Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1093 (2005) 59–68
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Application of high-performance anion-exchange chromatography with pulsed amperometric detection and statistical analysis to study oligosaccharide distributions – a complementary method to investigate the structure and some properties of alginates

Simon Ballance [a,*], Synnøve Holtan [a], Olav Andreas Aarstad [a], Pawel Sikorski [b], Gudmund Skjåk-Bræk [a], Bjørn E. Christensen [a]

[a] *Norwegian Biopolymer Laboratory (NOBIPOL), Department of Biotechnology, Norwegian University of Science and Technology (NTNU), Sem Saelands vei 6/8, N-7491 Trondheim, Norway*
[b] *NOBIPOL, Department of Physics, Norwegian University of Science and Technology (NTNU), N-7491 Trondheim, Norway*

Received 3 May 2005; received in revised form 13 July 2005; accepted 14 July 2005
Available online 18 August 2005

Abstract

Alginates comprised of essentially alternating units of mannuronic (M) acid-guluronic (G) acid (MG-alginate), and G-blocks isolated from a seaweed where subjected to partial acid hydrolysis at pH 3.5 The chain-length distribution of oligosaccharides in the hydrolysate were investigated by statistical analysis after their separation with high-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC–PAD). Simulated depolymerisation of the MG-alginate provided an estimate of the ratio between two acid hydrolysis rate constants ($p = 8.3 \pm 1$) and the average distribution of the MM linkages in the original sample of polysaccharide chains. In conclusion, we found HPAEC–PAD together with statistical analysis was a useful method to investigate the fine structure and some properties of binary polysaccharides.

Keywords: Acid hydrolysis; Carbohydrate; Chain-length distribution; Degree of polymerisation

1. Introduction

Statistical analysis of the chain-length distribution of oligosaccharides obtained from the partial depolymerisation of polysaccharides can yield information on the mechanism of their formation, and the structure of the polysaccharide they originated from [1]. Our current understanding of the complex three-dimensional structure of amylopectin in starch granules is supported by analysis of the chain-length distribution of its oligosaccharides generated by enzymatic hydrolysis [2]. A similar approach, but using acid rather than enzymatic hydrolysis, also confirmed that the glycosidic linkages in cellulose were most probably identical [3], and that alginate contained some block sequences comprised of alternating mannuronic (M) and guluronic (G) acid [4]. The conclusions of this latter study were reached by using a combination of gel-filtration chromatography and a kinetic theory to analyse the products of the partial mild acid hydrolysis of a fragment of alginate. Oligosaccharides were fractionated according to their chain-length and the yield of each fraction used in computer simulation. This enabled the statistical determination of the nearest neighbour frequencies in the starting polymer and ultimately a description of its sequential structure [4]. The success of this approach contributed to the understanding that the depolymerisation of natural alginates

* Corresponding author. Tel.: +47 73598225; fax: +47 73593337.
E-mail address: simon.ballance@biotech.ntnu.no (S. Ballance).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.07.051

in weakly acidic media was due largely to intramolecular catalysis of glycosidic cleavage by the carboxyl group in the respective aglycone units [5].

In a more recent study [6], we showed that high-performance anion-exchange chromatography (HPAEC) on an analytical column of IonPac AS4A pellicular resin, combined with pulsed amperometric detection, was a useful and sensitive tool for the quantitative chain-length analysis of a partially degraded de-acetylated bacterial (1 → 4)-β-D-mannuronan, referred to as mannuronan hereon (Fig. 1A). Statistical analysis of this distribution confirmed its structure and revealed that hydrolysis of the glycosidic linkage in the polymer chain occurred at random positions. This implied that the process of acid hydrolysis of mannuronan under mildly acidic conditions could be adequately described by one rate constant. It proved to be a useful complementary method to NMR spectroscopy [6].

The advantage of HPAEC using pellicular resins over other methods, including other forms of chromatography (such as that used by Larsen et al. [4]), is its superior ability to achieve baseline separation of defined populations of oligosaccharides from polydisperse preparations. Oligosaccharides, with a chain-length of up to 30–40 monomer units, have previously been routinely separated without extensive optimisation of the chromatographic conditions [7]. Even trace constituents are captured in the analysis. It is therefore not a surprise that numerous other studies have used this technique to assess the chain-length distribution of various preparations of polysaccharide hydrolysates and then used this information to comment on the properties of the original polymer (see review by Zhang and Lee [7]). The most intensively studied of these originate from starch (see review by Wong and Jane [8]).

Despite the potential, to our knowledge there have been few instances where HPAEC with pulsed amperometric detection (PAD) and statistical analysis have been applied to study the chain-length distribution of oligosaccharides originating from glycuronans, especially binary ones, such as most alginates. A major reason for this is often the difficulty in obtaining appropriate oligosaccharide standards of required purity. These are needed firstly to calibrate the molar response of the PAD as a function of chain-length and secondly, in the case of heteropolysaccharides, they are needed to assist in the assignment of peaks in the resulting chromatograms. However, these obstacles are not insurmountable. In an investigation of the distribution of methyl esters in poly-galacturonic acid, a lack of oligosaccharide standards was circumvented by indirectly calculating the PAD response, via UV detection of oligosaccharides tagged with a chromaphore at their reducing-end [9]. Other workers have used post-column enzyme reactors to convert eluting oligosaccharides into equivalent amounts of easily quantifiable monomers [10,11].

In this study, we have utilised and extended the previous application of HPAEC–PAD and statistical analysis that investigated the chain-length distribution of oligosaccharides from mannuronan [6]. We now analyse two different alginates whose basic properties have both been prior investigated by NMR. The first is an engineered linear binary hetropolysaccharide with a predominantly alternating [4)-β-D-Man*p*A-(1 → 4)-α-L-Gul*p*A-(1→]$_n$ structure referred to as MG-alginate hereon (Fig. 1B). This alginate was engi-

