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Isolation and characterization of alginate-derived oligosaccharides with root growth-promoting activities

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Abstract

Lytic digestion of poly(mannuronate), poly(guluronate), and alginate with an alginate lyase from *Alteromonas macleodii* was used to prepare mixtures of unsaturated oligosaccharides. Four oligosaccharides isolated from the alginate lyase-lysate by anion-exchange chromatography on Q-Sepharose were found to be the major components of the root growth-promoting lysate. The oligosaccharides were analyzed by NMR and SIMS and identified as di- and tri-saccaharides having *O*-(4-deoxy-L-*erythro*-hex-4-enopyranosyluronic acid)-1 → at the nonreducing terminus. The trisaccharides from the lysate were found to have root growth-promoting activity in a barley bioassay.

1. Introduction

Oligosaccharides of both fungal and plant origins, derived from β-glucan, xyloglucan, chitin, and pectin, have been reported to be potent signaling molecules that regulate growth, development and defense reactions in plants [1,2]. With respect to acidic oligosaccharides, a series of α-(1 → 4)-oligogalacturonic acids has been extensively studied and oligomers ranging in degree of polymerization (dp) from 2 to 20 have been shown to have a variety of biological activities [3–8]. By contrast, only very limited information is available on other uronic acids. Fett and Dunn [9] reported that bacterial alginate accumulated in water-soaked lesions on the leaves of plants infected with phytopathogenic bacteria. We also found that an alginate lyase-lysate (ALL), a lytic digest of alginate prepared with a lyase from *Alteromonas macleodii*, had growth-promoting effects on the elongation of several

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plants [10]. This information suggests that specific alginic acid oligosaccharides act as plant-signaling molecules similar to oligogalacturonic acids. For an assessment of the activities of the alginic acid oligosaccharides, it is important to work with pure samples and to use appropriate analytical methods for the identification of the oligosaccharides.

This report describes the isolation and structural characterization of unsaturated oligosaccharides generated during the endolytic depolymerization of alginate by lyase from *A. macleodii*, as well as the root growth-promoting activities of these compounds in a bioassay with barley seedlings.

2. Experimental

Materials.—Sodium alginate [average molecular weight of 9 600 ±300 and a ratio of guluronic acid to mannuronic acid (GulA/ManA) of 47/53] was purchased from Kimitsu Chemicals Ind. Co. (Tokyo, Japan). Poly(α-L-guluronate) (average dp 11) and poly(β-D-mannuronate) (average dp 10) were prepared from the corresponding homopolymeric block-regions of alginate by the method of Haug et al. [11]. Both 3-deoxy-D-*manno*-octulosonic acid (Kdo) and D-mannurono-3,6-lactone were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Clewat 32® (a complex of minor metals) was purchased from Nagase Sangyo Co. (Tokyo, Japan). Other chemicals and resins were obtained from commercial sources. Uronic acids in this report were used as the sodium salts unless stated otherwise.

Preparation of enzyme.—The method of Adachi et al. [10] was used to prepare an alginate lyase as follows. *Alteromonas macleodii* (FEARP-9218) was subcultured in a medium (40 mL in a 250-mL flask) that contained 0.5% sodium alginate, 0.5% peptone, 0.25% yeast extract, 0.4% NaCl, 0.037% $CaCl_2 \cdot 2H_2O$, 0.8% $MgSO_4 \cdot 7H_2O$, 0.07% KCl, 0.03% $NaNO_3$, 0.0025% NaH_2PO_4, 0.034% $NaHCO_3$, 0.002% Clewat 32®, and 0.002% ethylenediaminetetraacetic acid disodium dihydrate (EDTA · 2Na · $2H_2O$), for 17 h at 25°C with rotary shaking (180 rpm). The subculture was then used to inoculate the growth medium (1 L in a 2-L flask) which consisted of 1.0% sodium alginate, 1.0% peptone, 0.1% yeast extract, 1.4% NaCl, 0.15% $CaCl_2 \cdot 2H_2O$, 1.2% $MgSO_4 \cdot 7H_2O$, 0.2% K_2HPO_4, 0.007% Clewat 32®, and 0.007% EDTA · 2Na · $2H_2O$. The culture was incubated at 25°C for 17 h with rotary shaking (180 rpm). The resultant culture broth had an alginate lyase activity of 0.34 U/mL and was used for endolytic depolymerization of alginate, poly(guluronate), and poly(mannuronate).

