# Preparation and characterization of oligomannuronates from alginate degraded by hydrogen peroxide

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## Abstract

Macromolecular alginate extracted from brown seaweeds was treated with acid to generate mannuronate blocks. The resulting polymannuronates were degraded by hydrogen peroxide with a procedure optimized for obtaining high recovery of low molecular weight saccharides. The resulted mixture of different sized oligomannuronates was separated by gel chromatography on a Bio-gel P-4 column and Mono Q column. The purified oligomers were characterized by anion-exchange HPLC as well as spectrum analysis. This report provides a general procedure for generation and purification of oligomannuronates within the size of 2–7 sugar units from alginate.
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## 1. Introduction

Alginates are a family of linear polysaccharide produced by brown seaweeds and some bacterial species belonging to the genera *Pseudomonas* and *Azotobacter*. These polymers are composed of two hexuronic acids, β-D-mannuronic acid (ManA) and α-L-guluronic acid (GulA), linked by α1–4 bonds (Gacesa, 1988). The monomers are distributed in blocks of continuous ManA residues (M-blocks), GluA redsidues (G-blocks), or alternating residues (MG-blocks). Alginates isolated from different natural sources vary in the length and distribution of the different block types. The variability in monomer block structures in the polymer strongly affects the physiological and rheological properties, and the application of the polymer.

The polymer alginate has been used for a variety of industrial purposes, e.g. as a stabilizing, thickening and gelling agent in food production and pharmaceutical applications. Recent developments in using alginate as a drug carrier (Tonnesen and Karlsen, 2002), wound dressing (Thomas, 2000) and in transplantation therapy (Fu, Hsu, Chiou, & Hsu, 2003) have expanded the use of this polymer. Demonstration of anti-hypertension effect of a modified alginate-derived oligomer (PSS $M_W = 11,000$ Da, S% = 12%). (Wang, Liu, Zhang, & Zhang, 1992) first reported medical application of alginate products. However, functions of the degradation product of polysaccharide, oligohexuronic acid, have largely remained unexplored. Recent reports have suggested that alginate oligosaccharides have stimulation effects on human keratinocyte growth (Kawada et al., 1997), human endothelial cell migration (Kawada, Hiura, Tajima, & Takahara, 1999) and human skin fibroblast proliferation (Tajima, Inou, Kawada, Ishibashi, Takahara, & Hiura, 1999), indicating a potential interest in developing biological active molecules based on alginate-derived oligosaccharides.

Alginate can be depolymerized in several ways, including enzymatic and chemical treatments. Alginate lyases, a family of more than 50 difference species isolated from a wide range of organisms, including algae, marine invertebrates, and marine and terrestrial microorganisms, are characterized as either mannuronate (EC 4.2.2.3) or guluronate lyases (EC 4.2.2.11), which cleave the glycosidic 1→4 O-linkage by a β-elimination mechanism,

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resulting in 4.5-unsaturated, nonreducing terminus. Most of the lyases have a defined substrate specificity, being used in selectively generating specific blocks, however, the distinct characteristic of lyase depolymerized products is the unsaturated nonreducing terminus which is not always desirable. The alternative degradation methods are acidic hydrolysis, using HCl (Haug, Larsen, & Smidsrød, 1967; Rehm, 1998), $H_2SO_4$ (Muramatsu, Yamada, Date, & Yoshika, 1993) formic acid (Sherbrock-Cox, Russell & Gacesa, 1984) and oxalic acid (Haug, Larsen, & Smidsrød, 1966). Though the chemical procedures are convenient, the common disadvantage of acidic hydrolysis is its low recovery, particular in oligosaccharides.

