J Plant Growth Regul (2002) 21:209–215
DOI: 10.1007/s003440010060

J P G R
Journal of Plant Growth Regulation

Preparation and Isolation of Oligogalacturonic Acids and Their Biological Effects in Cockscomb (*Celosia argentea* L.) Seedlings

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Abstract

A mixture of oligogalacturonic acids, the partial degradation substances of polygalacturonic acid, promoted shoot growth in cockscomb (*Celosia argentea* L.) seedlings, which generally had a high sensitivity for growth-promoting substances. The effect of the mixture of oligogalacturonic acids on shoot growth of cockscomb was higher than that of the polygalacturonic acid at concentrations above 30 ppm. These oligomers were loaded onto an anion exchange column, DEAE Sephadex A-25, and separated into individual oligomer sizes using the NH_4HCO_3 eluent system. This separation method has the advantage of using NH_4HCO_3 as the eluent solution; NH_4HCO_3 in the sample solution is effectively removed by lyophilization. Each of the isolated oligogalacturonic acids gave a single band on a fluorophore-assisted carbohydrate electrophoresis (FACE), and they showed the *m/z* value which corresponded to their molecular ion peaks $[M\text{-}H]^-$ on a fast atom bombardment mass spectrometry (FAB-MS) analysis. These results showed that the successive chromatography method used in this study is well suited for the preparation of oligogalacturonic acid for the plant growth test. Furthermore, we showed that the effective degree of polymerization (DP) of oligogalacturonic acid was around 8 on shoot growth of cockscomb seedlings, and the effects of both smaller and larger oligogalacturonic acids were slightly lower than that of octa-galacturonide. Octa-galacturonide promoted shoot growth of cockscomb at concentrations above 10 μM, and showed a 66% promotion at the most effective concentration of 300 μM. Root growth was slightly inhibited at concentrations above 300 μM. These results suggest that DP around 8 of oligogalacturonic acids has the function to control shoot growth in cockscomb as a growth-promoting substance.

Key words: Anion exchange chromatography; *Celosia argentea* L.; Cockscomb; Degree of polymerization; Growth-promoting substance; Oligogalacturonic acid

Received: 6 July 2001; accepted: 29 May 2002; Online publication: 20 December 2002
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Introduction

Certain oligosaccharides that decomposed from polysaccharides in both plant and fungal origins have shown a variety of biological activities (Fry and others 1993; Dumville and Fry 2000). These oligosaccharides control defense responses, plant development, and fruit ripening (Grierson and Tucker 1983; Davis and others 1986; Hoson and Masuda 1991; Ridley and others 2001). There are also reports that oligosaccharides promote general plant growth in several plants (McDougall and Fry 1990; Yonemoto and others 1993; Natsume and others 1994; Yamaguchi and others 1996; Suzuki and others 2002). McDougall and Fry (1990) reported that xyloglucan oligosaccharides, produced by the action of fungal cellulase, promoted the elongation of the etiolated pea (*Pisum sativum* L.) stem segments in a straight-growth bioassay designed for the determination of auxins. These authors purified four growth-promoting oligosaccharides which shared a common glucose$_4$ · xylose$_3$ (XG7) core, and showed that the substituted oligosaccharides XG8 (glucose$_4$ · xylose$_3$ · galactose) and XG9n (glucose$_4$ · xylose$_3$ · galactose$_2$) were more effective than XG7 itself and XG9 (glucose$_4$ · xylose$_3$ · galactose · fucose). In the study of alginate, Yonemoto and others (1993) reported that an alginate lyase-lysate (ALL) had growth-promoting effects on the elongation of rice (*Oriza sativa*) and komatuna (*Bassica rapa* var. *pervidis*) seedlings. Furthermore, the growth-promoting trisaccharides having O-(4-deoxy-L-*erythro*-hex-4-enopyranosyluronic acid)-1 → at the nonreducing terminus were isolated and identified from ALL (Natsume and others 1994). In polygalacturonic acid, it has been reported that the mixture of oligogalacturonic acids, the decomposition products of polygalacturonic acid by pectinase, promoted the growth of lettuce seedlings (Yamaguchi and others 1996).