Assay of enzymic acitivity.—Alginate lyase activity was determined by measuring unsaturated termini by the thiobarbituric acid method with Kdo as the standard, as described by Brown and Preston [12]. One unit of activity was defined as the amount that generated 1 μmol of unsaturated termini in 1 min at 30°C.

Preparation of lyase-lysates of alginate, poly(α-L-guluronate) and poly(β-D-mannuronate).—Three kinds of lyase-lysate were prepared as follows by a modified method of Adachi et al. [10]. An aqueous solution (367 mL) of sodium alginate (50.0 g) was treated with alginate lyase (133 mL, 45.3 U) at 35°C and pH 7.0 for

20 h, and the mixture was then heated at 95°C for 15 min. After filtration, the filtrate was condensed to 70% (w/w) of solid concentration to give an alginate lyase-lysate. Similarly, poly(α-L-guluronate) lyase-lysate and poly(β-D-mannuronate) lyase-lysate were prepared from poly(α-L-guluronate) and poly(β-D-mannuronate), respectively.

Isolation of lyase products by anion-exchange column chromatography.—Isolation of lyase products was performed by chromatographic fractionation on a Bio Pilot system (Pharmacia, Uppsala, Sweden) equipped with a Q-Sepharose column. An aqueous solution [50 mL, 4% (w/w)] of each lysate was loaded onto the column (100 mm × 60 mm i.d.) after it had been equilibrated with deionized water; the column was then eluted with a linear gradient of 0 to 300 mM NaCl at a flow rate of 25 mL/min, and 25-mL fractions were collected. Measurement of the absorbance at 230 nm of each fraction generated the elution profiles shown in Figs. 2a–2c. The fractions under each peak shown in the profiles were collected and concentrated *in vacuo* to ca. 10 mL. After desalting of the concentrates with an electrodialyzer (Micro Acilyzer, Asahi Chemical Co., Tokyo, Japan), the residues were analyzed by analytical HPLC. When further purification was necessary, rechromatography was carried out under the same conditions and purified lyase products were obtained after freeze-drying.

Analyses of carbohydrate content.—Total uronic acid content was measured by the phenol–H_2SO_4 method [13] with D-mannurono-3,6-lactone as the standard. The unsaturated sugar content was determined by the thiobarbituric acid method [14] with Kdo as the standard. The reducing sugar content was measured by the Somogyi–Nelson method [15] with D-mannurono-3,6-lactone as the standard.

Analysis by HPLC.—HPLC was performed on a Dionex (Sunnyvale, CA, USA) BioLC Model 4500i system with a Model PAD pulsed amperometric detector consisting of an amperometric flow-through cell with a gold working electrode and a Model LCM-2 injector. The Dionex eluant-degas module was used to sparge and pressurize the eluants with He. For elution, eluant *A* was 200 mM NaOH containing 100 mM NaOAc and eluant *B* was 200 mM NaOH containing 1 M NaOAc. These solutions were prepared by suitable dilution of 10 M NaOH with distilled water filtered through a 0.45-μm membrane filter. The gradient used for analysis was eluant *A* with a linear increase in eluant *B* (to 100% eluent *B* in 35 min). Lyase-lysates were separated on a column (250 × 4.0 mm i.d.) of Dionex CarboPac-PA1 pellicular anion-exchange resin (10 μm) with an CarboPac-PA1 GUARD column (25 × 3.0 mm i.d.) at flow rate of 1 mL per min at 25°C. Chromatographic data were collected and plotted with a CR3A chromatointegrator (Shimadzu, Kyoto, Japan). For additional cochromatographic analysis of the purified oligosaccharides, HPLC was also carried out with the Waters Associates (Milford, MA, USA) instrument consisting of 600E system controller, 486 tunable absorbance detector and 740 data module under the following conditions: column, TSKgel DEAE-5PW (75 × 2.5 mm i.d., Tosoh Corp., Tokyo, Japan); mobile phase, a linear gradient from 100% *A*/0% *B* (v/v) to 0% *A*/100% *B* (v/v) over 60 min; solvent *A* was water and solvent *B* was 1 M NaCl; flow rate, 1.0 mL/min; detector, UV monitor at 230 nm and temperature 25°C.

