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Alkali modification of carrageenans. Part V. The iota–nu hybrid carrageenan from *Eucheuma denticulatum* and its cyclization to iota-carrageenan☆

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Abstract

A homogeneous iota/nu-hybrid carrageenan (71% iota- and 21% nu-) isolated from *Eucheuma denticulatum* was used as a model compound to study the cyclization reaction of α-D-galactose 2,6-disulfate units to 3,6-anhydro-α-D-galactose 2-sulfate. The rate of cyclization, at 70 °C, of this carrageenan is about 50 times faster than that of a porphyran (non-sulfated β-D-galactose linked to α-L-galactose 6-sulfate) and 210 times faster when compared with a lambda-carrageenan (2-sulfated β-D-units linked to α-D-galactose 2,6-disulfate). The use of this model compound confirms the previous hypothesis of the accelerating effect of the β-D-4-sulfate group as well as suggests the influence of the 2-sulfate of the α-D-galactose units on the $^4C_1 \rightarrow {}^1C_4$ chair forms interchange. The easy of cyclization indicates that to produce commercial iota-carrageenans milder alkaline treatments could be used, avoiding degradation and increasing the gel strength.

Keywords: Alkaline treatment; Cyclization rate; Iota-carrageenan; *Eucheuma denticulatum*

1. Introduction

Formation of 3,6-anhydro-α-D-galactopyranose units from α-D-galactopyranose 6-sulfate residues by alkaline treatment is an important and well-known reaction undergone by carrageenans (Percival, 1949; Turvey, 1965). We have studied the kinetics of this reaction in lambda- and kappa/iota-carrageenans (Ciancia, Noseda, Matulewicz, & Cerezo, 1993), lambda-derived oligosaccharides (Noseda and Cerezo, 1995) and porphyrans (Noseda, Viana, Duarte, & Cerezo, 2000). These works show the influence that the sulfate groups have on the cyclization rates. The presence of sulfate at C-2 of β-D-galactose unit as in lambda-carrageenans has a decelerating effect, whereas at C-4 as in carrageenans of the kappa family accelerates the cyclization reaction. Fig. 1 shows the disaccharide repeating units of the before mentioned polysaccharides.

In this paper we extended these studies to an iota-carrageenan that was extracted from the commercially cultivated algae *Eucheuma denticulatum*. Alkaline extraction of this red seaweed is commonly used for the industrial production of the gel forming polysaccharide iota-carrageenan (Van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002; Aguilan et al., 2003). Nevertheless, in spite of the high commercial value of the seaweed and of its polysaccharide, detailed studies have not been carried out on the structure of the biosynthesized carrageenan and on the best conditions for the alkaline cyclization of its 'precursor units' to produce a 'nearly pure' iota-carrageenan. Thus, in this paper the iota/nu hybrid carrageenan extracted from the seaweed with water at room temperature was worked out with modern techniques and used

☆ For Part IV, see Noseda et al. (2000).