In the previous study, we attempted to produce plant growth-promoting substances from tomato juice waste, peel, seed, and jelly matter, which was discarded from the juice factory (Suzuki and others 2002). After comparing various methods of extraction from discarded tomato waste, the acid extract from tomato juice waste significantly promoted the growth of some plant seedlings. Several separations and refinements of the acid extract showed that the components of the most effective fraction were a mixture of oligogalacturonic acids, 6–10° of polymerization (DP). However, the most effective DP of galacturonic acid for the promotion of plant growth has yet to be determined.

In this report, we prepared individual lengths of oligogalacturonic acid using a simpler method than before, and investigated what DP of galacturonic acid is the most effective on plant growth using the sensitive bioassay, the cockscomb (*Celosia argentea* L.) seedling test, for plant growth substances.

Methods

Chemicals

Polygalacturonic acid was purchased from ICN Biomedicals (CA, USA). DEAE Sephadex A-25 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS) and all the other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of Oligogalacturonic Acids

To test the effect of the mixture of oligogalacturonic acids on cockscomb growth, polygalacturonic acid (100 ml of a 1% solution) was autoclaved at 121°C for 40 min, and the solution was lyophilized.

For separation of the oligogalacturonic acids, polygalacturonic acid was hydrolyzed according to a modification of the procedure reported by Hotchkiss and Hicks (1990). Polygalacturonic acid (300 ml of a 1% solution) was adjusted to pH 4.4 with 1 N NaOH and autoclaved at 121°C for 40 min. Following hydrolysis, the solution was concentrated to a 100 ml solution, adjusted to pH 8.3 with NH_4, and filtered using an ultrafiltration membrane of *M*r 10^4 (Novacell 150, Filtron, MA, USA).

Separation and Isolation of Individual DP of Oligogalacturonic Acids

A column (ø2.6 × 38 cm) of DEAE Sephadex A-25 was equilibrated with starting solution (10 mM NH_4HCO_3, pH 8.2). The oligogalacturonic acids (100 ml) were slowly loaded onto the column at a flow rate of 100 ml h^{-1}. After washing the column with 800 ml of the solution, the oligogalacturonic acids were eluted with 3 L nonlinear gradient from 10 mM to 0.5 M NH_4HCO_3 (from 10 mM to 0.3 M in 1.2 L, and from 0.3 M to 0.5 M in 1.8 L). Each 20-ml fraction was collected by a fraction collector at the flow rate of 100 ml h^{-1}. The oligogalacturonic acid content was monitored by the Orcinol method (Miller and others 1951). The peak fractions were combined, concentrated, and lyophilized.

Fluorophore Assisted Carbohydrate Electrophpresis (FACE)

FACE was performed following the method of Jackson on a 40% acrylamide gel (Jackson 1990). ANTS labeled sugar (10 μg) was put on the gel and was electrophoresed at 100 V for 1 h, followed by 400 V for 1.5 h. After the electrophoresis, the gel was placed on a UV transilluminator and photographed through a yellow filter.

Fast Atom Bombardment Mass Spectrometry (FAB-MS) Analysis

Each oligogalacturonic acid sample (2–4 μg) was dissolved with 1 μl of water. The solution was mixed with 1 μl of glycerol and thioglycerol (1:1) on the probe tip and analyzed by FAB-MS (HX1010A, JEOL, Japan) in the negative mode.

Bioassay

The cockscomb test was used to compare the activities of the oligogalacturonic acids. The test has proven useful as a bioassay because the seedlings have a high sensitivity for growth-promoting substances (Yokotani-Tomita and others 1998). Eight cockscomb seeds were placed on a filter paper moistened with 0.5 ml of the test solution in a 33 mm Petri dish. The Petri dishes were kept in the dark at 25°C for 4 days, and then the shoot and root lengths were measured.

In the oat coleoptile elongation test designed for the determination of auxin activity (Wightman and Setterfield 1968), the oat (*Avena sativa* L.) seeds were sterilized in 1% sodium hypochlorite for 30 min, rinsed with running water for 1 h, and incubated in darkness at 25°C. After incubation for 5 days, uniform oat seedlings were selected under weak red light conditions. The apical 3 mm were discarded and the following 4 mm sections were collected using razors, and floated on distilled water. The coleoptile sections were transferred to 33 mm Petri dishes which contained 500 μl of the test solution. After 10 h incubation, the coleoptile section lengths were measured.