Hydrogen peroxide has been used for degradation of chitin and chitosan (Nordtveit, Vårum, & Smidsrød, 1994). Nordtveit et al. demonstrated that the viscosity of chitosan solution decreased more rapidly in the presence of hydrogen peroxide ($H_2O_2$) than acid degradation. In this study, we first report using hydrogen peroxide to degrade alginate-derived polymannuronate blocks. The resulted oligomannuronates were separated by gel chromatography, and the purified oligmers of 2–7 were characterized by anion exchange chromatography and Electrospray ionization mass spectrometry (ESI-MS). Further, we have demonstrated that hydrogen peroxide treated oligmannuronates can be radio-labeled by reduction reaction with $NaB^3H_4$. This procedure maybe generally adapted to labeling linear poly/oligo-saccharides after treatment with hydrogen peroxide.

## 2. Materials and methods

### 2.1. Preparation of polymannuronate blocks

Purified sodium alginate was purchased from Huanghai Alginate Industry (Qingdao, PR China). The alginate was dissolved in de-ionized water to a final concentration of 2% and hydrolyzed in 0.5 M HCl (final concentration) at 100 °C for 8 h. The solution was filtrated and the precipitation was collected and dissolved in 8% NaHCO₃. After adjusting pH to 2.85 with 4 M HCl, the solution was filtrated again. The depolymerized alginate was recovered by precipitation with 75% ethanol and dried.

### 2.2. Estimation of average molecular size

The recovered polymannuronate blocks were dissolved in 2.84% $Na_2SO_4$ at the concentration of 5 mg/ml. Twenty microliter of the sample was applied to a TSK gel G3000PW$_{XL}$ column (TOSOH, 7.8×300 mm) equilibrated in 2.84% $Na_2SO_4$ at a flow rate of 0.5 ml/min. The column is connected to GPC software (Longzhida Company, PR China) and the effluent was monitored by a 410 differential refractometer (RI detector).

### 2.3. Preparation of oligomannuronates by hydrogen peroxide

The polymannuronate blocks were dissolved in distilled water at a final concentration of 8%, to which hydrogen peroxide (30%) was added to a final concentration of 5% (v/v). The solution was kept at 90 °C under stirring for 0.5, 1, 2, and 4 h, respectively. The reaction was stopped by cooling down to room temperature. After removing the insoluble particles by filtration, the oligomannuronate was recovered by precipitation in 75% ethanol. The pellet was washed with ethanol and dried.

### 2.4. Chemical determination of hexuronic acid

The amount of hexuronic acids was determined using the carbazole reaction method described by Bitter and Muir (1962).

### 2.5. Radioisotope labeling of the oligomannuronates

Samples (1 mg) were dissolved in distilled water and adjusted to pH 9 using 0.01 M NaOH. The solution was reduced with 0.5 mCi $NaB^3H_4$ (Amersham Biosciences, Uppsala, Sweden; 55 Ci/mmol) for 15 h at room temperature at a total volume of 0.2 ml. Following acidification of the reaction mixtures with 4 M acetic acid, samples were neutralized with 4 M NaOH. ³H-labelled oligosaccharides were recovered by gel chromatography on a column (1×190 cm) of Sephadex G-15 in 0.2 M NH₄HCO₃. The pooled fractions were desalted and dried by lyophilization.

### 2.6. Analysis of oligomannuronates by gel chromatography

A portion of the ³H-labelled Sample (50,000 cpm) in 200 μl of water was applied on a Bio-gel P-4 (superfine) column (1×190 cm), operated at 4 °C. The column was eluted with 0.5 M NH₄HCO₃ at a flow rate of 2 ml/h. Fractions of 1 ml were collected and analyzed for radioactivity by scintillation counting (LS 6000IC, Beckman). For the non-labeled sample, 1 mg was dissolved in 200 μl of water and applied on the Bio-gel P-4 (superfine) column (1.6×170 cm), operated at room temperature. The column was eluted with 0.5 M NH₄HCO₃ at a flow rate of 3 ml/h. From each collected fraction, 50 μl of sample was taken for hexuronic acid analysis as described above.

