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Characterisation of methyl-ester distributions in galacturonan regions of complement activating pectins from the roots of *Angelica acutiloba* Kitagawa[a]

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On digestion with endo-α-(1 → 4)-polygalacturonase the complement activating pectins (AR-2II*a*, II*b*, II*c* and II*d*) from the roots of *Angelica acutiloba* Kitagawa gave long (PG-2) and short (PG-3) oligogalacturonide fractions in addition to rhamnogalacturonan cores possessing neutral carbohydrate side chains. HPLC analysis of 2-aminopyridine-labelled derivatives indicated that PG-3 from the four pectins contained mono- to octa-galacturonides of which penta- to octa-galacturonides were suggested to contain some methyl-esterified GalA. Three major partially methyl-esterified oligogalacturonides (oligo-1, 2 and 3) were purified from PG-3 of AR-2II*b*, and oligo-1, 2 and 3 were suggested to be tetra-, hepta- and hexa-galacturonides which had one, three and two methyl-esters, respectively.

Long oligogalacturonide fraction (PG-2) from AR-2II*d* contained hepta- to tetradeca-galacturonides possessing some methyl-esters. Carboxyl-reduction of the methyl-esterified GalA followed by lithium degradation gave only galactitol as the final product from PG-2 of AR-2II*d*, suggesting that methyl-esterified GalA in PG-2 of AR-2II*d* was attached to another methyl-esterified GalA through unesterified GalA in a similar way to the short oligo-galacturonides in PG-3 of AR-2II*a*–II*c*.

INTRODUCTION

Four kinds of complement activating pectins (AR-2II*a*, II*b*, II*c* and II*d*) have been isolated from the roots of *Angelica acutiloba* Kitagawa (Kiyohara *et al.*, 1988). AR-2II*a*–II*d* consists of a small proportion of 'ramified' regions (rhamnogalacturonan cores possessing neutral galactosyl and arabinofuranosyl side chains) and a large proportion of galacturonan region (polymerized α-(1 → 4)-linked galacturonic acid (GalA)) (Kiyohara *et al.*, 1988, 1989*b*; Kiyohara and Yamada, 1989). The 'ramified' regions are active sites for expression of complement activating activity. Although three active pectins (AR-2II*a*–II*c*) activate complement only through the classical pathway of the complement system, and AR-2II*d* activates through both classical and alternative pathways of the system, all the 'ramified' regions of the pectins activate both pathways (Kiyohara *et al.*, 1988). Therefore, it has been postulated that the galacturonan regions of AR-2II*a*–II*c* inhibited complement activating potency of their 'ramified' regions through alternative pathways but the galacturonan region of AR-2II*d* did not (Kiyohara *et al.*, 1988). *Endo*-α-(1 → 4)-polygalacturonase digestion suggests that the distribution of methyl-esters in the galacturonan region of AR-2II*d* was different from the other three active pectins (AR-2II*a*–II*c*) (Kiyohara *et al.*, 1988), and the results proposed that the distributions of methyl-ester groups in the galacturonan region have a regulatory effect upon complement activating potency of their 'ramified' regions.

In the present paper we describe the distribution of methyl-ester groups in the complement activating pectins (AR-2II*a*–II*d*).

[a]Studies on polysaccharides from *A. acutiloba*: Part XV.
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MATERIALS AND METHODS

Materials

The roots of *A. acutiloba* Kitagawa were purchased from Tochimoto-Tenkaido Co, Ltd (Japan). QAE-Sephadex, DEAE-Sephadex and Sephadex G-50 were obtained from Pharmacia, and Bio-gel P-4 (−400 mesh) and P-6 (200–400 mesh) from Bio-Rad. Pectinase from *Aspergillus niger* was purchased from Sigma, and *endo*-α-(1 → 4)-polygalacturonase (poly(1,4-α-D-galacturonide)glycanohydrolase, EC 3.2.1.15) was purified using the procedure of Thibault and Mercier (1977).

