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Influence of the Degree of Polymerization of Oligogalacturonates and of Esterification Pattern of Pectin on Their Recognition by Monoclonal Antibodies[1]

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ABSTRACT

The ability of galacturonic and oligogalacturonic acids with degrees of polymerization (DP) from 2 to 10 to inhibit the recognition of homopolygalacturonic acid by a monoclonal antibody specific for dimers of pectin (F Liners, J-J Letesson, C Didembourg, P Van Cutsem [1989] Plant Physiol 91: 1419–1424) has been tested by enzyme-linked immunosorbent assays. Oligomers of DP9 and above preincubated with the antibodies clearly inhibited the association between the antibodies and immobilized pectin. A minimum DP of nine consecutive galacturonic residues is thus necessary to be associated through calcium cations to form dimers. Randomly deesterified pectin was recognized by the antibody if its degree of methylesterification was <30%, whereas blockwise deesterified pectin was recognized up to 40% of methylesterification. The replacement of calcium ions by magnesium prevented the recognition of polygalacturonic acid by the antibody.

Pectic oligomers have been reported to have physiological effects on plant cells (9). These oligomers may be released by pectic enzymes secreted by invading pathogens and, in some cases, they elicit phytoalexin production in host plants. Phytoalexins are low mol wt molecules with antibiotic activities. Nothnagel *et al.* (17) described the purification of a pectic elicitor of phytoalexin accumulation in soybean (*Glycine max*). By fast atom bombardment-MS, they identified the most active elicitor fraction to be a dodeca-α-1,4-D-galacturonate. They did not exclude contamination by smaller oligogalacturonides.

Jin and West (10) separated oligogalacturonides by anion exchange chromatography. A minimum DP[2] of 9 was found to be necessary to elicit casbene synthase activity in castor bean (*Ricinus communis*). Trideca-α-1,4-D-galacturonide was the most active oligomer that they tested, and methylesterification of the carboxylate groups greatly diminished elicitor activity.

Davis *et al.* (6) separated unsaturated oligogalacturonide fractions released by the action of an α-1,4-D-endopectate lyase from *Erwinia carotovora*. The decagalacturonide fraction caused maximum accumulation of phytoalexin in cotyledons of soybean. Fractions rich in oligogalacturonides containing 9 or 11 residues enhanced the elicitor activity of the decagalacturonide-rich fraction. Fractions rich in DP from 3 to 8 residues and those with DP > 12 residues inhibited the elicitor activity of the decagalacturonide-rich fraction.

Branca *et al.* (3) tested oligogalacturonides for their ability to interfere with the IAA-induced elongation of pea. Maximum inhibition was shown by oligomers with DP > 8. Inhibition by longer fragments was much lower, and the products of the 24-h digestion of pectate and the unhydrolyzed pectate were ineffective.

Recently, lignin biosynthesis and isoperoxidase activity have been elicited in suspension cultures of castor bean by pectic fragments with an average DP of 7 (4).

Pectic fragments have also been involved in the regulation of plant morphogenesis. Tran Thanh Van *et al.* (23) showed that oligosaccharins released from sycamore cell walls by endo α-1,4-polygalacturonase modified the *in vitro* organogenesis of tobacco explants. Eberhard *et al.* (7) confirmed this result, but they observed the induction of different organogenetic changes. Therefore, pectic fragments may have effects only if their DPs are greater than a critical minimum. This could be related to their calcium binding ability. Kohn (12) and Kohn and Luknàr (13) claimed that a threshold value of approximately 15 to 20 residues is necessary for a cooperative binding of Ca^{2+} by oligogalacturonate in an "egg-box"-like system (8).

Powell *et al.* (18) calculated that sequences of seven or more consecutive free carboxyl groups along the participating faces of the chains are necessary for cooperative binding. For a twofold chain symmetry, this value corresponds to 14 residues on each chain.

In previous work (14, 15), MoAbs were produced that recognize a calcium-induced conformation of pectin. It was suggested that these antibodies were specific of pectic chains associated as dimers according to the "egg-box" model. The objectives of the present work were: (a) to determine the

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[2] Abbreviations: DP, degree of polymerization; DM, degree of methylesterification; PGA, polygalacturonic acid; PLL, poly-L-lysine hydrobromide; HRP-SAM, horseradish peroxidase-labeled sheep anti-mouse immunoglobulin; ABTS, 2,2'-azino-bis(3-ethylbenzthiazolinesulfonate); MoAb, monoclonal antibody; IgM, immunoglobulin M.

minimum DP of oligogalacturonates able to adopt the conformation recognized by these antibodies, and (b) to test the influence of the degree and pattern of methylesterification of pectins on recognition by the MoAbs. Results from these studies could contribute information to elucidate the size requirements for the elicitor and morphogenetic activities of oligogalacturonates.

