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Short communication

Analysis of oligomannuronic acids and oligoguluronic acids by high-performance anion-exchange chromatography and electrospray ionization mass spectrometry

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Abstract

Oligomannuronic acids and oligoguluronic acids were prepared by enzymatic hydrolysis of alginate with alginate lyases. The oligosaccharides generated up to degree of polymerization 16 were characterized by high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) and electrospray ionization mass spectrometry (ESI-MS). Acetate buffer linear gradients were used as mobile phases for separations of oligosaccharides. ESI-MS and HPAEC–PAD are very effective for the analysis and characterization of anionic oligosaccharides.

Keywords: Oligomannuronic acids; Oligoguluronic acids; Polysaccharides

1. Introduction

Alginic acid is a linear polymer composed of (1→4)-linked β-D-mannuronic acid and α-L-guluronic acid units arranged in homo- or heteropolymeric sequences [1,2]. Numerous recent studies have reported alginic acid hydrolysates and alginate-derived oligouronic acids as increasingly important bioactive polysaccharides [3,4]. Alginate-derived oligouronic acids play an important role in hypertension, such as PSS, which is a good medicine for hypertension [5]. Other reports have described their physiological activities on plant growth [6,7].

We can obtain oligoglycuronates by enzymatic hydrolysis. Alginate-degrading enzymes, alginate lyases, which are specific for PG [poly(α-L-guluronate) lyase, EC 4.2.2.11], and PM [poly(β-D-mannuronate) lyase, EC 4.2.2.3], to make 4-deoxy-*erythro*-hex-4-enpoyranuronosyl groups at their non-reducing ends by the β-elimination reaction, have been found in a variety of sources, including marine molluscs, bacteria, and brown algae [8,9]. These enzymes are often used to obtain fragments of alginate, since their eliminase activity introduces an unsaturated bond at the non-reducing terminus, thus providing oligomers with a convenient UV chromophore. Despite their frequent use in the biochemical literature, these digestion products have not been well characterized. It is essential to characterize the structure because the structure–activity relationship (SAR) is becoming more and more important in drug design.

HPLC is the main method for the analysis of oligosaccharides; we chose high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) due to its high sensitivity and resolution [10,11]. Acidic oligosaccharides have been less well studied by this technique, perhaps

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because of the high pH which is typically required, causing the anion oligosaccharides to be tightly retained in the stationary phase. Nevertheless, several reports have been documented in this area [7,12,13]. Electrospray ionization mass spectrometry (ESI-MS) has emerged in the last few years as a powerful technique for carbohydrate analysis [12,14,15]. It is difficult to prepare various kinds of oligouronic acid from acid or enzyme hydrolysates. We purified extracellular endo-alginate lyases from *Pseudomonas* sp. and used the enzyme to degrade alginate, separated the hydrolysate by Q-Sepharose anion-exchange chromatography, and obtained two kinds of mannuronic acid, which showed almost a single peak on HPAEC, and their molecular masses were determined by ESI-MS. Using these oligosaccharides as standard samples, we can compare the degree of polymerization (DP) of oligomannuronate and oligoguluronate hydrolysates by HPAEC.

2. Experimental

2.1. Reagents

Sodium alginate (Huanghai Alginate Industry), Q-Sepharose (Pharmacia, Sweden), NaOH and NaAc (Guaranteed Reagent, Merck, Germany)

2.2. Preparation of enzyme

A marine bacterium isolated from kelp was inoculated in a medium (50 ml in a 250-ml flask containing 0.5% sodium alginate, 0.5% peptone, 0.25% yeast extract, 1% NaCl, 0.1% $MgSO_4$) for 24 h at 25°C with rotary shaking (180 rpm) as the seed culture. The bacterial culture was inoculated in 3 l medium in a 5-l fermentation jar containing 1% sodium alginate, 0.5% peptone, 0.25% yeast extract, 1% NaCl, and 0.1% $MgSO_4$ for 24 h at 25°C. The culture broth was centrifuged at 10 000 *g* for 20 min at 4°C. Ammonium sulfate was added to the supernatant with constant stirring at 4°C, resulting in a 100% saturated solution. The solution was centrifuged at 10 000 *g* for 15 min at 4°C. The precipitate was dialyzed for 24 h against two changes (5 l each) of 0.02 *M* Tris–HCl buffer at pH 7.5 at 4°C. The dialyzed mixture was centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant was lyophilized and the resulting crude enzyme powder was stored at −18°C for future use.

