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Note

Preparation of high purity monodisperse oligosaccharides derived from mannuronan by size-exclusion chromatography followed by semi-preparative high-performance anion-exchange chromatography with pulsed amperometric detection

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ABSTRACT

Oligosaccharides of ([4)-β-D-Man*p*A-(→]$_n$) with a degree of polymerisation (DP) of 5, 10 and 15 were generated by partial acid hydrolysis of alginate mannuronan. These were subsequently purified by a combination of size-exclusion chromatography and semi-preparative high-performance anion-exchange chromatography with pulsed amperometric detection. The purity of the isolated oligosaccharides was greater than 96%. With automated operation of the chromatography system, milligram quantities can be generated over a period of a few days. Thus, our methodology now offers some significant advantages over earlier, including our own, protocols focused on uronic acid oligomers, where the final products are either not as pure or more starting material is needed to generate an equivalent yield of product. Removal of ammonium ions in collected fractions after size-exclusion chromatography and prior to freeze-drying was found to be essential to prevent the formation of imines and subsequent Maillard browning products.

In earlier work, we had achieved high resolution analytical separations of alginate oligosaccharides on Dionex™ AS4A pellicular resin with PAD.[1,2] However, during chromatography of ([4)-β-D-Man*p*A-(→]$_n$) oligosaccharides (hereafter referred to as M-oligosaccharides) that were pre-purified by size-exclusion chromatography (SEC) and freeze-dried in ammonium acetate, an unidentified carbohydrate peak was observed in the subsequent chromatogram.[1] The compound responsible for this peak eluted from the anion-exchange column just in front of the mannuronan oligosaccharide (Fig. 7 in[1]).

With these observations in mind, we have now sought to identify and remove this unwanted material because we now assess both SEC and semi-preparative HPAEC-PAD on Ion-Pac AS4A resin eluted with alkali as a method to efficiently prepare high purity, (>96% as assessed by analytical chromatography) essentially monodisperse M-oligosaccharides. The aim is to develop and evaluate the feasibility of a method to produce milligram quantities of M-oligosaccharides in the range of DP 5–15, which could subsequently be used as ultrapure substrates in a range of further experiments. The importance of this research is that it aims to build on, and make improvements to, similar methodologies, including our own,[1] employed in earlier work.

Treatment of oligosaccharides with AG-50W-X8 cation-exchange resin (H^+-form) followed by freeze-drying revealed only the presence of essentially monodisperse oligosaccharides as determined by analytical HPAEC-PAD (Fig. 1A). The same result was also obtained if a pooled oligosaccharide SEC fraction in 0.1 M ammonium acetate was injected directly onto analytical HPAEC-PAD (result not shown). It should be noted that oligosaccharides prepared via freeze-drying are in their H^+-form and may, if analogy is made to mannuronic acid-rich alginate, be unstable if stored at room temperature for long periods.[3] Preferably, they should be stored in the freezer (−18 °C) for a short time as possible or should be converted to their Na^+-form if stored in the freezer for a longer time.

It would seem that freeze-drying M-oligosaccharides in the presence of ammonia initially generates a reversible equilibrium at the reducing end residue of the oligosaccharide between mannuronic acid (H^+-form) and 1-deoxy-1-imino-D-mannuronic acid. Further dehydration may then proceed to generate a small quantity of Maillard browning products. It is this incorporation of nitrogen into the reducing end monomer that probably gave rise to a second peak in front of the main oligosaccharide peak in the HPAEC-PAD chromatogram (Fig. 1B). The mechanism is that of

