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METHYLATION ANALYSIS OF CARRAGEENANS FROM THE SEAWEED *IRIDAEA UNDULOSA*

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Abstract—Two carrageenans from *Iridaea undulosa*, isolated by precipitation of the crude polysaccharide at 0.70–1.05 M and 1.55–1.65 M KCl concentrations, were studied by methylation analysis. Acid hydrolysis of the methylated derivative of the less soluble carrageenan (molar ratio galactose: 3,6-anhydrogalactose: sulphate 1.00:0.50:1.20) yielded major amounts of 2,6-di-*O*-methylgalactose (51.3 mol %), 4,6-di-*O*-methylgalactose (25.6%) and 4-*O*-methylgalactose (51.3 mol%), 4,6-di-*O*-methylgalactose (25.6%) and 4-*O*-methylgalactose (13.4%). Minor quantities of 3-*O*-methylgalactose (4.6%) and 6-*O*-methylgalactose (3.2%) were found together with traces of 2,3,6- and/or 2,4,6-tri-*O*-methylgalactose, 2-*O*-methylgalactose and galactose. Oxidative acid hydrolysis produced 3,6-anhydro-2-*O*-methylgalactonic acid and 3,6-anhydrogalactonic acid in a molar ratio 3.5–4.0:1.0. The methylated derivative of the more soluble carrageenan (molar ratio galactose: 3,6-anhydrogalactose: sulphate 1.00:0.04:1.43) gave on acid hydrolysis, 2,3,4,6-tetra-*O*-methylgalactose (4.6%), 2,3,6-tri-*O*-methylgalactose (4.2%), 2,4,6-tri-*O*-methylgalactose (10.7%), 4,6-di-*O*-methylgalactose (24.1%), 3,6-di-*O*-methylgalactose (8.0%), 2,3-di-*O*-methylgalactose (3.4%), 2,4-di-*O*-methylgalactose (4.6%), 2,6-di-*O*-methylgalactose (4.2%), 3-*O*-methylgalactose (19.5%), 4-*O*-methylgalactose (9.6%), 6-*O*-methylgalactose (3.1%), galactose (3.4%) and traces of 2-*O*-methylgalactose.

INTRODUCTION

The carrageenan extracted from a sample of *Iridaea undulosa*, collected in winter on the shores of Patagonia (Argentina), was fractionated by precipitation at increasing potassium chloride concentrations [1]. We report the methylation analysis of a fraction precipitated with 0.70–1.05 M KCl (fraction A) (molar ratio galactose: 3,6-anhydrogalactose: sulphate 1.00:0.50:1.20) whose analytical and solubility properties are characteristic of an 'intermediate fraction' [2], and another carrageenan (fraction B) (molar ratio galactose: 3,6-anhydrogalactose: sulphate 1.00:0.04:1.43) which precipitated with 1.55–1.65 M KCl and had the composition of a 'soluble fraction' [2[(λ-carrageenan [3]).

RESULTS

After reducing with sodium borohydride to minimize alkaline degradation, fractions A and B were methylated by repeated additions of dimethyl sulphate and sodium hydroxide at room temperature and then at 40–50° in the former case and at −2 and 4° in the latter. Table 1 shows the conditions, yields and percentages of methoxyl at the different methylation steps of fractions A and B. Total yields for fractions A and B were 41.1 and 40.7%, respectively.

The composition of fractions A and B after each methylation step is shown in Table 2. The permethylated derivative of fraction A gave on acid hydrolysis, 2,6-di-*O*-methylgalactose (51.3 mol %), 4,6-di-*O*-methylgalactose (25.6%), 4-*O*-methylgalactose (13.4%), together with small amounts of 3-*O*-methylgalactose (4.6%), 6-*O*-methylgalactose (3.2%) and traces of 2,3,6- and/or 2,4,6-tri-*O*-methylgalactose, 2-*O*-methylgalactose and galactose. Oxidative acid hydrolysis yielded 3,6-anhydro-2-*O*-methylgalactonic acid and 3,6-anhydrogalactonic acid in a molar ratio 3.5–4.0:1.0. Permethylated fraction B produced on acid hydrolysis 2,3,4,6-tetra-*O*-methylgalactose (4.6%), 2,3,6-tri-*O*-methylgalactose (4.2%),

Table 1. Conditions, yields and percentages of methoxyl for the different methylation steps of carrageenan fractions A and B

Methylation*		Yield (%)	Methoxyl (%)	Temp.†
[1]		59.0	9.4	20°
[2]		82.7	13.0	40–50°
[3]		84.3	13.2	45–50°
	Total	41.1		
(1)		76.0	8.8	−2°
(2)		100.0	10.8	−2°
(3)		64.9	9.7	4°
(4)		82.5	8.8	4°
	Total	40.7		

* Numbers in brackets correspond to the methylation steps of fraction A and those in parentheses to fraction B.