General methods

Uronic acid was assayed by *m*-hydroxybiphenyl methods (Blumenkrantz & Asboe-Hansen, 1973) using GalA as a standard. The methyl-ester group was measured by the method of Wood & Siddiqui (1971) using MeOH as the standard. Poly- and oligosaccharides were hydrolysed with 2 M TFA at 121°C for 1·5 h, and the hydrolysates were analysed by TLC on cellulose (Merck) with EtOAc-C_5H_5N-HOAc-H_2O (5:5:1:3). Reducing sugars and uronic acids were detected with alkaline $AgNO_3$ (Trevelyan *et al.*, 1950) and *p*-anisidine HCl (Hough *et al.*, 1950), respectively. Sugars were converted conventionally into the alditol acetates, and analysed by GC, which was carried out using a Hewlett Packard model 5890 series II gas chromatograph equipped with an SP-2380 capillary column (30 m × 0·25 mm i.d., 0·20 μm film thickness, Supelco) and FID detector. Samples were injected with a splitless injector. The flow rate of carrier gas (He) was 0·9 ml/min. The temperature program was 60°C (1 min), 60 → 215°C (30°C/min), 215°C (18·8 min), 215 → 250°C (8°C/min) and 250°C (5·7 min). The molar ratios of sugars were calculated from the peak areas and the response factors on the FID of the corresponding alditol acetates.

Preparation of oligogalacturonides from complement activating pectins

Preparation of complement activating pectins
Complement activating pectins (AR-2II*a*–II*d*) were purified from the crude polysaccharide fraction (AR-1) of *A. acutiloba* Kitagawa by cetavlon (cetyltrimethylammonium bromide) precipitation and anion-exchange chromatography on DEAE-Sephadex as reported previously (Kiyohara *et al.*, 1988).

Endo-α-*(1 → 4)-polygalacturonase digestion*
AR-2II*a*–II*d* each were digested with *endo*-α-(1 → 4)-polygalacturonase in 50 mM acetate buffer (pH 4·2) for 4 days at 37°C. After neutralisation, the reaction mixture was fractionated on Sephadex G-50 in H_2O to obtain void volume fraction (PG-1), intermediate fraction (PG-2) and the lowest molecular weight fraction (PG-3). In order to de-esterify, the pectins were stirred in 0·2 M NaOH for 2 h at room temperature, and the de-esterified pectins were digested with the *endo*-polygalacturonase after neutralisation with AcOH.

*Purification of short oligogalacturonides from AR-2II*b
PG-3 (50 mg), obtained from AR-2II*b*, or AR-2II*b* de-esterified as above, was applied to a column (1·5 × 49 cm) of QAE-Sephadex A-25 equilibrated with 125 mM imidazole buffer (pH 7·0). After the column was washed with the same buffer (pH 7·0) oligogalacturonides were eluted with a linear gradient of imidazole buffer (pH 7·0) from 125 to 750 mM, and fractions *a*, *b* and *c* were obtained from PG-3 of AR-2II*b*. Fractions *a*, *b* and *c* each were further fractionated on Bio-gel P-6 equilibrated with 50 mM acetate buffer (pH 5·2) to obtain oligo-1, 2 and 3, respectively.

HPLC analysis of oligogalacturonides

HPLC analysis of 2-aminopyridine-labelled oligogalacturonides were performed according to the method of Maness and Mort (1989). Oligogalacturonides (1 mg) were heated in 500 μl of aqueous 2-aminopyridine (which contained 1 g of 2-aminopyridine, 0·8 ml of 6 N HCl and 1·6 ml of H_2O) at 65°C for 20 h. The solution was subjected to HPLC, which was performed using Asahi-pak ES-502N anion-exchange column (10 × 0·75 cm i.d., Asahi Chemical Industry Co., Ltd, Japan). Labelled oligogalacturonides were eluted from the column using a linear gradient 30–500 mM acetate buffer (pH 5·2) over 60 min at 1 ml/min, and detected at 290 nm. Standard oligosaccharides were prepared by partial acid hydrolysis (Robertsen, 1986). Briefly, polygalacturonic acid from orange (Sigma) was autoclaved in 100 mM acetate buffer (pH 4·0) at 121°C for 30 min, and the solution was acidified to pH 2·0 by addition of AcOH after cooling in an ice bath. The resulting precipitate was removed by centrifugation, and the supernatant was lyophilized. The oligogalacturonides in the lyophilisate were labelled with 2-aminopyridine as above.

