ALKALINE TREATMENT OF THE CARRAGEENANS FROM THE CYSTOCARPIC STAGE OF THE RED SEAWEED *IRIDAEA UNDULOSA*

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Abstract

The carrageenans from the cystocarpic stage from the red seaweed Iridaea undulosa were fractionated by means of potassium chloride precipitation. The soluble and insoluble fractions were treated with alkali. The products were refractionated with potassium chloride, giving rise to several fractions, being the main ones those precipitating at low concentrations of potassium chloride. Monosaccharide analyses, methylation data, spectroscopy and fractionation by anion-exchange chromatography show that, although κ/ι-carrageenans are the main components of the cystocarpic products, other polysaccharides like agarans, mannans, xylans, an α-(1→6)-glucan, and other glucans are minor-to-trace constituents of the matrices of this seaweed.

Resumen

Los carragenanos del estadio cistocárpico del alga roja Iridaea undulosa se fraccionaron por medio de precipitación con cloruro de potasio. Las fracciones insoluble y soluble fueron tratadas con álcali. Los productos se refraccionaron con cloruro de potasio, dando lugar a algunas fracciones, siendo las más importantes aquellas insolubles a bajas concentraciones de cloruro de potasio. El análisis de monosacáridos, los datos de metilación, los estudios espectroscópicos y el fraccionamiento por cromatografía de intercambio iónico demuestran que, si bien los κ/ι-carragenanos son los componentes principales del estadío cistocárpico, otros productos, como agaranos, mananos, xilanos, un α-(1→6)-glucano y otros glucanos, son constituyentes menores del sistema de polisacáridos matriciales de esta especie.

Introduction

Members of the family Gigartinaceae elaborate different carrageenans from karyotypically different generations [1-3]. The red seaweed *Iridaea undulosa* is an important source of carrageenans, which were widely studied [4-11]. First studies were

carried out on unsorted samples [4-9], but the latter ones were performed on sorted cystocarpic and tetrasporic samples [10,11]. Stortz and Cerezo determined that the cystocarpic thalli from *Iridaea undulosa* contain a gelling carrageenan with predominance of the κ/ι-type, and a soluble one with partially cyclized μ/ν-structure [10]. On the other hand, the tetrasporic samples were constituted mainly by λ-carrageenans, but also by a smaller proportion of L-galactose-containing polysaccharides [10]. The later appears in the potassium chloride 2 M-soluble fraction as a complex system composed by λ-carrageenans, agarans and other polysaccharides [12].

It may be expected to find similar unusual products (agarans and variants) within the cystocarpic carrageenans, as occurred with *Gigartina skottsbergii* [13]. However, in this case an alkaline treatment should be carried out to separate these products from the μ/ν-carrageenans, by converting them into gelling κ/ι-carrageenans. Herein, the results of the alkaline treatment of the soluble and gelling carrageenans, from cystocarpic *Iridaea undulosa*, are presented as well as a preliminary analysis of some fractions.

Results and Discussion

The cystocarpic carrageenans from *Iridaea undulosa* (**Cc**) were fractionated and treated as depicted in Scheme 1. Bulk precipitation of **Cc** with 2 M potassium chloride yielded 62 % of an insoluble product (**Ci**) and 31 % of a soluble product (**Cs**). The yield of the insoluble product is higher than that produced by stepwise precipitation [10], thus indicating a possible co-precipitation effect. Their analyses (Table 1) are very similar, as occurred with the stepwise-fractionated products [10]. The co-precipitation can occur either on the basis of a non-selective adsorption process or by a selective complexation. Alkaline treatment of **Ci** and **Cs** proceeded with excellent yields (93-95%) giving **CiT** and **CsT**. A decrease of the sulfate content is accompanied by a nearly concomitant increase of the 3,6-anhydrogalactose content. The molar ratios Gal/3,6-AnGal approaches unity in both treated fractions. According to the precursors previously found by NMR on fractions equivalent to **Cs** and **Ci**, but obtained by stepwise precipitation, the treated products should contain about 55-60% of structural units of κ-carrageenans and 40-45% of units of ι-carrageenans [11].

CiT and **CsT** were fractionated by precipitation with increasing concentrations of potassium chloride. Both yielded four fractions, namely those insoluble at 0.1 M, 1 M and 2 M potassium chloride, and that soluble in potassium chloride (maximum concentration used, 2 M). Table 2 shows the yields and analyses of those fractions.