### 2.7. Purification of oligomannuronates

Both radio-labeled and unlabeled oligomannuronates generated by treatment of hydrogen peroxide as describe previously were purified first on a Bio-gel P-4 column. The fractions with distinct peaks were pooled and desalted by lyophilization. The individual peaks were further purified by repeated gel chromatography.

## 2.8. Analysis of the purified oligomannuronates by ion-exchange chromatography

High-performance anion-exchange chromatography was performed on a ProPac™ PA-1 column (4×250 mm) equipped in a Gilson 204 series system (Gilson, USA) with an on-line Radiomatic™ Flow Scintillation Analyzer (500TR series, Packard, USA). The elution was carried out with a linear gradient of 0–0.5 M NaCl in 50 mM Tris–HCl buffer, pH 7.0 at a flow rate of 1.0 ml/min. The total elution volume was 120 ml.

## 2.9. Electrospray ionization mass spectrometry

Negative-ion electrospray mass spectrometry was conducted using a Gilson LC-MS instrument. Samples (0.5 mg) dissolved in 500 μl of water were applied to a reverse phase C-18 column (Chromolith™, 5 μm particle, 4.6×100 mm). The HPLC apparatus (Gilson series 322) equipped with an autosampler (Gilson 402 syringe pump, 233 XL online column switching, injection volume = 50 μl) was used. The mobile phase was a gradient of solvent A (0.05% formic acid in water) and solvent B (0.05% formic acid in acetonitrile), 5–50% B in 5 min at a flow rate of 4 ml/min. Detection was accomplished by using a diode array detector (Gilson series 170), storing the signal at a wavelength of 208 and 254 nm. The MS detector was Finnigan AQA, unipoint 3.0 LC/MS interface, negative 10 kV field. The mass range was from 120 to 1200 u.

## 3. Results and discussion

### 3.1. Preparation of oligomannuronates

After acid hydrolysis in 0.5 M HCl the resulting alginate fragments were recovered by precipitation with ethanol. The average molecular size of the fragments was ∼8000 Da (about 40 sugar units) as estimated by GPC analysis (Fig. 1). Circular Dichroism analysis showed that the fragments contained more than 95% of manuronate (data not shown). To find out an optimal degradation process, this poly-mannuronate was treated with hydrogen peroxide for 0.5, 1, 2 and 4 h, respectively, at 90 °C. After stopping the reactions by cooling down to room temperature, the degraded oligossccharides were recovered by ethanol precipitation. The recovery and size distribution of the oligosaccharides are summarised in Table 1.

We found that the depolymerization of polymannuronate in the presence of hydrogen peroxide ($H_2O_2$) was rapid, and in a time-dependent manner. Incubation of 0.5 h resulted in a more than 50% depolymerization with an equal level of recovery. Though the total recovery was decreased with prolonged treatment time, the proportion of low molecule fragments is enriched. Furthermore, depolymerization degree is dependent on the reaction time, therefore it is
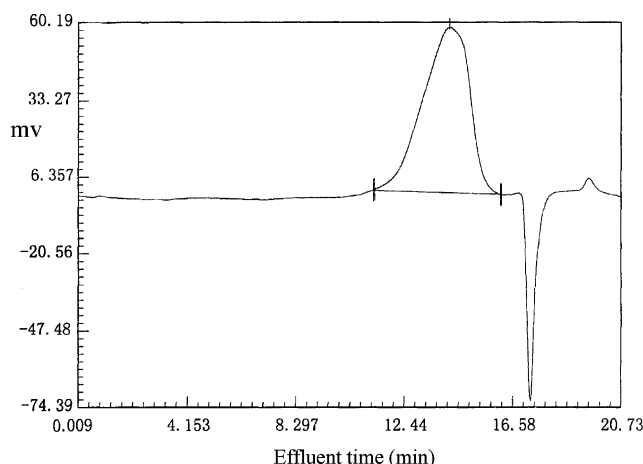


Fig. 1. GPC Chromatography of polymannuronates. The polymannuronate blocks were dissolved in 2.84% $Na_2SO_4$ and analyzed in an TSK gel G3000PW$_{XL}$ column. The column was eluted with 2.84% $Na_2SO_4$ at a flow rate of 0.5 ml/min and the effluent was monitored by a 410 differential refractometer (RI detector).

possible to generate different sized fragments by controlling reaction time. Since we are interested in obtaining oligomers within the range of 1–7, in the following experiments, a 2 h reaction time was used.