2.2.1. Assay of the enzyme activity

A substrate solution containing 0.2% sodium alginate and 2% NaCl in 0.05 *M* Tris–HCl buffer (pH 7.5) was prepared. Enzyme activity was detected at room temperature. A reaction mixture containing 2.8 ml substrate solution and 0.2 ml of the above-prepared diluted enzyme solution was mixed directly in a 10 mm quartz cuvette. Data were recorded every 6 s for the first 3 min. Enzyme activity was monitored by measuring the increase in absorbance at 230 nm using an UV spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to cause an increase of one unit of optical density at 230 nm under the above conditions [1].

2.3. Preparation of unsaturated oligomannurate and oligoguluronate

Alginate (5 g) was dissolved in 1000 ml of 50 m*M* Tris–HCl buffer (pH 7.5). A solution of alginate lyase from *Pseudomonas* sp. (1.0 ml, 0.5 unit) was added to the alginate solution. The reaction was then carried out at 30°C for 10 h. The enzyme reaction was stopped by heating the solution in boiling water for 10 min. After the solution had been filtered, the pH of the supernatant was adjusted to separate mannuronic acids and guluronic acids. Both fractions were neutralized (pH 7.0) with NaOH, and 5 ml of the concentrated hydrolysates of polymannuronate (PM) was placed on a column (30×1.6 cm) of Q-Sepharose which had previously been equilibrated with 0.5 *M* NaAc and then eluted by the gradient buffer (NaAc 0.5–1.0 *M*) at a flow-rate of 1.5 ml/min. This chromatographic operation was carried out at room temperature, and the eluent was fractionated into 4.5-ml aliquots. Uronate in each fraction was measured by the absorbance at 230 nm. Fig. 1 shows an elution profile of the hydrolysates of PM. Peaks 1 and 2 were pooled and concentrated. These two fractions, sample 1 and sample 2, were precipitated by alcohol three times to desalt, and then lyophilized.

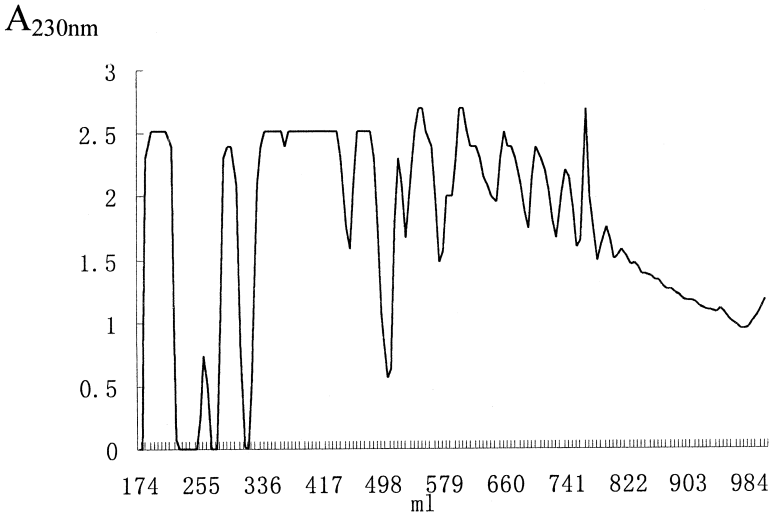

Fig. 1. Polymannuronate hydrolysate fractionation by anion-exchange chromatography on Q-Sepharose.

2.4. High-performance anion-exchange chromatography apparatus

The analytical system consisted of a Dionex DX-500 system (Sunnyvale, CA, USA) equipped with a CarboPac PA-1 guard precolumn (25×3 mm), a CarboPac PA-1 column (250×4 mm), a quaternary gradient pump, eluent degas (He) module, and a pulsed amperometric detector (with gold working electrode). The triple pulse sequence used for amperometric detection included the following potentials and durations: $E_1 = -0.15$ V (210 ms, integrating from 81 to 210 ms), $E_2 = +0.75$ V (180 ms), $E_3 = -0.35$ V (360 ms). To facilitate pulsed amperometric detection and minimize baseline drift, 100 m*M* NaOH was consistent in all processes. The alginate hydrolysate sample (1–50 μg/ml) was eluted at a flow-rate of 1.0 ml/min. The eluent buffers were obtained by adjusting the quaternary gradient pump with three kinds of buffers (data shown in Table 1).