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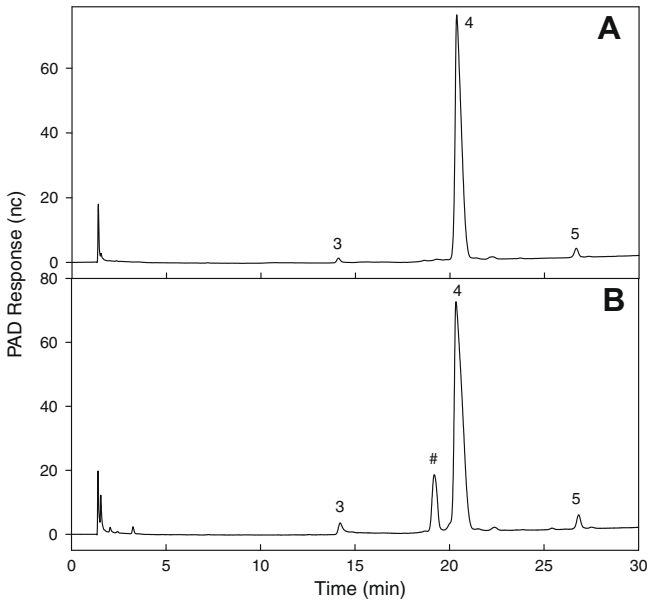

Figure 1. Analytical HPAEC-PAD chromatogram of an M-oligosaccharide standard of DP 4 recovered from SEC and (A) freeze-dried or treated with AG-50W-X8 cation-exchange resin prior to freeze-drying (B). Number annotation represents the chain length of oligosaccharides responsible for the peak, while # represents a peak from an oligosaccharide with 1-deoxy-1-imino-mannuronic acid or a Maillard browning product at the reducing end.

initial imine formation driven specifically by removal of water via freeze-drying. Ammonia (nucleophile) is generated upon lyophilisation of ammonium acetate followed by acid catalysis of the carboinolamine intermediate as the pH drops from 6.9 to a final pH of about 5 with subsequent removal of water.[4] Similar reaction products have been found, where aldoses have been heated under anhydrous conditions in the presence of ammonia and an acid catalyst.[5] Identification of such reaction products in this study was supported by evidence from mass spectroscopy (MS) and NMR.

Analysis by electrospray ionisation quadropole-time-of-flight (ESI-Q-TOF)-MS of an M-disaccharide sample isolated from SEC and freeze-dried in ammonium acetate detected $[C_{12}O_{12}H_{19}N–H]^-$ (monoisotopic $^{12}C/^{14}N$ calculated mass = 368.083) as a measured *m*/*z* of 368.082. This was in addition to the expected main molecular ion[1] of $[C_{12}O_{13}H_{18}–H]^-$ (monoisotopic $^{12}C/^{14}N$ calculated mass = 369.067) at *m*/*z* 369.067. The relative detected abundance ratio of these two ions was approximately 32:1. In contrast, MS analysis of an M-disaccharide treated with cation-exchange resin, or analysed in ammonium acetate after direct recovery from SEC without freeze-drying, did not detect any molecular ions at around *m*/*z* 368, only the main molecular ion at *m*/*z* 369 indicating the absence of nitrogen in these carbohydrates.

For ^{1}H NMR of an M-disaccharide sample isolated from SEC and freeze-dried in ammonium acetate, an aldimine proton was assigned to a doublet peak at 9.62 ppm in the 1D proton spectrum (Fig. 2A). In a ^{13}C-HSQC spectrum (Fig. 2E), this proton was correlated to a carbon atom at 145.56 ppm. These chemical shifts are similar to those of 9.50 ppm for ^{1}H and 146.70 ppm for ^{13}C predicted by ACD Labs version 5.0 software and are consistent with the literature values.[6] This assignment is also confirmed by the following: (1) in a 2D IP-COSY spectrum, there was a cross-peak from the proton on the imino-carbon to a proton with a chemical shift typical for a sugar moiety at 4.05 ppm (Fig. 2F), however, it was not possible to safely assign the whole spin system due to heavily overlapping sugar signals, (2) no peak was observed in the same