MATERIALS AND METHODS

Chemicals

PGA from orange, PLL (338,000 mol wt), and gelatin were purchased from Sigma. HRP-SAM was from Amersham (UK), and ABTS from Boehringer (Mannheim, Germany). The NUNC ELISA microtiter plates were supplied by GIBCO Europe SA (Gent, Belgium).

Pectic Samples

PGA had a mean DP of 35 as estimated by the ratio of total sugar to reducing sugar. Oligogalacturonates (DP2–DP10) were obtained by gel-permeation chromatography on a Bio-Gel P2 column as previously described (20). All the oligomers (except DP10) were rechromatographed on the same column. The purity of each oligomer was checked by HPLC on Nucleosil 10SB (8 mm i.d. × 30 cm) eluted with 0.15 M acetate buffer, pH 3.75, at a flow rate of 2.6 mL·min^{-1}. The pure fractions were immediately used for ELISA tests.

Pectins with a random (R10–R85) and blockwise (B30 and B40) distribution of free carboxyl groups were obtained by alkaline or enzymic deesterification, respectively. The preparation and the characteristics of these samples were described elsewhere (21). A highly esterified pectin (R95) was obtained by incubation of Sigma PGA (P-3889) in methanolic H_2SO_4 at 4°C for several weeks (5).

Direct ELISA: General Procedure

The experiments were performed as previously described (14). Fifty μL of PLL (0.05 mg·mL^{-1} in deionized water) were dispensed into each well of NUNC High Binding Capacity microplates and incubated for 1 h at room temperature. Identical volumes of a 200 μg·mL^{-1} PGA sample in a Ca/Na solution containing 0.5 mM $CaCl_2$ and 150 mM NaCl were dispensed and left overnight at 4°C. Nonspecific binding was blocked by incubating the wells for 2 h at 37°C with 200 μL of gelatin (200 μg·mL^{-1}) prepared in the same solution. After removal of the excess gelatin, 2F4 ascites purified on protein G (450 μg·mL^{-1}) were diluted 1:40 in the Ca/Na solution and added to the wells. The microplates were then washed six times with the Ca/Na solution using an Immunowash (NUNC) before addition of 50 μL of HRP-SAM-labeled sheep anti-mouse immunoglobulin (1:500 in Ca/Na solution) for 1 h at 37°C. After a second washing cycle, the binding of the antibodies was revealed by a chromogen-substrate solution made of ABTS (4.2 mg in 5.6 mL of citrate-phosphate buffer, pH 5.6) and H_2O_2 (final concentration 0.03%). The absorbance of the solution was measured after 15 min with a Titertek multiscan at 405 nm.

Competition ELISA Tests

Competition assays were first performed with 200 μL of 2F4 ascites diluted 80 times in the Ca/Na solution and preincubated with 200 μL of pectic material in the same solution overnight at 4°C. The final concentration of the pectic samples was 100 μg·mL^{-1}, except DP4, DP5, and DP6, which were prepared at 37, 45, and 84 μg·mL^{-1}, respectively. The pectic samples were omitted in the blanks. These pectin-antibody mixtures were then centrifuged for 10 min at 7,500*g* before dispensing the supernatants in microwells coated with PGA and blocked as described for the direct ELISA. Incubations with antibodies, washings, and detection were performed as described above. Dilutions of PGA, DP9, and DP8 in the preincubation step were tested by the same procedure.

To check the effect of the centrifugation step on the inhibitory capacity of PGA, DP9, and DP8, two other competition procedures were used: the first was performed as described above except that the centrifugation step was omitted. In the second method, the test galacturonides were added directly to PGA-coated wells blocked as usual, followed by the 2F4 antibodies. The binding competition was thus carried out with all components present in the wells. Dilutions of PGA, DP9, DP8, and DP7 were also tested by this competition assay.