In the rice microdrop test designed for determining gibberellin activity (Krishnamoorthy 1975), dwarf cultivar of rice (*Oryza sativa* L. 'Tan-ginbozu') seeds were sterilized in 1% sodium hypochlorite for 30 min, soaked in running water, and incubated in darkness at 25°C. After incubation for 2 days, the seedlings were transferred to 1% agar medium and incubated for 2 days under white light (20 μmol m^{-2} s^{-1}). Uniform seedlings with the second leaf emerged from the first leaf were selected, and 1 μl of the test solution was applied to the second leaf surface. After 3 days, the lengths of the second leaf sheath were measured.

Results and Discussion

Figure 1 shows the effects of the polygalacturonic acid and the mixture of oligogalacturonic acids on shoot and root growth of cockscomb. The promoting effect of the mixture of oligogalacturonic acids on shoot growth was higher than that of polygalacturonic acid at concentrations above 30 ppm, however, the mixture of oligogalacturonic acids had an inhibitory effect on root growth at concentrations above 100 ppm. There were two reports that the degradation of polygalacturonic acid has shoot elongation effects, however, there is no report as to what DP of galacturonic acid is most effective for plant growth (Yamaguchi and others 1996; Suzuki and others 2002). The optimum DP of galacturonic acid has not yet been determined because of the difficulties in preparing the large quantities (mg) of each oligogalacturonic acid and in separating it from the salt-conjugated form.

Acid oligomers have been separated by low or middle pressure anion-exchange chromatography with KCl salt, imidazole-HCl, or sodium formate buffer (Nagel and Wilson 1969; Jin and West 1984; Davis and others 1986). These methods can deal with large quantities of samples, however, they require a desalting process for the plant growth test. Similarly, a volatile buffer (ammonium formate) and Q-sepharose has been used for preparing oligogalacturonides (Spiro and others 1993), however, it is difficult to completely evaporate the formic acid (bp. 100.8°C) using an evaporator or lyophilization.

Other separation methods have been performed with thin-layer chromatography, gas chromatography and high-performance liquid chromatography (HPLC) using gel-filtration, and strong or weak anion-exchange (Doner and others 1988; Raymond and Nagel 1969; Thibault 1980; Voragen and others 1982). Recently, high-performance anion-exchange chromatography coupled with pulsed amperometoric detection (HPAEC-PAD) was used to separate oligogalacturonic acids (Hotchkiss and Hicks 1990; Lieker and others 1993). These methods can effectively separate the oligomers of the galacturonic acids, however, they cannot deal with large sample quantities. Meanwhile, Shimokawa and others (1996) reported the preparation of two series of oligoguluronic acids from sodium alginate by acid hydrolysis and enzymatic degradation. They pre-

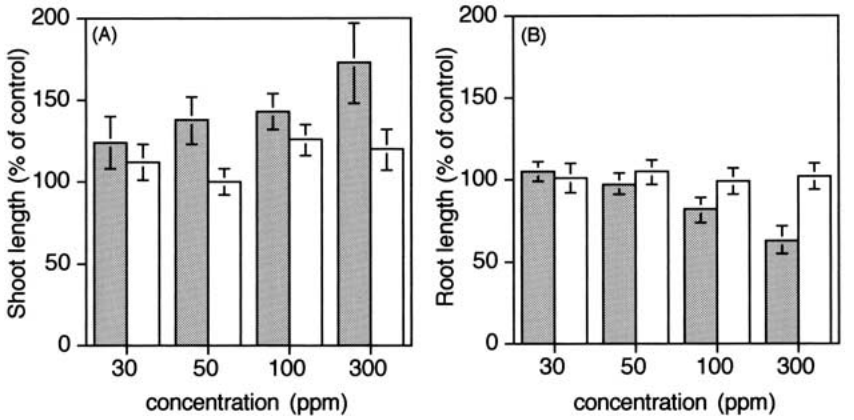

Figure 1. Effects of the mixture of oligogalacturonic acids (left column) and the polygalacturonic acid (right column) on shoot (**A**) and root (**B**) growth of cockscomb. Each value is the average of 16 seedlings. Bars indicate average ± S.E.