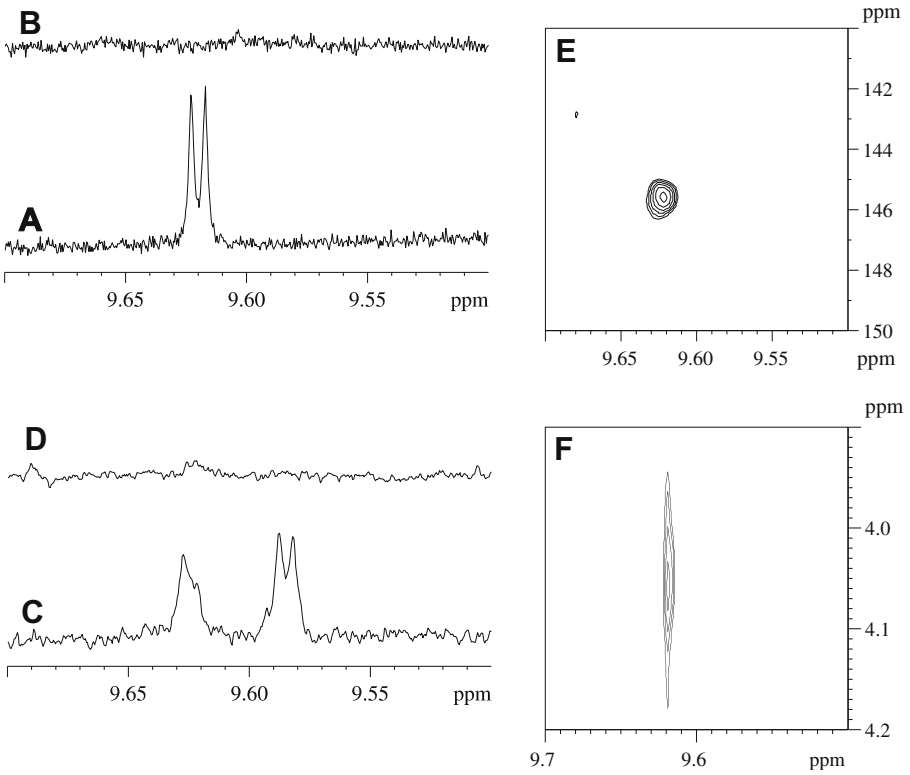

Figure 2. NMR spectra of different M- and G-disaccharides in DMSO-d_6 at 298 K. (A) M-Disaccharide recovered from SEC and freeze-dried in ammonium acetate. (B) M-Disaccharide treated with AG-50W-X8 cation-exchange resin prior to freeze-drying. (C) G-Disaccharide recovered from SEC and freeze-dried in ammonium acetate. (D) G-Disaccharide treated with AG-50W-X8 cation-exchange resin prior to freeze-drying. (E) ^{13}C-HSQC spectrum of an M-disaccharide with aldimine proton correlation to the aldimine carbon atom recovered from SEC and freeze-dried in ammonium acetate. (F) A part of a 2D IP-COSY spectrum with the cross-peak from the aldimine proton to the sugar moiety for M-disaccharide recovered from SEC and freeze-dried in ammonium acetate.

spectral region (Fig. 2B) in a corresponding sample treated with the cation-exchange resin. Similar observations were made for a M-trisaccharide (result not shown) and corresponding ([4)-α-L-Gul*p*A-(→]$_n$) or G-oligosaccharides (Fig. 2C and D) isolated in a parallel study. It was found that the imino-G oligosaccharide complexes were much less chemically stable than their M counterparts because two doublet peaks in the ^{1}H NMR spectrum at 9.58 ppm and 9.63 ppm (Fig. 2C) disappeared after 2 h. Addition of 10% D_2O to the sample did not lead to any change in these two peaks, indicating that both peaks stem from hydrogen atoms attached to a carbon atom. The origin of these two doublet peaks is at present uncertain.