Direct ELISA: Effect of Magnesium

In these tests, the wells were coated with PGA dissolved in Ca/Na and rinsed five times with a Mg/Na solution containing 0.5 mM $MgCl_2$ and 150 mM NaCl, and the test was completed with either the Ca or the Mg solution. Two controls consisted of tests performed completely in either Ca/Na or Mg/Na. The tests were simultaneously done with the 2F4 (0.1 mg·mL^{-1}, diluted 40×) and the 7F7 (unpurified ascites, diluted 40×) MoAbs, an IgM less sensitive to the presence of calcium (14).

RESULTS

Effect of the DP

Preincubation of the 2F4 MoAbs with DP1 to DP8 did not inhibit the recognition by these MoAbs of PGA adsorbed in the microwells (Fig. 1), even at high concentrations of DP8 (Fig. 2). In contrast, oligogalacturonates of DP9 as well as PGA associated in solution with the antibodies, precluding any further interaction of the MoAbs with PGA in the microplates. However, the results of Figures 1 and 2A, were obtained after a centrifugation step of the preincubated antibody-antigen mixture, which could have precipitated the antibodies cross-linked by the antigens and which could have removed antibodies from the supernatants used for the ELISA test. Examples are known where two antibodies can be bound and cross-linked by divalent antigens smaller than a galacturonide of nine residues (11).

This possibility was checked by comparing centrifuged and noncentrifuged mixtures in a competition test and by mixing the test antigens and the antibodies directly in the wells (Fig. 3). The supernatant of centrifuged mixtures of DP9 and MoAbs did not react with pectin in the microwells. The DP8

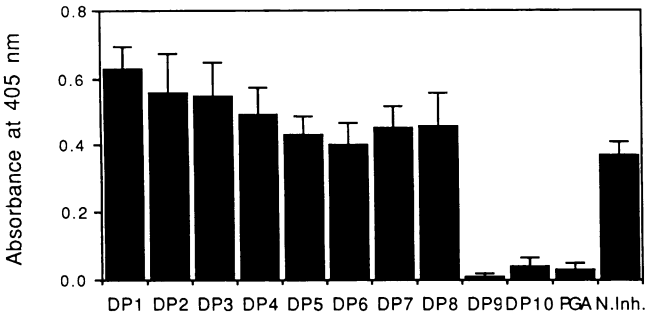

Figure 1. Competition ELISA test: Effect of the DP of pectic samples on the recognition of PGA by 2F4 MoAbs. The antibodies were incubated with either galacturonic acid (DP1), oligogalacturonates (DP2–DP10), or PGA. The resulting mixtures were centrifuged and the supernatants dispensed in PGA-coated microwells. Noninhibited antibodies were used as control (N.Inh.). The mean values (n = 12) of two independent assays are presented with the standard errors.

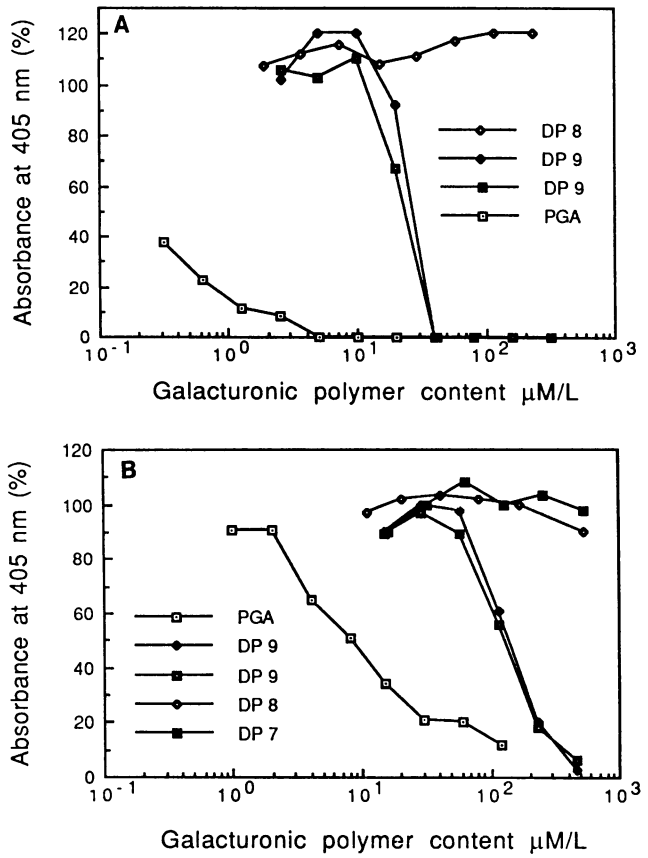

Figure 2. Competition ELISA test: A, effect of the concentration of PGA, DP9, and DP8 on the recognition of PGA by 2F4 MoAbs. The antibodies were incubated with different concentrations of PGA, DP9, and DP8. The resulting mixtures were centrifuged and the supernatants added to PGA-coated microwells. The mean absorbances (n = 6) are expressed as percentages of controls (antibodies not inhibited); B, the 2F4 MoAbs and various concentrations of PGA, DP9, DP8, or DP7 were added, without the preincubation step, to PGA-coated microwells. The mean absorbances (n = 2) are expressed as percentages of controls (antibodies not inhibited); the DP9 experiment has been duplicated.