† Other conditions of methylation were constant.

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Table 2. Composition of sugars produced during the methylation and hydrolysis of carrageenans*

Methylation†	2,3,4,6	2,3,6	2,4,6	2,6	4,6	3,6	2,3	2,4	2	3	4	6	Gal‡
[1]	-	–		38.1		-		–	10.7	35.3		15.9	-
[2]	–			65.6			-		4.2	22.4		7.7	
[3]	–		tr	51.3	25.6§		-		tr	4.6	13.4	3.2	tr
(1)	-		4.4	18.7		1.7		tr	8.4	53.0		13.5	
(2)			9.8	23.1		4.7		6.3	4.3	42.7		9.0	-
(3)			15.4	26.6		7.3	3.5	4.6	2.7	33.6		6.2	
(4)	4.6	4.2	10.7	4.2	24.1	8.0	3.4	4.6	tr	19.5	9.6	3.1	3.4

* The composition of the partially methylated galactoses produced by acid hydrolysis is given in mol %.

† Numbers in brackets correspond to the methylation steps of fraction A and those in parentheses to fraction B. In all the cases a 3% ECNSS-M column and acetylated alditols were used; in cases [3] and (4), a column packed with a mixture containing 0.2% poly(ethylene glycol adipate), 0.2% poly(ethylene glycol succinate) and 0.4% silicone XF-1150 and acetylated aldononitriles was also used.

‡ The peak corresponding to 3- and/or 4-*O*-methylgalactose in the 3% ECNSS-M column also includes galactose.

§ A molar ratio of 2:1 was determined by chromatography on a cellulose column.

2,4,6-tri-*O*-methylgalactose (10.7%), 4,6-di-*O*-methylgalactose (24.1%), 3,6-di-*O*-methylgalactose (8.0%), 2,3-di-*O*-methylgalactose (3.4%), 2,4-di-*O*-methylgalactose (4.6%), 2,6-di-*O*-methylgalactose (4.2%), 3-*O*-methylgalactose (19.5%), 4-*O*-methylgalactose (9.6%), 6-*O*-methylgalactose (3.1%), galactose (3.4%) and traces of 2-*O*-methylgactose.

DISCUSSION

From the structural point of view, fraction A has been classified as *ι*-type [1, 4]. Nevertheless, the characterization of 3,6-anhydro-2-*O*-methylgalactonic acid and 3,6-anhydrogalactonic acid in a molar ratio 3.5–4.0:1.0 suggests a *κ*-type structure [4]. Comparison of the 'ideal' molar ratio of *κ*-carrageenan (galactose:3,6-anhydrogalactose:sulphate 1:1:1) with that of fraction A (1.00:0.50:1.20) shows deviations from the 'ideal' structure. It was suggested [1] that, besides 4-linked galactose 6-sulphate, these deviations would include 4-linked non-sulphated galactose ('kinking residues') [5], 3-linked galactose 2-sulphate and non-sulphated galactose. 3-Linked galactose 2-sulphate was found in significant quantities and 3- and/or 4-linked non-sulphated galactose in trace amount.

The finding of major amounts of monomethylated galactose residues (21.2%) indicates that the units from which they derive determine the solubility properties of the carrageenan. They are, most probably, monosulphated branching points or disulphated units (not 4-linked galactose, 2,6-disulphate) [1]. Both types of units would increase the solubility of the molecule.

Fraction B should be classified, according to its solubility, as an 'intermediate fraction' [2], but the molar ratio galactose:sulphate (2:2.9), similar to that of the 'ideal' structure of *λ*-carrageenan (2:2.7) [3], the very low 3,6-anhydrogalactose content (1.6 weight %), and the relatively high percentages of galactose 6-sulphate and 2,6-disulphate [1] suggest a *λ*-type structure [3]. The finding of 3-linked galactose 2-sulphate and 4-linked galactose 2,6-disulphate, together with minor amounts of 4-linked galactose 6-sulphate, fits into that structure. Deviations are produced by 3-linked galactose 4-sulphate and 6-sulphate and non-sulphated galactose, and 4-linked galactose 2-sulphate and non-sulphated galactose.

Nevertheless, the main feature in the pattern of partially methylated sugars is the presence of monomethylated galactose units (32.2%). 3-*O*-Methylgalactose units derive from the 4-linked galactose 2,6-disulphate residues [1]; the remaining units must be produced from other disulphated residues or monosulphated branching points as previously discussed. The presence in this case of 4.6 mol % of 2,3,4,6-tetra-*O*-methylgalactose is consistent with the latter supposition. Thus, not only in *κ*-carrageenans [6], but also in the more soluble fractions the sulphate groups may be located in any of the possible sugar unit positions.