The reaction mechanism of the degradation by hydrogen peroxide remains unclear. The possible mechanism is that the hydroxyl radicals generated in the experimental system caused the polymer degradation. The hydroxyl free radicals abstracted the C-1 hydrogen atoms from the mannuronic acid residues first, then the molecules rearranged their structures and broken glycosidic bonds. The hydroxyl free radicals may prefer to attack the more mobile sequences close to the ends of the chains, however, the cleavage seems to be rapid, estimated by the high population of low molecular weight products with a relative short treatment time (for 0.5 h), suggesting a combination of random cleavages and chain-end scissions.

Since the reaction system contained $H_2O_2$, the aldehyde group in the new generated reducing end was largely oxidated to carboxyl as illustrated in Fig. 2. This structure was confirmed by analysis of ESI-MS.

### 3.2. Radioisotope-labelling of hydrogen peroxide fragmented oligomannuronates

To improve the analysis method for oligomers, instead of chemical detection for hexuronic acid by carbazole reagent,

Table 1
Hydrogen peroxide degradation of polymannurontes

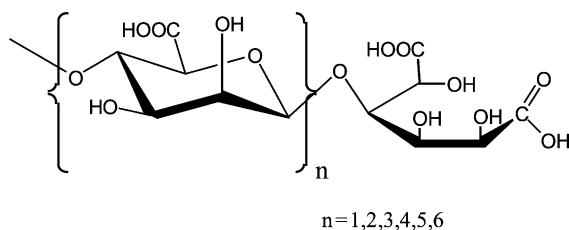| Sample | Reaction time (h) | Oligomers | Yield (%) |
|---|---|---|---|
| A | 0.5 | 1–15 | 58 |
| B | 1.0 | 1–10 | 46 |
| C | 2.0 | 1–7 | 21 |
| D | 4.0 | 1–5 | 13 |

Fig. 2. Deduced structure of oligomannuronates degraded by hydrogen peroxide. According to the results of ESI-MS and NMR analysis, the reducing end of most oligomers is reduced to a carboxyl.

we have labeled the oligomannuronates by reduction with $NaB^3H_4$. The samples C and D (Table 1), resulted from treatment with hydrogen peroxide for 2 respective 4 h, were selected for labeling. The oligomers (1 mg) dissolved in water were incubated with 0.5 mCi $NaB^3H_4$ at pH 9 for 15 h. After reduction, the free $NaB^3H_4$ was removed by applying the samples to a Sephadex G-15 column. The total labeled fragments were pooled and analyzed. The specific activity of the samples was $9.2 \times 10^6$ cpm/mg hexuronic acid (sample C) and $13 \times 10^6$ cpm/mg hexuronic acid (sample D), respectively. Radioisotope labeling technique offers the great advantage of high sensitivity for detection, while at same time not altering the properties of the labeled molecules. For the first time, we are able to demonstrate that oligosaccharides derived by treatment with hydrogen

peroxide are still reducible, indicating not all the reducing terminal mannuronic acid is reduced by hydrogen peroxide.