did not contain any epitope recognized by the antibodies, as evidenced by the large positive readings of the tests, whether centrifuged or not. In the direct competition assay, the DP9 and PGA efficiently decreased the association of the antibodies to the microwells, whereas DP8 was completely inefficient. When the antibodies were titrated with the test antigens (DP7, DP8, DP9, and PGA) directly in the coated microwells (Fig. 2B), they were 50% inhibited by PGA at a galacturonic acid content of 8 $\mu M \cdot L^{-1}$ and by DP9 at 120 $\mu M \cdot L^{-1}$. DP8 and DP7 at concentrations up to 550 $\mu M \cdot L^{-1}$ did not inhibit the 2F4 antibodies. At least nine galacturonate residues in pectic chains are, therefore, needed for recognition by the antibodies in a Ca 0.5 mM/Na 150 mM solution.

Effect of Methylesterification

Pectins with degrees of random methylesterification up to about 20 were recognized by the antibodies to the same extent as PGA (Fig. 4). At DM of approximately 30, the response of the MoAbs dropped to nearly 25% of its initial value. At DM of approximately 40 and higher, the pectins were not recognized. The absorbance of the ELISA tests of B30 and B40 samples was about four times higher than that of R30 and R40 samples.

Effect of Magnesium

When the test was completely performed with the Mg/Na solution (Mg only, Fig. 5), most of the 2F4 MoAb did not associate with PGA in the wells and was washed away during rinsing. The same result could have been obtained if PGA were leached from the wells by the $MgCl_2$ solutions. This was checked by coating the wells with PGA dissolved in the

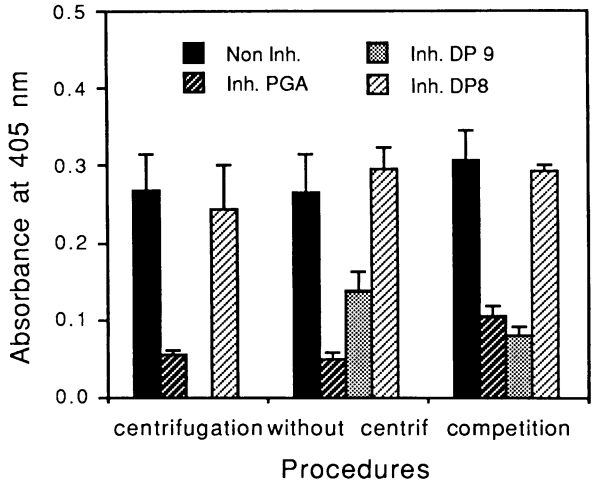

Figure 3. Comparison of three competition procedures by ELISA test: the 2F4 MoAbs were incubated with 320 µg/mL of PGA, DP9, or DP8. The resulting mixtures were then centrifuged and added to PGA-coated microwells (centrifugation); same procedure without centrifugation (without centrif); the antibodies and the pectic fractions were added directly to the microwells without the preincubation step (competition). Noninhibited antibodies were used as controls (Non Inh.). The mean absorbances (n = 6) are presented with the standard errors.

Ca/Na solution, washing with the Mg/Na solution before blocking with gelatin, and returning to the Ca/Na for the rest of the test (Ca/5Mg/Ca, Fig. 5). PGA was not completely desorbed by the magnesium solution because the absorbance of the microwells was still about 0.25. The same treatment performed with the 7F7 MoAb, which is much less sensitive to the presence of calcium, clearly confirmed that polygalacturonates were still present in the wells, but magnesium as the sole divalent cation prevented their recognition by the 2F4 MoAb. These results with the 7F7 antibodies allowed PGA bound directly to PLL to be distinguished from PGA associated indirectly by calcium to PLL-bound PGA. Only calcium-associated polygalacturonates were removed by the magnesium treatment. The antibody then recognized the remaining species: the PLL-bound PGA.