As expected, the main fractions are in both cases those precipitating at 0.1 M KCl. For **CiT**, this fraction represents 92% of its weight, and carries a composition very similar to that of the original fraction. The minor fractions arising from that product also show a similar galactose/3,6-anhydrogalactose/sulfate (G/A/S) ratio, though an increase of the sulfate content with the solubility is observed. Although D-galactose is the main, acid-stable monosaccharide, other sugars as arabinose and L-galactose appear in small amounts. Furthermore, in the 2 M soluble fraction **CiTs** the proportions of L-galactose, mannose and xylose appear to be significant.

CsT is even more heterogeneous. Slightly less product (81%) precipitates with 0.1 M KCl, and even that product contains minor amounts of glucose, arabinose and L-

galactose (Table 2). Another important fraction (**CsTi-1**, 9%) contains less 3,6-anhydrogalactose but more sulfate, glucose and L-galactose. At last, the soluble fraction (**CsTs**), also obtained with a 9% yield, is almost devoid of 3,6-anhydrogalactose, carries less sulfate, and a large heterogeneity of sugars is observed (Table 2): the D/L galactose ratio is *ca* 1.4, highly unusual for a carrageenophyte.

	Yield (%)	Molar ratio Gal[a]: AnGal: S	Ratio per 100 monosaccharide units 3,6-AnGal	Sulfate
Cc		1:0.57:1.14	36	73
Ci	62	1:0.59:1.14	37	72
CiT	93	1:0.96:1.07	49	55
Cs	31	1:0.55:1.23	35	79
CsT	95	1:0.97:1.08	49	55

Table 1. *Yields and analyses of the native carrageenan (**Cc**), as well as those obtained by KCl fractionation (**Ci** and **Cs**) and alkaline treatment (**CiT** and **CsT**)*

	Pption range (M KCl)	Yield(%)	Monosaccharides[a, b] (mole%) Ara	Xyl	Man	Glc	D-Gal	L-Gal	Molar ratio Gal:AnGal:S
CiT	-	-	-	-	1	1	95	3	1:0.96:1.07
CiTi-0.1	0.0 – 0.1	92	4	-	-	-	93	-	1:0.95:1.10
CiTi-1	0.1 – 1.0	5	tr	tr	1	tr	94	4	1:1.12:1.07
CiTi-2	1.0 – 2.0	1	tr	-	-	1	96	3	1:1.05:1.24
CiTs	2.0 sol.	2	2	7	14	2	58	16	1:1.13:1.52
CsT	-	-	2	-	Tr	5	84	7	1:0.97:1.08
CsTi-0.1	0.0 – 0.1	81	4	-	Tr	4	88	4	1:1.18:1.16
CsTi-1	0.1 – 1.0	9	2	2	1	11	77	7	1:0.76:1.55
CsTi-2	1.0 – 2.0	<1	2	2	5	16	66	8	1:1.03:0.89
CsTs	2.0 sol	9	2	6	2	22	38	28	1:0.18:0.71

Table 2. *Yields and analyses of the fractions obtained by alkali treatment and fractionation of carrageenans **Ci** and **Cs**. [a] Acid-stable monosaccharides. [b] Small amounts (ca 1%) of 3-O-methyl-D-galactose were found in samples **CiTi-0.1, CsT, CsTi-2** and **CsTs**, whereas 1% of 6-O-methyl-D-galactose appeared in **CiTi-0.1**.*

The presence of glucose may be an indicative of floridean starch contamination, although traces of other glucans were also found (see later). The presence of glucose in the main chain of galactans cannot be discarded either [12,14,15]. L-Galactose should be due to agarans, and mannose could be related to the mannans found in the fibrilar cell wall of *Iridaea undulosa* [16,17].

Mono-*O*-methylated sugars were not abundant: only trace amounts of 3-*O*-methyl-D-galactose were found in some fractions, together with its isomer methylated on position 6, in one fraction (Table 2). The monomethylated sugars are rare within the carrageenans, though they were mentioned in some variants from the order Halymeniales [1,18,19]. They are usually found in agarans [20], generally methylated at positions 2-

and 6. The presence of 3-*O*-methyl-D-galactose was only mentioned in some agaroids [13,21-24].