### 3.3. Characterization of the $^3$H-labelled oligomannuronates

$^3$H-labelled samples C and D were analyzed in a Bio-gel P-4 column that is calibrated with glucuronic acid (monomer) and a glucuronic acid- glucosamine disaccharide. About 50,000 cpm in 200 µl was applied to the column and eluted under the conditions described in Experimental Procedures. The elution profiles are shown in Fig. 3A and B. It is shown that sample C is composed of a mixture of 1–7-mers that have been well separated. The proportion of each oligosaccharide species is 15.6, 26.7, 20.4, 14.8, 11.1, 8.4, 3% for the 1, 2, 3, 4, 5, 6 and-7-mers, respectively, with a main population of 1–5-mers. While sample D contained a mixture of 1–5-mers, the proportion of each oligomer is 15.3, 34.5 26.7, 15.9 and 7.6% for the 1, 2, 3, 4 and 5-mers, respectively. The respective peaks were pooled and lyophilized and re-analyzed on the same column as shown in Fig. 4. Each sample gave a single peak at the expected elution volume.

The evaluation of the molecular size of the component in each peak was made by the method of Whitaker (1963). The peak designated as 1 (Fig. 3), which had the same elution volume as glucuronic acid, thus represents a monomer. The peak designated as 2 had a same elution volume as
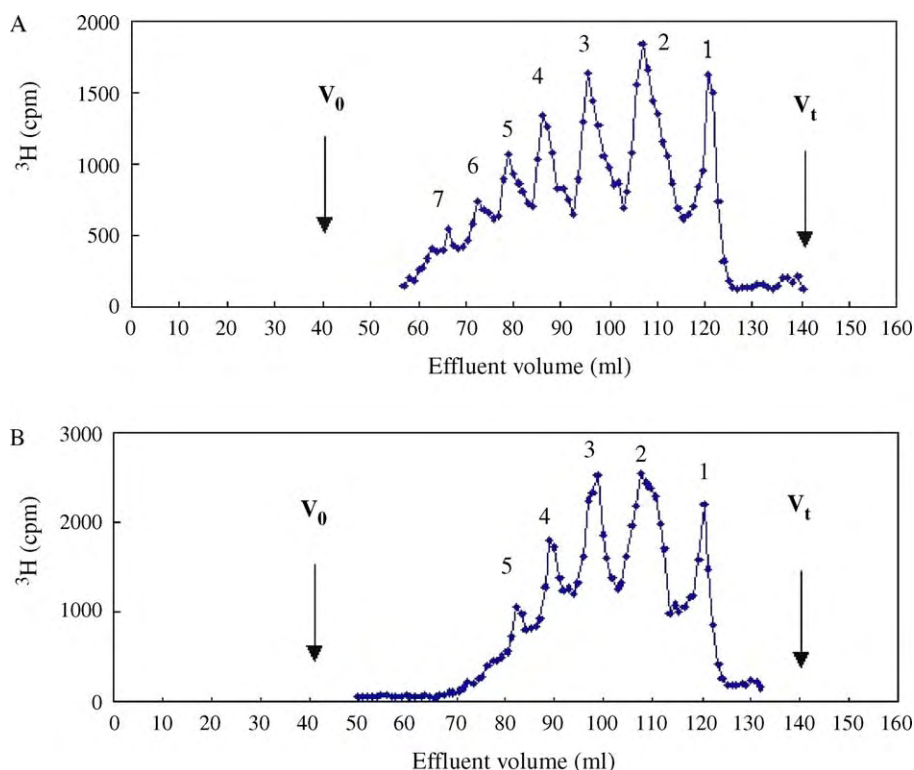