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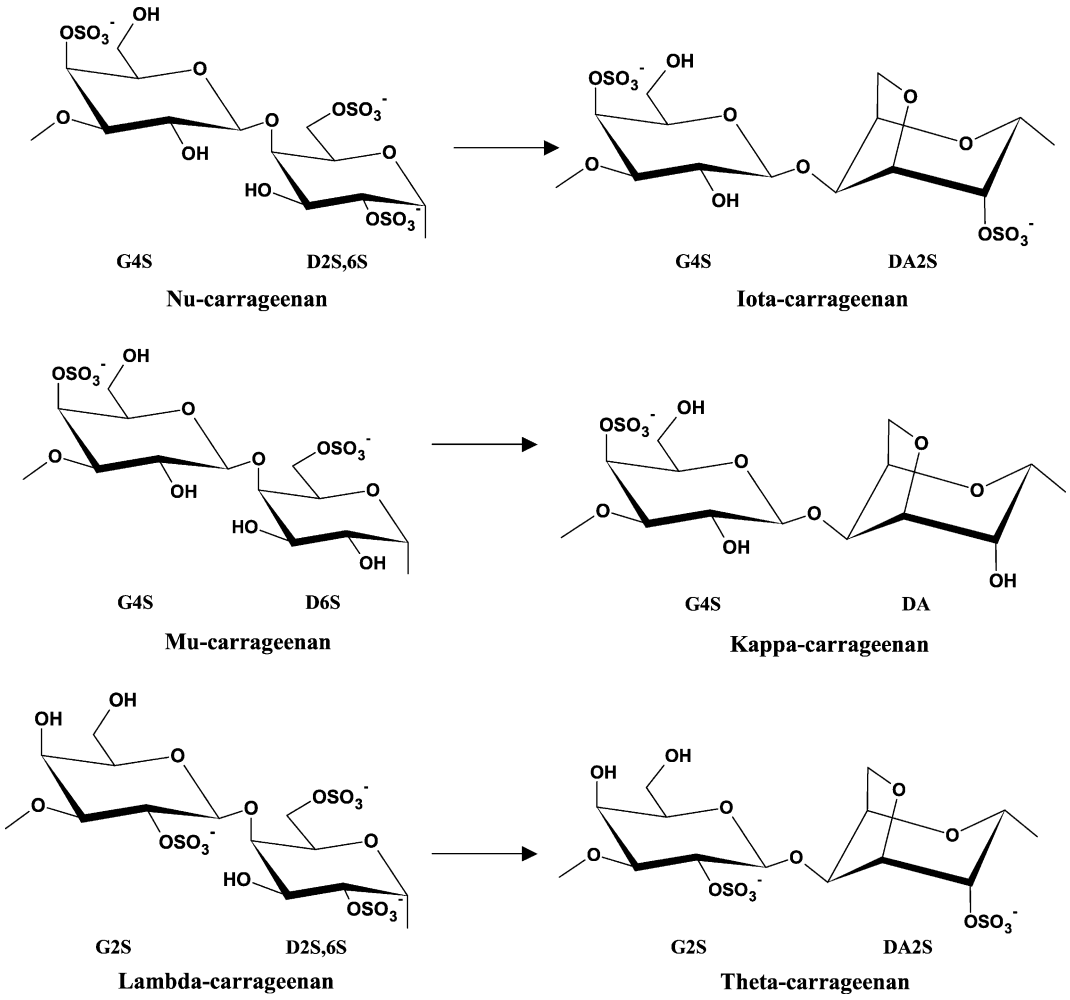

Fig. 1. Disaccharide repetitive structures of carrageenans.

as a model compound to study the kinetics of the cyclization of the (1→4)-linked α-D-galactopyranose 2,6-disulfate units to (1→4)-linked 3,6-anhydro-α-D-galactopyranose 2-sulfate residues, in a 'pure' nu-structure and to determine its rate constant and half-life.

2. Material and methods

2.1. Material

Commercial samples of *E. denticulatum* (N.L. Burman) F.S. Collins & Hervy from Philippines were obtained from Gelymar S.A. (Puerto Montt, Chile).

2.2. Extraction of polysaccharides

The milled seaweed was extracted with water (5 g% w/v) at room temperature (25 °C), with mechanical stirring, for 14 h. The residue was removed by centrifugation and the supernatant was poured into ethanol (three volumes) yielding the polysaccharide fraction ECW. This fraction was purified by redissolution in water, dialysis, centrifugation and lyophilization (yield 24%).

2.3. Alkaline treatment

The analytical alkaline treatment was carried out as described by Ciancia et al. (1993). 3 M sodium hydroxide (1 ml) was added to the sample (4 mg), previously dissolved in water (2 ml) and reduced with sodium borohydride (10% w/w), reaching the final concentration of M sodium hydroxide. This solution was then heated at 40, 50, 60 and 70 °C. Samples were taken at regular intervals, the reaction stopped by cooling in an ice bath and neutralized with M hydrochloric acid. 3,6-anhydrogalactose content was determined by resorcinol method (Yaphe, 1960). From these results, the rate constants and half-life were determined at each temperature. The preparative alkaline treatment of ECW was carried out at 80 °C in M sodium hydroxide. After 0.5 h of treatment the solution was cooled, neutralized, dialyzed and freeze-dried rendering the alkali-modified polysaccharide ECW-m (yield 80%).