Sugar[b] Methylated in	Fraction							
	CsTi-0.1	CsTi-1	CsTi-2	CsTs	CiTi-0.1	CiTi-1	CiTi-2	CiTs
Glucose								
2,3,4,6-	-	3	Tr	2	-	-	-	-
2,3,4-	Tr	-	Tr	7	-	-	-	-
2,4,6-	-	Tr	4	3	-	-	-	-
6-	-	Tr	Tr	5	-	-	-	-
3-	Tr	5	5	Tr	-	-	Tr	Tr
4-	2	Tr	4	-	-	-	-	-
-	-	Tr	Tr	5	-	-	-	-
Galactose								
2,3,46-	2	7	4	25	Tr	2	Tr	24
2,4,6-	16	13	-	4	10	3	5	-
2,3,6-	2	2	-	5	-	-	-	-
4,6-	Tr	Tr	3	-	-	-	-	-
2,6-	64	46	16	19	70	70	78	35
2,4-	5	9	23	-	6	8	6	15
2,3-	3	4	10	5	5	7	7	-
6-	Tr	3	15	3	Tr	2	Tr	Tr
3-	Tr	Tr	-	2	2	6	Tr	-
-	Tr	2	5	5	Tr	-	-	-
Mannose								
2,3,6-	-	-	4	-	-	Tr	-	12
3,6-	-	-	-	Tr	-	-	-	2
Xilose								
2,3,4-	-	-	-	-	-	-	-	6
Arabinose								
2,3	-	-	-	-	4	-	-	-

Table 3. *Methylation analyses[a] of the products obtained after KCl fractionation of* ***CsT*** *and* ***CiT****. [a]In mole/100 moles, normalized with respect to the monosaccharide content in Table 2. Proportions lower than 1.5% are marked as traces (tr). [b]Acid-stable, partially methylated monosaccharides.*

Both soluble products (**CsTs** and **CiTs**) concentrate the most heterogeneous composition. This may be easily explained for **CsTs**, as this fraction acts as the "garbage bin" of all the non-precipitating fractions. Although in small proportion, the presence of **CiTs** should be unexpected, considering that this fraction precipitated originally with potassium chloride (probably by co-precipitation) and remains soluble after alkaline treatment. Evidently, the co-precipitation effect acting in the native product disappears

for the alkali-treated product: possibly, the stability of the complex has decreased after the alkali treatment. Although both **CiTs** and **CsTs** carry agaran units (Table 2), a differential concentration of the other components is observed: while the former concentrates the mannans, the later is concentrating glucans. Xylose appears to be significant in both fractions. Mannans, xylans and xylomannans are polysaccharides usually present in the simpler orders Chaetangiales and Nemaliales [25-28].

The four fractions originated in **CsT** and **CiT** were submitted to methylation analyses. Results are shown on Table 3. The main product in most of the fractions is 2,6-di-*O*-methylgalactose, usual in cystocarpic carrageenans as it indicates 4-sulfated 3-linked units. However, the same unit may indicate 4-linked, 3-sulfated units. Some of the fractions (especially **CsTi-2** and **CiTs**) show important amounts of 2,4-di-*O*-methylgalactose, which suggests the presence of 3-linked, 6 sulfated units. These units should not be affected by alkaline treatment. Ayal and Matsuhiro [29] indicated the presence of these units in *Chondrus canaliculatus*. This sugar is also an important constituent of the corallinans from *Corallina officinalis* [30], and its presence is significant in the agarans found in tetrasporic *Iridaea undulosa* [12]. 2,4,6-tri-*O*-methylgalactose appears in the gelling fractions, suggesting that some of the typical units of the κ-family are not sulfated. The presence of terminal 2,3,4,6-tetra-*O*-methylgalactose in the soluble fractions is indicative of low molecular weights or branched products. This sugar also accompanies the presence of 2,4-di-*O*-methylgalactose in corallinans [30] and agarans from tetrasporic *Iridaea undulosa* [12]. The presence of 2,3-di-*O*-methylgalactose cannot be rationalized on the basis of 4-linked, 6-sulfated units, as they would have suffered alkaline cyclization. Thus, variant possibilities as 4-linked, 6-branched, or 6-linked, 4-sulfated units cannot be discarded. **CsTi-2** is especially rich in those unusual units. However, this fraction is a minor component of **CsT**.

A great variety of methylated glucoses were found in the methylation analysis of **CsT** fractions. Many of them may be indicating undermethylation. However, the presence of 2,3,4-tri-*O*-methylglucose in **CsTs** is suggesting the existence of a separate 6-linked glucan (see below). The mannose-containing fractions show this sugar mainly as its 2,3,6-derivative, suggesting once more the presence of a β-(1→4)-mannan, which was co-solubilized with the carrageenans. Its solubility may be due to a low molecular weight or to branching. The appearance of small amounts of 3,6-di-*O*-methylmannose in **CiTs** agrees with this suggestion.