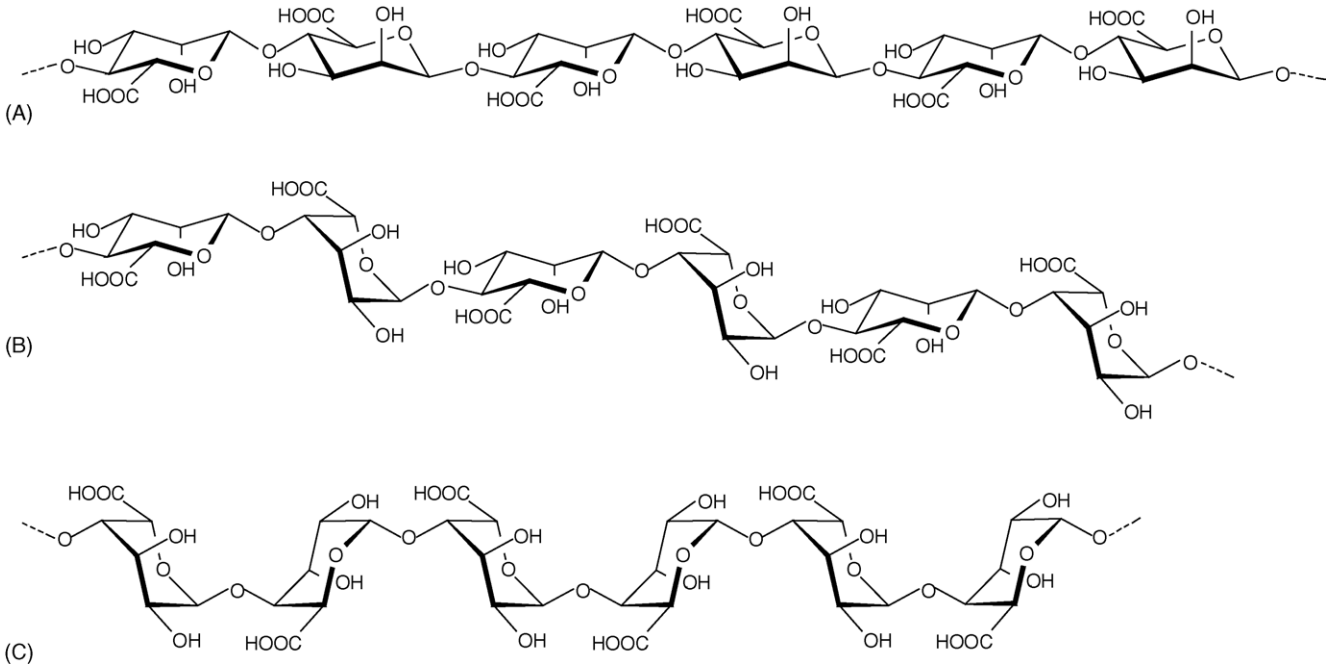

Fig. 1. Conformational representation of segments of mannuronan (A), MG-alginate (B) and G-blocks (C).

neered by in vitro epimerisation of every second M-residue in mannuroan with the recombinant C-5 epimerase AlgE4. Due to mode of action of this enzyme a fully epimerised mannuronan has a molar fraction of guluronic acid (F_G) of 0.47. It is thought that blocks of repeating MG units with a number average length >20 [12] are flanked by short [4)-β-D-Man*p*A-(1→]$_n$ (M-blocks; Fig. 1A). Therefore, while the majority of the polymer comprises two types of glycosidic linkage, β-D-Man*p*A-(1 → 4)-α-L-Gul*p*A (MG) and α-L-Gul*p*A-(1 → 4)-β-D-Man*p*A (GM), it also contains a minor amount of a third linkage of β-D-Man*p*A-(1 → 4)-β-D-Man*p*A (MM). In solutions with a pH from 2 and 4 these linkages are hydrolysed at very different rates. The GM linkage is hydrolysed the fastest to generate a majority of MG-oligosaccharides with M at their non-reducing end [4,5]. The second glycuronan we study has been extracted from seaweed alginate and comprises predominantly contiguous units of [4)-α-L-Gul*p*A-(1→]$_n$ and approximately 4% residual M. We refer hereon to this polymer as G-block seaweed alginate (Fig. 1C).

We now show for both partially acid hydrolysed glycuronans, fractionated by HPAEC and detected by PAD, that it is possible to separate and assign major oligosaccharide fractions. This study demonstrates it is possible to determine the yield of each chain-length fraction via calculation of their respective relative molar response factors (MRF_{rel}). Statistical analysis of the resulting chain-length distribution can then provide information on its number and weight-average chain-length, the structure of the parent polysaccharide the oligosaccharides originated from, and the process of their formation by acid hydrolysis. In addition, for the MG-alginate, further statistical analysis of chain-length distribution by simulated depolymerisation yields an estimate of the ratio between two acid hydrolysis rate constants. Using the same method we also attempt to provide additional information on the average distribution of the MM linkages in the original polysaccharide chains.

2. Materials and methods

2.1. Preparation of MG-alginate and isolation of G-block seaweed alginate

MG-alginate was prepared by in vitro epimerisation of mannuronan with AlgE4. It had a number average chain-length (DP_n) of 2400. The synthesis, de-acetylation and epimerisation of the mannuronan to generate the MG-alginate is described elsewhere [13–15]. It had a F_G of 0.47 and F_{GG} of 0, as determined by ^{1}H and ^{13}C NMR [15] indicating there were still minor contiguous sequences of M monomers that had not hosted AlgE4. G-blocks were isolated from the stipe alginate of the seaweed *Laminaria hyperborea*. Purification was carried out as described earlier [16,17] to yield a polydisperse preparation with a F_G of 0.96 (F_M = remainder) and a DP_n of 42 as estimated by ^{1}H NMR [6].

2.2. Partial acid hydrolysis and preparation of purified oligosaccharides

Five hundred milligrams of MG-alginate or 100 mg of G-blocks was dissolved in water to a concentration of 3.5 and 5 mg/ml, respectively. The pH was then adjusted to 5.6 with 0.1 M HCl, prior to de-oxygenation with nitrogen, followed by incubation at 95 °C for 3.5 h to undergo pre-hydrolysis. The sample was then cooled, pH adjusted to 3.6 with 0.1 M HCl, degassed and hydrolysed as before for a further 3.8 or 12 h for MG-alginate or G-blocks, respectively. After this period the sample was again cooled, neutralised by addition of 0.1 M NaOH, and freeze-dried. These polysaccharide hydrolysates comprised a distribution of oligosaccharides. Hydrolysates of mannuronan were produced in a similar way to that outlined by Campa et al. [6]. These were used as a positive control and for comparison. All hydrolysates were stored in the freezer prior to either direct use, or fractionated according to their chain-length on three columns of preparative grade Superdex 30 (2.6 cm × 60 cm, serially connected) eluted with 0.1 M NH_4Ac (pH 6.9) to yield oligosaccharide standards (mostly in their H^+-form) in a similar manner to that described before [6]. Analysis of these oligosaccharides by ^{1}H and ^{13}C NMR spectroscopy together with electrospray ionisation mass spectrometry (ESI-MS) confirmed their identity and average chain-length to range from 2 to 15 [15].

2.3. HPAEC–PAD

A stock solution of 0.01 mg/ml α-D-Gal*p*A-(1 → 4)-D-Gal*p*A was prepared in 1.5 mM NaOH for use as an internal standard. Oligosaccharide standards (0.4–2 mg) were dissolved in the internal standard stock solution to a final concentration of 0.05 mg/ml. Polysaccharide hydrolysates (ca. 1 mg) were dissolved in the internal standard stock solution to a concentration of 1 mg/ml. The chromatography system and conditions used are identical to those described earlier [6]. In this study, however, buffer A comprised 0.1 M NaOH, buffer B contained 1 M NaAc in 0.1 M NaOH and linear gradients of acetate were produced by increasing the concentration of buffer B from 0 to 70% over 80 min. The detection system and pulsed amperometric waveform was identical to that described earlier [6].

Each batch of oligosaccharide standards and polysaccharide hydrolysates, derived from the three different polysaccharide preparations, was analysed in 1 day over a consecutive period of 3 days. Analysis was repeated in three consecutive replicate series. Samples were prepared independently at the start of each day. The buffers were refilled prior to the start of each analysis series from a freshly pre-prepared stock. The gold electrode was not cleaned at any time during the analyses but the combination pH/Ag/AgCl electrode was replaced once. All analysis was completed within a period of 2 months. Integration of peaks in the resulting chromatograms was made with PeakNet software following man-

ual adjustment of the baseline and peak threshold. Integrated areas of multiple peaks observed for each chain-length were summed.