Methylation analysis

Samples were methylated once by the method of Hakomori (1964), and methylated products were recovered using a Sep-pak C_{18} cartridge (Waters Associates) using the procedure of Waeghe and co-workers (1983), except that the samples were eluted only with EtOH. The methylated products were reduced with $NaBD_4$ in THF-EtOH (7:3) for 18 h at room temperature, followed by incubation at 80°C for 1 h (Waeghe *et al.*, 1983). The methylated and carboxyl-reduced products were hydrolysed with 2 M TFA at 121°C for 1·5 h, and derived into alditol acetates which were analysed by GC and GC-MS as described previously (Yamada *et al.*, 1991).

Carboxyl-reduction of methyl-esterified GalA in oligogalacturonides, and their lithium degradation

Methyl-esterified GalA in PG-2 was carboxyl-reduced by the method of Maness *et al.* (1990). Briefly, PG-2 (10 mg) from AR-2II*d* was dissolved in *M* imidazole buffer (pH 7·0, 2 ml) and the solution was incubated in an ice bath for 1 h after $NaBH_4$ (40 mg) was added to the solution. Excess $NaBH_4$ was decomposed by addition of AcOH, and salt was removed by passing through AG50WX8 (H^+) resin (Bio-Rad).

The partially carboxyl-reduced product was subjected to lithium degradation according to the procedure of Lau *et al.* (1987), and the reduced product (8 mg) from PG-2 was suspended in ethylenediamine (5 ml). Several pieces of lithium metal (Sigma) were then added and stirred for 1 h, after which time the colour of the reaction mixture changed to dark blue. After the reaction product was refrigerated, lithium was decomposed by addition of H_2O. Solutions were removed by evaporation, and the products were desalted by AG50WX8 (H^+) resin. The products were further reduced with $NaBH_4$ in 2 M NH_4OH, and desalted.

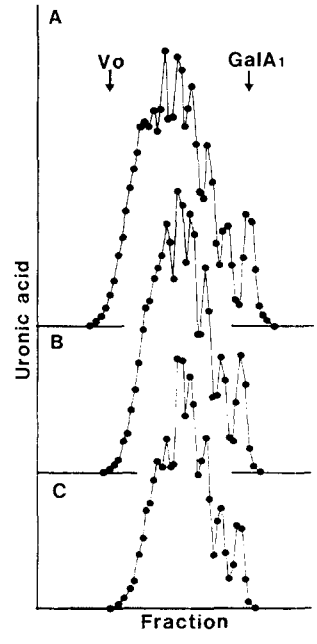

Fig. 1. Gel filtration patterns of short oligogalacturonides in PG-3 obtained from AR-2II*a* (A), AR-2II*b* (B) and AR-2II*c* (C) on Bio-gel P-4. Vo, void volume; $GalA_1$, eluting position of GalA.