The soluble products were sub-fractionated by means of anion-exchange chromatography. As previously suggested, the exchange resin was "diluted" with neutral gel in order to maximize the elution of the highly sulfated products [12]. The chromatographic elution profile of **CsTs** is shown on Figure 1. The products were regrouped in five fractions. Table 4 indicates the yields and monosaccharide analyses of the fractions. The main fraction (**Fs-0.2**), obtained in 43 % yield, is mainly composed by glucose and galactose. The following fraction (**Fs-0.5**) is also glucose-rich. These products exhibit a galactose D/L ratio close to 1, typical of agarans. By increasing the ionic strength, the products become more sulfated, and the proportion of "rare" sugars such as L-galactose, xylose and glucose diminishes, giving rise to products with

characteristics closer to those of carrageenans. Methylation analysis of the five fractions did not give clear-cut results, due to undermethylation of the galactose constituents.

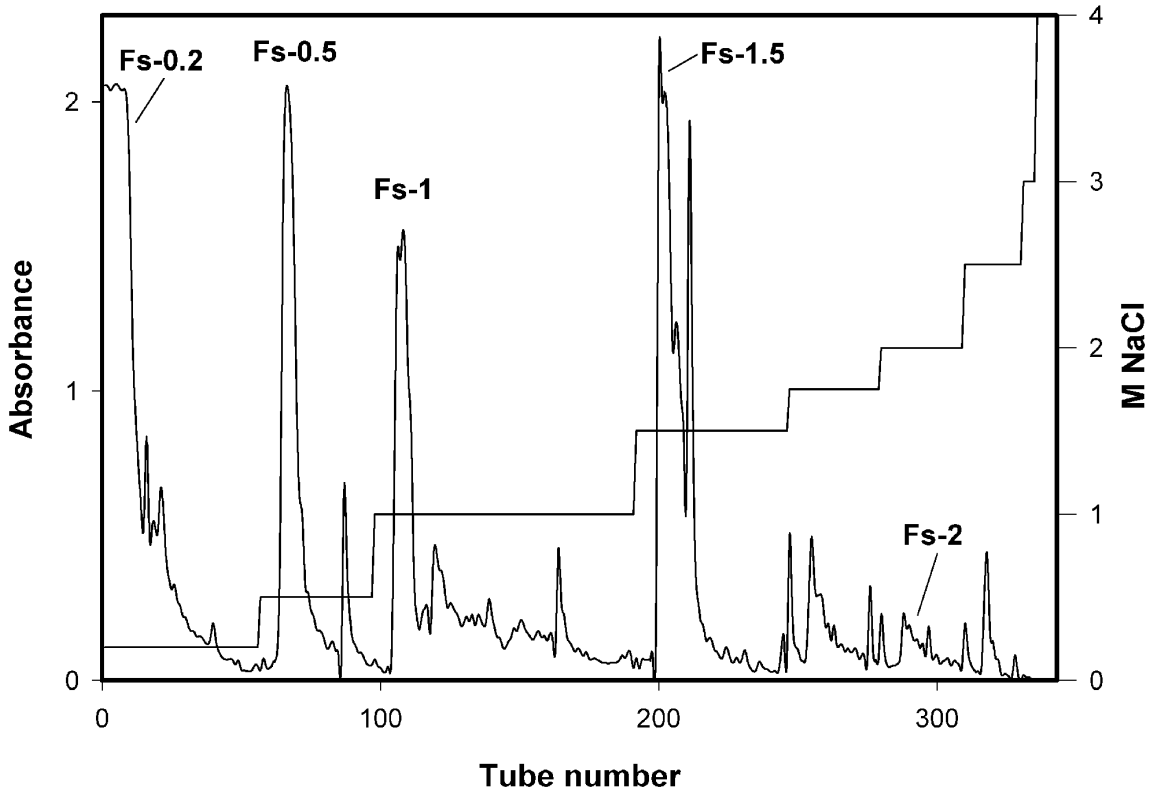


Figure 1. *Elution profile of the anion-exchange chromatography of fraction* ***CsTs*** *on DEAE-Sephadex A-50/Sephadex G-100.*

However, two different kinds of glucans in **Fs-0.2** and **Fs-0.5**, as 2,3,4- and 2,3,6-tri-*O*-methylglucose were the predominant derivatives in each fraction, respectively. Thus, **Fs-0.2** should contain a 6-linked glucan, while **Fs-0.5** should contain a 4-linked glucan. The ^{13}C-NMR spectrum of the main fraction **Fs-0.2** (Figure 2) confirms the presence of a 6-linked glucan, as well as determines its α-configuration. The signals of a typical dextran [31] are observed, while the remaining components of the sample do not give rise to any recognizable signal.