The greater solubility of some carrageenans was explained not only by higher sulphate and lower 3,6-anhydrogalactose content in the more soluble ones, but also by the presence of higher amounts of 'kinking residues' [2, 5]. The results given in Table 2 support this hypothesis but also indicate two other factors which would promote solubility, namely: the diversification of the structural units and the presence of major amounts of disulphated residues and/or branching points. Both factors would influence the conformation of the molecule so as not to allow the K^+-induced double helix formation [7].

EXPERIMENTAL

General methods. For PC see [13]. The partially methylated aldoses were analysed by GLC as: a, TMSi alditols [8] (stainless steel column packed with 3% OV-101, 1.80 m × 1.8 mm, temp. programmed from 120° to 210° at 40/min, and N_2 at 40 ml/min; injector temp. 170°, dual FID temp. 340°); b, acetylated alditols [9] (stainless steel column packed with 3% ECNSS-M on Gas-Chrom Q, 1.80 m × 1.8 mm, at 180° isothermally, and N_2 at 24 ml/min; injector temp. 210°, FID temp. 210°); and c, acetylated aldononitriles [10] [stainless steel column packed with a mixture containing 0.2% poly(ethylene glycol adipate), 0.2% poly(ethylene glycol succinate), and 0.4% silicone XF-1150 on Gas-Chrom P (100–120 mesh), 1.80 m × 1.8 mm, at 180° isothermally, and N_2 at 27 ml/min; injector temp. 230°, FID temp. 250°]. Galactonic acids were analysed as acetylated methyl esters [11] on a glass column packed with 3% OV-101, 1.20 m × 2 mm, at 150° isothermally, and N_2 at 27 ml/min; injector temp. 200°, FID temp. 240°. MS and computerized GC–MS were performed at 70 eV. GC–MS was carried out using: (a) a glass column packed with

3% ECNSS-M on Gas-Chrom Q, 1.20 m × 2 mm, for the acetylated alditols and acetylated aldononitriles; and (b) a glass column packed with 2% OV-101, 1.20 m × 2 mm, for the acetylated methyl esters. Methoxyl was determined by the method of Belcher *et al.* [12]. The solutions were coned under red pres. at 35–40°.

Methylation of fractions A and B and identification of the acid-stable sugars. Fractions A and B were methylated and further submitted to acid hydrolysis as described elsewhere [13, 14] (Table 1). *Fraction A.* PC of the hydrolysate of permethylated fraction A (solvent A, reagent a) gave 5 compounds: R_f 0.60 (R_f of 2,4,6-tri-*O*-methylgalactose 0.61), 0.40 (R_f of 2,6-di-*O*-methylgalactose 0.41), 0.31 (R_f of 4,6-di-*O*-methylgalactose 0.29), 0.11 (R_f of 3-, 4- and 6-*O*-methylgalactose 0.13), and 0.02 (R_f, galactose 0.02). GLC of the mixture of TMSi alditols showed the presence of trimethylated (traces), dimethylated (82.8%), monomethylated (17.2%) galactitols, together with traces of galactitol. The mixture of partially methylated sugars (0.220 g) was chromatographed on a cellulose column (70 × 4.5 cm, solvent A) 10 ml fractions were collected, examined by PC (solvent A, reagent 1) and combined into 5 large fractions *Fraction 1* (56 mg), R_f 0.75–0.79. PC and GLC (TMSi alditols) showed the absence of methylated sugars and therefore the fraction was a mixture of degradation products, possibly formed from the 3,6-anhydrogalactose residues. *Fraction 2* (16 mg). PC showed a major compound, R_f 0.76 together with traces of compounds with R_f 0.61 and 0.40. The substance with R_f 0.61 was identified by PC and GLC (TMSi alditols and acetylated alditols) as 2,3,6- and/or 2,4,6-tri-*O*-methylgalactose, while that with R_f 0.40 was identified in the next fraction. *Fraction 3* (65 mg). PC gave one component with R_f 0.41 which was identified by PC, GLC (TMSi alditols) and MS (acetylated alditols) as 2,6-di-*O*-methylgalactose. *Fraction 4* (32 mg). PC showed one substance with R_f 0.34 which was identified by PC, GLC (TMSi alditols) and MS (acetylated alditols) as 4,6-di-*O*-methylgalactose. *Fraction 5* (41 mg). PC and GLC (TMSi alditols) showed that this fraction was composed of monomethylated galactoses together with traces of dimethylated galactoses and galactose. PC (triple development) showed a strong spot $R_{2,6}$ (relative to 2,6-di-*O*-methylgalactose) 0.44. Standards of 3-, 4- and 6-*O*-methylgalactose had $R_{2,6}$ 0.45–0.47. GLC and GC–MS of the acetylated aldononitriles showed that the major monomethylated sugar was 4-*O*-methylgalactose with minor amounts of 3-*O*-methylgalactose and traces of galactose. GLC and GC–MS of the acetylated alditols indicated that 6-*O*-methylgalactose and traces of 2-*O*-methylgalactose were also present.