2.4. Determination of relative molar response factors

In order to take into account that each oligosaccharide standard was not pure but contained small amounts of ± one monomer residue in chain-length, relative molar response factors (MRF_{rel}) were determined for each standard in the chain-length range 2–15 by using linear equations [6]. First a unique relative response factor RF (f_x) was assigned to each oligosaccharide chain-length by $f_x = (A_x/A_{i.s.})(m_{i.s.}/m_x)\, fx_{i.s.}$, where m is the sum of moles for each chain-length in the sample; A is the integrated area of peak(s) from HPAEC–PAD assigned to each chain-length and i.s. denotes the internal standard. It then goes that the relative molar response factor $(MRF_{rel}) = (A_x/A_{i.s.})(m_{i.s.}/m_x)$. After determining MRF_{rel} for each sample in each batch, the mean MRF_{rel} ($n = 3$) for each series was plotted against chain-length. MRF_{rel} dependence on chain-length was then described by fitting the data firstly to a standard first order logarithmic function, and where applicable, to an equation that gave a good description of each MRF_{rel} data set. This relationship was used to calculate MRF_{rel} for oligosaccharides of longer chain-length (>15), as well as for the monomer.

2.5. Statistical analysis of chain-length distribution

Calculated and extrapolated MRF_{rel} were used to calculate the number and weight chain-length distributions of oligosaccharides in the polysaccharide hydrolysates ($n = 3$) together with their corresponding averages as described previously [6]. For comparison, the number-average chain-length (DP_n) was also measured with ^1H NMR as described previously [6,15]. Kuhn equations were plotted using the degree of chain scission (α) calculated directly from the experimental data points [4,6]. In the case of the MG-alginate the α-value was weighed to reflect the relative abundance of oligosaccharides in two populations within the chain-length distribution. As an additional test (for G-block oligosaccharides only), the Kuhn equation was also fitted to the experimental data points using a least squares approach.

2.6. Simulation of the depolymerisation of MG-alginates

Depolymerisation of MG-alginates were simulated using custom developed software and a statistical approach (Monte Carlo simulation) similar to what has been described for the enzymatic hydrolysis of chitosans [18]. Acid hydrolysis was simulated as a chemical reaction with an activation barrier dependant on whether the linkage was MG or GM. First an ensemble of ∼10,000 MG-alginate chains with a DP_n of 2400 was generated using a most probable distribution with a polydispersity index of 2 [19]. In simulations which take into account the presence of MM linkages in the polymer chain, short M-blocks with a variable length (see below), but complying to a total abundance of 6% of the mass as dictated from a pre-determined F_G of 0.47, were inserted at random positions. In the simulation process a polymer chain was first selected at random (step A). This was followed by random selection of one linkage within this chain (step B). The selected linkage was hydrolysed with a probability dependent on the sequence of adjacent units (step C). These probabilities were p_{GM}, p_{MG} and p_{MM} for GM, MG and MM linkages, respectively. The steps A to C were repeated until the required final DP_n (α) was achieved. In the final step of the simulation, the distribution of predicted oligosaccharide fractions was calculated and compared with that obtained from the experimental hydrolysis. The agreement was quantified by a means of a error function (S_{err}) defined in Eq. (1), where W_i^{obs} and W_i^{cal} are observed and calculated weight fractions of ith oligosaccharide. Only the chain-length fractions were used for which response factors had been experimentally determined ($i_{min} = 2$; $i_{max} = 15$).

$$S_{err} = \frac{\sum_{i=i_{min}}^{i_{max}} (W_i^{obs} - W_i^{cal})^2}{\sum_{i=i_{min}}^{i_{max}} (W_i^{obs})^2} \quad (1)$$

Calculations were performed as a function of DP_n and no rate constant (time dependence) was used. As a consequence, to describe the hydrolysis of MG-alginate under given experimental conditions, it is sufficient to determine the characteristic ratio: $p = p_{GM}/p_{MG}$ and then the hydrolysis reaction can be described using only one variable. The value of the p-parameter is determined by minimising the value of S_{err} [18]. For $p = 1$, the model predicts a standard Kuhn distribution of the reaction products, as expected for random hydrolysis of a homopolymer with uniform linkages. It is important to note that the value of p depends on the experimental conditions, and here it is determined only for the hydrolysis reaction described above. For simulations that included extra M-units, the experimentally determined probability of hydrolysing MM linkages (p_{MM}) was used. Since it has recently been determined that MM linkages are hydrolysed under the conditions used in this study approximately four times slower than GM linkages [15] we set $p_{MM} = 0.25 p_{GM}$. In simulations II–IX (Table 1) the polymer chain was constructed by inserting M-blocks with a given length and at random positions to achieve an $F_G = 0.47$. The number of M-blocks, and therefore an average distance between them, depends on the block length (Table 1). Each average M- and MG-block length, expressed as the number of monomers in the respective block, were determined in the simulation process. F_{MM}, F_{MMM}, F_{MMMM} values were calculated using the average M-block length and assuming a 'most probable distribution' of block sizes [20].

Table 1
The difference (S_{err}) between the experimentally determined and simulated (I–IX) weight distribution of chain-lengths in the partial hydrolysis products of an MG-alginate as a function of the average M-block length

Simulation	F_G	F_M	F_{MM}	F_{MMM}	F_{MMMM}	$F_M > 4$	[a]Average MG-block length	[a]Average MG-block length	p	S_{err}
I	0.5	0.50	0.000	0.000	0.000	0.000	DP_n	0[b]	5.7	2.65
II	0.47	0.53	0.060	0.000	0.000	0.000	16	2[c]	24.5	9.43
III	0.47	0.53	0.005	0.004	0.004	0.047	100	12.8	6.5	1.52
IV	0.47	0.53	0.010	0.008	0.007	0.036	98	7.3	6.9	1.37
V	0.47	0.53	0.017	0.012	0.009	0.021	56	4.5	7.7	1.14
VI	0.47	0.53	0.020	0.013	0.009	0.018	48	4.0	7.4	1.04
VII	**0.47**	**0.53**	**0.025**	**0.015**	**0.008**	**0.012**	**38**	**3.4**	**8.3**	**1.01**
VIII	0.47	0.53	0.031	0.015	0.007	0.007	32	3.0	8.8	1.12
IX	0.47	0.53	0.036	0.014	0.006	0.004	26	2.7	10.0	1.53

In all simulations, except simulation I which represents a hypothetically pure MG-alginate with an F_G of 0.5, the molar fraction of guluronic acid (F_G) was set at the experimentally determined value of 0.47. S_{err} is an error function as defined in Section 2, and p represents the calculated ratio between the two acid hydrolysis constants p_{GM} and p_{MG}. F_{MM}, F_{MMM}, F_{MMMM} and $F_M > 4$, respectively, represent the molar fraction of pure mannuronic acid dimers, trimers, tetramers, and oligomers larger than tetramers in the partial hydrolysate of each simulation. Simulation VII (bold type) highlights the simulation parameters that provide the best agreement between observed and predicted chain-length distributions.

[a] Expressed as monomer units.

[b] Result for the hypothetically pure MG-alginate which does not contain M–M linkages.