The other soluble fraction, **CiTs** was also fractionated by anion exchange chromatography. Table 5 shows the yields and monosaccharide composition of the fractions. Many different fractions were obtained; only two of them appear in significant proportions. The heterogeneity is marked, and no direct relationship between the ionic strength and composition can be rationalized. Furthermore, it is noteworthy the high amounts of mannose in charged products. This may be suggesting the presence of sulfated mannans as a minor constituent of the matrix polysaccharides of this seaweed. Those products appear usually within the orders Chaetangiales and Nemaliales [25-28].

Fraction	Yield (%)	Monosaccharides[a] (mole %)								Sulfate (% SO_3Na)
		Rha	Ara	Xyl	Man	Glc	D-Gal	L-Gal	3-*O*-Me-D-Gal	
Fs-0.2	43	2	1	10	2	33	23	27	2	8
Fs-0.5	9	-	2	12	6	42	20	17	1	10
Fs-1	17	-	-	7	1	3	63	25	1	17
Fs-1.5	23	1	2	2	1	3	66	25	1	26
Fs-2	7	1	-	1	3	6	82	5	2	22

Table 4. *Yields and composition of the main fractions isolated by ion exchange chromatography of* ***CsTs.*** *[a] Acid-stable monosaccharides.*

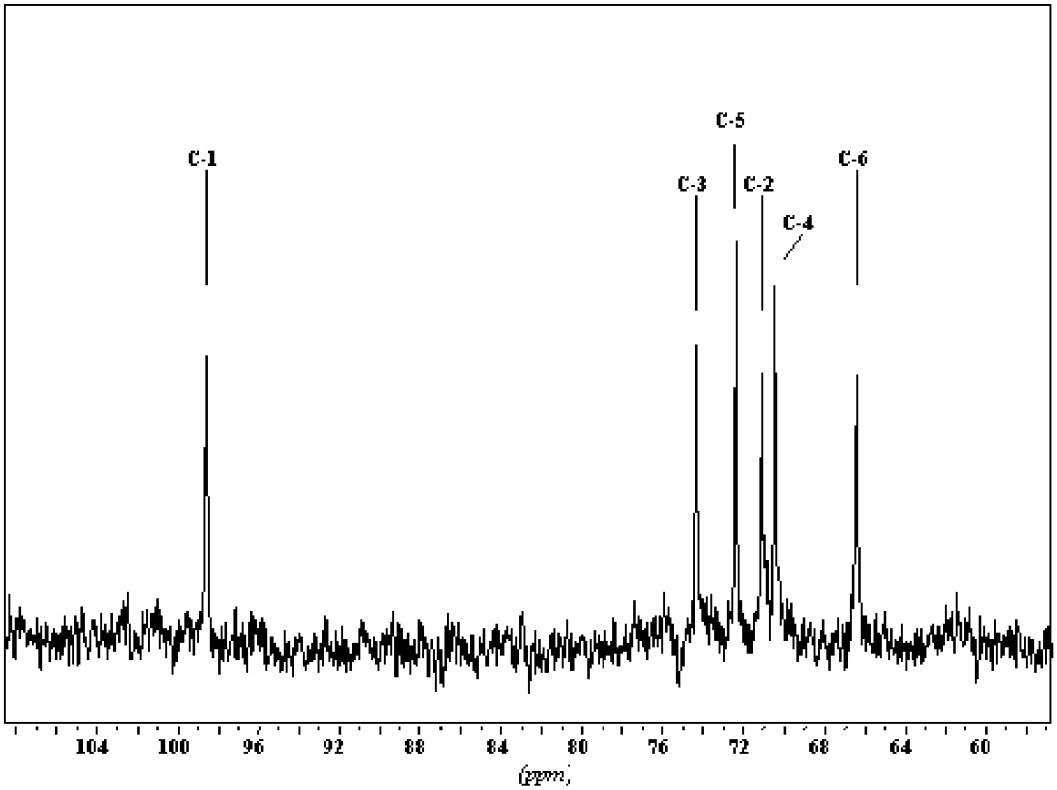


Figure 2. *^{13}C-NMR spectrum of fraction* ***Fs-0.2****.*

No mannans were detected within the Florideophycidae, with the exception of the fibrilar mannans found in the species under study [16,17]. Glucose also appears in large amounts in some of the products eluting at high NaCl concentrations, consequently suggesting its presence in a sulfated polysaccharide. Methylation analysis of some fractions showed signs of undermethylation. However, the presence of 2,6-di-*O*-methylgalactose in the last-eluting fractions is consistent with carrageenan features. The main derivatives