2.5. Electrospray ionization mass spectrometry

Negative-ion electrospray mass spectra were obtained using a Micromass Quattro GC–MS–MS instrument, with ion-spray source (Analytic, USA). Data acquisition and manipulation was accomplished using the Micromass MassLynx software package, including MaxEnt data analysis software. Sample solutions (in MeOH–water, 1:1) were directly injected and the flow-rate was 0.16 ml/h. The scanning range was from 40 to 2000 u; the scanning speed was 100 u/min. Nitrogen was used to keep the pressure at 0.5–0.7 mPa.

3. Results and discussion

3.1. Isolation of oligoglycuronates

Alginate is a kind of anionic polysaccharide, and

Table 1
Eluent conditions for HPAEC[a]

Time (min)	A (%)	B (%)	C (%)
Init	80.00	10.00	10.00
0.00	80.00	10.00	10.00
1.00	80.00	10.00	10.00
2.00	80.00	10.00	10.00
14.00	0.00	10.00	90.00
22.00	0.00	10.00	90.00
22.10	0.00	100.00	0.00
27.90	0.00	100.00	0.00
28.00	80.00	10.00	10.00
30.00	80.00	10.00	10.00

[a] Eluent A, water; eluent B, 100 m*M* NaOH; eluent C, 1 *M* NaAc.