[c] Polymer in which extra M-units are distributed in mono-disperse M-blocks containing two M-units.

3. Results and discussion

3.1. Retention time on the anion-exchange resin as a function of chain-length and sequence

Analysis of oligosaccharide standards and polysaccharide hydrolysates by HPAEC–PAD showed it was possible to obtain a high-resolution separation of oligosaccharides according to chain-length (Fig. 2). This is because retention time increases as the number of negatively charged functional groups concurrently increase [21]. We managed to separate oligosaccharides by their chain-length until they exceeded between 30 and 35 monomer units (Fig. 2A–C).

From examination of Fig. 2D, it is seen that chains of equivalent length but different monomer sequence also have different retention times on the anion-exchange resin. Oligosaccharides of mannuronan are more strongly retained than those of the same chain-length derived from G-blocks. But epimerisation by AlgE4 of every second M-residue in mannuronan results in only a minor decrease in retention time of ensuing oligosaccharides (Fig. 2A). On closer inspection of the MG-oligosaccharides with odd-numbered chain-lengths (Figs. 2D, 3B and D) it is those with the highest number of M which are retained the longest. The physical and chemical factors, other than just monomer sequence, that may explain these differences in retention are complex and not fully understood [7,21]. It is therefore beyond the scope of this article to invoke a detailed discussion on this subject. However, on the basis of monomer sequence, the retention trends described here can assist in the confident assignment of some of the observed chromatographic peaks.

From inspection of the chromatogram of the MG-alginate hydrolysate (Fig. 2B), it is quite evident that oligosaccharides with even-numbered chain-lengths were more abundant than their odd-numbered counterparts. This is because to generate an even-numbered chain-length two identical linkages must be cleaved. Since the GM linkage is hydrolysed the fastest, the majority of these oligosaccharides would favourably have an M at their non-reducing end and G at their reducing end [5]. Indeed, from examination of the chromatogram obtained after HPAEC–PAD of the ^{1}H NMR assigned dimer (Fig. 3A), it is safe to assign the major peak to MG (non-reducing residue on the left). The slightly smaller peak, which elutes about a minute later, must therefore arise from GM. This trend is repeated for other larger even-numbered chains (Fig. 3C). For MG-oligosaccharides of equivalent sequence it is those with an M at their reducing end that are retained on the AS4A resin the longest. From the parallel analysis of pure M-oligosaccharides of the same monomer length (not shown), a third minor peak in the chromatogram of the ^{1}H NMR assigned dimer fraction is firmly assigned as MM (Fig. 3A).

For those oligosaccharides with an odd-number of monomers in their chain (Fig. 3B and D), roughly half should have M at their non-reducing end. This is because to generate an odd numbered oligosaccharide both a GM and an MG linkage must be broken. It is therefore not a surprise that two major peaks of similar size are observed in the chromatogram of the odd-numbered oligosaccharides (Fig. 3B and D). Given what we now know about retention time as a function of sequence we can easily assign these two peaks. Oligosaccharides responsible for the second of the two major peaks must have terminal residues of M and overall comprise one less G residue than M. The opposite is true for the oligosaccharides responsible for the other major peak. These oligosaccharides elute earlier because they have a G residue at each end and overall comprise one less M (Fig. 3B and D).

Assignment of oligosaccharides from G-blocks (Fig. 3 E–H) is even easier as there is only one major peak observed for each standard/chain-length. It is firmly assigned as a pure G-oligosaccharide. Each oligosaccharide standard, both MG-

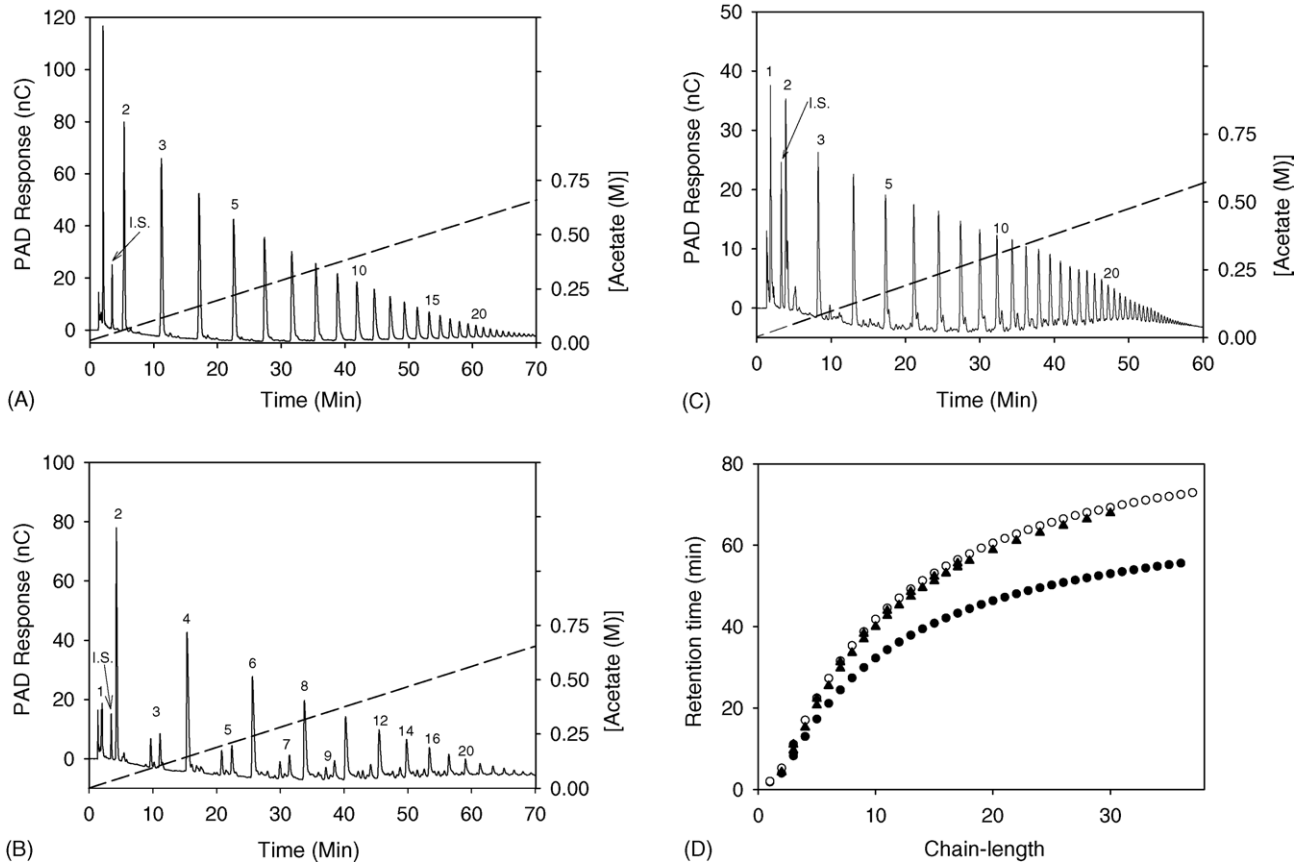

Fig. 2. Typical examples of HPAEC–PAD chromatograms of the oligosaccharide hydrolysis products generated by the partial acid hydrolysis of mannuronan (A), MG-alginate (B) and seaweed alginate G-blocks (C). The abbreviation i.s. denotes the internal standard and the dashed line shows the acetate gradient formed in 0.1 M NaOH at a flow rate of 1 ml min^{-1} which was used to elute the IonPac AS4A resin column. Peak labels refer to assigned chain-length. Panel (D) shows the mean ($n = 3$) oligosaccharide retention time as a function of chain-length. Open circles (○), filled circles (●) and filled triangles (▲) represent oligosaccharides originating from mannuronan, seaweed alginate G-blocks and MG-alginate, respectively.