becoming from mannose and glucose are methylated at positions 2, 3 and 6, suggesting the presence of 4-linked mannans and glucans. However, branching and sulfation are not precluded by the analysis. Xylose appears methylated at positions 2,3,4- and 2,3-, suggesting that it may appear as branches of the galactans, glucans and/or mannans, but also that a separate 4-linked xylan may exist. In the minor fractions **Fi-12** and **Fi-15**, galactose is present only as its L-isomer. In red seaweeds no galactan has been found with a galactose D/L-ratio lower than 1.

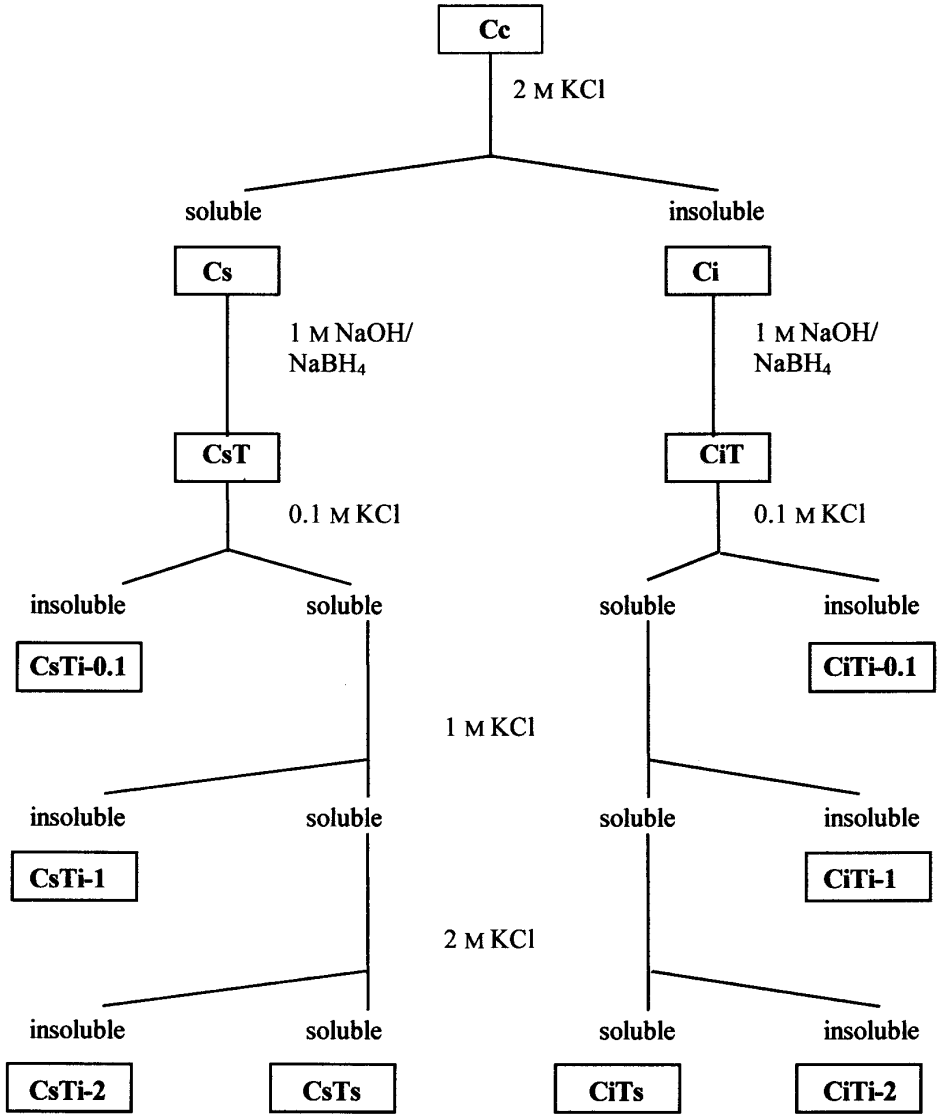
Fraction	[NaCl] (M)	Yield (%)	Molar ratio G:A:S[b]	Monosaccharides[a] (moles %)							
				Rha	Ara	Xyl	Man	Glc	D-Gal	L-Gal	3-Me Gal
Fi-1	0.2	31	1:0.08:0.07	4	1	14	12	61	3	4	-
Fi-2	0.2	5	1:0.19:0.19	7	1	26	23	19	9	12	2
Fi-3	0.5	3	1:0.37:0.36	7	1	29	30	18	10	4	1
Fi-4	1.0	1	1:0.21:0.40	8	1	32	27	15	8	7	3
Fi-5	1.5	5	1:0.65:0.24	1	-	19	19	9	35	17	2
Fi-6	1.5	2	1:0.04:0.00	3	-	22	27	7	25	17	-
Fi-7	1.5	31	1:0.92:1.87	1	1	16	13	9	36	23	1
Fi-8	1.75	4	1:0.13:0.23	1	5	23	16	5	31	19	-
Fi-9	1.75	4	1:0.47:0.38	1	2	20	4	3	-	68	2
Fi-10	1.75	2	1:0.37:0.39	2	3	26	10	39	11	8	1
Fi-11	2.0	2	1:0.25:0.75	2	3	26	10	39	11	8	1
Fi-12	2.0	2	1:2.12:0.48	1	1	7	19	12	1	58	1
Fi-13	2.5	5	1:1.02:0.50	1	1	13	26	6	52	1	-
Fi-14	3.0	2	-	-	-	-	39	17	32	13	-
Fi-15	4.0	1	1:0.95:0.40	2	-	1	57	13	1	26	-

