	19.4	59.1	1.7	3.7	19.3	7.7	8.5
F5	1.0	12.6 (15.6)	11.3	23.3	5.2	tr	11.3	5.4	54.8
F6	1.2	9.6 (11.9)	4.8	16.4	8.4	2.6	9.0	6.6	57.0
F7	1.4	24.2 (30.2)	2.6	2.4	5.3	1.6	2.8	3.2	84.7
F8	2.0	7.4 (9.2)	19.9	46.4	21.7	tr	20.4	1.6	9.9
F9	3.0	2.9 (3.7)	30.4	85.5	5.6	tr	4.7	1.0	3.2

[a] In parentheses, percentage of the recovered.
[b] Included for comparison.
[c] Percentages lower than 1% are given as trace (tr).

and 3,6-anhydrogalactose, as well as higher xylose contents, were found; these fractions also contained from significant to major amounts of 2-*O*-methyl- and/or 6-*O*-methyl-galactose.

Results from methylation analysis of fraction F1 are given in Table 3; in spite of showing major amounts of the methylated derivatives usually expected for classical carrageenans (2,3,6- and 2,4,6-tri-*O*-methyl-, 2,6-di-*O*-methyl-, and 3-*O*-methyl-galactose, comprising 64.7% of the methylated sugars), its low sulfate (3.8%, Table 6), 2-*O*-methyl-3,6-anhydrogalactose (3.9%) and 3-*O*-methyl-galactose (2.8%) content preclude its analysis as a carrageenan/agaroid. The percentage of terminal xylose could have been underestimated, considering that the percentage of xylose detected in the monosaccharide composition determination of F1 (Table 6) is much higher (28.9%). This discrepancy has been previously observed and was attributed

Table 6
'Carrageenan' monosaccharide compositions (mol/%) of the fractions obtained by ion-exchange chromatography of E1F3

Fraction	Yield [a,b] (%)	Gal	6-*O*-Me-Gal	2-*O*-Me-Gal	3,6-AnGal	Xyl	Sulfate (SO_3Na) (%)	Gal [c]:3,6-AnGal molar ratio
E1F3	78.3 (99.0)	49.0	15.1		27.9	8.1	19.7	1:0.43
F1	15.6 (32.9)	64.9	tr	3.9	2.3	28.9	3.8	1:0.03
F2	1.3 (2.7)	58.8	3.9	7.1	9.7	20.5	7.8	1:0.13
F3	4.2 (8.8)	53.9	2.3	2.3	31.4	10.1	17.5	1:0.53
F4	1.4 (2.9)	66.9	1.8	tr	22.6	8.7	21.2	1:0.32
F5	7.9 (16.6)	36.5	16.8	tr	25.1	21.6	18.1	1:0.47
F6	4.1 (8.6)	38.4	19.5	6.0	20.9	15.2	11.2	1:0.33
F7	3.7 (7.8)	15.8	34.6	10.4	18.3	20.9	17.0	1:0.30
F8	6.6 (13.9)	50.0 [d]	24.0	1.7	22.5	1.8	22.1	1:0.20
F9	2.7 (5.7)	88.3	5.8		4.9	1.0	32.3	1:0.05

[a] Calculated after discounting the glucose content.
[b] In parentheses, percentage of the recovered.
[c] It includes galactose, 6-*O*-methylgalactose, and 2-*O*-methylgalactose.
[d] 39.9% of D-galactose and 10.1% of L-galactose.

to the volatility of the acetylated 2,3,4-tri-*O*-methylxylitol that is lost, in part, during the sample workup [33]. However, even in that case, no structure can be assigned to this fraction. An ill-defined ^{13}C NMR spectrum of F1 (not shown) indicated the presence of significant quantities of β-D-xylose.

3. Discussion

Red algal galactan sulfates have traditionally been classified as either agaroids or carrageenans, which are respectively extracted from agarophytes or carrageenophytes. However, galactan sulfates that do not conform to this classification scheme have been found in several cryptonemialean algae [34]. These polysaccharides appear to contain both D- and L-derivatives, with a predominance of D-galactose units that cannot make pairs with L-galactose residues in constructing the agaroid backbones. If an alternating structure is supposed, the excess of D-galactose should be attributed to carrageenan structures. Recent structural studies have also demonstrated the cooccurrence of agaroid and carrageenan structures in *Rhodomela larix* and *Digenea simplex* (Ceramiales) [34].

