Note
Preparation of Two Series of Oligo-guluronic Acids from Sodium Alginate by Acid Hydrolysis and Enzymatic Degradation

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The objective of this study was to prepare two series of authentic oligo-guluronic acids from sodium alginate. Oligo-guluronic acids (DP = 1–9) were prepared from an acid hydrolysate of poly-guluronic acid by successive chromatographies of Bio-Gel P-6 and Q Sepharose Fast Flow. Oligo-guluronic acids having 4-deoxy-t-erythro-hex-4-enopyranosyluronic acid residues at the non-reducing end (DP = 2–7) were prepared from the enzymatic degradation products of the poly-guluronic acid in the same manner. Each of the isolated oligo-guluronic acids gave a single band on fluorophore-assisted carbohydrate electrophoresis. These results suggest that successive chromatographies used in this study are well suited for the preparation of alginate-derived oligouronic acids.

Key words: alginate; alginate lyase; oligo-guluronic acids; fluorophore-assisted carbohydrate electrophoresis

Alginites are hetero-polysaccharide composed of (1→4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues, and are widely used in the food, cosmetic, and pharmaceutical industries. As the enzymatic degradation of the alginate has the capability of expanding the potential use of the alginate, an alginate-degrading enzyme, alginate lyase, has recently been the focus of intense research interest. However, there are only a few studies on the substrate specificity of the alginate lyase, because of the difficulty of preparing various kinds of oligouronates to be used as oligomeric substrates of alginate lyase. Therefore, we now report a method for the preparation of two series of oligouronic acids, namely oligo-guluronic acids and oligo-guluronic acids having 4-deoxy-t-erythro-hex-4-enopyranosyluronic acid (J) residues at the non-reducing end (which are designated J-oligo-guluronic acids).

Sodium alginate (Duck Algin 350-G, the mannnuronic acid guluronic acid ratio = 0.2) was produced by Kibun Food Chemifa Co., Ltd. Poly-gulurionate (Poly-G) was prepared from the sodium alginate by the method of Haug et al., and circular dichroism analysis established that the Poly-G contained 79% 1-guluronic acid.

A homologous series of oligo-guluronic acid was isolated from an acid hydrolysate of the Poly-G. The Poly-G was hydrolyzed by a modification of the procedure of Hotchkiss and Hicks. One hundred ml of 1% poly-G (adjusted to pH 4.0 with 0.3 N HCl) was hydrolyzed at 121°C for 30 min in an autoclave. After cooling, the sugar solution was neutralized with dilute NaOH solution and concentrated to 15 ml. Figure 1 shows the fluorophore-assisted carbohydrate electrophoresis (FACE) of the hydrolysate. FACE was done by the method of Jackson on a 30–40% polyacrylamide gradient gel. The result shows that oligo-guluronic acids having a broad range of DPs (lane 4 in Fig. 1) were generated from the poly-G (lane 3 in Fig. 1) by the acid hydrolysis. In addition, the poly-G used in this study had heterogeneity in size, and such heterogeneity was in good agreement with the report of Haug.

Oligo-guluronic acids in the acid hydrolysate of poly-G were separated by gel filtration chromatography by the method of Muramatsu et al. with a slightly modification. After passing the hydrolysate through a membrane filter (0.45 μm, Millex-HV, Millipore), the concentrated hydrolysate was put on a column (10 × 100 cm) of Bio-Gel P-6 (extra fine, Bio-Rad) equilibrated with 50 mM phosphate buffer (pH 7.0) containing 0.02% NaN3 at a flow rate of 0.5 ml/min. This chromatographic operation was done at 55°C, and the eluate was fractionated into 10-ml portions. Total sugar in each fraction was measured by the phenol-sulfuric acid method, and the sugar composition in each fraction was

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Abbreviations: DP, degree of polymerization; FACE, fluorophore-assisted carbohydrate electrophoresis; ANTS, 8-amino-naphthalene-1,3,6-trisulfonic acid; J, 4-deoxy-t-erythro-hex-4-enopyranosyluronic acid; NMR, nuclear magnetic resonance; FAB-MS, fast atom bombardment-mass spectrometry.

Fig. 1. FACE of the Products Produced from Poly-G by Acid Hydrolysis and Enzymatic Degradation.

FACE was done by the method of Jackson on a 30–40% polyacrylamide gradient gel. About 20mg as total sugar was put on the gel, and was electrophoresed at 100 V for 1 h, followed by 400 V for 1.5 h. After electrophoresis, the gel was placed on a UV transilluminator and photographed through a yellow filter. Lane 1, maltopentaose (lower) and maltodecaose (upper); lane 2, ladder marker of glucose and maltotriose; lane 3, the poly-G prepared from sodium alginate; lane 4, an acid hydrolysate of poly-G; lane 5, enzymatic degradation products of poly-G with Flavobacterium alginate lyase.
examined by FACE. Figure 2 shows an elution profile of the acid hydrolysate of poly-G. A good resolution was achieved within mono- and oligo-guluronic acids up to DP 5. After repeating the procedures described above, electrophoretically identical fractions were combined and concentrated, and incompletely separated oligo-guluronic acid fractions (DP = 6–9) were further rechromatographed on the Bio-Gel P-6 column in the same manner. The individual oligo-guluronic acid fraction (DP = 1–9) thus obtained was desalted by anion-exchange chromatography by the following.