2.4. Chemical analyses

The monosaccharide composition of ECW and ECW-m was determined after reductive hydrolysis (Stevenson & Furneaux, 1991; Falshaw & Furneaux, 1994) by GC. The sulfate content was determined as described by Dodgson and Price (1962).

2.5. Optical rotation analysis

Optical rotation of aqueous solution of polysaccharide samples (0.2%), were measured at 20 °C, using a 10 cm cell and sodium D line (589.3 nm) with a Rudolph Autopol III automatic polarimeter.

2.6. Fourier-transform infrared analysis (FT-IR)

The FTIR spectra of KBr pellets of polysaccharides (2/100 mg KBr) were recorded in a Perkin–Elmer Series 2000 FTIR spectrophotometer (eight scans, at a resolution of 4 cm^{-1}) scanning between 4000 and 400 cm^{-1}.

2.7. Methylation analysis

Methylation analysis was carried out by the method of Ciucanu and Kerek (1984) on the triethylammonium salts of the carrageenans (Stortz & Cerezo, 1993) using methyl iodide. The permethylated polysaccharides were hydrolyzed following the reductive hydrolysis procedure (Stevenson & Furneaux, 1991; Falshaw & Furneaux, 1994) and analyzed by GC and GC-MS as partially methylated alditol acetates. Iota- and nu-structure percentages were calculated from the amount of 3,6-anhydrogalactose and 3-*O*-methyl galactose, respectively, in ECW and from 2,6-di-*O*-methyl galactose (iota-) in ECW-m.

2.8. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis

GC analyses were carried out with a HP-5890 gas chromatograph equipped with a flame ionization detector (FID), using a fused silica capillary column (30 m × 0.25 mm) coated with DB-225. Chromatography was run isothermically at 210 °C. Both injector and FID temperature were at 250 °C. Nitrogen was used as carrier gas at a flow rate of 1 ml/min and a split ratio of 100:1. GC-MS analyses were performed using a Varian 3300 chromatograph and a Finnigan Mat ITD spectrometer. The chromatograph was programmed to run at 50 °C for 1 min, then 50–220 °C at 40 °C/min. Helium was used as the carrier gas at 1 ml/min.

2.9. Nuclear magnetic resonance spectroscopy (NMR) analysis

For NMR spectroscopic analysis the lyophilized sample was dissolved in D_2O (20 mg/0.6 ml). The NMR spectrum of the solution was recorded at 70 °C using a Bruker Avance DRX400 NMR spectrometer. ^{13}C NMR spectra of ECW and ECW-m were obtained using a multinuclear inverse detection 5 mm probe. Chemical shifts are expressed in ppm using acetone as internal standard at 30.2 ppm.

2.10. High-pressure size-exclusion chromatography (HPSEC) analysis

HPSEC analysis was carried out with a 2 mg/ml solution of polysaccharide, using a multidetection equipment with a Waters 2410 differential refractometer (RI) and a Wyatt Technology Dawn F multiangle laser light scattering (MALLS) detector adapted on-line. Four Waters Ultrahydrogel 2000/500/250/120 columns were connected in series and coupled to the multidetection equipment. A 0.1 M $NaNO_3$ solution, containing NaN_3 (0.5 g/l), was used as eluent.

3. Results

The seaweed was extracted with water at room temperature and the polysaccharide was recovered by precipitation with ethanol and purified by redissolution and dialysis. The polysaccharide fraction (ECW), homogeneous as determined by HPSEC-MALLS, was obtained with 24% yield, containing 30.7% of sulfate (2.2 mol of sulfate every disaccharide unit), galactose and 3,6-anhydrogalactose as monosaccharide constituents (Table 1).

Table 1
Yield, analysis and monosaccharide compositions of native and permethylated ECW and ECW-m

Sample	Yield[a] (%)	$[\alpha]_D^{20}$ (°)	SO_3Na (%)	Monosaccharide (mol%)		Methylation analysis (mol%)				
				AnGal[b]	Gal[c]	2,6-MGal[d]	6-MGal[e]	3-MGal[f]	2-MGal[g]	AnGal
ECW	24	+29.0	30.7	36.1	63.9	46.9	2.3	10.3	5.1	35.4
ECW-m	80	+16.5	24.3	49.0	51.0	46.8	0.8	1.3	2.8	48.3

[a] Related to milled seaweed submitted to extraction (ECW) and polysaccharide mass initially submitted to preparative alkaline treatment (ECW-m).
[b] 3,6-Anhydrogalactose.
[c] Galactose.
[d] 2,6-Di-*O*-methyl galactose.
[e] 6-*O*-Methyl galactose.
[f] 3-*O*-Methyl galactose.
[g] 2-*O*-Methyl galactose.