and G-, contains a small amount of oligosaccharide ± one monomer residue (Fig. 3) together with a host of smaller peaks whose identity is uncertain. The former is a result of the gel-filtration step [6] but the latter may have several origins. Firstly, all the polysaccharides examined were not compositionally pure (see Section 2). It is likely that some of the extra peaks arise from minor oligosaccharide fractions of irregular sequence. Proton NMR assignments of the MG-oligosaccharide standards from gel-filtration confirmed that short chain-length fractions contained some MM sequences. These are more abundant in oligosaccharides with an odd number of residues in their chain [15]. This phenomenon can be explained by the different degradation rates of glycosidic linkages together with the length of M-blocks in the polymer. An interspersion of MM dimer sequences within the MG-chain would give irregular odd oligosaccharides with the more rapid degradation of GM than MM and MG linkages. Perhaps this observation explains why, in HPAEC–PAD analysis of these oligosaccharides, more small peaks flank the major peaks in odd-numbered chains (Fig. 3B and D) than in even-numbered ones (Fig. 3A and C). In addition it is also likely that some of these additional peaks in the oligosaccharide standards (mostly H^+-form) arise from their acid-catalysed modification during their formation and storage. After paper chromatography of acid-generated alginate oligosaccharides it was suggested that modification occurred at the reducing end [4]. Whether alkaline β-elimination during HPAEC–PAD analysis also makes a contribution cannot be definitively ruled out, but it seems unlikely (see also [6]). This process should generate monomers but these were not observed to any extent in the chromatograms of purified standards (Fig. 3).

3.2. Relative molar response factors

Fig. 4A shows the mean ($n = 3$) MRF_{rel} for each chain-length obtained from the three glycuronans. Overall they are quite well described by a standard first order logarithmic function $y = y_0 + a \ln x$, where MRF_{rel} versus chain-length plotted on a logarithmic axis has a linear relationship ($R^2 = <0.96$). We observed over the 2-month study period that the PAD response per oligosaccharide batch, even when corrected against the internal standard, decreased with each

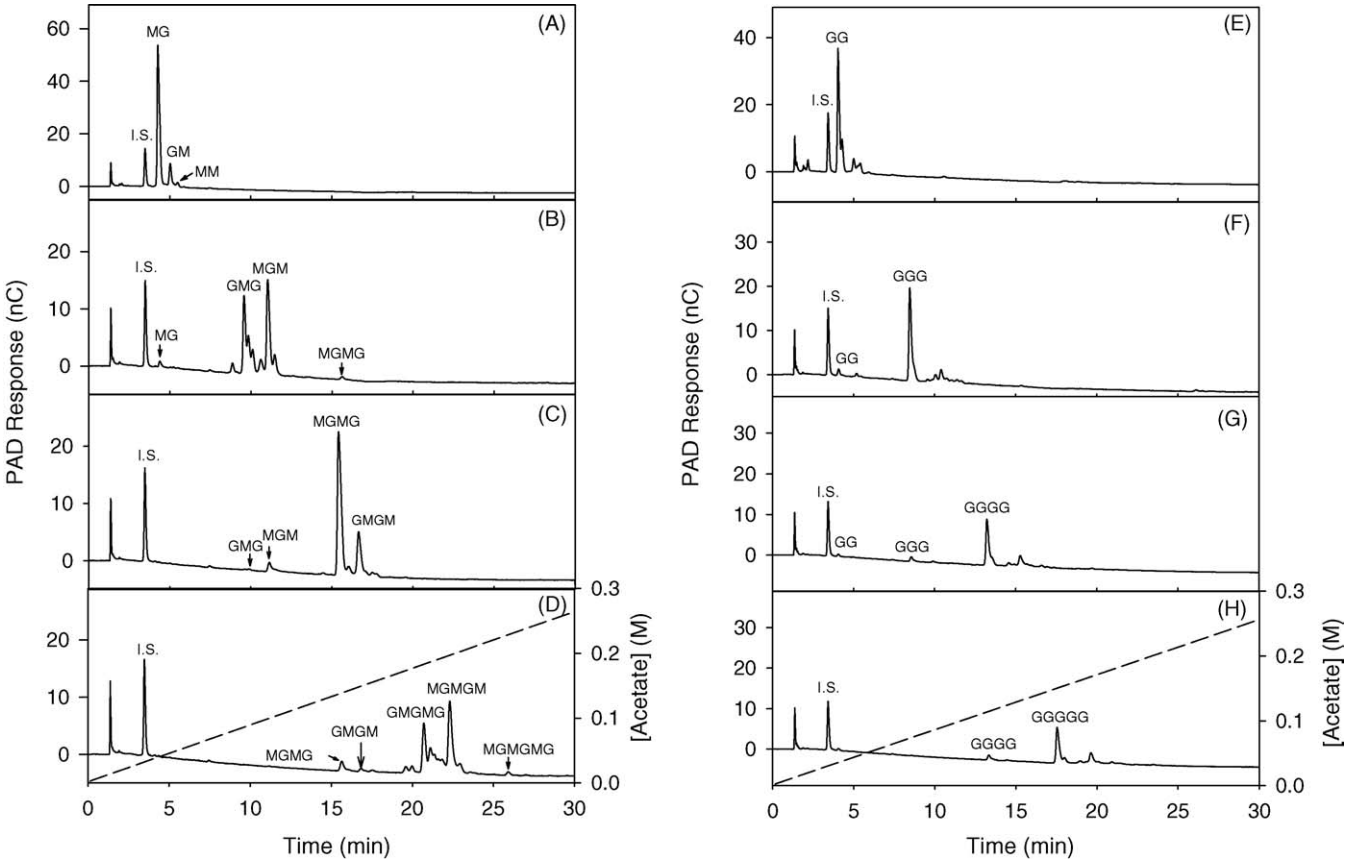

Fig. 3. Typical examples of HPAEC–PAD chromatograms of oligosaccharide standards DP 2–5 originating from MG alginate (A–D) and seaweed alginate G-blocks (E–H). The abbreviation i.s. denotes the internal standard and the dashed line shows the acetate gradient formed in 0.1 M NaOH at a flow rate of 1 ml min^{-1} which was used to elute the IonPac AS4A resin column. Other peak labels refer to the assigned identity of oligosaccharides responsible for the peak.

successive replicate series analysed. A gradual decrease in carbohydrate peak areas over time due to working electrode wear/recession is typical when, as in this study, positive electrode cleaning potentials (+0.6 V, 120 ms) are used [22]. However, the overall MRF_{rel} trends were consistent and highly reproducible with G-oligosaccharides having slightly lower response factors than their M and MG counterparts. The gradient (a), obtained from a fit of a first order logarithmic function to the calculated MRF_{rel} values for each oligosaccharide batch in each consecutive analysis series shows each of the three successively analysed hydrolysis samples fitted well to a straight line ($R^2 \leq 0.98$; Fig. 4A inset). In addition each line had a similar gradient (Fig. 4A; inset). This serves to validate the mean values ($n = 3$) and trends presented in Fig. 4A and B as representative. Our study agrees with the view that as long as a representative internal standard is used when calculating MRF_{rel} then it does not matter whether the gold electrode is partially recessed, fouled, or even sanded and re-polished at an interval during analysis [23]. Why G-oligosaccharides have an overall lower MRF_{rel} value than corresponding M-oligosaccharides is at present unknown. As with the factors that govern the subtle differences in retention times on the anion-exchange resin more than one mechanism may be responsible. These factors include rates of diffusion, physical shape and chemical composition [24].