With the methodology in place to limit the formation of imines/Maillard browning contaminants, M-oligosaccharides with a purity of >96% (Table 1, Fig. 3D–F) and a degree of polymerisation of 5, 10 and 15 were recovered from collected fractions after chromatography on a semi-preparative Ion-Pac AS4A column (Fig. 3A–C) eluted with NaOH/NaAc. The detected minor impurities were not identified, but may represent possible degradation/reaction products either before, during or after chromatography. Once an assessment of purity has been made via analytical chromatography for collected fractions over the target peak on one run from the semi-preparative column, this information can be overlaid onto all successive collections using the Chromeleon software chromatogram overlay function. Even if a small error/mis-judgement is made in collecting/ pooling fractions then oligosaccharide purity should be still very good. In all the experiments we have done, the purity of recovered oligosaccharides has never been less than 91% even for oligosaccharides of DP >15. The fact that the chromatogram obtained from the semi-preparative column does not look very nice in terms of peak symmetry and shape (Fig. 3A–C) is because the column is overloaded and the active surface of the PAD is saturated with eluting oligosaccharides. But this is not a problem. Chromatograms of a similar kind have been obtained before.[7] On the negative side, pellicular resin columns typically have a very low loading capacity,[8–10] but this disadvantage can be overcome with automated operation. In this respect, it is possible to collect a few milligrams of highly pure oligosaccharides over the duration of a few days without excessive time demands on personnel (Table 1).

Table 1
Typical purity and yield data for M-oligosaccharides of DP 5, 10 and 15 purified by semi-preparative HPAEC-PAD

Degree of polymerisation	Material injected per run (mg)	Purity of material recovered (%)	Yield per run (μg)	Purified material recovered as a fraction of total injected	Theoretical yield per day (mg)
5	0.5	99	480	0.96	15.5
10	0.25	97	185	0.74	4.8
15	0.1	96	48	0.48	1.05