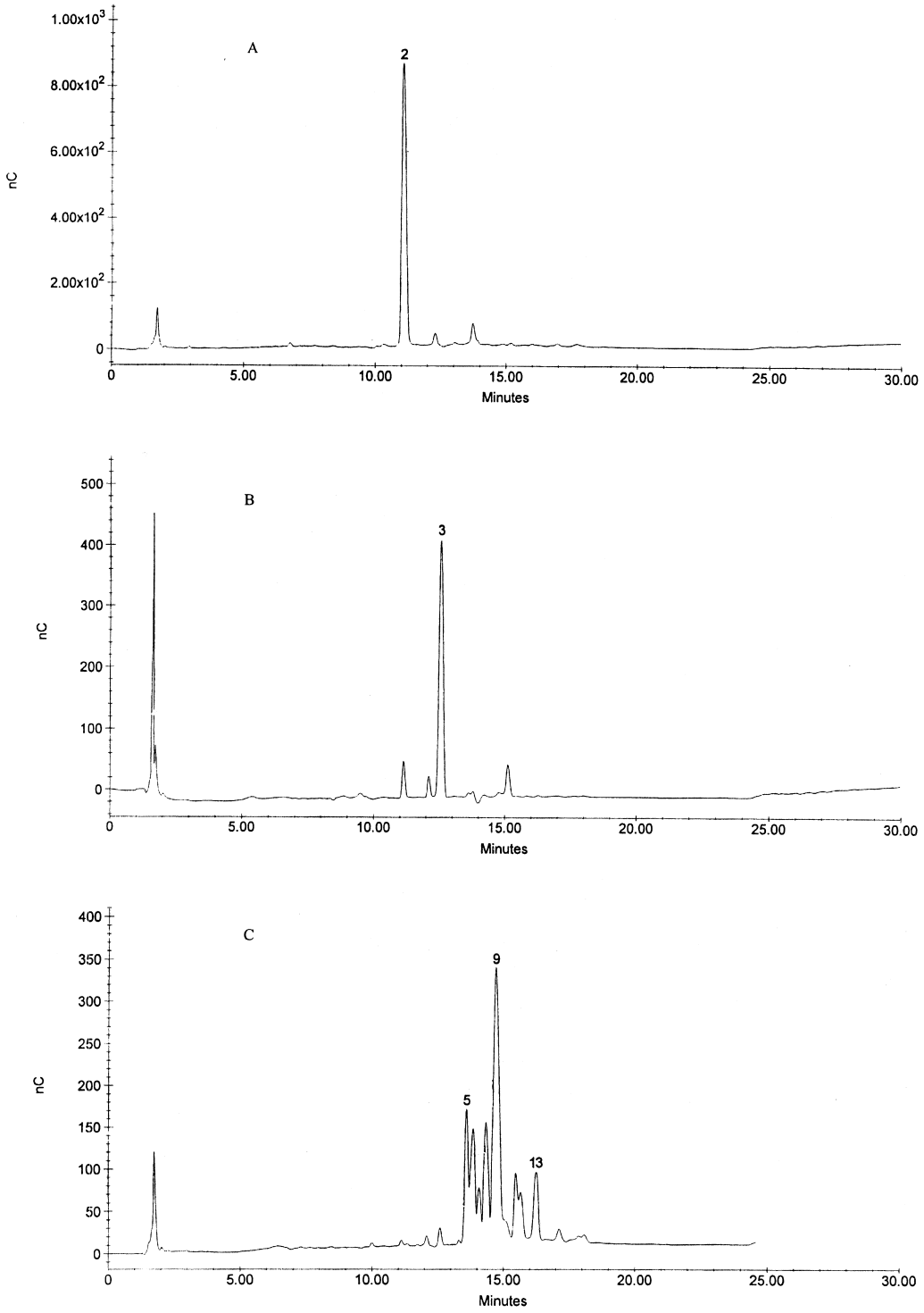

Fig. 2. Oligomannuronates and samples 1 and 2 by HPAEC. (A) Sample 1; (B) sample 2; (C) hydrolysates of polymannuronate.

alginate lyases can degrade alginate to produce many kinds of oligosaccharides of different molecule masses. The distribution of oligosaccharides was investigated by HPAEC. The profile shown in Fig. 5 indicates that the enzyme hydrolysate was a complicated mixture of many components. It is very difficult to obtain polysaccharides in single DP. The alginate-degrading enzymes cleave the glycosidic linkage of alginate by a β-elimination reaction and produce oligouronic acids with a 4,5-unsaturated uronic acid residue at the non-reducing end [16,17], which can easily be detected by UV spectrometry at 230 nm. In order to isolate purified components, we used gel filtration chromatography and anion-exchange chromatography. Although the enzyme hydrolysate profile on gel filtration chromatography (Sephacryl S-200) was a single peak (data not shown), its HPAEC figure gave many peaks. Therefore, anionic polysaccharides cannot be separated successfully by gel filtration chromatography on a Sephacryl S-200 column. Enzyme hydrolysates of polymannuronate on Q-Sepharose ion-exchange chromatography are shown in Fig. 1. This was a good way to produce pure oligoglycuroates. The first two peaks were collected. After concentration, the samples were precipitated by alcohol three times to desalt, and then lyophilized. Samples 1 and 2 showed a main peak on HPAEC; the other peaks were the mobile phases and neglectable peaks. Therefore, two single DP samples were obtained (Fig. 2A and B).

3.2. ESI-MS to determine the M_r of mannuronates

The molecular masses of two purified mannuronates were examined by ESI-MS. Producing multiple ion peaks is a property of ESI-MS. The major peaks