Spectroscopic analyses.—^{1}H NMR, ^{13}C NMR, and heteronuclear $^{1}H-^{13}C$ chemical shift correlation spectroscopy ($^{1}H-^{13}C$ COSY) spectra were recorded in D_2O on a Jeol GX-400 spectrometer. The chemical shifts were referenced indirectly to Me_4Si by setting ^{1}H from HOD at 4.8 ppm and ^{13}C from 1,4-dioxane at 67.4 ppm. Secondary ion mass spectroscopy (SIMS) was performed with a Hitachi M-80A spectrometer.

Bioassay.—Barley seeds (*Hordeum vulgare L.* cv. Minori) were surface-sterilized with 0.05% (v/v) NaOCl and allowed to germinate on a tray in darkness. After 16 h, three germinated seeds were placed on an agar bed in a test tube that contained a solution of the sample to be tested at 600 ppm and 1/1000-fold diluted Hyponex® solution (Hyponex Co., Tokyo, Japan). After 12 days in culture, the longest portion of each radicle was measured. Growth rates were calculated as percentages by comparing average values from five replicate experiments to control values (alginate and deionized water instead of solutions of samples).

3. Results and discussion

Alginate lyase-lysate (ALL).—Commercial sodium alginate was depolymerized by treatment with a culture broth from *A. macleodii* to give an ALL. The bioassay showed that ALL caused 1.7 times the control root growth in barley. Analysis of the carbohydrate content revealed that ALL consisted of uronic acids (92.8%) that contained reducing sugar (23.5%) and unsaturated sugar (20.0%). This result suggested that the major components of ALL would be unsaturated oligoglycouronates and so we attempted the isolation and structural characterization of the biologically active compounds.

Isolation of oligoglycuronates.—The distribution of oligosaccharides in ALL was investigated by analytical HPLC with an anion-exchange column (CarboPac-PA1) and a pulsed amperometric detector (PAD). The profile shown in Fig. 1a indicated that ALL was a complicated mixture of many components. In order to isolate purified components, direct fractionation of the major products was attempted on a preparative anion-exchange column of Q-Sepharose. However, this attempt did not succeed because the first chromatographic fractionation, shown in Fig. 2a, resulted in such imperfect separation that the major peak area ratio of each of the combined fractions (54–65, 66–75, and 76–86 in Fig. 2a) was less than 60% of the fraction and the purified components could not be isolated by further rechromatography.

Recently, Lieker et al. [16] reported a finding that is useful for anion-exchange chromatographic analysis of oligoglycuronates. They found that the capacity factors depended on chain length and the nature of carbohydrate constituents and that the capacity factors of a homologous series of oligoglycuronates were linearly correlated with chain length. Furthermore, we assumed that ALL would contain a considerable number of homo-oligomers because alginate consists of homopolymeric block-regions of poly(guluronate) and poly(mannuronate), together with heteropolymeric blocks with an alternating sequence of guluronic acid and man-