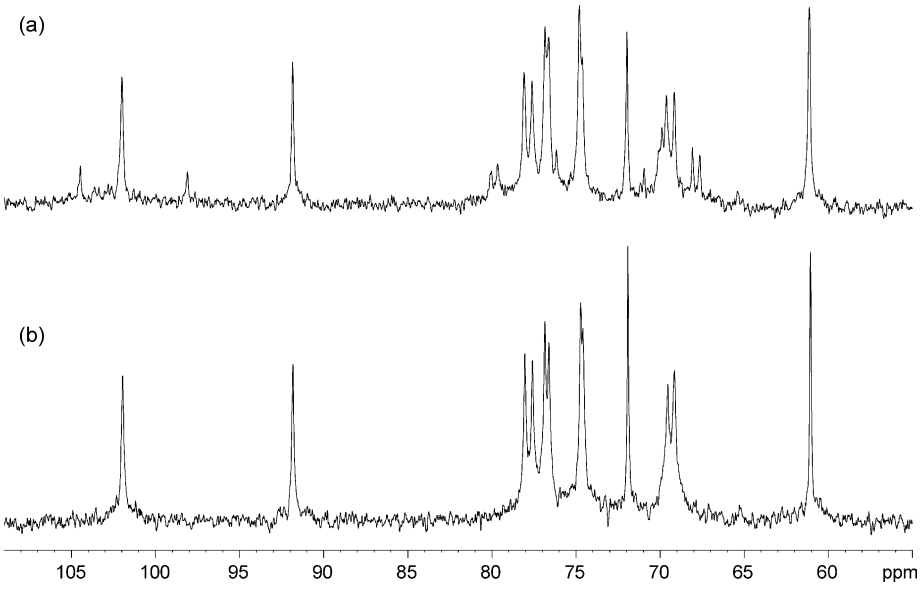

Fig. 2. ^{13}C NMR spectra of partially cyclized nu-carrageenan (ECW) before (a) and after (b) alkaline treatment (ECW-m).

This product was submitted to a preparative alkaline treatment for 0.5 h, giving a homogeneous fraction ECW-m, with 80% yield and 24.3% of sulfate. Its ^{13}C NMR spectrum (Fig. 2b) showed 12 absorption peaks typical of an iota-carrageenan (Usov & Shashkov, 1985) different from the original one (ECW, Fig. 2a), in which this pattern of absorption was shown together with a minor one of a nu-carrageenan (Stortz, Bacon, Cherniak, & Cerezo, 1994).

Both FTIR spectra were very similar, with an increase in the absorptions at 928.7 and 805.1 cm^{-1} in the alkali-treated derivative corresponding to the 3,6-anhydro ring and sulfate linked to C-2 of 3,6-anhydrogalactose, respectively (Bellion, Brigand, Prone, & Bociek, 1983; Prado-Fernández, Rodríguez-Vásquez, Tojo, & Andrade, 2003). No signals corresponding to nu-carrageenan (absorptions of equatorial and primary sulfate groups) were detected in the spectrum of the original product in spite of the 10.3% of 2,6-disulfated α-D-galactopyranosyl units, determined by methylation analysis (Table 1). Comparison of the percentages of 3,6-anhydrogalactose in the original and methylated products indicates that no cyclization was produced during the methylation. Methylation analysis of both, original and alkali-treated products (ECW and ECW-m) also indicate the presence of 46.9–46.8% of 2,6-di-*O*-methyl galactose and 35.4 and 48.3%, respectively, of 3,6-anhydrogalactose (Table 1), showing that the original one contained about 71% of iota-structure (compositional analysis showed 72% of iota-structure, Table 1) together with approximately 21% of nu-backbone while the treated one contained 94% of iota-structure. The alkaline derivative showed together with the increase in 3,6-anhydrogalactose, indicative of the cyclization of the 4-linked 2,6-disulfated α-D-galactose units into the 2-sulfate 3,6-anhydrogalactose, traces of 3-*O*-methyl galactose (1.3%), compatible with a slightly incomplete cyclization (Table 1). Small amounts of 6-*o*-methyl galactose (2.3–0.8%) and 2-*O*-methyl galactose (5.1–2.8%) detected for both fractions could suggest the presence of unusual units such as 3-linked 2,4-disulfate β-D-galactose (alternatively 4-linked 2,3-disulfate α-D-galactose residues) and 4-linked 3,6-disulfate α-D-galactose (alternatively 3-linked 4,6-disulfate β-D-galactose), respectively.

