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Journal of Chromatography A, 1059 (2004) 83–87
JOURNAL OF CHROMATOGRAPHY A
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Characterisation of ι-carrageenans oligosaccharides with high-performance liquid chromatography coupled with evaporative light scattering detection

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Received 28 June 2004; received in revised form 29 September 2004; accepted 30 September 2004

Abstract

Enzymatically digested oligo-ι-carrageenans were separated with liquid chromatography, coupled to evaporative light scattering detection. As expected, compared to oligo-κ-carrageenans, the additional sulphate group in the neocarrabiose unit of ι-carrageenans significantly modified the separation mechanisms on ion-exchange chromatography, porous graphitic carbon and ion-pair chromatography. The oligomers were then isolated and characterised off-line with electrospray ionisation mass spectrometry in positive-ion mode. The tetrasaccharide, hexasaccharide and octasaccharide that were identified were associated with protonated heptylamine molecules whose number depended on the number of sulphate groups.

Keywords: ι-Carrageenans; Sulphated oligosaccharides; Porous graphitized carbon; Carbohydrates

1. Introduction

Recently, enzymatically digested oligosaccharides of κ-carrageenan were separated on anion-exchange and porous graphitic carbon (PGC) columns coupled with evaporative light scattering detection (ELSD) [1]. In that work, we pointed out the difficulty of coupling the chromatographic systems with electrospray ionisation mass spectrometry (ESI-MS) due to high salt concentration. Therefore, a new method was developed based on ion-pair liquid chromatography [2] with heptylamine as ion-pairing agent (5 mM, pH 4). The oligosaccharides of κ-carrageenan were separated, isolated and characterised off-line by ESI-MS in positive-ion mode. This method proved to be able to separate the oligomers of κ-carrageenan up to dotriacontasaccharide $(A\text{-}G4S)_{16}$.

The enzymatically digested ideal neocarrabiose unit of κ-carrageenan (A-G4S) is sulphated only in position C_4 of the galactose unit (G), whereas the ideal neocarrabiose unit of ι-carrageenan (A2S-G4S) has an additional sulphate group in position C_2 of the 3,6-anhydrogalactose unit (A) (Fig. 1).

We now present the separation of oligosaccharides of ι-carrageenan with ion exchangers, PGC and C_{18} columns, all coupled with ELSD. The oligomers were then isolated and characterised off-line with ESI-MS in the positive-ion mode.

2. Experimental

2.1. Apparatus

The liquid chromatographic apparatus consisted of a Thermoseparation (Les Ulis, France) model P4000 inert quaternary gradient pump, a Rheodyne (Berkeley, CA, USA) Model 7125 injector with a 20 μL sample loop and an ELSD system (Sedere, Alfortville, France) Model Sedex 75. The ELSD set-

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doi:10.1016/j.chroma.2004.09.093

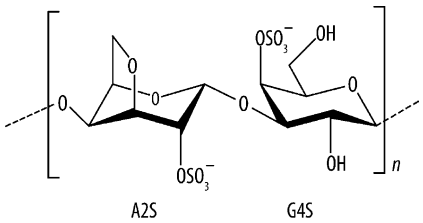

Fig. 1. Ideal neocarrabiose unit (A2S-G4S) of ι-carrageenans.

tings were as follows: photomultiplier range, 10; evaporative drift tube temperature, 47 °C; nebulisation gas pressure, 3.5 bar.

The columns used were as follows: Nucleosil Anion II (250 mm × 4 mm i.d., particle size 10 μm) from Macherey-Nagel (Düren, Germany); IC-A1S (100 mm × 4.6 mm i.d., particle size 10 μm) from Shimadzu (Kyoto, Japan); Biobasic-AX (50 mm × 2.0 mm i.d., particle size 5 μm) from Hypersil (Runcorn, UK); Hypercarb (100 mm × 2.1 mm i.d., particle size 5 μm) from Hypersil; Spherisorb ODS1 (250 mm × 4 mm i.d., particle size 5 μm) from Phase Sep (Düren, Germany). Dead volume on each column was determined by injecting xylose.

