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Monoclonal Antibodies against Pectin

Recognition of a Conformation Induced by Calcium

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ABSTRACT

Monoclonal antibodies have been produced that recognize a conformation of homopolygalacturonic acid (pectic acid) induced by an optimum concentration of calcium and sodium of about 1 and 150 millinormal, respectively. The epitope recognized is probably part of the dimers of pectin chains associated according to the 'egg box' model.

Pectins constitute one of the main matrix components of higher plant cell walls. Their backbone results essentially from 1–4 polymerization of α-D-galacturonic acid monomers, interspersed with neutral sugar blocks and/or monomers (*e.g.* L-rhamnose). They can be branched, acetylated, and methylated (3). Pectins in plant cell walls have been shown to act like cation exchangers and to adsorb cations selectively (16). Homopolygalacturonic acid (pectic acid) is able to associate intermolecularly in presence of calcium ions. Morris *et al.* (10) and Powell *et al.* (11) have proposed a two-step model of cooperative association between homopolygalacturonic acid chains through calcium bonds. In that model, there would be an initial dimerization of pectin chains of 2_1-helical symmetry by cooperative binding of Ca^{2+} to the inner faces of the chains (Fig. 1). The association would be cooperative because binding of the first cation between any pair of chains causes alignment which facilitates binding of the next, and so on along the sequence. Sufficient oxygen atoms on each chain are suitably placed to form a complete coordination sphere. This form of association has been named 'egg-box' (2). Subsequent Ca^{2+}-induced aggregation of these preformed dimers in tetramers, hexamers, etc., could occur, but the binding energy of these subsequent associations would be lower than for dimer formation. The associations between dimers would thus be far weaker and more tenuous than those between participating chains within dimers.

The biochemical methods of extraction, purification, and analysis of pectic as well as hemicellulosic polysaccharides are particularly long and/or difficult (selective extractions; ion exchange and gel permeation chromatographies; GC-MS, and NMR). These methods do not always allow a complete description of the primary structure of the polysaccharides. Nearly nothing is known about their secondary and tertiary structures (8). At the present time, several cytochemical methods exist for studying polysaccharides in plant cells. The periodic acid shiff methods and the gold-labeled lectins and enzymes have proved to be good tools for localizing pectins and hemicelluloses from plant cell walls, although they suffer from some limitations (17).

MoAbs[1] are highly specific for minute amounts of conformational and even sequential epitopes. They can distinguish between the D and L isomers of a sugar. Few reports exist on the production of antibodies against plant polysaccharides. Vreeland (19) has produced a polyclonal serum against alginates. Kaku *et al.* (4) raised sera against α-L-arabinofuranose. Moore *et al.* (9) used sera to label a xyloglucan and a rhamnogalacturonan I on *Acer pseudoplatanus*. Vreeland *et al.* (20) have prepared MoAbs which specifically identify gelling and nongelling alginate block substructures. No MoAbs have been produced against homopolygalacturonic acid. This paper describes the production and the characterization of such MoAbs.

MATERIALS AND METHODS

Chemicals

HRP-SAM and HRP-DAR were purchased from Amersham Belgium. These reagents were titrated for optimal reactivity. Alginic acid was from Fluka. PGA from orange (98% purity), methylated BSA, OVA, and PLL (338,000 mol wt) were from Sigma. 2,2′-azino-bis(3-ethylbenzthiazolinesufonate) was from Boehringer and diaminobenzidine from Sigma.

Antigens

Homopolygalacturonic acid was used as antigen either alone or coupled to a carrier protein, methylated BSA or OVA, to confer immunogenicity. PGA was coupled to methylated BSA (18). The periodate-lysine fixation method (7) was used to fix PGA to OVA. Excess fixative was removed by overnight dialysis against phosphate buffer (50 mM, pH 7.4).

Immunization Protocol

BALB/C mice and rabbits were raised and maintained in a laboratory animal colony. Antisera were raised in rabbits

[1] Abbreviations: MoAb, monoclonal antibody; Ig, immunoglobulin; HRP, horseradish peroxidase; HRP-SAM, HRP-labeled sheep anti-mouse IgG; HRP-DAR, HRP-labeled donkey anti-rabbit IgG; PGA, polygalacturonic acid; OVA, ovalbumin; PLL, poly-L-lysine hydrobromide; TBS, Tris-buffered saline.

against PGA (with or without carrier proteins) following the procedure of Moore *et al.* (9). In brief, antigen emulsified in Freund's complete adjuvant was injected intradermally in multiple sites along the back of the animals. Rabbits were boosted with the same dose in Freund's incomplete adjuvant 1, 2, and 9 weeks later. Mice were immunized in the same way, except that all the injections were performed intraperitoneally at half of the dose. Antibody titers were determined as described below. Sera from uninjected animals were used as negative controls.