With the exception of mannuronan, which is shown for comparison, it seems the MRF_{rel} for the monomer fraction is significantly underestimated when extrapolated by the first order linear function (Fig. 4A). This has serious adverse consequences when calculating chain-length distributions and averages particularly were DP_n is <10. G-block oligosaccharides, and the extrapolated monomer fractions, were better, but not perfectly described, by a second order logarithmic fit where $MRF_{rel} = y_0 + a(\ln x) + b(\ln x)^2$; $R^2 = 0.96$ (Fig. 4B). MRF_{rel} for MG-oligosaccharides fitted better to a sigmoidal distribution where $MRF_{rel} = a/1 + e^{-(x-x_0)}/b$; $R^2 = 0.97$ (Fig. 4B). Extrapolation of response factors for oligosaccharides with chain-lengths >20 are also prone to some uncertainty [6] and should be taken into consideration when assessing samples with an average DP_n >10.

Comparison of calculated values of MRF_{rel} for corresponding M-oligosaccharides (Fig. 4) to those from a previous study [6] show they are about twice the size. The reason for this discrepancy is that the molecular weight of a GalA monomer was erroneously used in the earlier calculations [6] instead of the correct molecular weight of the internal

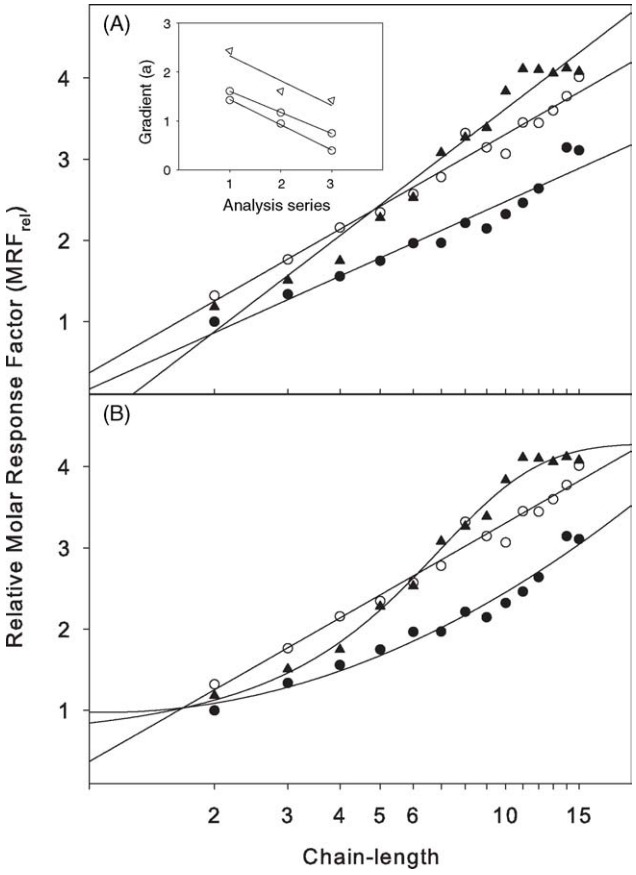

Fig. 4. Mean (n = 3) relative molar response factors (MRF_{rel}) determined for oligosaccharide standards (DP 2–15) originating from mannuronan (open circles, ○), seaweed alginate G-blocks (filled circles, ●) and MG-alginate (filled triangles, ▲). (A) A first order logarithmic function is fitted to the data. (A) Inset (top left) is a plot of the gradient (a), calculated from the fit of the first order logarithmic function for each oligosaccharide batch in each consecutive analysis series. (B) A second order logarithmic function and a sigmoidal function are fitted to the seaweed alginate G-blocks and MG-alginate MRF_{rel}, respectively.

GalA dimer standard. However, since the particular molecular weight of the internal standard is only a scaling factor, and scale is only relative, it has no influence on the use of the MRF_{rel} values in calculating chain-length distributions and associated averages.

3.3. Chain-length distributions of partially hydrolysed MG-alginate and G-blocks

The number and weight chain-length distribution is shown in Fig. 5. Two distinct populations of MG-oligosaccharides are evident in their chain-length distribution (Fig. 5A and B). Each population exclusively comprises odd and even-numbered chain-lengths and each is well described by a Kuhn equation (lines in Fig. 5A and B) calculated using the same α-value (see Section 2). The Kuhn equation also explains the observed number and weight distribution of chains for oligosaccharides generated by the partial acid hydrolysis of G-blocks (Fig. 5C and D). It is therefore assumed that these G-oligosaccharides were generated via the random hydrolysis of glycosidic linkages. For the MG-alginates depolymerisation is more complex. It involves a combination of two hydrolysis processes which are simultaneously operating at different rates. However, glycosidic cleavage in each process is random and it is this that generates the chain-length populations described by the Kuhn equation. A comparison between the DP_n of the polysaccharide hydrolysates calculated via HPAEC–PAD and ^{1}H NMR spectroscopy shows reasonable agreement (Table 2).

Table 2
Estimated DP_n after partial acid hydrolysis of MG-alginate and G-blocks as determined by ^{1}H NMR and HPAEC–PAD

	HPAEC–PAD		^{1}H NMR	
	DP_n	DP_w	DP_n	DP_w
G-blocks	7.5	13.0	8.1	–
MG-alginate	5.8	10.1	6.5	–

3.4. Simulated depolymerisation of MG-alginate

Fig. 6 shows a comparison between experimentally determined weight fractions of oligosaccharide fractions in the hydrolysate from an MG-alginate with an F_G of 0.47 and final DP_n = 5.8 to that calculated by simulated depolymerisation (simulation I, Table 1) of a hypothetically pure MG-alginate (F_G = 0.5, black bars) to the same final DP_n. The ratio between GM and MG hydrolysis rates, p was determined by minimising the difference between observed and predicted weight fractions of oligosaccharides expressed by S_{err} (Eq. (1)). The best agreement is obtained for p = 5.7, which means that the M–G linkages are hydrolysed 5.7 times slower than GM. However, this calculation does not take into account that the MG-alginate used in the experiment is not pure, and it contains 6% extra M-units distributed in short blocks. To more accurately simulate our polymer, and assess the influence of extra M-units within the polymer chain, a number of additional calculations were carried out. To begin with we simulated a polymer in which all the extra 6% M-linkages were randomly distributed as mono-disperse dimers (simulation II, Table 2). We then repeated the simulations for polymers in which M-blocks have length distribution calculated using average M-block length and assuming a 'most probable distribution' of block sizes [20].