Even in those seaweeds traditionally accepted as carrageenophytes, the isolation of L-galactose-containing fractions upsets this simple picture of agaroid–carrageenan polysaccharides. L-Galactose-containing fractions were isolated from cystocarpic samples of *Gigartina skottsbergii*, after alkaline treatment and further fractionation with potassium chloride of both κ/ι-carrageenans and of partially cyclized μ/ν-carrageenans [6,8]. Galactans with similar characteristics have been isolated from cystocarpic samples of other Gigartinaceae [10]. In these studies, major emphasis was given to the presence of L-galactose and/or 3,6-anhydro-L-galactose, although the existence of carrageenan 'hybrid' structures or unusual units, like single stubs of xylose and/or galactose, were noted.

The tetrasporic carrageenans of *Iridaea undulosa* [7] and *Gigartina skottsbergii* [9] have fractions soluble in 2 M potassium chloride, which comprise L-galactose-containing galactans, together with carrageenan structures and unusual units. The *I. undulosa* fraction was further studied by anion-exchange chromatography [7] and shown to be composed of the expected λ-carrageenan, together with mixtures (or hybrids) of λ structures and agaroids. In the agaroid moiety, the 3-linked 6-substituted units and possibly 4-linked 3-substituted residues, predominate, a pattern common to corallinans [35] and other red seaweed galactans obtained from several species of the Halymenaceae [36,37]. These fractions differ from corallinans in that they are complex mixtures of products of low molecular weight, extractable with water at room

temperature and with high dispersion of structures.

In the case of those fractions obtained from cystocarpic plants of the Gigartinaceae, they are extracted with carrageenans at room temperature and co-precipitate with them during their isolation. They complex with partially cyclized κ/ι- or μ/ν-structures, but not with the wholly cyclized carrageenans, and the only way, at the moment, to separate them from carrageenans is through alkaline treatment of the sample and insolubilization of the now wholly cyclized κ/ι-carrageenans with potassium chloride. In carrageenans from tetrasporic stages of these seaweeds, they are isolated as the soluble fraction remaining, after precipitation of the λ-carrageenans with high concentrations of potassium chloride, and they are recognized by their low optical rotation.

The case of *K. alvarezii* is different, because the major polysaccharides are only extracted with hot water. Therefore, the above-mentioned agaroid–carrageenan fractions are easily obtained by treatment of the seaweed with water at room temperature.

Linkage analysis of E1, E1F3, E1F3T, E1F3T3, and F1 (Tables 3 and 4), as well as the corresponding ^{13}C NMR spectra (Fig. 1) and the increase of L-galactose (Table 2) show that the complexity of the samples increases through the fractionation and cyclization–fractionation processes, possibly by the loss of the more regular molecules during dialysis.

The dextran-like structure of the glucan present in fractions F5–F7, its yields higher than that expected from the percentage of glucose in the starting product (E1F3), and its elution at high concentrations of sodium chloride together with charged fractions in spite of being a neutral product, would suggest an artifact washed off from the column. This conclusion does not agree with the well-defined and separated peaks in the general elution pattern, which show no bleeding of the column (Fig. 2). A similar product was identified in a fraction obtained from the red seaweed *I. undulosa*, which was soluble in KCl 2.0 M (M.L. Flores, personal communication).

In summary, the highly valuable red seaweed *K. alvarezii* synthesizes not only major amounts of the gel-forming commercial κ-carrageenan, but also small quantities of a family of low-molecular-weight, non-gelling carrageenans and agaroids. These carrageenans showed molecular weight, compositional and structural dispersion around a basic κ-pattern. The main differences from an ideal κ-structure are: (a) low percentages of 3,6-anhydrogalactose and the presence of precursor units; (b) small amounts of unusual units, as non-sulfated 3-linked β-D-galactose and its 2,4- and 4,6-disulfated forms, and 4-linked α-D-galactose; (c) significant amounts of ι-structure, and (d) important amounts of 6-*O*-methyl-β-D-galactose (4-sulfate) in the polysaccharide backbone. Agaroids are suggested by the presence of 4-linked α-L-galactose and, possibly, of 4-linked 3-substituted and 3-linked 2- and 6-substituted galactose residues. The predominance of D-galactose suggested the coocurrence of carrageenan and agaroid structures.

4. Experimental

Material.—Samples of *K. alvarezii* were imported from the Philippines to Argentina by Soriano S.A.

