_root growth-promoting activity of unsaturated oligomeric uronates from alginate on carrot and rice plants_ 

**Xu Xu, Yoshiko Iwamoto, Yoshie Kitamura, Tatsuya Oda, and Tsuyoshi Muramatsu**

1Division of Biochemistry, Faculty of Fisheries, Nagasaki University, Nagasaki 852-8521, Japan
2Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8521, Japan

Received April 7, 2003; Accepted June 11, 2003

The root elongation activity of unsaturated oligomeric uronates from alginate on carrot and rice plants was investigated. Unsaturated oligomeric uronates were prepared by digesting polymannuronate (PM) and polyguluronate (PG) with an alginate lyase purified from _Pseudoalteromonas_ sp. strain No. 272. The root elongation activity was measured by elongation in length of carrot- and rice-excised root incubated in the B5-medium containing 0.8% agar in the dark. PM and PG showed no activity, but the enzymatic digestion mixtures of PG had promoting activity on roots of both plants at a final concentration of 0.5 mg/ml. The maximum activity was obtained at 0.75 mg/ml. The dependence of activity on degree of polymerization of the uronates was tested and the pentamer was most active, but the mechanism of the action of unsaturated uronates on the cells remains to be solved.

**Key words:** root elongation; uronate oligomer; alginate; carrot; rice

Alginates are generally extracted from marine brown algae and widely known to be a copolymer of α-L-guluronic (G) and its C5 epimer β-D-mannuronic (M), arranged in homopolymeric G blocks, M blocks, and random heteropolymeric G and M stretches. Alginates extracted from seaweed are widely used in the food industry, biotechnology, for medical purpose _etc._ Over the last decade, the oligosaccharides derived from alginate have been investigated as new functional materials. Akiyama _et al._ reported the effects of depolymerized alginites on the growth of _bifidobacteria._ Yonemoto _et al._ studied the promotion of germination and shoot elongation of some plants by alginate oligomers. It has been reported that the tri-saccharides from the enzyme-digested alginate oligomers promoted barley root elongation. Iwasaki _et al._ have reported that an alginate oligosaccharide mixture had promoting activity toward the root growth of lettuce seedlings. The tri-, tetra-, penta-, and hexasaccharides had especially strong activity. The effects of oligomeric saccharides from alginate on plant growth is very significant in the use of marine biomass and mass production of useful plants. However, in most of these studies, the mixtures of alginate oligomers, being mixtures of mannuronic and guluronic acid oligomers, have been used and the plants tested have been limited.