The cyclization reaction of ECW follows, as the previous ones (Ciancia et al., 1993), a pseudo first-order kinetics as determined by the plot ln $(A_o - A_\infty)/(A_t - A_\infty)$ as a function of time (Fig. 3). Table 2 shows the rate constant and half-life for ECW in M sodium hydroxide, at different temperatures, compared with those previously reported for different carrageenans (Ciancia et al.), carrageenan derivatives (Noseda & Cerezo, 1995) and porphyran (Noseda et al., 2000). The cyclization was too fast to be measured at 80 °C. At 60 and 70 °C (Table 2) the reaction constants are about 25 and 50 times higher than that of porphyran PC75, an agaran without sulfation on the β-D-unit and 6-sulfation on the α-L-residue. Table 2 also shows that the cyclization of ECW is about 160–210 times faster than that of a lambda-carrageenan ($1T_2$) with non-sulfated and 2-sulfated β-D-units and 2,6-disulfated α-D-residues (Ciancia et al.).

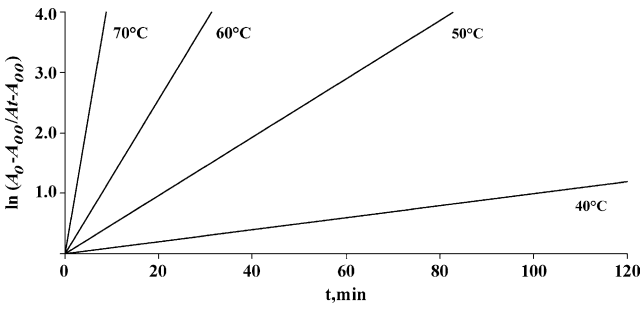

Fig. 3. Determination of the rate constants of the cyclization reaction for nu-carrageenan ECW, at 40, 50, 60 and 70 °C.

Table 2
Cyclization reactions for nu-carrageenan ECW in M NaOH, at different temperatures

Temperature (°C)	Rate constant k (10^4 s^{-1})				$t_{½}$ (min)			
	ECW	$1C_3$[a]	PC75[b]	$1T_2$[a]	ECW	$1C_3$	PC75	$1T_2$
40	2.0	–	–	–	69.4	–	–	–
50	7.0	2.3	–	0.04	15.8	49.9	–	3000
60	21.0	4.8	1.0	0.14	5.4	24.0	139.0	850
70	77.0	13.0	1.5	0.35	1.5	9.0	77.0	320
80	–	26.0	4.9	0.67	–	4.5	23.0	170
90	–	59.0	11.0	1.11	–	2.0	10.5	130

[a] $1C_3$: Partially cyclized mu/nu-carrageenan and $1T_2$: lambda-carrageenan (Ciancia et al., 1993).
[b] PC75: Porphyran (Noseda et al., 2000).

4. Discusión

The carrageenan from *E. denticulatum* (former *E. spinosum*) was studied firstly by Anderson, Dolan, and Rees (1973). They obtained, by hot water extraction, an iota/nu-hybrid carrageenan (using the current nomenclature) with about 8–9% of precursor, 2,6-disulfate α-D-galactose units and small amounts (6 and 4%, respectively) of non-sulfated (1→3)- and (1→4)-linked residues.