General methods.—Galactose was analyzed by the phenol–sulfuric acid method [38] without previous hydrolysis of the polysaccharide. Galactose content was corrected for the presence of 3,6-anhydrogalactose, which was determined independently by the resorcinol method [39]. Sulfate was determined turbidimetrically [40]. The molecular weight was estimated by the method of Park and Johnson [41], while the protein content was determined by the method of Lowry et al. [42]. Optical rotations (Na D-line) were measured in a Perkin–Elmer 343 polarimeter using 0.2–0.4% solutions of the polysaccharides in water. For GLC, alditol acetates were obtained by reductive hydrolysis and acetylation of the samples [43].The ratio D:L-galactose was determined by the method of Cases et al. [44] and the ratio 3,6-anhydro-D:L-galactose was estimated by the method of Errea et al. [45].

Extraction.—The plants, previously milled (42 g) were extracted with water (2.1 L) at room temperature (rt) with mechanical stirring for 24 h. The residue was removed by centrifugation and the supernatant was concentrated, dialyzed (molecular-weight cut off 6000–8000) and freeze-dried. The residue was extracted once more in the same way.

Fractionation with potassium chloride.—The polysaccharide (0.1–3.5 g) was dissolved in water (0.04–1.4 L, 0.25%). Solid, finely divided KCl was added in small portions with constant and violent mechanical agitation so that the concentration was increased by 0.1 M each time. After each addition, stirring was continued for 5–16 h to ensure equilibration of the system; the upper limit of KCl concentration was 2.0 M. The precipitates, as well as the residual solutions were dialyzed (molecular-weight cut-off for E1F1–E1F3, 6000–8000; for E1F3T1–E1F3T3, 3500; and for E1T1–E1T4, 1500); concentrated and freeze-dried.

Alkaline treatment of fractions.—The sample (235–390 mg) was dissolved in water (94–195 mL) and $NaBH_4$ (10–20 mg) added. After 24 h at rt, 3 M NaOH was added (47–98 mL) with a further quantity of $NaBH_4$ (5–10 mg). The solution was heated at 80 °C for 3 h. The solution was cooled to rt, dialyzed (molecular-weight cut-off 6000–8000), concentrated and freeze-dried.

Fractionation of E1F3.—For the analytical fractionation a column (1.0×18 cm) was filled with the following mixture: 18 mL of DEAE-Sephadex A-25 was stabilized in water and boiled with 1.5 g of Sephadex G-50. E1F3 (15 mg) was dissolved in water, which was used as first eluant, then increasing concentrations of NaCl were applied; the upper concentration was 4 M. Fractions of 1.7 mL were isolated, and the aliquots were assayed by the phenol–sulfuric acid method [29]. After obtaining blank readings, the eluant was replaced by another with higher concentration of NaCl.

For the preparative fractionation, a column (1.0×60 cm) was filled with the mixture indicated above. Fractionation of E1F3 (300 mg) was carried out as described before, but in this case, fractions of 4.1 mL were isolated.

Methylation analysis.—The sample (3–10 mg) was converted into the corresponding triethylammonium salt and was methylated by the Hakomori procedure as described in Refs. [11,43]. The methylated samples were derivatized to the acetylated alditols as described for the polysacharides [43]. A portion of methylated E1F3 was hydrolyzed with TFA for 2 h at 120 °C, and the partially methylated sugars were converted into the corresponding aldononitrile acetates [46]. Fraction F7 was methylated in two steps: a first step using the method of Haworth [47] and then by the Hakomori procedure, as described above.

Ethylation analysis.—The sample (3–10 mg) was converted into the corresponding triethylammonium salt and was ethylated as described by Cases et al. [48]. The ethylated samples were submitted to a reductive hydrolysis and acetylation [43] to give the corresponding alditol acetates.

GLC.—GLC of the alditol and aldononitrile acetates was carried out on an Hewlett–Packard 5890A gas–liquid chromatograph equipped with a flame ionization detector and fitted with a fused-silica column (0.25 mm i.d. × 30 m) WCOT-coated with a 0.20 μm film of SP-2330. For the acetylated alditols, chromatography was programmed from 200 (5 min hold) to 230 °C at 2 °C min^{-1}; for the partially methylated and ethylated alditol acetates chromatography was carried out running from 180 (5 min hold) to 210 °C at 1 °C min^{-1}, then from 210 to 230 °C at 2 °C min^{-1}.

The partially methylated aldononitrile acetates were run by temperature programming for 200–230 °C at 1 °C min^{-1}. N_2 was used as carrier at a flow rate of 1 mL min^{-1}, the split ratio was 80:1, and the head pressure was 15 psi. The injector and detector temperature was 240 °C.