Flow-rates were 1 mL min^{-1} for the Nucleosil Anion II, IC-A1S and Shperisorb ODS1, 0.2 mL min^{-1} for the Biobasic AX and Hypercarb columns (for chromatographic conditions, see figure legend).

Data acquisition of the chromatograms was performed using EZChrom Elite Client/Server software, version 2.5, Scientific Software (Pleasanton, CA, USA).

2.2. Reagents

Deionised water was obtained by an Elgastat UHQ II system (18 MΩ) from Elga (Antony, France). HPLC-grade acetonitrile (MeCN) and methanol (MeOH) were from J.T. Baker (Noisy le Sec, France). Analytical-grade ammonium acetate, heptylamine ($C_7H_{15}NH_2$), pentylamine, triethylamine and formic acid were from Fluka (St. Quentin Fallavier, France).

2.3. Preparation of oligosaccharides

Oligo-ι-carrageenans were produced according to Rochas and Heyraud [3] using recombinant ι-carrageenase from *Alteromonas carrageenovora* [4]. They were prepared by enzymatic digestion with ι-carrageenase.

All analytical samples of hydrolysates of ι-carrageenan were injected at a concentration of 1220 ppm. For the isolation part, the mixture of oligo-ι-carrageenans was injected in a concentration of 10 000 ppm (0.2 mg of oligo-ι-carrageenans mixture injected). In both cases (analytical and isolation part), the solvent of the HPLC samples was water.

The oligo-ι-carrageenans were collected using a split with a ratio of 1:10. The mobile phase of each oligomer was evaporated under a gentle stream of N_2 at room temperature and then 0.5 mL of MeCN–water (80:20) were added in the residue.

2.4. Mass spectrometry

The ESI-MS system used was a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a pneumatically assisted electrospray ionisation source. Data acquisition and processing were performed using MASSLYNX 4.0 software. The analyte was introduced into the mass spectrometer via the ESI probe with a Harvard Apparatus pump 11 (Harvard Apparatus, MA, USA) with a flow-rate of 5 μL min^{-1}.

ESI-MS conditions were as follows: N_2 was used as both nebulising gas and desolvation gas at flow rates of 50 L h^{-1} and 500 L h^{-1}, respectively. The electrospray capillary voltage was 2.75 kV, and the cone voltage was 32 V. The radio frequency (RF) Lens 1 was set at 0.0, the aperture was set at 0.0, and the RF Lens 2 was at 1.0. The source operated at a temperature of 130 °C and the desolvation temperature at 150 °C. Full scan mode was used, and both positive and negative ionisation modes were tried. The mass scan range was 300–4000 u, for 1 min total scan time, with 3 s scan time and 0.1 s inter-scan time. The molecular mass of oligo-ι-carrageenans was calculated with Macisotopes software, version 1.2.

3. Results and discussion

3.1. Anion-exchange columns

Fig. 2a presents the separation of oligo-ι-carrageenans on the Nucleosil Anion II column. A concentration of 800 mM of ammonium acetate in a gradient mode was necessary to elute the oligomers. A high baseline drift was observed, as already shown with the separation of oligo-κ-carrageenans [1]. This drift is caused by a bleeding of the column, as has been previously noticed on aminopropyl column with hydroorganic mobile phases, and although it can be subtracted, it is not compatible with ESI-MS [5]. Therefore, the baseline (Fig. 2a, grey line) was subtracted from the chromatogram.

IC-A1S is a polymer type column, which does not present the problems encountered with the Nucleosil Anion II. Fig. 2b depicts the separation results of oligo-ι-carrageenans on this column. A concentration of 125 mM of ammonium acetate i.e., less than the concentration on Nucleosil Anion II, was necessary to elute the oligomers of ι-carrageenan up to octasaccharide $(A2S\text{-}G4S)_4$. However, owing to lack of efficiency, no further peak could be detected.

Fig. 2c depicts the separation of oligosaccharides of ι-carrageenans on the Biobasic-AX column. A concentration of 700 mM of ammonium acetate in a gradient mode was necessary to elute the oligomers.