Fig. 3. Gel chromatography of $^3$H-labeled oligomannuronates. The products of polymannuronates treated with hydrogen peroxide for 2 h (A) and 4 h (B) were reduced with $NaB^3H_4$. The resulting mixtures of oligomers were analyzed on a Bio-gel P-4 column. The samples were eluted with 0.5 M $NH_4HCO_3$ at the flow rate of 2 ml/min. The peak numbers represent the size of the oligomers.
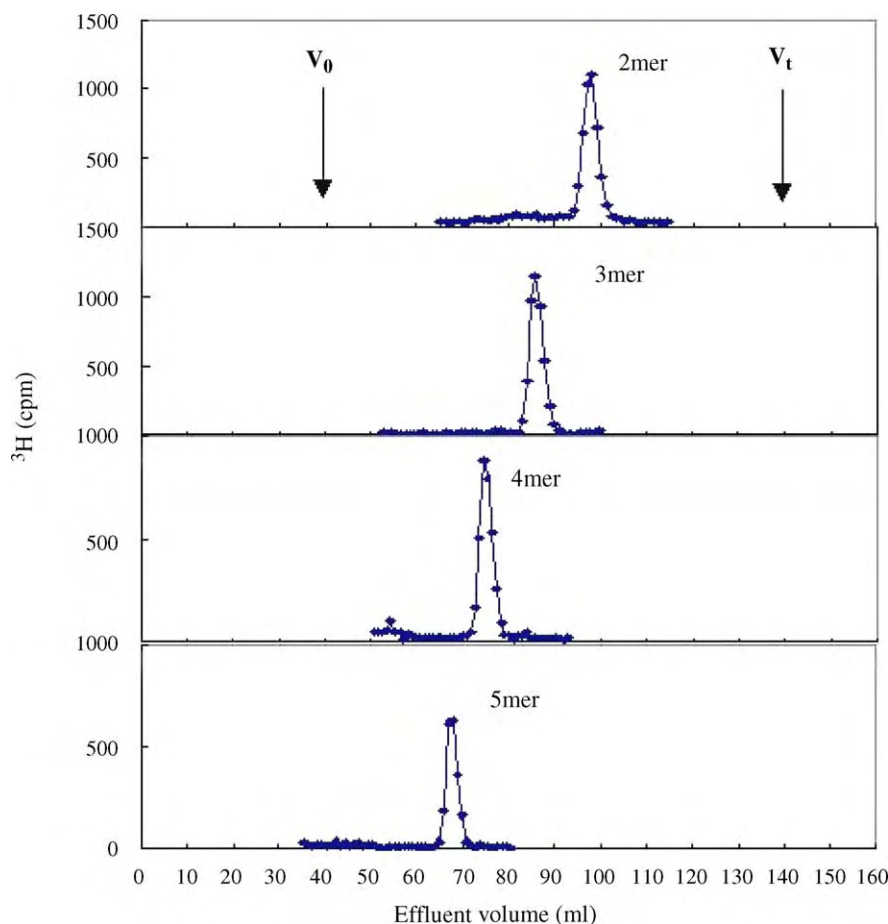
Fig. 4. Analysis of ^{3}H-labeled 2–5 mer on Bio-gel P-4 column. The pooled fractions of oligomannuronates were analyzed on the Bio-gel P-4 column at the same condition as described in the legend to Fig 3. The symmetric sharp peaks in each panel show the oligomers eluted at the positions corresponding to their size.

the glucuronic acid-glucosamine disaccharide. A good correlation between $\text{Log}\,M_r$ of the oligomers and their elution volumes was observed for both samples. Thus peak 1 to peak 7 in Fig. 3A are deduced as monomer to heptamer and peak 1 to peak 5 in Fig. 3B are monomer to pentamer. These results indicate that 1–7-mers of unsubstituted sugars can be well separated in the Bio-gel P4 column at a low flow rate.

To confirm that oligomer fractions collected from gel chromatography each contains a single component, the [3]H -labeled oligomers (2, 3, 4 and -5-mer) were further analyzed on a ProPac™ PA-1 column (anion-exchange). An analytical example of the 5-mer shows a single sharp peak (Fig. 5).