RESULTS AND DISCUSSION

It has been reported that similar or greater numbers of GalA in AR-2II*a*–II*c* were methyl-esterified than in AR-2II*d* (Kiyohara *et al.*, 1988). However, three complement activating pectins (AR-2II*a*–II*c*) from *A. acutiloba* gave large proportions of short oligogalacturonide fractions (PG-3) and small proportions of long oligogalacturonide fractions (PG-2) in addition to the 'ramified' regions (PG-1) on digestion with *endo*-α-(1 → 4)-polygalacturonase (ratio of yield; PG-3:PG-2 = 1·0:0·3–0·4), whereas AR-2II*d* gave more PG-2 and less PG-3 than AR-2II*a*–II*c* (PG-3:PG-2 = 1·0:6·0). When the short oligogalacturonide fractions (PG-3) from AR-2II*a*–II*c* were compared by gel filtration on Bio-gel P-4, these fractions seemed to contain similar sizes (degree of polymerization: 1–8) of oligogalacturonides (Fig. 1). After short oligogalacturonides in PG-3 obtained from AR-2II*c* and II*d* were labelled with 2-aminopyridine, according to the method of Maness & Mort (1989), the labelled oligogalacturonides were analysed by HPLC. As shown in Fig. 2, PG-3 from both AR-2II*c* and II*d* contained tri- to octa-galacturonides. These results suggested that *endo*-α-(1 → 4)-polygalacturonase digestion released similar sizes of short oligogalacturonides from the four active pectins. Thibault and Mercier have reported that *endo*-α-(1 → 4)-polygalacturonase cleaved α-(1 → 4)-polygalacturonic acid polymer into mono- to tetra-galacturonides (Thibault & Mercier, 1978). Because oligogalacturonides were digested into mono- to tetra-galacturonides when PG-3 from AR-2II*a*–II*d* were re-digested with the *endo*-polygalacturonase after

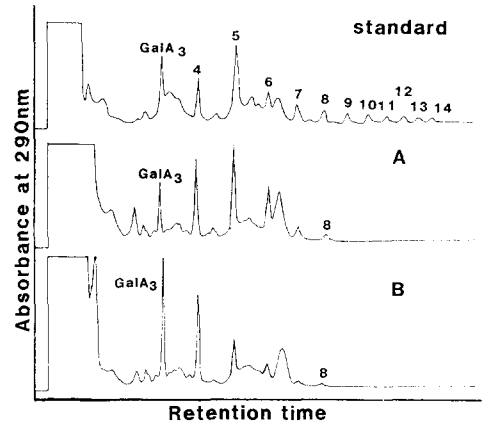

Fig. 2. Elution patterns of 2-aminopyridine-labelled short oligogalacturonides from PG-3 of AR-2II*c* (A) and AR-2II*d* (B). Number-average degree of polymerisation of oligogalacturonides.

de-esterification (data not shown), it was assumed that oligogalacturonides over tetragalacturonide in PG-3 from AR-2II*a*–II*d* were resistant against *endo*-α-(1 → 4)-polygalacturonase. *Endo*-α-(1 → 4)-polygalacturonase from *A. niger* is able to cleave α-(1 → 4)-linkages of GalA but not to cleave α-(1 → 4)-linkages of methyl-esterified GalA (Thibault & Mercier, 1978), therefore it was suggested that oligogalacturonides over tetragalacturonides were methyl-esterified.

In order to clarify methyl-esters of the short oligogalacturonides in PG-3, oligogalacturonides of PG-3 from AR-2II*b*, which was the major active pectin, were

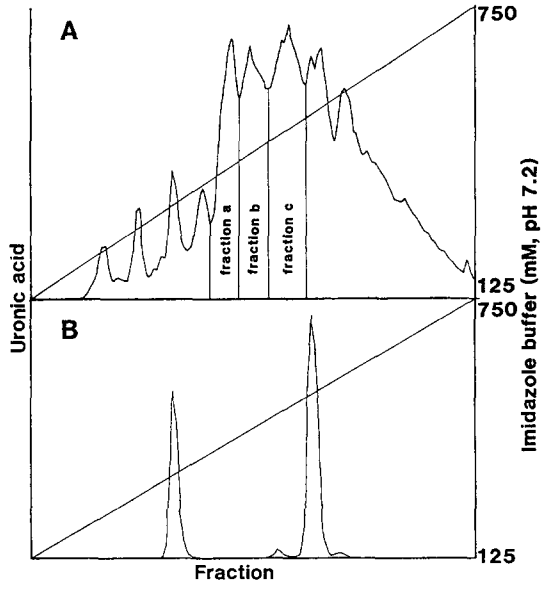

Fig. 3. Anion-exchange chromatography of short oligogalacturonides in PG-3 obtained from AR-2II*b* (A) and de-esterified AR-2II*b* (B) on QAE-Sephadex A-25.