3.2. Porous graphitic carbon column

On PGC, it was shown that in order to elute and separate the oligomers of κ-carrageenan, it was necessary to

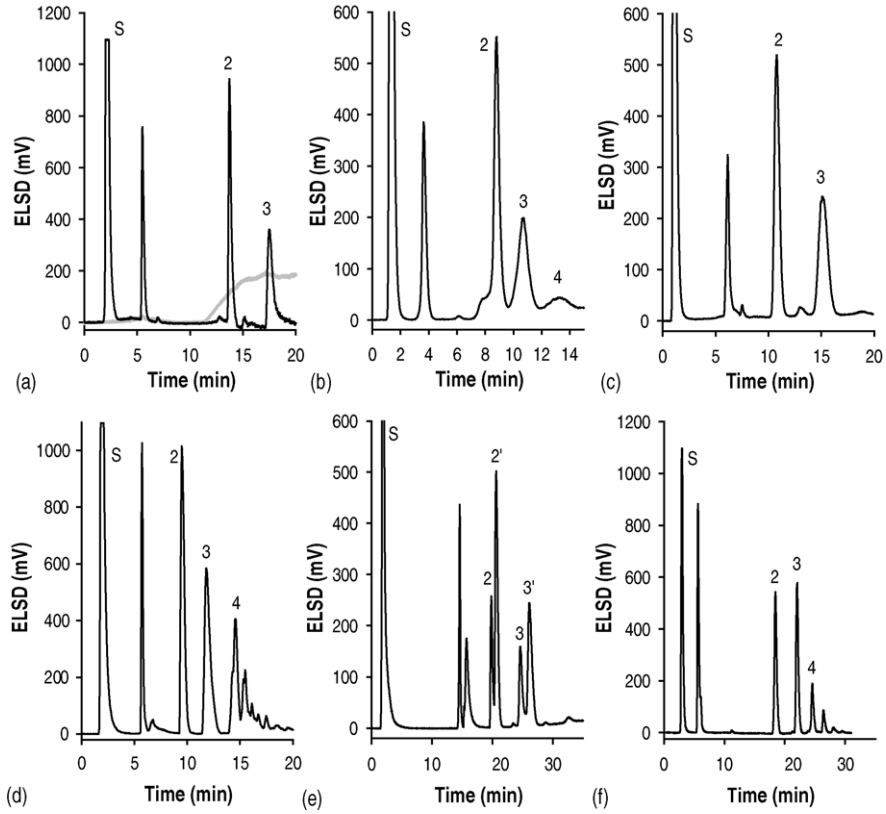

Fig. 2. Chromatograms of hydrolysats of ι-carrageenans (a) on Nucleosil Anion II column; (b) on IC-A1S column; (c) on Biobasic-AX column; (d,e) on PGC column; (f) on Spherisorb ODS1 column. Experimental conditions: (a) eluent A, water; eluent B 1 M CH_3COONH_4; eluent C, MeCN. Gradient program: 0–8 min, 80–56% of A, 0–24% of B, 20% of C; 8–15 min, 56–10% of A, 24–70% of B, 20% of C; 15–20 min, 80% of B, 20% of C. (b) Eluent A, water; eluent B, 500 mM CH_3COONH_4; eluent C, MeCN. Gradient program: 0–5 min, 60–54% of A, 0–6% of B, 40% of C; 5–20 min, 45–35% of A, 15–25% of B, 40% of C. (c) Eluent A, water; eluent B, 1 M CH_3COONH_4. Gradient program: 0–4 min, 100–73% of A, 0–27% of B; 4–20 min, 73–30% of A, 27–70% of B. (d) Eluent A, water; eluent B, 700 mM CH_3COONH_4; eluent C, MeCN. Gradient program: 0–20 min, 90–20% of A, 0–70% of B, 10% of C. (e) Eluent A, water; eluent B, 700 mM CH_3COONH_4; eluent C, MeOH. Gradient program: 0–10 min, 90% of A, 10% of C; 10–11 min, 0–13% of B, 10–15% of C; 11–35 min, 13–70% of B, 15% of C. (f) Eluent A, water; eluent B, 20 mM heptylammonium formate (pH 4); eluent C, MeCN. 0–30 min, 95–45% of A, 5% of B, 0–50% of C. S designates salt.

use an organic and an electronic modifier at the same time [1].