### 3.4. Purification of the oligomannuronates

To obtain chemical amount of oligmannuronates, preparative separation of sample B (1 mg) was performed at the same way as that for the labeled-materials. Interestingly the separation of 2–7-mers (Fig. 6) was as good as that of the analytical run, though the chemical amount of hexuronic acid applied is 1000-fold more than

the analytical separation. This result shows that the separation potential of the Bio-gel P4 is high, and a large-scale purification of the oligomers is possible by scaling up the column volume.
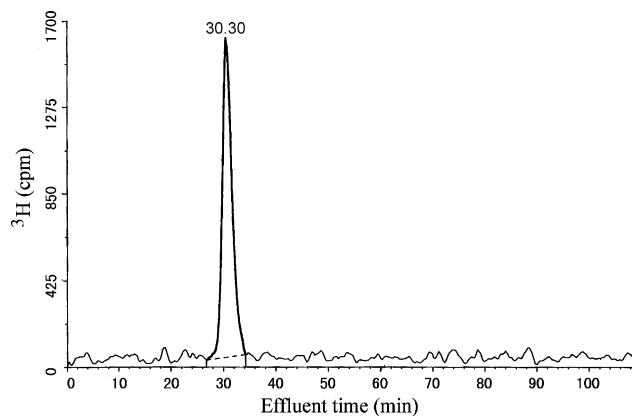


Fig. 5. Analysis of ^{3}H-labeled 5 mer on anion exchange column. The purified 5 mer was analyzed on a ProPac™ PA-1 column eluted with a linear gradient of 0–0.5 M NaCl in 50 mM Tris–HCl buffer, pH 7.0 at a flow rate of 1.0 ml/min. A single symmetric peak is obtained at the salt concentration of 0.15 M.
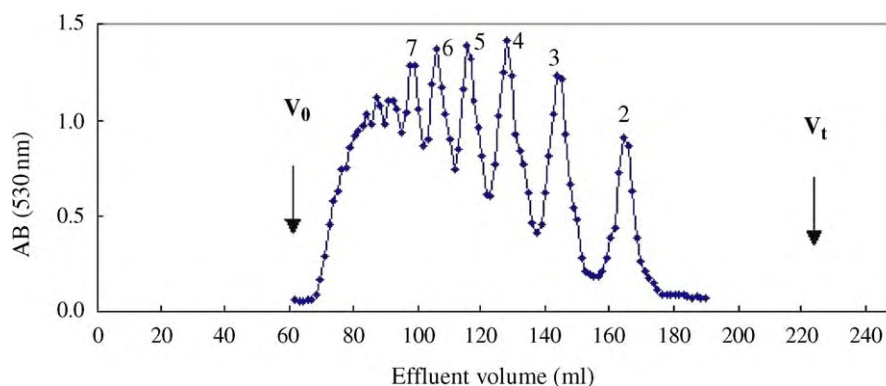
Fig. 6. Separation of oligommanuronates on Bio-gel P-4 column. One milligram of the sample treated with hydrogen peroxide for 1 h was applied to the column and eluted with 0.5 M NH$_4$HCO$_3$ at the flow rate of 3 ml/min. The fractions of 1 ml were collected. A sample of 50 μl was taken for hexuronic acid analysis by the carbazole reaction. The absorption at 530 nm was plotted.

### 3.5. Characterization of the purified oligomannuronates

To further confirm the molecule size of the purified unlabeled oligomers, samples of 2, 3, 4, 5, 6 and -7 mer were subjected to mass spectrometry analysis. Fig. 7 shows an example of analysis result of 5-mer. The peak at $m/z$ 914.0 corresponds to the molecular ion peak $[M-1]^{-1}$ of the oxidation degradation pentamannuronate. The peak at 883.9 was $[M-CH_2O-H]^{-1}$, that at 853.9 was $[M-C_2H_4O_2-H]^{-1}$, that at 823.8 was $[M-C_3H_6O_3-H]^{-1}$, that at 737.8 was $[M-Man\ A-H]^{-1}$, that at 456.7 was $[M-2H]^{-2}$, and that at 304.1 was $[M-3H]^{-3}$. Therefore, the $M_r$ of 5-mer is 914.6 (Fig. 7). Combined with the absence of the $Mr30$ (CH$_2$O), $Mr60$ (C$_2$H$_4$O$_2$) and the $M_r90$ (C$_3$H$_6$O$_3$) peaks, these data confirm the 1–4 type glycosidic linkages in the oligomer (Quéméner, Désiré, Debrauwer, & Rathahao, 2003). The ESI-MS result suggests that the oligomannuronates degraded by oxidation had a structure as shown in Fig. 2. The NMR analysis showed

that the oxidation occurred on the reducing-end C1, which is oxidised to a carboxyl group (data not shown).