There was no satisfactory agreement between simulated and experimentally observed weight fractions of each oligosaccharide fraction when all the M-blocks are mono-disperse dimers (simulation II, S_{err} = 9.43). This is because, in a polymer with an F_G = 0.47, the average minimum MG-block length is 16 monomer units. More importantly such a polymer would contain a large number of M-blocks at an average interval of one for every eight repeating MG-units. This leads to an almost equal number of odd and even-numbered oligosaccharides being released from the hydrolysis of only one linkage type. Hydrolysis of only the GM linkages can

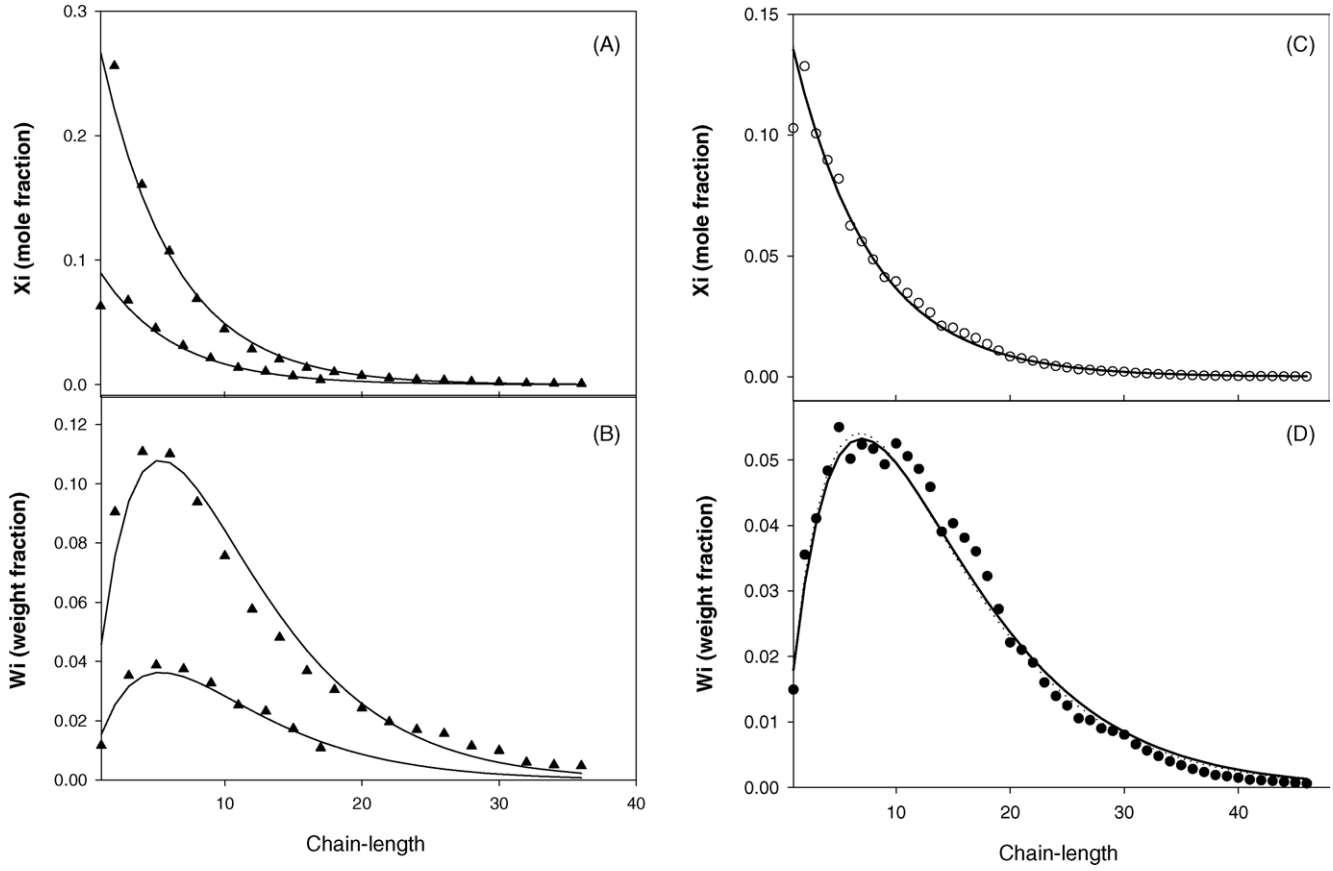

Fig. 5. Number (A and C) and weight (B and D) distribution of oligosaccharides chain-lengths generated by the partial acid hydrolysis of seaweed alginate G-block (filled circles, ●) and MG-alginate (filled triangles, ▲). Kuhn equations for $DP_n = 7.7$ and 5.8 calculated from the raw data for G-block oligosaccharides and MG-alginate oligosaccharides are, respectively, fitted as black lines. In the latter case the fit has been weight adjusted and applied to two populations of even and odd numbered chain-lengths within the chain-length distribution. Small dashes represent the Kuhn equation that best describes the data in terms of a least squares fit.

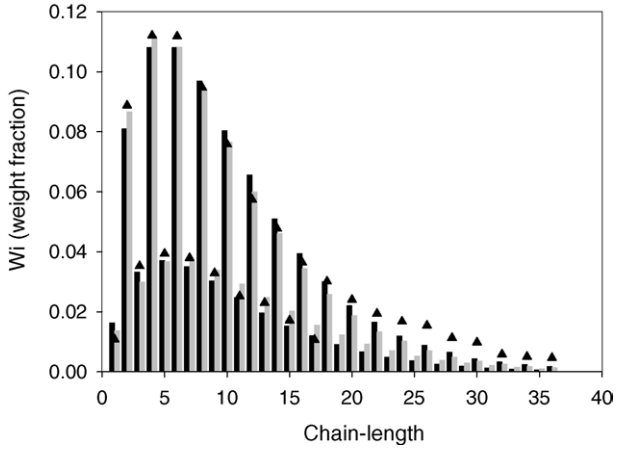

Fig. 6. Weight fraction of MG-oligosaccharide chain-lengths obtained by hydrolysing the starting polymer to an average $DP_n = 5.8$. Experimental values (filled triangles, ▲) were obtained using the described HPAEC–PAD method. Filled bars represent the simulated MG-oligosaccharide chain-length fractions assuming the acid depolymerisation of a hypothetically pure MG-alginate ($F_G = 0.5$) and gives a ratio between GM and MG hydrolysis rate as $p = 5.7$. Grey bars represent the same simulation but for an MG-alginate ($F_G = 0.47$) with an average M-block length of 3.4 monomer units. The ratio between GM and MG hydrolysis rates was $p = 8.3$.

produce both odd and even-numbered chain-lengths. For example: MGMGMMG for a heptamer, and MGMGMG for a hexamer. For this simulation (Table 1) $p = 24.5$. In a parallel study using the same starting MG-alginate ($F_G = 0.47$), similar conditions of partial hydrolysis, and ^{1}H NMR to directly determine p, a value of 10.7 was obtained [15].