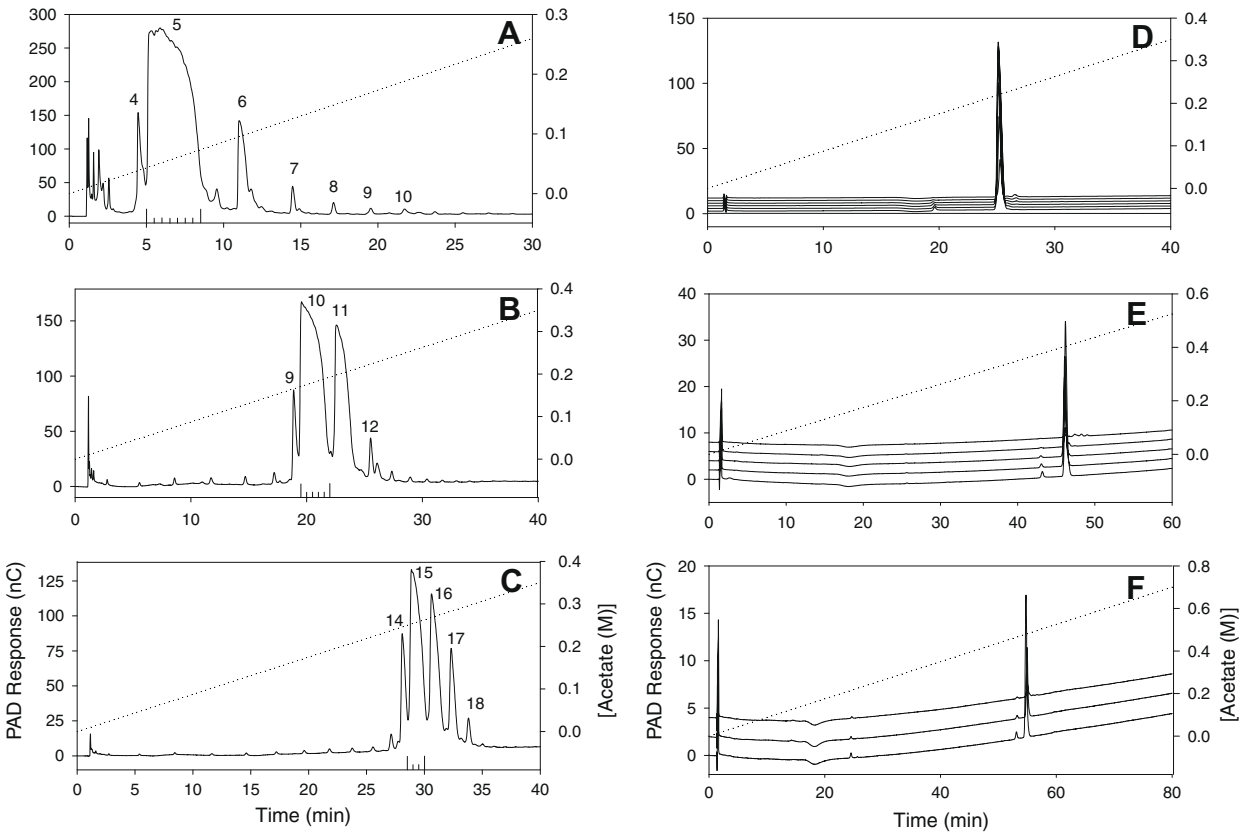

Figure 3. Typical semi-preparative HPAEC-PAD chromatograms (A–C) of M-oligosaccharides recovered from SEC and treated with AG-50W-X8 cation-exchange resin. The nominal DP was 5 (A), 10 (B) and 15 (C) from a total injection mass of 0.5, 0.25 and 0.1 mg, respectively. Collected fractions are represented by the bars pointing upwards from the *x*-axis. Number annotation represents the DP of oligosaccharides responsible for the peak. Analysis by analytical HPAEC-PAD chromatography (D–F) of fractions collected from the semi-preparative HPAEC-PAD chromatography with a nominal DP of 5 (D), 10 (E) and 15 (F). The detector responses of successive analysed fractions are plotted with an off-set of 2 *n*C. The dotted line is the gradient of acetate.

Overall, our chromatography methodology compares favourably to an earlier study, where oligogalacturonic acids with a DP from 7 to 21 were simultaneously collected after PAD detection from a total of 90 runs (7.5 days continuous run-time) on a preparative (21 × 250 mm) CarboPac PA-1 resin column.[7] Between 0.5 and 6.1 mg of oligosaccharides with a purity of 90+% were obtained from a total injection of 4.5 g start material at 50 mg per injection.[7] In the current study, 100–500 times less material is injected on to the column (Table 1), and this gives a much improved resolution for baseline separation of each oligosaccharide chain length (Fig. 3) leading to the recovery of products with an improved degree of purity (>96%) even if the yield per run is quite low (Table 1). In addition, much less starting material is required, which means an earlier round of oligosaccharide preparation clean-up is not too demanding.

Degradation of collected fractions is minimised because they are neutralised a few seconds after eluting from the column by mixing with acetic acid. No evidence was found of significant alkali degradation or 'peeling' by β-elimination during semi-preparative runs. This is in agreement with earlier observations.[1,10] Although not done in this study, desalting prior to final freeze-drying can be conducted by dialysis against distilled water to give oligosaccharides in their more stable Na^+-form.

1. Experimental

1.1. Preparation of milligram amounts of pre-purified oligosaccharides

Oligosaccharides (DP 2-18) were prepared as described earlier by partial acid hydrolysis of mannuronan followed by neutralisation, freeze-drying and finally SEC in 0.1 M ammonium acetate at pH 6.9 on Superdex 30.[1,2] Collected fractions were pooled to yield semi-purified oligosaccharide fractions of 50–100 mL, which were predominantly in their NH_4^+-form. These were subsequently passed through a 1.6 × 18 cm column of 20–50 mesh AG-50W-X8 cation-exchange resin (BioRad) in its H^+-form at a flow rate of 3–5 mL/min. This treatment removed and exchanged NH_4^+ for H^+ to generate acetic acid and oligosaccharides in their H^+-form. The sample was immediately frozen and freeze-dried prior to short-term storage (a maximum of one-two months) at −18 °C. After each round, the cation-exchange resin was regenerated with 10 column volumes of 0.1 M HCl followed by 20 column volumes of water.