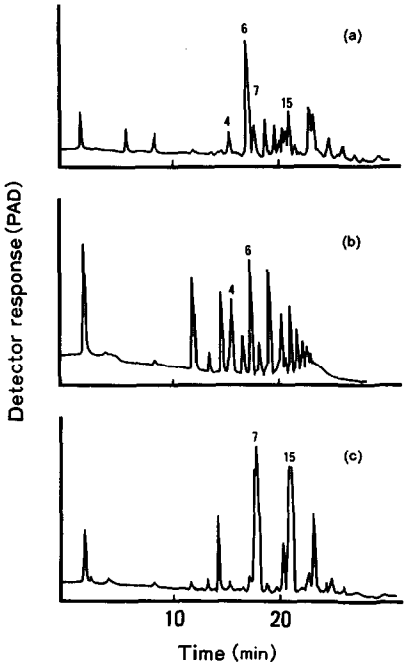

Fig. 1. HPLC profiles of: (a) alginate lyase-lysate, (b) poly(guluronate) lyase-lysate, and (c) poly(mannuronate) lyase-lysate. Numbers indicate the designated component numbers of purified alginate-derived oligosaccharides.

nuronic acid [17]. These considerations suggested that some peak components of ALL in Fig. 1a might be identified by virtue of chromatographic properties identical to those of homo-oligomers that might be expected to be more easily purified from two kinds of lyase-lysate, namely, those of the homopolymeric poly(guluronate) and poly(mannuronate), derived from alginate.

Therefore, poly(guluronate) and poly(mannuronate), prepared from alginate in advance by the method of Haug et al. [11], were treated with the culture broth of *A. macleodii* in the same way as alginate to generate a poly(guluronate) lyase-lysate (GLL) and a poly(mannuronate) lyase-lysate (MLL), respectively. As shown by the analytical HPLC chromatograms (Figs. 1a–1c) and by the preparative chromatograms (Figs. 2a–2c), peak separation in the case of both GLL and MLL was better than that in the case of ALL. In fact, when the fractionations were carried out by preparative chromatography, as shown in Figs. 2b and 2c, the major peak area ratio of the fractions (40–50, 51–57, and 58–65 of GLL; and 50–61, 62–73, and 74–84 of MLL in Figs. 2b and 2c) constituted 52–83% of the fraction. Components with the largest peak areas in these six sets of pooled fractions could be purified by further rechromatography. Each purified compound gave a single peak on HPLC with different column systems (CarboPac-PA1 and TSKgel DEAE-5PW), and cochromatographic analysis on the two different columns using the

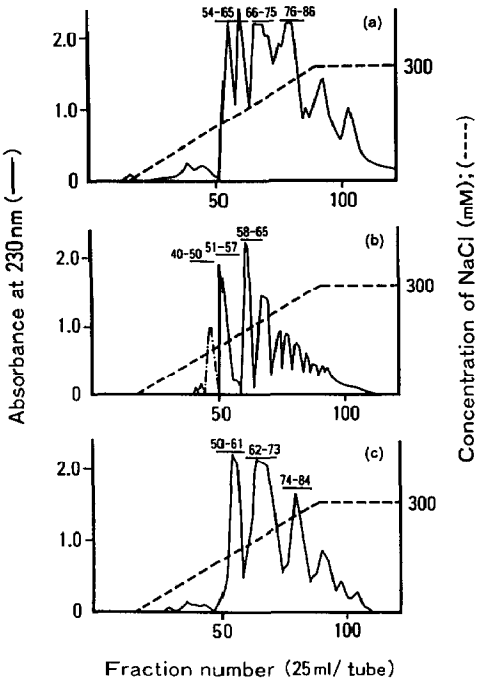

Fig. 2. Fractionation by anion-exchange chromatography on Q-Sepharose of: (*a*) alginate lyase-lysate, (*b*) poly(guluronate) lyase-lysate, and (*c*) poly(mannuronate) lyase-lysate. Column fractions indicated by numbers were monitored by measuring the absorbance of unsaturated oligomers at 230 nm, except in the case of fractions 40–50, shown by an alternating dotted and dashed line (·–·–·) in Fig. 2b, in which total uronic acids were monitored at 480 nm.

purified compounds showed that ALL contained compounds **4**, **6**, **7**, and **15** which corresponded to peak numbers 4, 6, 7, and 15 in Figs. 1a–1c, respectively. Table 1 shows HPLC data of the oligosaccharides in ALL, GLL, MLL, and their fractions separated by Q-Sepharose chromatography.