DISCUSSION

Whatever the type of ELISA performed, with or without centrifugation of the preincubation mixture of test antigens and antibodies, or direct incubation in the microwells, the results showed that oligogalacturonates shorter than DP9 were not recognized by the antibodies. The centrifuged DP9-MoAb mixture (Fig. 3) did not react in the assay. Either it contained no antibodies, which implies that DP9 has at least two epitopes for cross-linking the antibodies that precipitated on centrifugation, or the binding sites of the antibodies in the transferred mixture were already occupied by tightly bound DP9 oligomers and were completely blocked. This last hypothesis can be easily ruled out because noncentrifuged mixtures of MoAbs and DP9 gave a positive reading after transfer to the microtiter wells. In this case, competition led to some of the antibodies releasing the short galacturonides and binding to the pectic polymer coating of the wells instead. Competitive transfer seems likely because the galacturonides of nine residues bound weakly to antibodies compared to the pectic polymer: 50% inhibition of the antibodies occurred at a DP9 molar concentration 15 times higher than PGA (Fig. 2B). We conclude that the DP9 oligomers contain at least two epitopes for cross-linking the antibodies.

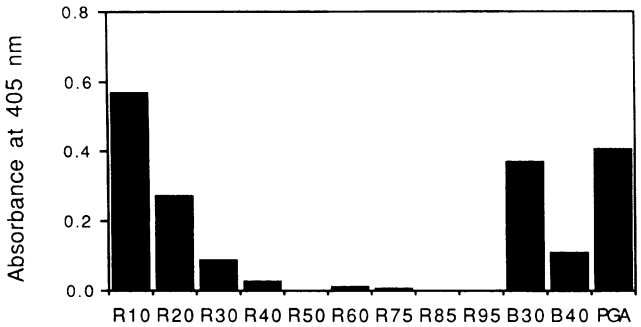

Figure 4. Direct binding of 2F4 MoAbs to pectins of varying degrees DM immobilized on ELISA plates: Randomly deesterified pectins with DM 9.7% (R10), 21.4% (R20), 27.3% (R30), 38.1% (R40), 48.3% (R50), 58.2% (R60), 72.1% (R75), 83% (R85), and 95% (R95). Blockwise deesterified pectins with DM 27.7% (B30) and 39.7% (B40); polygalacturonic acid is indicated as PGA. Values represent the means of duplicates.

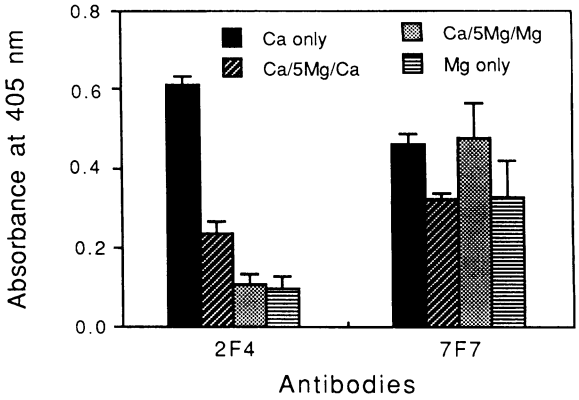

Figure 5. Direct binding of 2F4 and 7F7 MoAbs to PGA immobilized on ELISA plates: effect of either Ca^{2+} or Mg^{2+} in the cationic solutions. Microwells were coated with PGA in Ca/Na solution. After five washings with the Mg/Na solution, the tests were completed with Ca/Na (Ca/5Mg/Ca) or Mg/Na (Ca/5Mg/Mg) solutions. The controls consisted of tests performed with Ca/Na (Ca only) or Mg/Na (Mg only). The mean values (n = 6) are presented with the standard errors.

This is consistent with the observation that the predominant antigenic determinants of polysaccharides often consist of short oligosaccharides one to five sugars long (2). Moreover, the fact that PGA, a rather long polymer, is recognized at a lower concentration than DP9, suggests that the epitope does not include the terminal residues of the chains, because a much higher concentration of PGA would be necessary for the antibodies to be inhibited to the same extent as by the DP9. This rules out the possibility that the additional galacturonic acid unit creates a sequential epitope at either end of the oligomer.