In this paper, we deal with the effects of isolated unsaturated mannuronate and guluronate oligomers with various DP from alginate, which were prepared by enzymatic digestion of alginate, on root elongation of carrot and rice. Sodium alginate (1000 cps grade) was obtained from Nacalai Tesque Inc., Kyoto, Japan, and used without further purification. Carrot (_Daucus sativa_ L) seed was obtained from Tohoku Seed Co., Ltd., Tochigi, Japan. Rice (_Oryza sativa_ L) seed was obtained from Ishikawa Agricultural College, Nonoichi, Ishikawa, Japan. All other chemicals were the purest grade commercially available. Purification of the enzyme from _Pseudoalteromonas_ sp. strain No. 272 was done as described previously, and the properties of the enzyme are shown in the previous paper. Preparations of poly α-1,4-L-guluronate (PG) and poly β-1,4-D-mannuronate (PM) were done from the sodium alginate by the method of Haug _et al._ The purity of PG or PM was checked by circular dichroic spectral analysis with a Jasco spectropolarimeter J500A coupled with a data processor, based on the method of Morris _et al._ An amount of 5 g of PG or PM was dissolved in 50 ml of H_2_O. The enzyme solution (0.05 mg/ml, 0.08 ml) was added to PG or PM solution pre-incubated at 30°C. The enzyme digestion of PG and PM was stopped by adjusting pH to 4.0 with 0.1 N HCl, and after 15 min, the solution was neutralized with 0.1 N NaOH. After filtering the solution through a membrane (pore size, 0.45 μm, Advantec Tokyo), 20 ml of the solution was put on a column (8.8 × 95 cm) of Bio Gel P-6 previously equilibrated with 50 mM phosphate buffer.
(pH 7.5) and the sample was eluted with the same buffer at a flow rate of 1.2 ml/min. The eluate was fractionated into 10-ml portions. Unsaturated uronates in each fraction were measured by absorbance at 235 nm with a Hitachi spectrophotometer U-2001. Each peak was pooled and lyophilized. The degree of polymerization was routinely measured by the method of Whitaker by using Blue dextran 2000 and galacturonic acid for calibration of the column. To remove the overlapped neighbouring oligomers as contaminants, the individual peak was further rechromatographed on the Bio Gel P-6 column in the same manner as above. The lyophilized sample from the second Bio Gel P-6 column was dissolved in a small amount of water and the resulting phosphate that crystallized upon cooling the solution was removed. Each oligosaccharide solution was further chromatographed to remove residual phosphate on a Bio Gel P-2 column (2.5 × 95 cm) with water as the eluent at a flow rate of 0.5 ml/min. Phosphate in fractions eluted from the column was measured by the molybdenum blue method. Trimmers or more than trimers of saccharides could be freed from phosphate by this method. Finally, the saccharide-containing fraction was pooled, lyophilized, and used throughout the experiments. Agar and B5 media were prepared as follows: agar powder (0.8% w/v) was mixed in twice distilled H2O (DDH2O) and sterilized in an autoclave at 121°C for 15 min and molten agar was poured in Petri dishes (90 × 20 mm). Petri dishes were kept at 25°C in the dark. B5 salts were mixed in DDH2O and the pH was adjusted to 5.8-6.0 with 0.1 N NaOH. The medium containing 0.8% (w/v) agar was sterilized in autoclave as mentioned above. Alginate uronate powder was dissolved in pure water to reach of concentration of 2 mg/ml. Different concentrations of this solution was added to the B5 medium (0.25-2 mg/ml) and poured into Petri dishes. Carrot seeds were sterilized by placing them in running water for 4 h before treatment with ethanol for 30 s. Subsequently the seeds were incubated in 0.5% (v/v) sodium hypochlorite solution initially for 10 min in a vacuum and then for 45 min on a magnetic stirrer, followed by soaking in 0.1% (w/v) HgCl2 solution for 2 min. Similarly, the rice seeds were sterilized by treating with HgCl2 for 25 min after removing the cover. The treated seeds were thoroughly rinsed in sterilized water for 4 times. The surface-sterilized seeds were placed in Petri dishes containing agar medium. The roots (1.5-2 cm) emerging from seeds were cut into 0.8-1.2 cm lengths and cultured on B5 medium containing various concentrations (0.25-2 mg/ml) of alginate uronates. Ten roots were placed in each dish and numbered. These Petri dishes were incubated at 25°C under the dark conditions after the original length of isolated roots was measured. To prevent contamination, measurement of root length should be done without opening the Petri dishes by using a thread cut into the same length as that of the root at 4 or 5-day intervals. The effects of uronates on the root elongation were analyzed with Dunnett’s multiple comparison test, at p < 0.05 and p < 0.01 as statistically significant.

The enzymatically digested PG and PM were separated on a Bio Gel P-6 column it showed a good correlation between the molecular weight of each oligosaccharide and its elution volume. After desalting, the trimer to nonamer of G (G3-G9) and of M (M3-M9) were obtained. In this study, we selected the carrot as a representative of dicotyledons and rice as a representative of monocotyledons to compare the effects. We tried to find a method to search for effects of uronates on seedlings at first. The fluctuation of the growth rate of the roots was observed when the roots themselves originated from seedlings were used for measurement of the growth. However, we found that with excised roots, the growth rate became more synchronized and more accurately measurable. It could be due to the fact that the seedling produces many kinds of factors by itself to influence the growth of roots. However, the excised root is very simple and its growth relies much more on exterior conditions. Thereby, we used excised roots as material for the experiments. There has been no study about using excised roots to investigate the effects of saccharide on the growth rate of plant roots previously.