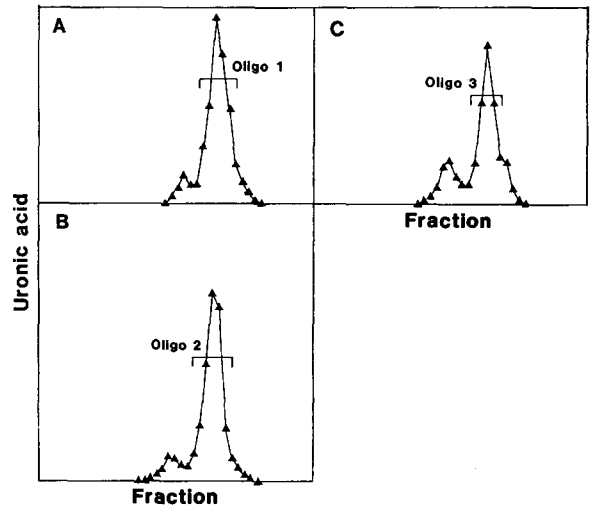

Fig. 4. Gel-filtration patterns of fractions *a* (A), *b* (B) and *c* (C) from Fig. 3(A) on Bio-gel P-6.

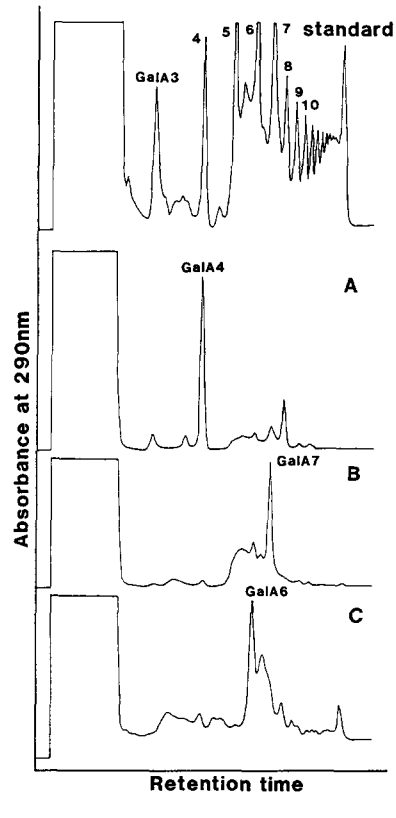

Fig. 5. HPLC patterns of 2-aminopyridine-labelled oligogalacturonides (oligo-1, 2 and 3) from Fig. 4. A, labelled oligo-1; B, labelled oligo-2; C, labelled oligo-3.

purified by anion-exchange chromatography. PG-3 from AR-2II*b* was applied to QAE-Sephadex equilibrated with 125 mM imidazole buffer (pH 7·2), and oligogalacturonides were eluted by linear gradient of imidazole buffer (125–750 mM). As shown in Fig. 3(A), PG-3 from AR-2II*b* gave several kinds of oligogalacturonides, whereas PG-3 from de-esterified AR-2II*b* eluted two kinds of oligogalacturonides which were suggested to be tri- and tetra-galacturonides, respectively, by the analysis with HPLC (Fig. 3(B)). This result indicated that the oligogalacturonides, which were not obtained from de-esterified AR-2II*b*, were methyl-esterified oligogalacturonides. The major fractions *a–c* from native AR-2II*b* (Fig. 3A) were further analysed. Fractions *a–c* each were fractionated on Bio-gel P-6, and each gave oligo-1, 2 and 3 as major oligogalacturonides (Fig. 4). Contents of oligo-1 ~ 3 in the galacturonan region of AR-2II*b* were 11·8, 14·0 and 19·1%, respectively. Oligo-1, 2 and 3 consisted of terminal and 4-linked GalA by methylation analysis (data not shown), and 38·2, 40·0 and 38·1% of GalA in oligo-1–3 were methyl-esterified, respectively. Oligo-1–3 were labelled with 2-aminopyridine, and analysed by HPLC. From the comparison of retention times of oligo-1–3 with those of 2-aminopyridine-labelled standard oligogalacturonides, oligo-1–3 were considered to contain mainly tetra-, hepta- and hexa-galacturonides, respectively (Fig. 5). From the chain-length and content of methyl-ester, one or two GalA in oligo-1, three GalA in oligo-2, and two GalA in oligo-3 were considered to be methyl-esterified.