Because the DP8 is not recognized by the 2F4 antibody, the addition of one galacturonic unit to the DP8 creates two or more antigenic sites. As it is unlikely that several epitopes are localized on the same additional galacturonic unit, the presence of a ninth uronic residue could rather induce a conformation of the whole molecule that creates the antigenic sites in presence of calcium ions. The appearance of the epitopes between DP8 and DP9 seems to be an "all or none" mechanism, suggesting a cooperative change of conformation.

Pectins are stiff molecules and homopolygalacturonic sequences may be considered as rigid linear chains with restricted flexibility due to the *trans* diaxal α-(1, 4)-glycosidic bonds (1, 12). These rigid molecules cannot form intramolecular chelates of calcium, but readily associate into chain dimers even when the molar ratio of calcium to monovalent cations in the solution is as low as 0.006:3 (16). Accordingly, the epitope recognized by the MoAbs in the Ca/Na solution used for the ELISA tests should be part of dimerized polygalacturonic chains.

An alternative interpretation could be that the antibodies bind to single pectic chains and require calcium as a cofactor. For example, it is well known that many enzymes acting on phosphorylated compounds such as ATP have a quasi-absolute requirement for magnesium as a cofactor. This inter-

pretation is not valid in our case, because, in the competition tests of Figure 3, both DP8 and DP9 were incubated with the antibodies in identical Ca/Na solutions. Calcium ions are, therefore, not necessary for the 2F4 antibody itself but are necessary to induce the epitopes recognized by the 2F4 antibody.

The small positive readings of the first two competition assays performed with PGA (Fig. 3) could originate from the polydisperse nature of the pectin used. The DP35 measured by colorimetric methods is only an average value. Possibly longer and shorter chains could form dimers and bind an antibody. Upon transfer into the microwells, some of these "incomplete dimers" could have associated through their "sticky" ends to the pectic coating of the wells carrying an immunoglobulin.

Thibault and Rinaudo (21) studied the calcium and sodium activity coefficients and transport numbers of solutions of randomly and blockwise deesterified pectins. A tight association between calcium ions and randomly esterified pectins can occur only if the DM is < 30%. This closely matches our present results showing that recognition of pectins by the antibodies is also lost between DM20 and DM40. In these samples, the length of the sequences of nonmethylesterified galacturonic residues is too short to form calcium-induced associations that could resist thermal agitation. If the methyl groups are randomly distributed, the probability of finding nine consecutive unmethylesterified residues in a pectin with a DM of 10% is $(0.9)^9 = 39\%$; if the DM is 40%, the probability of finding the same sequence is $(0.6)^9 = 1\%$, which is only slightly detected by our ELISA assay. In contrast, pectins with blocks of free carboxyl groups are able to associate intermolecularly through calcium bridges up to a higher DM (12, 21). The blockwise esterified pectins (B40 and B30, Fig. 5) are detected by the MoAbs as slightly methylesterified or unesterified samples, respectively, confirming results obtained previously by potentiometry and conductivity (21, 22).

Transport parameter determination showed that the Mg^{2+} ion activity in a solution of PGA has a value predicted from the Manning's theory (21), demonstrating that Mg^{2+} is unable to induce intermolecular cross-links between pectic molecules. The absence of recognition of magnesium pectate confirms that the anti-pectin antibodies are specific to polygalacturonic chains associated intermolecularly.

Clearly, calcium induces a supramolecular conformation of the pectic chains, possibly according to the "egg box" model. In that model, the pectic chains form stable dimers in presence of calcium ions. These dimers can exist in solution, and gelation can be obtained by further association of dimers into multimers in the presence of additional calcium ions (18). In our opinion, the 2F4 antibody is specific for these dimers. According to our results, the minimum length necessary for oligogalacturonates to form dimers in presence of calcium is nine. Two DP9 chains would thus associate intermolecularly by forming five calcium bridges.

This critical value of DP9 is less than the values of about 15 to 20 proposed by Kohn and Luknàr and confirmed by Ravanat and Rinaudo (19) on the basis of calcium activity coefficient measurements. However, all the DPs were not studied, and the results of their studies can be interpreted as showing a transition zone between DP 5 and 20, rather than a critical DP value. The critical value of DP14 found by Powell *et al.* (18) results from the best fit of data of circular dichroism changes between solution and gel with a model based on a statistical distribution of methyl esters on the pectic chains. However, our antibodies recognize dimers in solution but not multimers in a gel (14). Very probably, the association of dimers into multimers is only possible with longer DPs, because a DP9 dimer has only four available carboxylate anions on each of its external faces, which is too short even for dimer formation.

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