Each fraction was diluted with water until the conductivity of the solution became lower than 1.1 millimho. After dilution, the pH of the solution was adjusted to 8.2 with dilute ammonia water, and put on a column (3 x 19 cm) of Q Sepharose Fast Flow (Pharmacia) equilibrated with 0.1 M NH₄HCO₃ at a flow rate of 1 ml/min. The oligo-guluronic acid in the column was eluted by a linear gradient from 0.1 to 1.0 M NH₄HCO₃. The peak fractions were combined, concentrated, and lyophilized. The yields of oligo-guluronic acids from 10 g of the poly-G were as follows: G₁, 30 mg; G₂, 40 mg; G₃, 152 mg; G₄, 234 mg; G₅, 396 mg; G₆, 192 mg; G₇, 60 mg; G₈, 40 mg; G₉, 40 mg.

On the other hand, a homologous series of Δ-oligo-guluronic acid was produced by enzymatic degradation of poly-G. The poly-G (2 g) was dissolved in 100 ml of 10 mM phosphate buffer (pH 7.0). A solution of alginate-degrading enzyme from Flavobacterium multivolum (10 ml, containing 14 units of alginase lyase activity, Nagase Biochemicals Ltd.) was added to the poly-G solution. Then, the reaction was done at 37°C for 30 min. The enzyme reaction was stopped by heating the solution at 100°C for 10 min. Lane 5 in Fig. 1 shows that the enzyme decomposed the poly-G and produced oligouronic acids having DPs below 10. The sugar solution so obtained was diluted with 50 mM phosphate buffer (pH 7.0) containing 0.02% NaN₃ to give a concentration of 0.5% of uronic acid, because the viscosity of the solution was considerably higher than that of the acid hydrolysate. The diluted sugar solution was filtered through a membrane filter (0.45 μm). Then, Δ-oligo-guluronic acids in the sugar solution were separated on the Bio-Gel P-6 column as described above. Fifteen milliliters of the sugar solution were put on the column at a time. Each Δ-oligo-guluronic acid fraction (DP = 2–7) obtained above, contained oligo-guluronic acid having the same DP, which was produced from the non-reducing end of the poly-G by enzymatic digestion. In addition, Δ-oligo-guluronic acid fractions (DP > 3) were contaminated with appreciable amounts of neighboring oligomers besides the desired sugar. Therefore, each Δ-oligo-guluronic acid was further purified and desalted on Q Sepharose Fast Flow column by the same manner described above, except for the use of a longer column (3 x 70 cm). An elution profile of Δ-tetra-guluronic acid (ΔG₄) is shown in Fig. 3, as an example of such purification of Δ-oligo-guluronic acid. Penta-guluronic acid (ΔG₅) and Δ-penta-guluronic acid (ΔG₅) were removed by this chromatography. Similarly, the other individual Δ-oligo-guluronic acids were purified, and peak fractions of desired sugars were combined, concentrated, and lyophilized. The yields of Δ-oligo-guluronic acids from 2 g of the poly-G were as follows: ΔG₁, 7 mg; ΔG₂, 35 mg; ΔG₃, 100 mg; ΔG₄, 84 mg; ΔG₅, 33 mg; ΔG₆, 19 mg.

The FACEs of isolated oligouronic acids, namely oligo-guluronic acids (DP = 1–9) and Δ-oligo-guluronic acids (DP = 2–7), are shown in Fig. 4. Each of the isolated oligouronic acids gave a single band on FACE.

FAB-MS spectra were recorded with a JEOL HX110A mass spectrometer (JEOL, Tokyo) operating in the negative-ion mode with accelerating voltage of 10 kV. A portion (1 μl) of the
ammonium-oligo-saccharides in water was mixed with 1 μl of glycerol and thioglycerol (1:1, v/v) on the probe tip. Each of the oligo-guluronic acids gave the m/z value corresponding to the molecular ion peak [M – H]⁺ of ammonium salts on FAB-MS analysis, these were free from β-o-mannouronic acid in ¹H-NMR spectra.¹⁵,¹⁶

Gel chromatography using Bio-Gel columns has been developed as an effective technique for the fractionation of oligosaccharides.¹⁰ and Muramatsu et al.¹⁰¹ reported the separation of oligo-guluronic acids (DP = 1–7) and oligo-mannuronic acids (DP = 1–6) by chromatography on a Bio-Gel P-2 column. In the case of a Bio-Gel P-6 column, oligo-guluronic acids smaller than nona-uronic acid were separated. On the other hand, it is difficult to isolate the J-oligo-guluronic acids by using gel chromatographic technique alone, because the products of enzymatic degradation of poly-G contain oligo-guluronic acids. The oligo-guluronic acid and J-oligo-guluronic acid having the same DPs, however, were separated by anion-exchange chromatography on a Q Sepharose Fast Flow column using NH₄HCO₃ solution as a eluent. Moreover, there is a advantage in using the eluent system, as NH₄HCO₃ is effectively removed by lyophilization. In conclusion, successive chromatographies on Bio-Gel P-6 and Q Sepharose Fast Flow, used in this study, are well suited for the preparation of alginate-derived oligouronic acids.

References