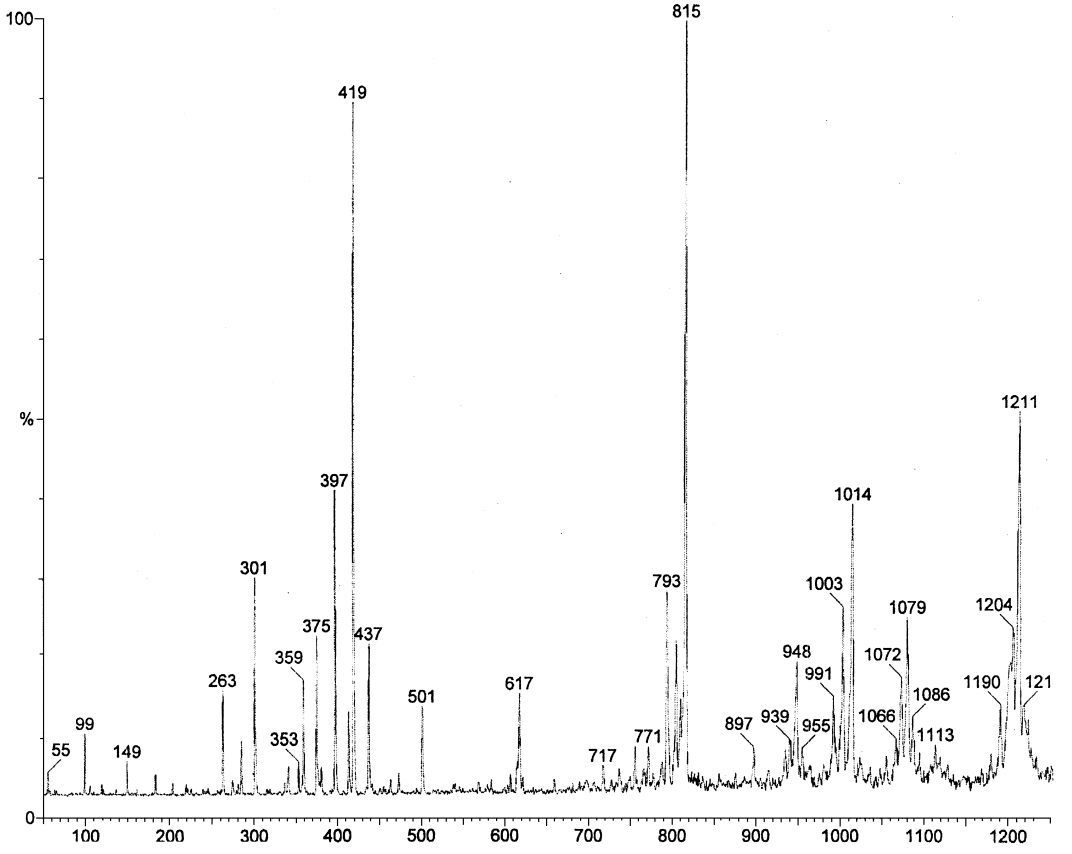

Fig. 3. ESI-MS of sample 1.

were the multiple ion peaks, which could be used to further interpret the molecular mass. The peak at m/z 397 corresponds to the molecular ion peak $[M+1]^+$ of the sodium salt of unsaturated dimannuronate. The peak at 419 is [M+Na], that at 815 is [2M+Na], and that at 1211 is [3M+Na]. Therefore, the M_r of sample 1 is 396 (Fig. 3). The M_r of sample 2 is 572, which means that the sample is a trimannuronate with two sodium ions (Fig. 4).

3.3. Analysis of oligomannuronic acids and oligoguluronic acids by high-performance anion-exchange chromatography

HPAEC is widely used in the analysis of polysaccharides because of its excellent sensitivity and resolution [10,11]. Neutral carbohydrates have been successfully determined by HPAEC with an alkaline mobile phase, but this did not work for anionic polysaccharides. Arland et al. [13] reported that the HPAEC–PAD technique is useful for the analysis of anionic polysaccharides. The retention time of oligosaccharides on an anion-exchange column depends on their pK_a value in the eluent. The lower the pK_a, the longer the retention time. This means that anionic polysaccharides have a longer retention time than neutral ones. All of the hydroxyl groups on residues contribute to the pK_a value. The hydroxyl (α- or β-) at the reducing terminal has the most acidic pK_a; the others are 2-OH>6-OH>3-OH>4-OH in neutral polysaccharides. Because of the different linkages between the residues, oligosaccharides have different numbers of hydroxyl groups at different positions. This causes oligosaccharides to have high selection on an anion column. Generally, the more residues it has, the longer the retention time [18–21]. It is necessary to select different eluent and eluting