Table 5. *Yields and composition of the main fractions isolated by ion exchange chromatography of* ***CiTs.*** *[a] Acid-stable monosaccharides. [b] G:A:S= galactose/3,6-anhydrogalactose/sulfate*

Experimental

Material. The cystocarpic carrageenans from *Iridaea undulosa Bory* were obtained as described elsewhere [10,16,17].

Fractionation and alkaline treatment of the cystocarpic carrrageenans. The whole carrageenans from cystocarpic *Iridaea undulosa* (**Cc**, 12 g) [10] were fractionated in bulk with 2 M KCl. The precipitate and the supernatant were separately dialyzed and lyophilized, yielding products **Ci** and **Cs**. Both products were treated with 1 M NaOH for 5 h at 80 °C in the presence of $NaBH_4$. The treated products (**CsT** y **CiT**), obtained after dialysis and lyophilization, were re-fractionated with KCl at increasing concentrations, from 0.1 M to 2 M (Scheme 1).



Scheme 1

General analyses. Total carbohydrates were determined by the phenol-H_2SO_4 method [32], 3,6-anhydrogalactose by the resorcinol-HCl method [33], and the percentages of sulfate were measured by turbidity measurement [34] after hydrolysis with 1 M HCl. Hydrolysis of the polysaccharides was carried out with 2 M CF_3COOH (90 min, 120 °C) Hydrolyzates were converted into the aldononitrile acetates [35] and analyzed by GLC using a capillary column (30 m × 0.25 mm) coated with SP-2330 (0.20 μm) on a HP-5890 Gas Chromatograph equipped with a flame ionization detector (FID). Nitrogen was used as the carrier gas, with a flow rate of 1 mL/min and a split ratio of 100:1. Chromatography runs were isothermal at 220 °C, while the injector and detector were set

at 235 °C. The configuration of the constituting monosaccharides was determined after conversion into the corresponding aminoalditols and GLC analysis [36].

Anion-exchange chromatography of the products. The fractions soluble in 2 M KCl (**CsTs** and **CiTs**) originated in the alkaline treatment of the soluble and insoluble carrageenans from *Iridaea undulosa*, were subfractionated by anion exchange chromatography on DEAE Sephadex A-50/Sephadex G-100: the gel was prepared with 32 ml of DEAE-Sephadex A-50 in 0.2 M NaCl and 1.8 g of Sephadex G-100 in excess NaCl 0.2 M, after boiling [12]. The gel was poured into glass columns (1.5 x 32 cm), used to achieve the separation. 169 mg of **CsTs** and 69 mg de **CiTs** were applied to the column, previously dissolved in 0.2 M NaCl. Elution was carried out by stepwise addition on increasing concentrations of NaCl until 4 M. Three-ml fractions were collected, and dosed by the PhOH-H_2SO_4 method [32]. According to the chromatographic profile, see Figure 3, the fractions were grouped, dialyzed (mol.wt. cutoff 3,500) and lyophilized.