A better agreement between the experimental and simulated data is obtained when a hypothetically pure MG-alginate has a poly-disperse distribution of M-blocks inserted into its polymer chain, shown as grey bars in Fig. 6. The S_{err} is at a minimum, and 60% lower than what was obtained for a hypothetically pure MG-alginate, when the average M-block length is 3.4 monomer units (simulation VII, Table 1, bold type). For this average length, M-blocks are distributed as 41% dimer, 25% trimer, 13% tetramer and the remainder as pentamers or larger (corresponding F_{MM}, F_{MMM}, F_{MMMM} values are shown in Table 1). Furthermore, the average MG-block length of this polymer is around 38 monomer units (Table 1). This value is in good agreement with that previously determined for the same polymer by NMR (see Table 1 in [12]). The calculated ratio between GM and MG hydrolysis rates under the specific condition of our hydrolysis is $p = 8.3 \pm 1$ (estimated error). This is also in good

agreement with the value measured independently using ^{1}H NMR [15].

4. Conclusion

HPAEC–PAD is a useful tool to determine the distribution of oligosaccharides derived from linear binary polysaccharides, such as alginate. Together with other complementary methods, such as NMR, chain-length analysis can reveal physical and structural details of the parent polysaccharides. Confident peak assignments of some major oligosaccharide fractions were made by exploiting mild partial acid hydrolysis as a method of depolymerisation. However, other than on the basis of sequence composition we did not attempt to explain the various chemical and physical factors that governed oligosaccharide retention on the anion-exchange resin.

Because of the presence of several unidentified smaller peaks in the chromatograms integrated peak areas for each chain-length had to be combined in order to calculate the relative molar response factor with some accuracy. A similar approach to circumventing the influence of acid-catalysed modification of alginate oligosaccharides was successfully applied before [4]. Using MRF_{rel} and extrapolation for the monomer and chain-length fraction <15 we calculated chain-length distributions and associated averages which correlated well with complementary measurements made with ^{1}H NMR. The chain-length distribution of the MG-alignate hydrolysate contained two populations, one comprising even-numbered and one comprising odd-numbered chain-lengths, both of which were explained by a Kuhn equation. On the other hand, one Kuhn equation explained the entire chain-length distribution observed for the G-blocks. The gross structure of the starting polymers was therefore confirmed. Further statistical simulation of the depolymerisation of alginates compared to the experimentally determined distributions shed further light on the fine structure of the MG-alginate. For the hydrolysis conditions applied it allowed us to give an estimate of $p = 8.3 \pm 1$ for the ratio between the hydrolysis constants of the GM and MG linkage. We tentatively propose this polysaccharide preparation to have an average MG-block length of 19 dimer units separated by regions of MM linkages with an average M-block length of 3.4 monomer units.

One disadvantage of using PAD for detection is the initial time-consuming procedure of having to calibrate the PAD response for the different oligosaccharide sequences and chain-lengths. But once this is done, a calibration standard can be made and stored for future use. If this approach is developed further then it may be possible to investigate the average fine structure of many other types of binary polysaccharide as well as alginate. HPAEC–PAD analysis could especially offer an advantage over current NMR spectroscopy methods, where the characteristic NMR resonance of particular diad and triplet sequences [25–27] are small and therefore difficult to accurately integrate.

Acknowledgements

We thank FMC Biopolymers and Norwegian Research Council under the Centre for Biopolymer Engineering at NOBIPOL, NTNU (Grant No. 145945/130) for financial support. We also thank Kurt Ingar Draget and Anne Sissel Duun for producing the G-block seaweed alginate, Espen Granum for proof reading the manuscript, and various members of NOBIPOL for useful discussions and comments.

References

[1] H.O. Bouveng, B. Lindberg, Adv. Carbohydr. Chem. 15 (1960) 53.
[2] S. Hizukuri, Carbohydr. Res. 147 (1986) 342.
[3] K. Freudenberg, G. Blomquist, Ber. B68 (1935) 2070.
[4] B. Larsen, O. Smidsrød, A. Haug, T. Painter, Acta Chem. Scand. 23 (1969) 2375.
[5] O. Smidsrød, B. Larsen, T. Painter, A. Haug, Acta Chem. Scand. 23 (1969) 1573.
[6] C. Campa, A. Oust, G. Skjåk-Bræk, B.S. Paulsen, S. Paoletti, B.E. Christensen, S. Ballance, J. Chromatogr. A 1026 (2004) 271.
[7] Y. Zhang, Y.C. Lee, in: Z. El Rassi (Ed.), Carbohydrate Analysis by Modern Chromatography and Electrophoresis (Journal of Chromatography Library, vol. 66), Elsevier, Amsterdam, 2002, p. 207.
[8] K.-S. Wong, J.-L. Jane, in: Z. El Rassi (Ed.), Carbohydrate Analysis by Modern Chromatography and Electrophoresis (Journal of Chromatography Library, vol. 66), Elsevier, Amsterdam, 2002, p. 403.
[9] A.J. Mort, F. Qui, N.O. Maness, Carbohydr. Res. 247 (1993) 21.
[10] L.A. Larew, D.C. Johnson, Anal. Chem. 60 (1988) 1867.
[11] J. Emneus, L. Gorton, Anal. Chem. 62 (1990) 263.
[12] C. Campa, S. Holtan, N. Nilsen, T.M. Bjerkan, B.T. Stokke, G. Skjåk-Bræk, Biochem. J. 381 (2004) 155.
[13] H. Ertesvåg, G. Skjåk-Bræk, in: C. Bucke (Ed.), Methods in Biotechnology, vol. 10, Humana Press, Totowa, 1999, p. 71.
[14] M. Gimmestad, H. Sletta, H. Ertesvåg, K. Bakkevig, S. Jain, S. Suh, G. Skjåk-Bræk, T. Ellingson, D. Ohman, S. Valla, J. Bacteriol. 185 (2003) 3515.
[15] S. Holtan, Q. Zhang, W.I. Strand, G. Skjåk-Bræk., in preparation.
[16] A. Haug, O. Smidsrød, Acta Chem. Scand. 20 (1966) 183.
[17] A. Haug, B. Larsen, O. Smidsrød, Acta Chem. Scand. 21 (1967) 691.
[18] P. Sikorski, B.T. Stokke, A. Sørbotten, K.M. Vårum, S.J. Horn, V.G.H. Eijsink, Biopolymers 77 (2005) 273.
[19] C. Tanford, Physical Chemistry of Macromolecules, Wiley, New York, 1961, p. 710.
[20] P.J. Flory, Principles of Polymer Chemistry, Cornell University Press, New York, 1953, p. 672.
[21] Y.C. Lee, Anal. Biochem. 189 (1990) 151.
[22] Dionex Corporation, Technical Note 21, 1998, p. 4.
[23] J.S. Rohrer, J. Thayer, M. Weitzhandler, N. Avdalovic, Glycobiology 8 (1998) 35.
[24] W.R. LaCourse, in: Z. El Rassi (Ed.), Carbohydrate Analysis by Modern Chromatography and Electrophoresis (Journal of Chromatography Library, vol. 66), Elsevier, Amsterdam, 2002, p. 905.
[25] H. Grasdalen, B. Larsen, O. Smidsrød, Carbohydr. Res. 56 (1977) C11.
[26] H. Grasdalen, B. Larsen, O. Smidsrød, Carbohydr. Res. 68 (1979) 23.
[27] H. Grasdalen, Carbohydr. Res. 118 (1983) 225.