The long oligogalacturonide fraction (PG-2) from AR-2II*d* was also labelled with 2-aminopyridine, and analysed by HPLC. PG-2 from AR-2II*d* mainly contained hepta- to tetradeca-galacturonides (Fig. 6A), and 34·8% of GalA in PG-2 was methyl-esterified. When PG-2 from AR-2II*d* was re-digested with the *endo*-polygalacturonase after de-esterification, higher molecular weight oligogalacturonides (>*dp* 5) were digested giving lower molecular weight oligogalacturonides (≤*dp* 4) (Fig. 6(B)). This suggests that the

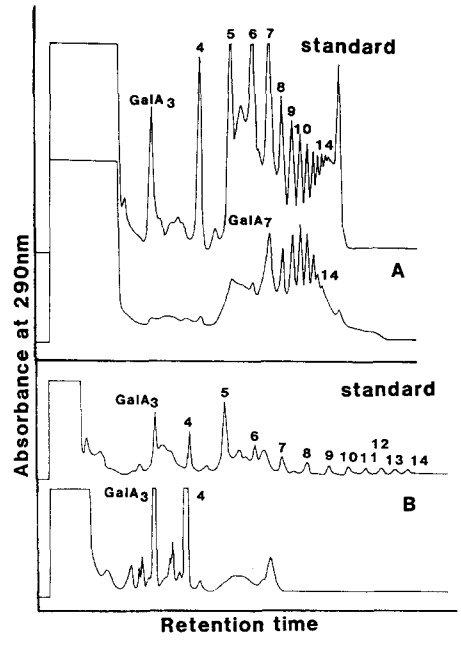

Fig. 6. (A) HPLC pattern of 2-aminopyridine-labelled long oligogalacturonides obtained from PG-2 of AR-2II*d*. (B) HPLC of 2-aminopyridine-labelled oligogalacturonides derived from de-esterified PG-2 of AR-2II*d* by redigestion with *endo*-α-(1 → 4)-polygalacturonase. Number-average degree of polymerisation of oligogalacturonides.

hepta- to tetradeca-galacturonides were also partially methyl-esterified. In order to clarify the distribution of methyl-esterified GalA in the long oligogalacturonides of PG-2, methyl-esterified GalA in the oligogalacturonides were carboxyl-reduced into Gal with $NaBH_4$ by the method of Maness *et al.* (1990). TLC analysis of the hydrolysates indicated that the product consisted of Gal and GalA, whereas the hydrolysates of PG-2 consisted only of GalA. Gal in the product was thought to be derived from methyl-esterified GalA in PG-2 by reduction. When the uronic acid content of the products was assayed by the colorimetric method (Blumenkrantz & Asboe-Hansen, 1973), 34·2% of GalA was decreased by the reduction of PG-2, suggesting that most of methyl-esterified GalA in PG-2 was reduced by this reduction. The reduced product from PG-2 was further treated with lithium in ethylenediamine in order to degrade the remaining GalA (unesterified GalA) in the product of PG-2, according to the procedure of Lau *et al.* (1987). The lithium-treated product was reduced with $NaBH_4$ to reduce the glycosyl residues which were attached with GalA. It was thought that oligogalactosylgalactitols could be detected in the product if methyl-esterified GalA was linked with another methyl-esterified GalA directly. However, the hydrolysates from the product consisted only of galactitol but not Gal. After the lithium-treated product from PG-2 was directly acetylated without hydrolysis, the acetylated derivatives were analysed as alditol acetate by GC, and only galactitol hexaacetate was detected. These results suggested that a methyl-esterified GalA in the long oligogalacturonides of PG-2 was attached with another methyl-esterified GalA through unesterified GalA.