The carrageenan from *E. denticulatum* has become most valuable to industry since it produces a 'nearly ideal' iota-carrageenan upon alkali modification, and the seaweed is now currently farmed in the Philippines and other countries of the Far East. Its high commercial value has promoted studies on the alkali-treated modification (Santos, 1989; Aguilan et al., 2003) but they did not deepen into the structure of the original product.

Thus, extraction of the seaweed with water at 115–120 °C gave a polysaccharide (40–50% yield), which was worked out through its alkali-treated derivative (Aguilan et al., 2003). This alkaline-modified product contains not only galactose and 3,6-anhydrogalactose but also a small amount of xylose, which is not usual in carrageenans. Methylation analysis showed the expected iota-structural units but also small amounts of 3,6-anhydrogalactose, (1→4) linked α-D-galactose, a disulfated unit and non-reducing end-chain galactose. As the carrageenans are non-branched linear polysaccharides the last residue (3.5%), indicating a molecular weight about 4.6 kDa, suggests degradation during the extraction or the alkaline treatment of the original carrageenan.

The extraction of the carrageenan from *E. denticulatum* was carried out different from previous works this time at room temperature on the basis that the distribution of the precursor unit in the carrageenan molecules would produce, even if with lower yield, a sample with higher amounts of 2,6-disulfate α-D-galactopyranosyl units and, as a consequence, higher solubility.

The product obtained was 92% an iota/nu-hybrid carrageenan with approximately 21% of nu-structure (see Experimental) and only 7% of 'unusual units'. Thus, it was a good natural model to study the cyclization rate of the 2,6-di-*O*-sulfated α-D-galactopyranosyl units linked to β-D-galactose 4-sulfate residues. The cyclization of the nu-structure in the carrageenan from *E. denticulatum* was the fastest determined (Table 2). Comparison of its rate constant with that obtained in the cyclization of $1C_3$ (the nearest value, Table 2) is not straightforward due to the structural complexity of $1C_3$ (it contains non-sulfated and 4-sulfated β-D-units together with 6- and 2,6-sulfated α-D-residues (Ciancia et al., 1993)) but a rough appreciation of the data (Table 2) suggests that the cyclization of a 'pure' mu-carrageenan should be slower than that of a 'pure' nu-carrageenan. This difference could be rationalized supposing that the ionization of the C-3–OH in the α-units (the repulsive interaction between the negative charges in the C-2 and C-3 groups of the pyranose cycle is the driven force for the exchange of the $^4C_1 \rightarrow {}^1C_4$ chair forms, for the mechanism of the alkaline cyclization, see refs. Percival, 1949; Turvey, 1965) could be suppressed more efficiently by the C-2 hydroxide anion (C-2–O^-) in a mu-carrageenan (the C-2 hydroxyl of the same unit would ionize first due to its higher acidity (Lenz, 1960; Sugihara, 1953)) than by the C-2 sulfate anion (C-2–O–SO_3^-) in a nu-carrageenan, considering that this last group would situate the negative charge farther apart.

The extraction from *E. denticulatum* of an iota/nu-hybrid carrageenan with a higher percentage of nu-precursor units, and therefore more adequate for the kinetic studies, by choosing the conditions of the extractions exemplify the importance of this step in the study of the structurally dispersed polysaccharides. Alkaline extractions of seaweeds producing carrageenans of the kappa family must be avoided, even at room temperature, as the products obtained could be different of the native ones. The ease with which the cyclization reaction takes place in the carrageenans of the kappa family (this paper and Ciancia et al., 1993) indicates that the alkaline treatments used industrially to increase the gelling properties of crude carrageenans could be carried out under milder conditions giving products with lower degradation and, consequently, higher gel strengths.

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References

Aguilan, J. T., Broom, J. E., Hemmingson, J. A., Dayrit, F. M., Montaño, M. N. E., Dancel, M. C. A., Niñonuevo, M. R., & Furneaux, R. H. (2003). Structural analysis of carrageenan from farmed varieties of Philippine seaweed. *Botanica Marina*, *46*, 179–192.

Anderson, N. S., Dolan, T. C. S., & Rees, D. A. (1973). Carrageenans. Part VII. Polysaccharides from *Eucheuma spinosum* and *Eucheuma cottonii*. The covalent structure of ι-carrageenan. *Journal of Chemical Society*, *19*, 2173–2176.