No positive effect on root elongation was observed with excised roots of carrot and rice cultured on B5 medium containing PM or PG before depolymerization at 0.5 mg/ml in a final concentration. However, excised roots of carrot and rice which were placed on B5 medium containing an unsaturated oligoguluronates mixture from PG at 0.5 mg/ml in a final concentration showed a pronounced positive effect on root growth as compared to the control (Fig. 1). By contrast, oligomannuronate has apparently no or little positive effect on root elongation. It has been reported that alginate oligomers prepared by enzymatic digestion shows growth-promoting activity on plants and alginites as polymers before depolymerization had no effect. Thus, it seems likely that alginate oligomers, but not alginites as a polymer, are mainly responsible for the bioactivities in these cases. In our experiment, we have found that enzymatically depolymerized guluronate oligomers apparently had a positive effect on the root elongation of carrot and rice. By contrast, PG before depolymerization were ineffective on root elongation, as well as PM. These results suggest that oligomer formation through the enzymatic digestion of PG is essential for the root elongation.

The effects of the concentration of the unsaturated oligoguluronate mixtures are shown in Fig. 2. The enzymatically depolymerized unsaturated oligoguluronate mixtures at 0.75 mg/ml in a final
concentration were mostly effective. Carrot root elongation was directly related to the increase in concentration of the unsaturated oligoguluronate mixtures up to 0.75 mg/ml. However, in rice, the response was not regular, but the maximum positive effect was also observed at 0.75 mg/ml of the unsaturated oligoguluronate mixtures, although the test was done for 8 days of culture.

What is more, the excised roots of carrot and rice were placed in Petri dishes of control and B5 medium containing the unsaturated oligoguluronates with various DPs (G3-G9) at 0.75 mg/ml for the final concentration. It was observed that all from G3 to G9 had positive effects on the root elongation. The maximum elongation was found with G5 (Fig. 3). The effect decreased with increasing or decreasing of DP on both sides at G5. Moreover, the effect of the unsaturated oligoguluronates on the root elongation of carrot was clearer than that of rice. In summary, it was observed that the pentaguluronate had the highest activity, and the others were less than the activity with DP5. Compared with what has been
reported previously, for example, the enzyme-treated alginate oligomers with DP 3 had a promotion activity on barley root elongation.\textsuperscript{4} Iwasaki et al. have reported that unsaturated alginate oligosaccharide mixtures promote the root growth of lettuce seedlings. The tri-, tetra-, penta-, and hexasaccharides have especially strong activity.\textsuperscript{5} But in these experiments, there was not much discipline or systematization shown. Especially, the separate effects of mannuronates or guluronates with different degrees of polymerization was not clearly shown. The effects of the unsaturated alginate oligomers with different DP apparently depends on the plant species to be tested. In our experiment, the unsaturated pentaguluronate was found to be most active for the root elongation of carrot and rice. By contrast, PM and PG had no effect on the root elongation, as mentioned above. Very recently, we showed that enzymatically depolymerized alginate oligomers caused cytotoxic cytokine production in human mononuclear cells.\textsuperscript{10} The result shows that enzymatically depolymerized guluronate and mannuronate oligomers are active, but not PM and PG, while the action mechanism on the cells is still unknown. The differences in these biological activities of alginate, PM, PG, and unsaturated alginate oligomers might be partly explained on the basis of the conformation of L-guluronic acid in a $^{13}C_4$ buckled polymeric form and of D-mannuronic acid in a $^{14}C_4$ extended polymeric form, and on the basis of multiple steric sites for saccharide recognition ability on the cell surface.

References