Structural analysis.—SIMS and high-resolution NMR techniques were employed to determine the structures of the purified oligomers. The general approach involved an investigation of molecular ions in SIMS spectra and assignment of peaks in the ^{13}C and ^{1}H NMR spectra, from which the chain lengths, relative stereochemistry, and anomeric configuration of the constituent glycosyl residues were identified. Table 2 shows the spectral data together with the assignments of the nonreducing, intermediate or reducing glycosyl residues. These assignments were made from ^{1}H–^{13}C COSY experiments and from comparisons to published data related to monomers [18,19] and oligomers [12,19–22] containing L-guluronate, D-mannuronate, and 4,5-unsaturated uronate residues. The ^{1}H–^{13}C COSY spectrum of **15** is shown in Fig. 3 as a typical example.

The SIMS spectra of purified **4**, **6**, **7**, and **15** provided corroborating evidence related to chain lengths: the values for m/z of 397 and 595 in Table 2 correspond

Table 1
HPLC data for the oligosaccharides (**4**, **6**, **7**, and **15**) in alginate lyase-lysate (ALL), poly(guluronate) lyase-lysate (GLL), poly(mannuronate) lyase-lysate (MLL) and their fractions separated by Q-Sepharose chromatography

Column (detector) of HPLC	Oligosaccharide	Retention time (min)	Peak area ratio (%) [a]						
			ALL	GLL			MLL		
				Intact	Fr. 51–57	Fr. 58–65	Intact	Fr. 62–73	Fr. 74–84
CarboPac	4	15.19	3.6	6.3	52.4				
PAI	6	17.01	12.4	11.2		66.8			
(PAD)	7	17.55	6.6				13.5	67.3	
	15	20.78	9.3				29.9		52.4
TSKgel	4	19.40	7.7	14.6	64.4				
DEAE-SPW	6	22.97	12.7	24.5		70.7			
(UV)	7	21.17	10.1				24.4	75.5	
	15	23.88	10.3				40.9		62.1

[a] Values indicate percentages relative to the total area after HPLC.

to the molecular ion peaks $[M + H]^+$ of sodium salts of unsaturated diglycuronate and triglycuronate, respectively (Table 2).

In the low-field region of the NMR spectra, **4**, **6**, **7**, and **15** each showed three characteristic resonances of a ^1H signal at 5.77–5.89 ppm (d, J 3.10–4.36 Hz) and two ^{13}C signals at 108.6–110.1 and 144.0–146.1 ppm. These three low-field resonances are indicative of nonreducing 4,5-unsaturated glycuronate residues which are reported to have the H-4 [12,22], C-4 [22], and C-5 Ref. [22] signals in similar regions, and unambiguous assignments of these signals were made from ^1H–^{13}C COSY experiments. Anomeric C-1 and H-1 resonances of nonreducing 4,5-unsaturated residues were assigned to resonances at 100.8–101.5 and 5.13–5.28 ppm, respectively. The spectral data assigned to the nonreducing termini were very similar (Table 2). Kiss [23] reported in his review that β-eliminative degradation of both D-glucopyranuronate and L-idopyranuronate gave 4,5-unsaturated termini with the same L-*threo* configuration, and the corresponding L-*erythro* terminus was obtained from alginate. These data support the conclusion that products of digestion by *A. macleodii* lyase, namely **4**, **5**, **7**, and **15**, from both poly(L-guluronate) and poly(D-mannuronate), had the same nonreducing terminus of *O*-(4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid)-1 → (Δ-). The low coupling constants (J ~ 0 Hz) of the H-1 signals support the hypothesis that the termini had α-L-linkages. The *erythro* configuration was inferred from the fact that the coupling constants between H-2 and H-3 were 4.0–4.9 Hz and differed from the large value for the corresponding α-L-*threo* residue reported by Iwahara [24].