With the oligo-ι-carrageenans, when 10% of MeCN was used as organic modifier in the mobile phase, the elution process resulted in co-elution of the oligosaccharides (Fig. 2d) and when its concentration was decreased, the oligomers did not elute. The use of MeOH as mobile phase resulted in anomer separation (Fig. 2e) as in the case of the oligo-κ-carrageenans with MeCN.

Comparing the separation of the oligo-ι-carrageenans with that of oligo-κ-carrageenans, a lower concentration of organic modifier for the former was necessary to elute the

Table 1
Main ions identified for the oligo-ι-carrageenans in the positive-ion mode

Oligosaccharide $(A2S\text{-}G4S)_n$		*m/z* found	*m/z* calcd[a]
Tetrasaccharide ($n=2$)	$[(A2S\text{-}G4S)_2 + (C_7H_{15}NH_3^+)_6]^{2+}$	821.4	821.3
	$[(A2S\text{-}G4S)_2 + (C_7H_{15}NH_3^+)_3 + 2H\text{-}2SO_3]^+$	1136.2	1136.3
	$[(A2S\text{-}G4S)_2 + (C_7H_{15}NH_3^+)_4 + H\text{-}SO_3]^+$	1331.3	1331.4
	$[(A2S\text{-}G4S)_2 + (C_7H_{15}NH_3^+)_5]^+$	1526.5	1526.5
Hexasaccharide ($n=3$)	$[(A2S\text{-}G4S)_3 + (C_7H_{15}NH_3^+)_7 + H\text{-}SO_3]^{2+}$	1071.5	1071.8
	$[(A2S\text{-}G4S)_3 + (C_7H_{15}NH_3^+)_8]^{2+}$	1169.5	1169.4
	$[(A2S\text{-}G4S)_3 + (C_7H_{15}NH_3^+)_7]^+$	2222.4	2222.7
Octasaccharide ($n=4$)	$[(A2S\text{-}G4S)_4 + (C_7H_{15}NH_3^+)_9 + H\text{-}SO_3]^{2+}$	1419.6	1419.9
	$[(A2S\text{-}G4S)_4 + (C_7H_{15}NH_3^+)_{10}]^{2+}$	1517.6	1517.5
	$[(A2S\text{-}G4S)_4 + (C_7H_{15}NH_3^+)_9]^+$	2918.5	2918.9

[a] calcd: calculated.

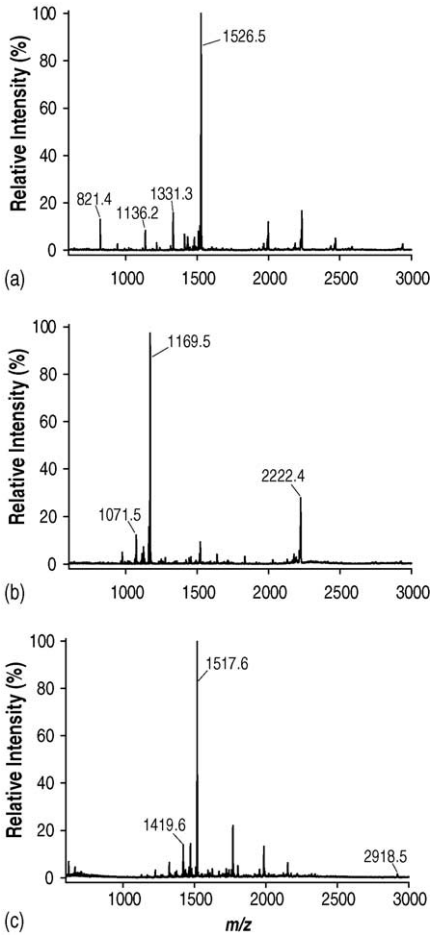

Fig. 3. Mass spectra of the enzymatically digested tetrasaccharide (a); hexasaccharide (b); and octasaccharide (c) of ι-carrageenans.