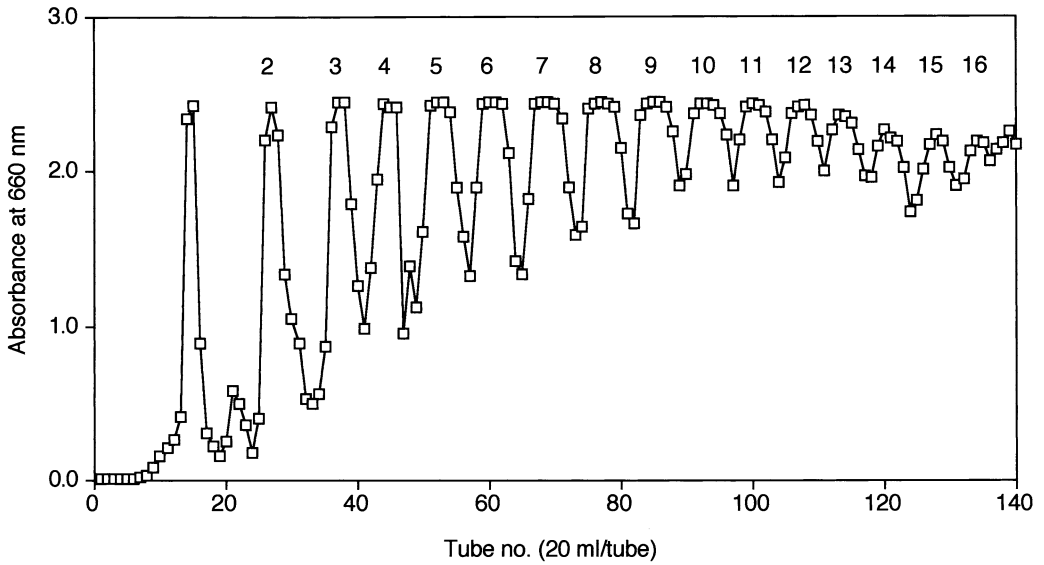

Figure 2. Chromatogram of the oligogalacturonic acids with NH_4HCO_3 as the eluent. Numbers indicate the DP of oligogalacturonic acids.

pared oligoguluronic acids and oligoguluronic acids having 4-deoxy-L-*erythro*-hex-4-enopyranosyluronic acid residues at the non-reducing end, and separated them by anion exchange chromatography on a Q Sepharose Fast Flow column using NH_4HCO_3 solution as the eluent. The advantage of NH_4HCO_3 is that it is effectively removed by lyophilization.

We prepared a mixture of the oligogalacturonic acids by acid hydrolyzing polygalacturonic acid and then separated it into individual oligomers. Figure 2 shows an anion exchange chromatogram (DEAE A-25) of the oligogalacturonic acids with NH_4HCO_3 as the eluent. The yields of the oligogalacturonic acids derived from 3 g of polygalacturonic acid was as follows: Gal A_2, 280 mg; Gal A_3, 76 mg; Gal A_4, 32 mg; Gal A_5, 58 mg; Gal A_6, 58 mg; Gal A_7, 57 mg; Gal A_8, 51 mg; Gal A_9, 44 mg; Gal A_{10}, 40 mg; Gal A_{11}, 33 mg; Gal A_{12}, 29 mg; Gal A_{13}, 16 mg; Gal A_{14}, 17 mg; Gal A_{15}, 12 mg; Gal A_{16}, 10 mg. The FACEs of the isolated oligogalacturonic acids (DP 2-16) are shown in Figure 3. Each of the isolated oligogalacturonic acids gave a single band on FACE, and they showed an *m/z* value that corresponded to their molecular ion peaks $[M\text{-}H]^-$ on FAB-MS analysis (data not shown). These results showed that the successive chromatography method used in this study is well suited for the preparation of oligogalacturonic acid for the plant growth test. According to the research by Spiro and others (1993), these oligogalacturonic acids pre-