The structures of the intermediate and reducing glycosyl residues were anticipated from the starting materials and the spectral data provided additional evidence. Based on the data reported by Grasdalen et al. [20], the intermediate residues of **6** and **15** were deduced to be → 4)-*O*-(α-L-gulopyranosyluronic acid)-(1 → (-GulA-) and → 4)-*O*-(β-D-mannopyranosyluronic acid)-(1 → (-ManA-), respectively. The anomeric conformations of reducing termini were determined from

Table 2
Molecular ions in SIMS, chemical shifts in the ^{13}C and ^{1}H NMR spectra, and assignments of glycosyl residues of purified alginate-derived oligosaccharides

Product [a]	Mol. ion (m/z)	Chemical shift, δ, ppm (coupling constant, Hz)									Glycosyl residue [c]
		^{13}C NMR						^{1}H NMR			
		C-1	C-2	C-3	C-4	C-5	C-6	H-1 ($J_{1,2}$)	H-3 ($J_{2,3}$) [b]	H-4 ($J_{3,4}$)	
4	397	101.2	67.6	63.1	110.1	144.0	168.5	5.28 (~ 0)	4.33 (4.9)	5.91 (4.4)	Δ-
		94.1	69.6	70.6	80.4	73.6	n.d.	4.94 (8.4)			-GulA
6	595	101.5	68.0	63.6	108.9	145.7	170.2	5.21 (~ 0)	4.37 (4.6)	5.89 (4.1)	Δ-
		102.0	65.9	69.9	80.8	68.0	176.5	5.03 (4.2)			-GulA-
		94.4	70.2	71.1	81.4	74.5	176.2	4.91 (8.6)			-GulA-
7	397	100.8	67.8	64.4	108.6	146.1	170.2	5.20 (~ 0)	4.46 (4.1)	5.82 (3.9)	Δ-
		94.2	70.1	71.2	79.7	74.1	177.1	5.27 (3.0)			-ManA
		97.7	72.1	72.6	79.2	77.1	176.6	4.84 (~ 0)			-ManA′
15	595	101.1	67.7	64.7	108.7	146.1	170.2	5.13 (~ 0)	4.48 (4.0)	5.77 (3.1)	Δ-
		100.8	71.1	72.4	79.0	76.8	176.3	4.71 (~ 0)			-ManA-
		94.6	70.0	71.5	79.4	73.7	177.0	5.24 (2.5)			-ManA
		94.7	71.7	72.6	79.1	77.2	176.5	4.94 (~ 0)			-ManA′

[a] Products are identified by numbers that correspond to the peak numbers in Table 1.

[b] Observed as triplets.

[c] Abbreviations: Δ, O-(4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid)-1 →; -GulA-, → 4)-O-(α-L-gulopyranosyluronic acid)-(1 →; -GulA, → 4)-O-β-L-gulopyranuronic acid; -ManA-, → 4),-O-(β-D-mannopyranuronic acid)-(1 →;-ManA → 4)-O-α-D-mannopyranuronic acid; and -ManA′, → 4)-O-β-D-mannuronic acid.