Although oligo-1, 2 and 3 and PG-3 from AR-2II*a*–II*c* were not subjected to a reduction of methyl-esterified GalA followed by lithium degradation, the present results assumed that methyl-esterified GalA in PG-3 of AR-2II*a*–II*c* also attached with another methyl-esterified GalA in a manner similar to PG-2 from AR-2II*d*. Frequencies of methyl-esters in oligogalacturonides from AR-2II*b* and II*d* are shown in Table 1. It was indicated that oligo-1-3 from AR-2II*b* and long oligogalacturonide fraction (PG-2) from AR-2II*d* each contained about half the methyl-esters in their galacturonan regions. The present results suggested that the *endo*-α-(1 → 4)-polygalacturonase-resistant oligogalacturonides attached to each other through *endo*-α-(1 → 4)-polygalacturonase-sensitive oligogalacturonides. Thibault has reported that the *endo*-α-(1 → 4)-polygalacturonase from *A. niger* could recognize tetra-α-(1 → 4)-polygalacturonide as the

Table 1. Frequency of methyl-esters in oligogalacturonides of AR-2II*b* and AR-2II*d*

Oligogalacturonide	Structural unit of oligogalacturonide[a]	Yields (%)[b]	Frequency of methyl-ester[c]
AR-2II*b*			
Oligo-1	$(MeGalA)_1(GalA)_3$	11·8	4·4/33·0
Oligo-2	$(MeGalA)_3(GalA)_4$	14·0	5·6/33·0
Oligo-3	$(MeGalA)_2(GalA)_4$	19·1	7·2/33·0
		total 44·9	17·2/33·0
AR-2II*d*			
Long oligogalacturonide (PG-2)	$(GalA)_m(MeGalA)_1(GalA)_n$[d]	85·7	29·8/50·0

[a] MeGalA: methyl-esterified GalA.
[b] Contents in galacturonan region of AR-2II*b* and AR-2II*d*.
[c] Methyl-ester content (%) in oligogalacturonide per methyl-ester content (33% in AR-2II*b*; 50% in AR-2II*d*) in galacturonan region.
[d] $m, n < 4$.

minimum substrate (Thibault, 1982). Therefore it was assumed that the partially methyl-esterified oligogalacturonides, such as oligo-1-3 were attached to each other through unesterified oligogalacturonides more than tetragalacturonide in AR-2II*a*–II*c*, whereas the esterified oligogalacturonides might be linked to each other less through unesterified oligogalacturonides than tetragalacturonides in AR-2II*d*. These suggested that galacturonan regions of AR-2II*a*–II*c* contained more highly methyl-esterified and short oligogalacturonide blocks than that of AR-2II*d*. During the preparation of this manuscript, Mort *et al.* (1993) reported that low methyl-esterified pectin contains higher contents of unesterified GalA blocks (>tetragalacturonide) than highly esterified pectin. Because the content of methyl-esters in AR-2II*d* was higher (50%) than those of AR-2II*a* and II*b* (33%) (Kiyohara *et al.*, 1988), the present results seemed to agree well with those of Mort *et al.*, and differences of methyl-ester distribution between AR-2II*a* and II*b*, and AR-2II*d* seemed to be due to chain length of unesterified oligogalacturonide units. Although AR-2II*c* and II*d* had almost the same methyl-ester contents, great differences might be present on methyl-ester distributions in highly methyl-esterified galacturonan regions.

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