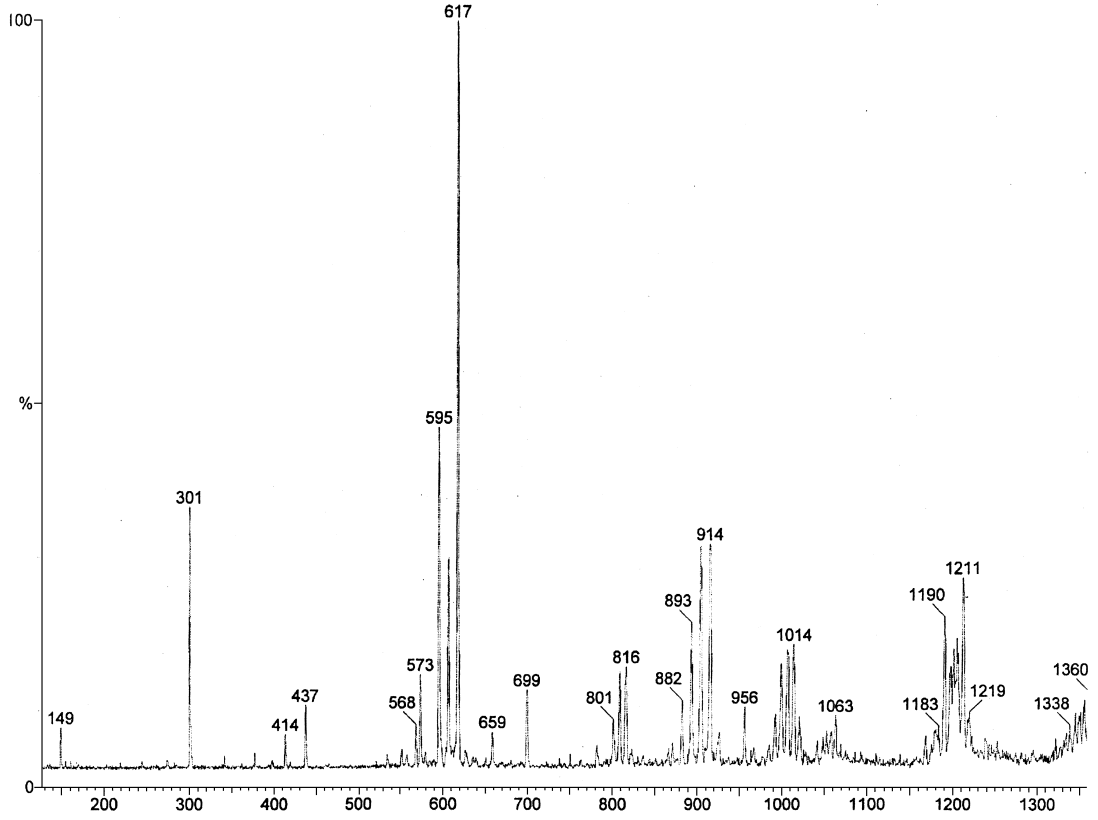

Fig. 4. ESI-MS of sample 2.

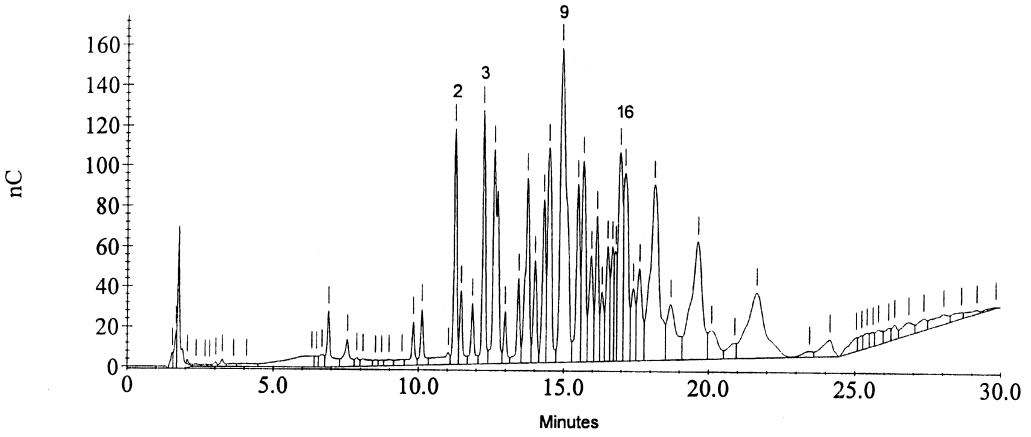

Fig. 5. HPAEC of oligoguluronate hydrolysate.

conditions in different oligosaccharide systems. Ac^- can reduce the retention time of oligosaccharides while increasing the concentration of NaAc. Acetate buffer gradations were utilized as mobile phase with a constant concentration of NaOH, which was necessary for the sensitivity of the detector. Using samples 1 and 2 as standard samples, we can determine the DP of other peaks (Fig. 5). The retention time and DP of fractions are shown in Table 2.

The results described indicate that HPAEC–PAD on CarboPac-PA-1 and anion-exchange column chromatography on Q-Sepharose is valuable for the analysis and isolation of alginate-derived oligosaccharides. ESI-MS is effective for the determination of the molecular mass of polysaccharides.

Table 2
Retention time and DP of different oligouronic fractions

Retention time (min)	DP of oligouronic acid
11.2	2
12.6	3
13.5	5
14.9	9
16.2	13
17.1	16

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