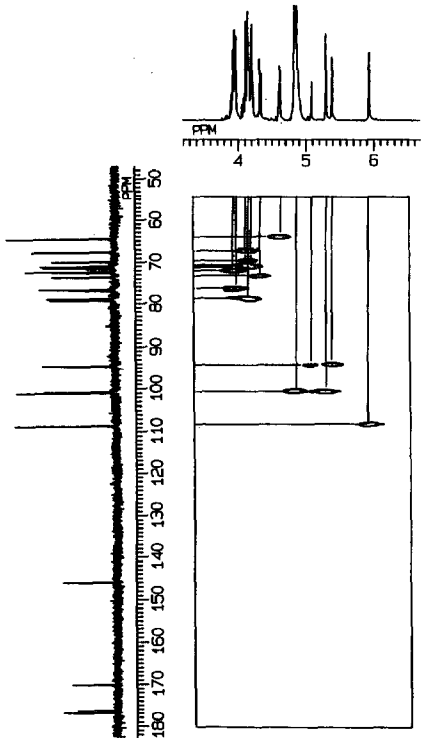

Fig. 3. $^{1}H-^{13}C$ COSY spectrum of **15**, an anomeric mixture of *O*-(4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid)-(1 → 4)-*O*-(β-D-mannopyranuronic acid)-(1 → 4)-*O*-α-D-mannopyranuronic acid (Δ-ManA-ManA) and the β anomer (Δ-ManA-ManA').

their H-1 signals in comparison with reported data for guluronate [17,18] and mannuronate [17]. As shown in Table 2, poly(guluronate)-derived oligomers **4** and **6** each had a large $J_{1,2}$ value (8.35–8.60 Hz), which indicated that the reducing residue existed as a → 4)-*O*-β-L-gulopyranuronic acid (-GulA) unit with a *trans*-diaxial arrangement of H-1 and H-2. In contrast, the poly(mannuronate)-derived unsaturated oligomers **7** and **15** each gave two kinds of H-1 signal, δ 5.24–5.27 ($J_{1,2}$ 3.01–2.49 Hz) and 4.94 ppm ($J_{1,2}$ ~0 Hz), which indicated → 4)-*O*-α-D-mannopyranuronic acid (-ManA) and the β anomer (-ManA') residues, respectively, because an axially oriented H-1 in the β anomer resonates at higher field than the equatorial H-1 of the α anomer [17]. The ratio of α to β anomer was estimated to be ~4:1 by reference to the corresponding anomeric proton signals.

The accumulated evidence described above suggests the structures for the alginate-derived oligosaccharides: **4**, Δ-GulA; **6**, Δ-GulA-GulA; **7**, a mixture of Δ-ManA and Δ-ManA'; and **15**, a mixture of Δ-ManA-ManA and Δ-ManA-ManA'. Furthermore, Table 1 shows that the predominant components of ALL were the triglycuronates designated as **6** and **15**.

Bioassay.—In order to investigate the biological activity of the predominant components in ALL, the triglycuronates designated **6** and **15**, on promotion of root growth in barley were examined under the conditions described in the Experimental section. The average root lengths for groups of seedlings treated with **6**, **15**, ALL, alginate, and deionized water were 69 ± 10, 68 ± 14, 76 ± 19, 56 ± 8, and 45 ± 6 mm, respectively. The results show that the addition of compounds **6** and **15** significantly ($p < 0.05$) increased the root growth rates to 153 ± 22 and $151 \pm 31\%$, respectively, of the both control rates (alginate, $128 \pm 18\%$ and deionized water instead of test sample solutions, $100 \pm 13\%$). Although these values were a little low in comparison with the value obtained with ALL ($169 \pm 42\%$), it was confirmed that the unsaturated triglycuronates, namely **6** and **15**, had root growth-promoting activities in barley.

Our results indicate that trisaccharides derived from alginate have root growth-promoting activity in barley. This is the first report, to our knowledge, that alginate-derived oligosaccharides have biological activity in plants. It is tempting to speculate that the oligosaccharides may also act as plant signaling molecules similar to oligogalacturonic acids. For future investigations of the functions of these compounds and for assessment of structure activity relationships, the results described herein indicate that HPLC on Carbopac-PA1 and anion-exchange column chromatography on Q-Sepharose will be valuable for the analysis and isolation of alginate-derived oligosaccharides.

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