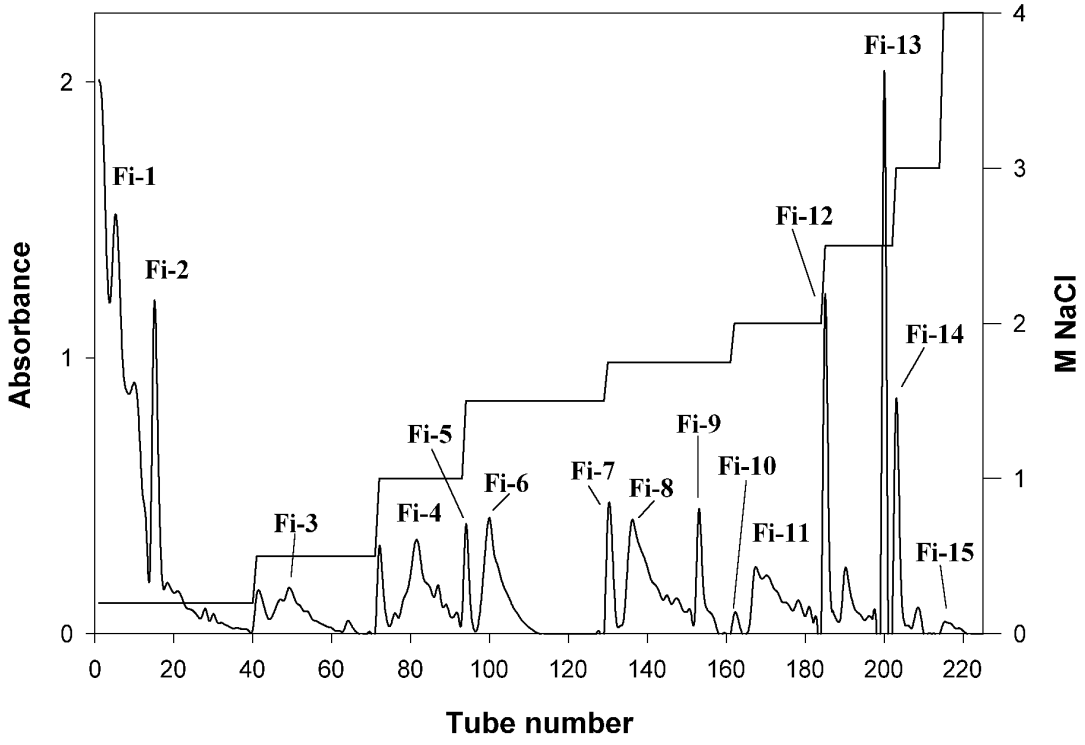


Figure 3. *Elution profile of the anion-exchange chromatography of fraction* ***CiTs*** *on DEAE-Sephadex A-50/Sephadex G-100.*

Methylation analysis. Methylation was carried out as described previously [10], by the action of sodium methylsulfinylmethanide or sodium hydroxide, and then methyl iodide, on the triethylammonium salts of the polysaccharides [37]. The products were recovered

by dialysis and lyophilization. The partially methylated monosaccharides were analyzed as their corresponding alditol acetates by GLC and GLC-MS.

^{13}C-NMR spectroscopy. The ^{13}C-NMR spectrum of fraction **Fs-0.2s** was carried out on a sample dissolved in 0.4 ml of a 1:1 mixture of H_2O / D_2O, at 50.32 MHz on a Bruker ACE-200 apparatus, using a pulse angle of 90°, no relaxation delay and 6,000 scans. Chemical shifts were determined with relation to dioxane as external standard, and referred to TMS by adding 67.4 ppm. Total proton decoupling, and deuterium-lock were used.

Conclusion

It was usually stressed that carrageenans are the only polysaccharides present in the Gigartinaceae. Many studies have been devoted to show their chemical structure, physical properties, and biological activity. This work confirms that, even though carrageenans are by far the most important polysaccharides, a variety of other constituents are present, some in trace amounts, in their matrices.

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