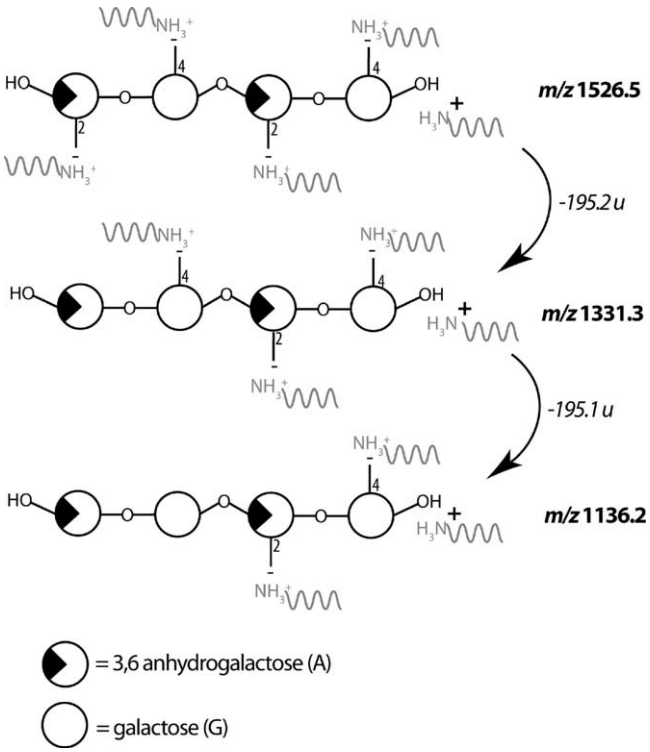

Fig. 4. Fragmentation scheme of the tetrasaccharide of ι-carrageenan. The number on the structures designates the position of the sulphate group (SO_3^-).

oligomers in the same salt gradient. Probably, the addition of an extra sulphate group in the neocarrabiose unit of ι-carrageenan significantly changes the hydrophobic phenomena [6], and therefore the separation mechanism.

3.3. Ion-pair chromatography and characterisation

Both with anion-exchange and PGC columns, we could not detect any peak further than the octasaccharide $(A2S\text{-}G4S)_4$. Ion-pair chromatography is an alternative method that could resolve this issue.

Fig. 2f depicts the separation of oligomers of ι-carrageenan on the Spherisorb ODS1 column. The peaks were identified by ESI-MS in positive-ion mode as $(A2S\text{-}G4S)_2$, $(A2S\text{-}G4S)_3$ and $(A2S\text{-}G4S)_4$ (Fig. 3; Table 1). The other peaks in the end of the chromatogram did not elute when overloading the column. As in the case of oligo-κ-carrageenans [2], the higher the molecular weight of the oligomer, the more the heptylamines attached to the molecule. In fact, this association is related to the number of sulphate groups present in the oligomer. The latter was observed in fast atom bombardment MS where the number of associated sodium ions increased with the number of sulphate groups [7].

In the tetrasaccharide $(A2S\text{-}G4S)_2$, a loss of 195 u from the main ion ($m/z = 1527$) was assigned as a loss of sulphate group associated with a heptylamine molecule. It was reported that neutral sulphate groups are more fragile than the charged ones, and in this case, there is a tendency towards sulphate loss from the oligosaccharides [8,9]. Fig. 4 represents a fragmentation scheme of the tetrasaccharide. Each sulphate group was associated with a protonated heptylamine molecule, while an extra protonated heptylamine molecule created the positive charge. The neutralised sulphate groups were dissociated from the molecule, creating a loss of 195 u. At the present time, the order of dissociation of the sulphate groups or the position of the extra heptylamine molecule have not yet been determined.

In conclusion, the chromatographic analysis of enzymatically digested oligomers of ι-carrageenans, compared to the oligomers of κ-carrageenans requires different chromatographic conditions due to the additional sulphate groups.

Acknowledgement

The authors acknowledge the Board of State Scholarships Foundation of Greece for financial support.

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