GLC–MS.—GLC–MS was performed on an HP 5890A gas–liquid chromatograph equipped the SP-2330 (see above) interfaced to a Trio-2 VG Masslab mass spectrometer working at 70 eV. Helium was used as the carrier gas.

FTIR.—Fourier-transform infrared spectra were recorded with a 510P Nicolet FTIR spectrophotometer, using films prepared by drying

aqueous solutions of the polysaccharides, at 4000–250 cm^{-1}, 32–64 scans were taken with a resolution of 2–4 cm^{-1}.

^{13}C NMR spectroscopy.—100 MHz ^{13}C NMR spectra of E1F3 and E1F3T were recorded at 70 °C, in 1:0.25 water–D_2O solutions, with external reference of TMS. The parameters were as follows: pulse angle 90°, acquisition time 0.6 s, relaxation delay 4.5 μs, spectral width 32 kHz and scans 82,924 and 152,457, respectively. 50 MHz ^{13}C NMR spectra of E1, E1T, E1F3T3, and E2 were recorded at rt, in 1:1 water–D_2O solutions, with external reference of TMS. Specific parameters included a pulse angle 90°, acquisition time of 0.74 s, no pulse delay, a spectral width of 11 kHz and 300,000–320,000 scans. 50 MHz ^{13}C NMR spectra of F6, F7, and F8 were recorded in the above described conditions, the number of scans was 80,000–165,000.

Acknowledgements

This work was supported by grants from CONICET. The authors are indebted to Mr Carlos Soriano (Soriano S.A., Argentina) for supplying the seaweed material, to Professor Masao Ohno for his help with its classification, and to Dr Miguel D. Noseda for the 100 MHz ^{13}C NMR spectra of E1F3 and E1F3T.