1.2. Semi-preparative HPAEC-PAD

Oligosaccharides of DP 5, 10 and 15 were dissolved in MQ-water to a concentration of 10 mg/mL. 0.5, 0.25 or 0.1 mg of DP 5, 10 or 15 were injected, respectively, via a 0.1 mL loading loop. The chromatography unit consisted of a Dionex BioLC system (Sunnyvale, CA, USA) coupled to a Dionex AS50 autosampler and a Gilson 204 fraction collector fitted with 40 mL tubes containing 0.25 mL 1 M acetic acid. The LC system was equipped with an IonPac AS4A (9 × 250 mm) anion-exchange column connected to an IonPac AG4A (4 × 50 mm) guard column. Chromatography was performed at room temperature at a calculated optimal flow rate of 5 mL/min. The eluents were held in four 2 l plastic bottles purchased from Dionex™, all were connected to the pumps via their own eluent supply tubing. All contained 0.1 M NaOH (eluent A), while the fourth (eluent B) also contained 1 M acetate. Linear gradients of acetate were generated as described in the results. Column effluent was monitored with a pulsed amperometric detector on an Au working electrode and Ag/AgCl reference electrode. The sequence potentials applied to the Au electrode were as for Waveform A.[11] Data acquisition and analysis were performed on Chromeleon 6.7 software. Fractions of 2.5 mL were collected every 0.5 min over the target peak, the first run of a series of several automated runs were analysed by analytical HPAEC-PAD, and pure fractions of the target pooled. It was possible to collect several sequential runs without replacing the tubes in the fraction collector. All collected fractions had a final pH of 6.5–7.

1.3. Analytical-HPAEC-PAD

Quantitative and qualitative analysis was conducted as described before for purity assessment of semi-preparative fractions and pools generated therefrom.[1] Waveform A was applied as the sequence potential to the electrode.

1.4. NMR spectroscopy

All homo- and heteronuclear NMR spectra were recorded on a Bruker Advance 600 spectrometer equipped with a 5 mm z-gradient TXI(H/C/N) cryoprobe. The NMR data were processed with the Bruker XWINNMR Ver. 3.6 software, and the spectral analysis was done with CARA Ver. 1.4.[12] Samples of oligosaccharides (DP 2-3) from SEC chromatography, either treated or untreated with cation-exchange resin, were chosen so as to readily detect the resonances from the reducing-end[1]. They were prepared by dissolving 1.5–4 mg in 500 μL 99.9% DMSO-d_6 or in 550 μL 99.9% DMSO-d_6 mixed with 10% of 99.9% D_2O. Homonuclear 1D, 2D in-phase correlation spectroscopy (IP-COSY) and heteronuclear ^{13}C single quantum coherence (HSQC) spectra were recorded at 298 K.[13] The residual proton and carbon signal from DMSO-d_6 were used for spectra calibration.

1.5. Mass spectroscopy

M-Disaccharides 0.5–1 mg/mL, analogous to those analysed by NMR, were dissolved in water or in case of the sample taken directly from SEC in 0.1 M ammonium acetate. 3 μL were injected at a flow rate of 0.25 mL/min via an Agilent 1200 HPLC connected to an Agilent 6520 Q-TOF (quadropole time-of-flight) mass spectrometer with negative mode electrospray ionisation. Automatic calibration of the mass axis was performed with continuous infusion of an Agilent reference mixture through a second nebulizer needle in the ion source. Agilent masshunter workstation software was used for data analysis.

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