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Positional isomers of sulfated oligosaccharides obtained from agarans and carrageenans: preparation and capillary electrophoresis separation

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Abstract—Partial reductive hydrolysis was used to produce oligosaccharide alditols from repetitive sulfated galactans obtained from four Rhodophyta species: κ-carrageenan (from *Kappaphycus alvarezii*), θ-carrageenan (*Gigartina skottsbergii*—alkali-treated λ-carrageenan), agarose 6-sulfate (*Gracilaria domingensis*), and pyruvylated agarose 2-sulfate (*Acanthophora spicifera*—alkali-treated pyruvylated agaran sulfate). Each hydrolyzate was submitted to anion-exchange and gel-filtration chromatography, and the isolated oligosaccharide alditols were identified by 1D and 2D NMR spectroscopy and by ESI mass spectrometry. The positional isomers of the sulfated oligosaccharide alditols were then completely resolved by capillary electrophoresis in a borate buffer. Attempts to correlate the availability of the hydroxyl groups for borate complexation with the relative migration of the oligosaccharides are presented.

Keywords: Capillary electrophoresis; Oligosaccharide alditol structure; Positional isomers; Borate complexation; Carrageenans; Agarans

1. Introduction

Galactans biosynthesized by red seaweeds (Rhodophyta) are essentially constituted of disaccharide repeating units of 3-linked β-D-Gal*p* and 4-linked α-Gal*p*, and in many cases, the latter residue appears as 3,6-AnGal*p*. These galactans are classified in accordance with the enantiomeric configuration of the α-units, which are L- in agarans and D- in carrageenans.[1] Most of these polysaccharides are found as anionic polymers with varying degrees of sulfation. Furthermore, some algal species produce carrageenans or agarans with an almost idealized sulfation pattern, for example, *Kappaphycus alvarezii* (Gigartinales) produces mainly κ-carrageenan (alternating 3-linked β-D-Gal*p* 4-sulfate and 4-linked 3,6-An-α-D-Gal*p*).[2–9] The use of partial hydrolysis methods on repetitive agarans and carrageenans has been an attractive way to obtain oligosaccharides with a specific sulfation positioning in relatively high yields.[10–13] These oligosaccharides can be utilized as useful standards for analytical techniques in the study of complex algal galactans, such as carrageenans with hybrid sulfation patterns and D,L-hybrid galactans.[14–17]

Red seaweed galactans are also potential sources of different positional isomers and/or diasteroisomers of sulfated oligosaccharides. Therefore, a technique capable of resolving complex mixtures of isomeric oligosaccharides is desirable for the study of carrageenan and agaran-derivative oligosaccharides. For this purpose, capillary electrophoresis (CE) has proved to be a powerful technique that allows rapid separations with high resolution and sensitivity.[18] CE methods have been developed to separate acidic oligosaccharides derived from chondroitin sulfate,[19] hyaluronan,[20,21] heparan sulfate,[22] κ-carrageenan,[23] and pectins.[24] Mixtures of isomers of oligosaccharides containing sialic acid residues[25] and positional isomers of sulfated

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monosaccharides[26] have also been successfully resolved by the use of CE. In addition, a large number of CE separations of carbohydrates have been carried out using borate buffers. These separations are based on the fact that borate anions form negatively charged complexes with polyhydroxy compounds, thus increasing the selectivity of their separation.[27–40]

We now describe the preparation and NMR/MS characterization of oligosaccharide alditols produced from four repetitive red seaweed galactans (two carrageenans and two agarans). Furthermore, we demonstrate CE separation of positional isomers of sulfated oligosaccharide alditols using a borate buffer. Attempts to correlate hydroxyl groups availability to form borate complexes with the relative migration of the oligosaccharides are presented.

2. Experimental

2.1. Extraction and processing of the galactans

Selected strains from tetraspores of *Kappaphycus alvarezii* were obtained in the laboratory and cultivated in the sea (Ubatuba, São Paulo, Brazil). Plants were then collected, washed with running tap water, sun dried, milled, and extracted with water (1.5% w/v) at 65 °C. Insoluble residual material was removed by centrifugation, and the supernatant was dialyzed and freeze-dried. The extracted polysaccharides were submitted to alkaline treatment[41] giving κ-carrageenan as the main polysaccharide.