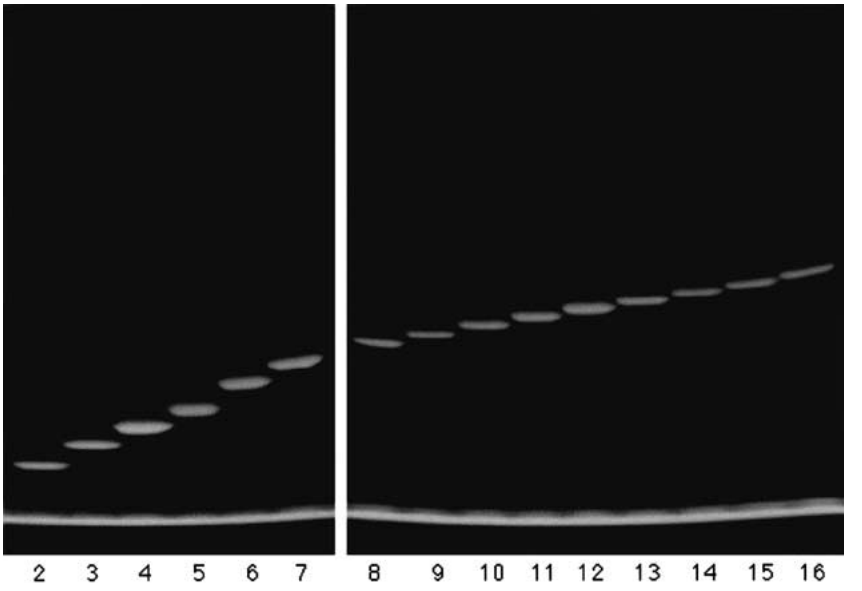

Figure 3. FACEs of the isolated oligogalacturonic acids (DP 2-16). The vertical axis indicates the DP of oligogalacturonic acids.

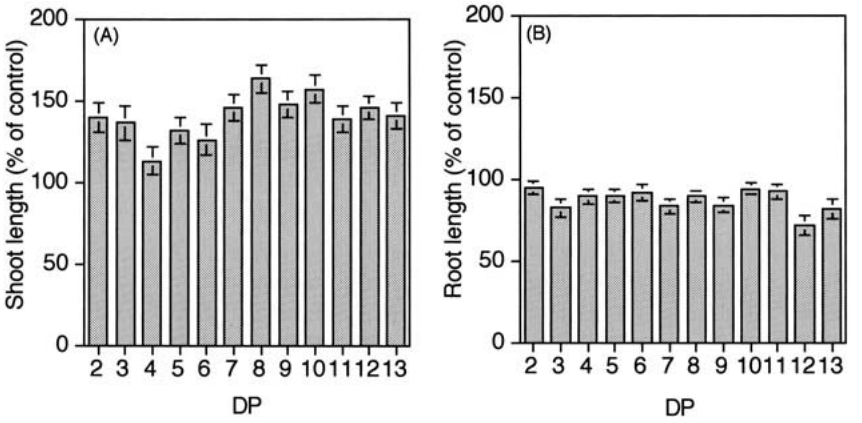

Figure 4. The effects of oligogalacturonic acids (DP 2-13) on shoot (**A**) and root (**B**) growth of cockscomb at a concentration of 100 μM. Each value is the average of 48 seedlings; bars indicate average ± S.E.

pared by column chromatography, not by HPLC-PAD, contain Gal A polymers ending with galactaric acid, the C1-oxidized derivative of Gal A. However, the amount of galactaric acid is very low and the contribution of galactaric acid in the plant growth test should also be very low. The details of the effect of galactaric acid including with oligogalacturonic acids on plant growth may require future investigation.

Figure 4 shows the effects of the oligo- galacturonic acids (DP 2-13) on the cockscomb growth at a concentration of 100 μM. The oligogalacturonic acids (DP 2-13) showed a promoting effect on the shoot growth in cockscomb. The most effective DP of the oligomers was 8 in this test, and a significant difference between octa-galacturonide and other oligomers was recognized ($P < 0.05$, *t*-test). Similar results were also seen at concentrations of 30 and 300 μM (data not shown). Figure 5 shows the effects of octa-galacturonide on shoot and root growth of cockscomb seedlings. Octa-galacturonide promoted shoot growth of cockscomb at concentrations above 10 μM, and showed a 66% promotion at the most effective concentration of 300 μM. On the other hand, root growth was slightly inhibited at concentrations above 300 μM.