### 4. Conclusion

The aim of this study was to generate low-molecular and size-defined oligomannuronates from alginate extracted from brown seaweeds. The results of the study have shown that the low-molecular weight mannuronates can be prepared by treatment with hydrogen peroxide. This method is simple and easy to control by adjusting reaction time. The most important advantage is the relative high recovery of low molecular sized oligosaccharides. Furthermore we found the reducing end C-1 position was mostly oxidised to a carboxyl group, however, there are still non-reducing sugar units in the oligomers that can be further reduced by NaB$^3$H$_4$, probably in a lactone form. Reduction with NaB$^3$H$_4$ resulted in labeling of the oligosaccharides,



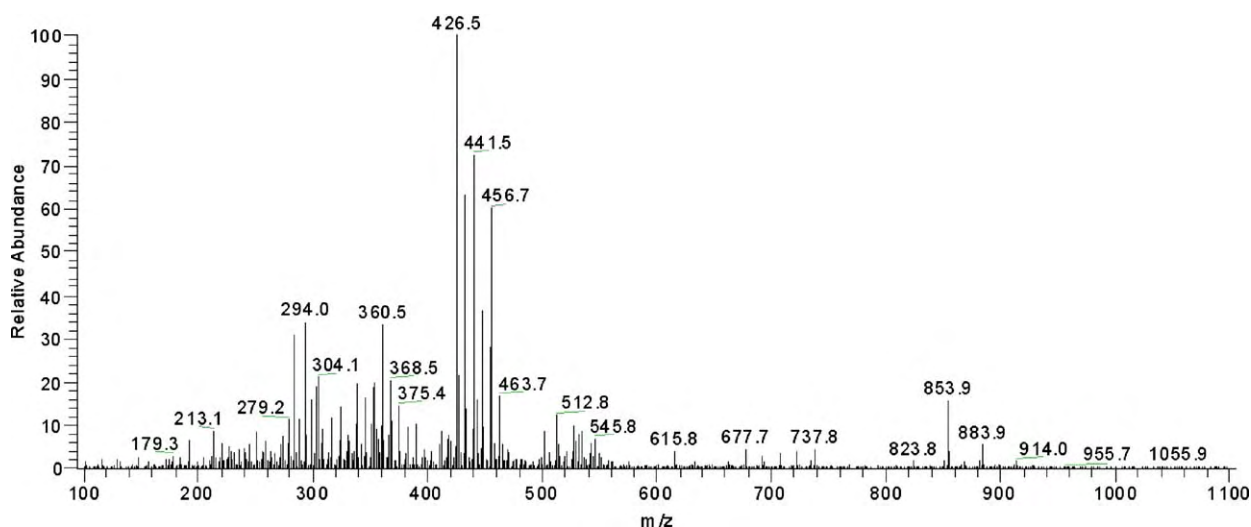Fig. 7. EIS-MS analysis of oligomannuronate 5 mer. The peak at $m/z$ 914.0 corresponds to the molecular ion peak $[M-1]^{-1}$ of the 5 mer. The ionic fragments of the 5 mer were also detected.

providing a general method for labeling of oligosaccharides treated with hydrogen peroxide. This method is special useful when analysis of a trace amount of oligosaccharides is under question. The one-step gel chromatographic procedure on Bio-Gel column for purification of oligomannuronates can be applied to purification of oligomers derived from other polysaccharides.

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