The composition of the mixtures produced by hydrolysis of the methylated carrageenans after each methylation step and the final identification of the partially methylated galactose sugars was carried out by GLC and GC–MS (acetylated alditols) (Table 2).

Fraction B. The composition of the mixtures produced by hydrolysis of the methylated carrageenans after each methylation step and the final identification of the partially methylated galactose sugars was carried out by GLC and GC–MS (acetylated alditols and acetylated aldononitriles) (Table 2).

Oxidative hydrolysis of permethylated fraction A. Permethylated fraction A was subjected to an oxidative acid hydrolysis as described elsewhere [13, 14]. The mixture of aldonic acids (0.300 g) was fractionated on a cellulose column (50 × 2.4 cm, solvent B). Fractions of 5 ml were collected and those of similar composition (by PC in solvent B) were combined to give the following six fractions: *Fraction 1* (15 mg). This gave a negative reaction with reagent b and reagent c and is presumed to be a mixture of degradation products formed from the unhydrolysed 3,6-anhydrogalactose residues in the first step of the oxidative hydrolysis. *Fraction 2* (11 mg). PC (reagent b) showed a spot with R_f 0.76. After alk. treatment a spot with R_f 0.43 (reagent c) appeared: GLC and GC–MS (acetylated methyl esters) showed that it was 3,6-anhydro-2-*O*-methylgalactonic acid. *Fraction 3* (80 mg). PC (reagent b) showed a spot with R_f 0.73. After alk. treatment a major spot with R_f 0.43 (reagent c) appeared. This fraction was again passed through the same column and 70 mg of pure 3,6-anhydro-2-*O*-methylgalactonic acid were obtained (GLC and GC–MS). *Fraction 4* (80 mg). R_f 0.43. GLC and GC–MS showed that it was 3,6-anhydro-2-*O*-methylgalactonic acid *Fraction 5* (55 mg), R_f 0.43 and 0.32, was a mixture of similar amounts of 3,6-anhydro-2-*O*-methylgalactonic acid and 3,6-anhydrogalactonic acid (GLC and GC–MS). *Fraction 6* (29 mg), R_f 0.32, was pure 3,6-anhydrogalactonic acid (GLC and GC–MS).

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REFERENCES

1. Matulewicz, M. C. and Cerezo, A. S. (1980) *J. Sci. Food Agric.* **31**, 203.
2. Pernas, A. J., Smidsrod, O., Larsen, B. and Haug, A. (1967) *Acta Chem. Scand.* **21**, 98.
3. Rees, D. A. (1963) *J. Chem. Soc.* 1821.
4. Anderson, N. S., Dolan, T. C. S and Rees, D. A. (1973) *J Chem. Soc. Perkin Trans.* **1**, 2173.
5. Lawson, C. J., Rees, D. A., Stancioff, D. J and Stanley, N. F. (1973) *J. Chem. Soc. Perkin Trans.* **1**, 2177.
6. Painter, T. J. (1966) *Proceedings of the Vth International Seaweed Symposium* (Young, E. G. and McLachlan, J. L., eds.) p. 305. Pergamon, London.
7. Penman, A. and Rees, D. A. (1973) *J. Chem. Soc. Perkin Trans.* **1**, 2182.
8. Sweeley, C. C., Bentley, R., Makita, M. and Wells, W. W. (1963) *J. Am Chem. Soc.* **85**, 2497.
9. Björndal, H., Lindberg, B. and Svensson, S. (1967) *Acta Chem. Scand.* **21**, 1801.
10. Lance, D. J and Jones, J. K. N. (1967) *Can. J. Chem.* **45**, 1995.
11. Anderson, N. S., Dolan, T. C. S. and Rees, D. A. (1968) *J. Chem. Soc. C* 596.
12. Belcher, R., Fildes, J. E. and Nutten, A. J. (1955) *Analyt. Chim Acta* **13**, 16.
13. Cerezo, A. S. (1973) *Carbohydr. Res.* **26**, 335.
14. Cerezo, A. S. (1974) *Carbohydr. Res.* **36**, 201.