Bellion, C., Brigand, G., Prome, J. C., & Bociek, D. W. S. (1983). Identification et caractérisation des précurseurs biologiques des carraghénanes par spectroscopie de R.M.N.-^{13}C. *Carbohydrate Research*, *119*, 31–48.

Ciancia, M., Noseda, M. D., Matulewicz, M. C., & Cerezo, A. S. (1993). Alkali-modification of carrageenans: mechanism and kinetics in the kappa/iota, mu/nu and lambda-series. *Carbohydrate Polymers*, *20*, 95–98.

Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, *131*, 209–217.

Dodgson, K. S., & Price, R. G. (1962). A note on the determination of the sulphate ester of sulphated polysaccharide. *Biochemical Journal*, *84*, 106–110.

Falshaw, R., & Furneaux, R. H. (1994). Carrageenan from the tetrasporic stage of *Gigartina decipiens* (Gigartinaceae Rhodophyta). *Carbohydrate Research*, *252*, 171–182.

Lenz, R. W. (1960). Distribution of methoxyl groups in the methylation of the monosodio derivative of methyl alfa-D-glucopyranose and cellulose. *Journal of American Chemical Society*, *82*, 182–186.

Noseda, M. D., & Cerezo, A. S. (1995). Alkali modification of carrageenans-II. The cyclization of model compounds containing non-sulfated β-D-galactose units. *Carbohydrate Polymers*, *26*, 1–3.

Noseda, M. D., Viana, A. G., Duarte, M. E. R., & Cerezo, A. S. (2000). Alkali modification of carrageenans. Part IV. Porphyrans as model compounds. *Carbohydrate Polymers*, *42*, 301–305.

Percival, E. J. V. (1949). Carbohydrate sulfates. *Quarterly Reviews*, *3*, 369–384.

Prado-Fernández, J., Rodríguez-Vásquez, J. A., Tojo, E., & Andrade, J. M. (2003). Quantitation of κ-, ι- and λ-carrageenans by mid-infrared spectroscopy and PLS regression. *Analytica Chimica Acta*, *480*, 23–37.

Santos, G. A. (1989). Carrageenans of species of *Eucheuma* J. Agardh and *Kappaphycus* Doty (Solieriaceae, Rhodophyta). *Aquatic Botany*, *36*, 55–67.

Stevenson, T. T., & Furneaux, R. H. (1991). Chemical methods for the analysis of sulfated galactans from red algae. *Carbohydrate Research*, *210*, 277–298.

Stortz, C. A., Bacon, C. F., Cherniak, R., & Cerezo, A. S. (1994). High field NMR spectroscopy of cystocarpic and tetrasporic carrageenans from *Iridaea undulosa*. *Carbohydrate Research*, *261*, 317–326.

Stortz, C. A., & Cerezo, A. S. (1993). The system of carrageenans from cystocarpic and tetrasporic stages from *Iridaea undulosa*: fractionation with potassium chloride and methylation analysis of the fractions. *Carbohydrate Research*, *242*, 217–227.

Sugihara, M. J. (1953). Relative reactivities of hydroxyl groups in carbohydrates. *Advances in Carbohydrate Chemistry*, *8*, 1–44.

Turvey, J. R. (1965). Sulfates of the simple sugars. *Advances in Carbohydrate Chemistry*, *20*, 183–218.

Usov, A., & Shashkov, A. S. (1985). Polysaccharides of algae. 34. Detection of iota-carrageenan in *Phyllophora brodiaei* (Turn.) J.Ag. (Rhodophyta) using ^{13}C-NMR spectroscopy. *Botanica Marina*, *28*, 367–373.

Van de Velde, F., Knutsen, S. H., Usov, A. I., Rollema, H. S., & Cerezo, A. S. (2002). ^{1}H and ^{13}C high resolution NMR spectroscopy of carrageenans: application in research and industry. *Trends in Food and Technology*, *13*, 73–92.

Yaphe, W. (1960). Colorimetric determination of 3,6-anhydrogalactose and galactose in marine algal polysaccharides. *Analytical Chemistry*, *32*, 1327–1330.