Oligogalacturonic acids have been recognized as regulators for several physiological processes in plants, including induction of phytoalexin, aproteinase inhibitor, and lignification (Darvill and others 1992; Fry and others 1993). During the study of phytoalexin, Nothnagel and others (1983) provided the evidence that the most active elicitor in soybean (*Glycine max* L.) was a molecule composed of 12 galacturonosyl residues. Jin and West (1984) demonstrated that the trideca-galacturonide was the most active elicitor of casbene synthetase activity in castor beans (*Ricinus communis* L.). For the

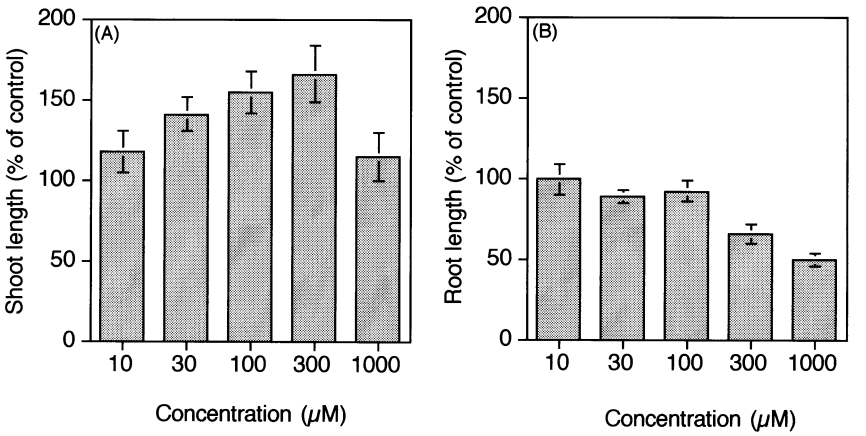

Figure 5. Effects of octagalacturonide on shoot (**A**) and root (**B**) growth of cockscomb. Each value is the average of 16 seedlings; bars indicate average ± S.E.

induction of lignin in cucumber, the most active oligomer had about 11 galacturonosyl units (Robertsen 1986). In studies of phytoalexin and lignin production, it is known that oligogalacturonic acids of DP 8-14 have the highest activity. Compared with those phytoalexin and lignin production effects of oligogalacturonic acid, the specificity of oligomer size required for an effect on shoot growth of cockscomb might be low (Fig. 4). The result indicated that the mode of action for shoot growth effect might be different from the actions of phytoalexin and lignin production. Additionally, we investigated the effects of octa-galacturonide on the oat coleoptile elongation test for auxin activity and the rice microdrop test for gibberellin activity, however, both oat coleoptile section and rice second leaf sheath were not affected by octa-galacturonide concentrations of 10–1000 μM (data not shown). These results appear to suggest that oligogalacturonic acids have no roles related to auxin or gibberellin activity.

In this study, we showed that the most effective DP of oligogalacturonic acid was around 8 for shoot growth in cockscomb seedlings, and the effects of both smaller and larger oligogalacturonic acids were slightly less than that of octa-galacturonide. On the other hand, there was also a negative effect on plant growth; oligogalacturonic acids inhibited pea stem segment elongation induced by auxin (Branca and others 1988).

Previously, we reported that the acid water extract from tomato juice waste has a species-selective effect on tested plant growth (Suzuki and others 2001). The most effective components in the acid water extract were almost all oligogalacturonic acids (DP 6-12) by HPAEC-PAD analysis (Suzuki and others 2002). The acid extract promoted the shoot and root growth of tomato, Chinese cabbage, corn, and radish, but not the growth of oat seedlings (Suzuki and others 2001). These results indicate that oligogalacturonic acids have different effects on plant species and their organs. In some plants, oligogalacturonic acids might bind with inorganic substances in the rhizosphere, making absorption of these substances easier, and promoting shoot or root growth. Further details of the function and mechanism of action of octa-galacturonide are under study.

ACKNOWLEDGMENTS

The authors wish to thank the editor and reviewers in Journal of Plant Growth Regulation for useful suggestions.

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