Samples of the tetrasporic phase of *Gigartina skottsbergii* (Gigartinales) were collected in Bahia Camarones (Chubut Province, Argentina), and the extraction of λ-carrageenans, as well as their alkaline treatment, were carried out as previously described.[42] Alkaline treatment of λ-carrageenan renders θ-carrageenan.

Agarose 6-sulfate was obtained from *Gracilaria domingensis* (Gracilariales) as previously described.[43] The powdered alga was submitted to an aqueous extraction (90 °C, 4 h), and the soluble agarose 6-sulfate was precipitated with ethanol (3 v), followed by dialysis and freeze-drying.

The extraction and alkaline treatment of the *Acanthophora spicifera* (Ceramiales) agaran sulfate was as previously described.[13] This process renders a repetitive agaran with sulfate groups mainly at C-2 of the β-D-Gal*p* unit with a minor amount of 4,6-*O*-linked pyruvic acid acetal that could be described as pyruvylated agarose 2-sulfate.[44]

2.2. Production and purification of the oligosaccharides

Samples of agarose 6-sulfate (360 mg), κ- (400 mg), and θ-carrageenans (400 mg) were partially depolymerized by partial reductive hydrolysis.[10] Each sample was dissolved in water (30 mL), the solution was heated to 60 °C, and 2.70 g of 4-methylmorpholine·borane (MMB) complex (97% Sigma–Aldrich) was then added, followed by 2 M CF_3COOH (10 mL). The mixtures were maintained at 65 °C for 8 h, and the acid was then evaporated by co-distillation with water. For θ-carrageenan four extra hours at 65 °C were necessary to complete the partial hydrolytic process. Each hydrolyzate was then resuspended in water (~4 mL) and applied to a DEAE-Sephadex A-25 (Cl^-) column (2.5 × 12 cm × 33 mL/h). Elutions were carried out with water and then with a continuous NaCl gradient (0–0.15 M) utilizing a Pharmacia Biotech pump P-1 for flux and gradient control. Anion-exchange fractions were desalted by aqueous elution on a BioGel P-2 column (1.5 × 100 cm × 20 mL/h). The column eluents were analyzed for carbohydrates by the phenol–sulfuric acid method.[45]

Agarobiitol 2^2-sulfate and $4^2,6^2$-*O*-(1-carboxyethylidene)-agarobiitol were produced and purified as previously described from the alkali-treated agaran sulfate of *A. spicifera*.[13]

2.3. Nuclear magnetic resonance spectroscopy

For NMR experiments, a portion of each dried sample (2–8 mg) was exchanged with deuterium by repeated evaporations in D_2O, and then dissolved in 99.99% D_2O (0.35 mL). NMR spectra were obtained with a Bruker Advance DRX 400 spectrometer equipped with a 5-mm inverse probe. 1D ^1H, ^{13}C, DEPT and 2D ^1H, ^1H COSY, TOCSY and ^1H, ^{13}C HMQC spectra were obtained at a base frequency of 100.63 MHz for ^{13}C and 400 MHz for ^1H nuclei. Chemical shifts are reported relative to an internal acetone standard at 2.225 and 30.20 ppm for ^1H and ^{13}C NMR spectra, respectively. ^{13}C NMR DEPT spectra were obtained at $\theta z = 135°$, where *C*H and *C*H_3 signals appear in a positive phase with *C*H_2 in a negative phase. For ^1H NMR experiments, the DO*H* signal was suppressed by low-power irradiation during relaxation. NMR experiments were performed at 25 °C for **KA**, **TA**, **TB**, **GA**, and **WB**, 40 °C for **WA**, and 50 °C for **KB**.

2.4. Electrospray-ionization mass spectrometry

The ESIMS equipment used was a Micromass Quattro LC–MS/MS triple quadrupole mass spectrometer. Data acquisition and processing were performed using Maslynx 3.2 software. Samples were diluted in 7:3 CH_3CN/H_2O at 1 mg/mL and introduced into the spectrometer by a syringe pump (KD Scientific Inc.). N_2 was used as nebuliser (83 L/h) and desolvation gas (309 L/h). The source was operated at 150 °C with a desolvation temperature of 250 °C. The pressure in the analyzer