References

[1] M. Glicksman, Red Seaweed Extracts, in M. Glicksman (Ed.), *Food Hydrocolloids*, Vol. 2, CRC Press, Boca Raton, FL, 1983, pp. 73–114.
[2] N.S. Anderson, T.C.S. Dolan, D.A. Rees, *J. Chem. Soc., Perkin Trans. 1*, (1973) 2173–2176.
[3] C. Bellion, G. Brigand, J.-C. Prome, D. Welti, S. Bociek, *Carbohydr. Res.*, 119 (1983) 31–48.
[4] C. Rochas, M. Rinaudo, S. Landry, *Carbohydr. Polym.*, 10 (1989) 115–129.
[5] R.A. Hoffmann, M.J. Gidley, D. Cooke, W.J. Frith, *Food Hydrocoll.*, 9 (1995) 281–289.
[6] M. Ciancia, M.C. Matulewicz, A.S. Cerezo, *Phytochemistry*, 34 (1993) 1541–1543.
[7] C.A. Stortz, M.R. Cases, A.S. Cerezo, *Carbohydr. Polym.*, 34 (1997) 61–65.
[8] M. Ciancia, M.C. Matulewicz, A.S. Cerezo, *Phytochemistry*, 45 (1997) 1009–1013.
[9] M.D. Noseda, Polissacarídeos sulfatados da fase tetrasporofítica de Gigartina skottsbergii (Rhodophyta, Gigartinales), Ph.D. Thesis, Federal University of Parana, Curitiba, Brasil, 1994.
[10] J.S.Craigie, H.Rivero-Carro, Abstracts of Papers, XIVth International Seaweed Symposium, Brest–St.Malo, France, August 1992, Abstr. 71.
[11] C.A. Stortz, A.S. Cerezo, *Carbohydr. Res.*, 242 (1993) 217–227.
[12] M.C. Matulewicz, M. Ciancia, M.D. Noseda, A.S. Cerezo, *Phytochemistry*, 28 (1989) 2937–2941.
[13] M. Ciancia, M.C. Matulewicz, A.S. Cerezo, *An. Asoc. Quim. Argent.*, 83 (1995) 103–108.
[14] T. Chopin, E. Whalen, *Carbohydr. Res.*, 246 (1993) 108–120.
[15] J.N.C. Whyte, S.P.C. Hosford, J.R. Englar, *Carbohydr. Res.*, 140 (1985) 336–341.
[16] A. Choivitti, A. Bacic, D.J. Craik, S.L.A. Munro, G.T. Kraft, M.-L. Liao, *Carbohydr. Res.*, 299 (1997) 229–243.
[17] A.I. Usov, M.Ya. Elashvili, *Bot. Mar.*, 34 (1991) 553–560.
[18] A.I. Usov, A.S. Shashkov, *Bot. Mar.*, 28 (1985) 367–373.
[19] C. Rochas, M. Rinaudo, M. Vincendon, *Int. J. Biol. Macromol.*, 5 (1983) 111–115.
[20] C.W. Greer, C. Rochas, W. Yaphe, *Bot. Mar.*, 28 (1985) 9–14.
[21] M. Ciancia, M.C. Matulewicz, P. Finch, A.S. Cerezo, *Carbohydr. Res.*, 238 (1993) 241–248.
[22] P.J. Archbald, M.D. Fenn, A.B. Roy, *Carbohydr. Res.*, 93 (1981) 177–190.
[23] A. Chiovitti, M.L. Liao, G.T. Kraft, S.L.A. Munro, D.J. Craik, A. Bacic, *Bot. Mar.*, 39 (1996) 47–59.
[24] M.L. Liao, A. Chiovitti, S.L.A. Munro, D.J. Craik, G.T. Kraft, A. Bacic, *Carbohydr. Res.*, 296 (1996) 237–247.
[25] A.I. Usov, A.S. Shashkov, *Bot. Mar.*, 27 (1984) 189–202.
[26] R. Falshaw, R.H. Furneaux, H. Wong, M.-L. Liao, A. Bacic, S. Chandrkrachang, *Carbohydr. Res.*, 286 (1996) 81–98.
[27] K. Bock, C. Pedersen, H. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 193–225.
[28] R.H. Furneaux, I.J. Miller, T.T. Stevenson, *Hydrobiologia*, 204/205 (1990) 645–654.
[29] C.A. Stortz, M.R. Cases, A.S. Cerezo Jr., Red seaweed galactans. Methodology for the structural determination of corallinan, a different agaroid, in R.R. Townsend, A.T. Hotchkiss Jr. (Eds.), *Techniques in Glycobiology*, Marcel Dekker, New York, 1997, pp. 567–593.
[30] M. Lahaye, W. Yaphe, C. Rochas, *Carbohydr. Res.*, 143 (1985) 240–245.
[31] M.R. Cases, C.A. Stortz, A.S. Cerezo, *Carbohydr. Polym.*, 34 (1997) 61–65.
[32] F.R. Seymour, R.D. Knapp, S.H. Bishop, *Carbohydr. Res.*, 51 (1976) 179–194.
[33] R. Falshaw, R.H. Furneaux, I.J. Miller, *Bot. Mar.*, 42 (1999) 431–435.
[34] R. Takano, K. Hayashi, S. Hara, *Recent Res. Dev. Phytochem.*, 1 (1997) 195–201.
[35] M.R. Cases, C.A. Stortz, A.S. Cerezo, *Int. J. Biol. Macromol.*, 16 (1994) 93–97.
[36] I.J. Miller, R. Falshaw, R.H. Furneaux, *Carbohydr. Res.*, 268 (1995) 219–232.
[37] I.J. Miller, R. Falshaw, R.H. Furneaux, J.A. Hemmingson, *Bot. Mar.*, 40 (1997) 119–127.
[38] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, *Anal. Chem.*, 28 (1956) 350–356.

[39] W. Yaphe, G.P. Arsenault, *Anal. Biochem.*, 13 (1965) 143–148.
[40] K.S. Dodgson, R.G. Price, *Biochem. J.*, 84 (1962) 106–110.
[41] J.T. Park, M.J. Johnson, *J. Biol. Chem.*, 181 (1949) 149–151.
[42] H.O. Lowry, A. Rosenbrought, L. Farr, F. Smith, *Anal. Chem.*, 29 (1951) 350–356.
[43] T.T. Stevenson, R.H. Furneaux, *Carbohydr. Res.*, 210 (1991) 277–298.
[44] M.R. Cases, A.S. Cerezo, C.A. Stortz, *Carbohydr. Res.*, 269 (1995) 333–341.
[45] M. Errea, M. Ciancia, M. Matulewicz, A.S. Cerezo, *Carbohydr. Res.*, 311 (1998) 235–238.
[46] C.A. Stortz, M.C. Matulewicz, A.S. Cerezo, *Carbohydr. Res.*, 111 (1982) 31–39.
[47] W.N. Haworth, *J. Chem. Soc.*, (1915) 8–16.
[48] M.R. Cases, C.A. Stortz, A.S. Cerezo, *J. Chromatogr. A*, 662 (1994) 293–299.