MoAbs

Two weeks after the last injection, one of the mice was given repeated intraperitoneal injections with daily doses of PGA-methylated BSA mixture according to the method of Stähly *et al.* (12). One day after the last injection, spleen cells were collected and fused with SP_2/O-Ag 14 myeloma cells. The fusion protocol of Köhler and Milstein (5) was followed with minor modifications. Hybridoma cells were grown in RPMI 1640 (GIBCO) basal medium supplemented with 5% fetal calf serum and 10% horse serum. Supernatants from fused cells were screened for antibody production 2 weeks later by a solid phase ELISA. Positive clones of interest were recloned at least two times by limiting dilution onto rat peritoneal lavage feeder cells and then stored under liquid nitrogen. Antibody containing ascitic fluid was produced in BALB/C mice by conventional methods.

Screening of Antibodies

Dot Blotting

Polyclonal antibodies were screened using a dot blot assay (15). Nitrocellulose paper (Bio-Rad) was used in a Bio-dot apparatus (Bio-Rad) to adsorb 0.5 μg of PGA in each well. Horse serum 10% in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) was used to block nonspecific protein binding to the nitrocellulose. The paper was then incubated in immune or control sera diluted in TBS-1% horse serum for 1 h. After washing in TBS-0.1% Tween 20, blots were incubated in HRP-DAR or HRP-SAM (1/500 in TBS-1% horse serum) for 1 h. Following an other TBS wash, antibody binding was detected by HRP substrate color development (Tris 50 mM, diaminobenzidine 0.05%, H_2O_2 0.03%, pH 7.5). The reaction was stopped by immersing the sheet in HCl 0.1 N. All operations were performed at room temperature.

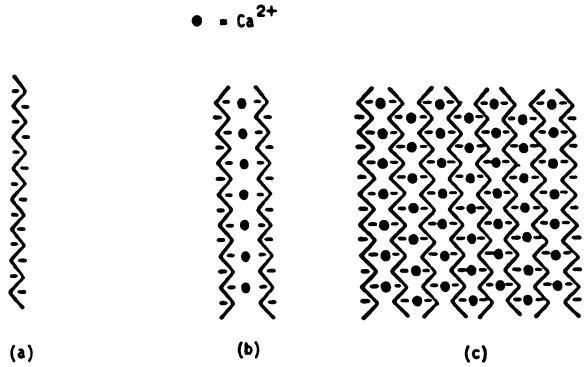

Figure 1. Two-step model of cooperative association between homopolygalacturonic acid chains through calcium bonds: (a) isolated pectin chains in absence of divalent cations, (b) dimer of pectin chains in presence of small amounts of bivalent cations, and (c) multimers in more concentrated divalent cationic solutions (*e.g.* Ca^{2+}).

ELISA (General Protocol)

Unless otherwise stated, High Binding Capacity microplates (NUNC) were pretreated for 60 min at room temperature with 50 μL per well of a 50 μg/mL polylysine-HBr solution. After removal of the excess PLL, wells were coated with 50 μL of a 2:1 mixture of PGA (200 μg/mL) and cationic solution (*e.g.* $CaCl_2$ 1 mN plus NaCl 150 mN) respectively, and left overnight at 4°C. Unbound antigen was flicked off from the plate and nonspecific binding was blocked by incubating the wells 2 h at 37°C with gelatin (200 μg/mL in cationic solution). After removal of the excess gelatin, a first step antibody (antiserum or hybridoma culture supernatant) was added at 50 μL per well and incubated 1 h at 37°C. The plate was then washed ten times with the cationic solution using an Immunowash 8 (NUNC). Fifty μL of the second antibody (HRP-SAM or HRP-DAR, 1/500 in cationic solution) were incubated for a further hour at 37°C. After a second washing cycle, the binding of the antibodies was revealed by a chromogen-substrate solution (2,2′-azino-bis(3-ethylbenzthiazolinesulfonate 4.2 mg in 5.6 ml of citrate-phosphate buffer, pH 5.6). The absorbance of the solution was measured after 15 min with a Titertek multiscan at 405 nm. All data presented are averages of six values, except for Figure 3 and 4, where duplicates were obtained.

Inhibition Experiment

In this test, 62 μL of supernatant from each of the three clones were incubated for 1 h at room temperature with 145 μL of a (Ca^{2+} 1 mN/Na^+ 150 mN) cationic solution and 289 μL of antigen (pectin or alginate). These mixtures were then used as primary antibody solutions in a conventional ELISA test to detect pectin adsorbed onto the wells. The absorbance measured at the end of the assay was thus inversely proportional to the concentration of test antigen in the incubation solution.

RESULTS

Polyclonal Sera

The only sera to react with pectin on a dot blot test down to a 1/320 dilution were those from rabbits immunized with methylated BSA-coupled pectin (Fig. 2). Sera from rabbits injected with pectin-OVA responded weakly down to a 1/40 dilution. The pure pectic acid did not trigger the production of any specific antibody. The homopolygalacturonic acid is probably not immunogenic by itself and needs to be coupled to a carrier protein. Similar observations have been reported: the fungal polysaccharides do not stimulate the production of antibodies by animals and coupling with proteins or inoculation with whole cells is the only way to produce sera against

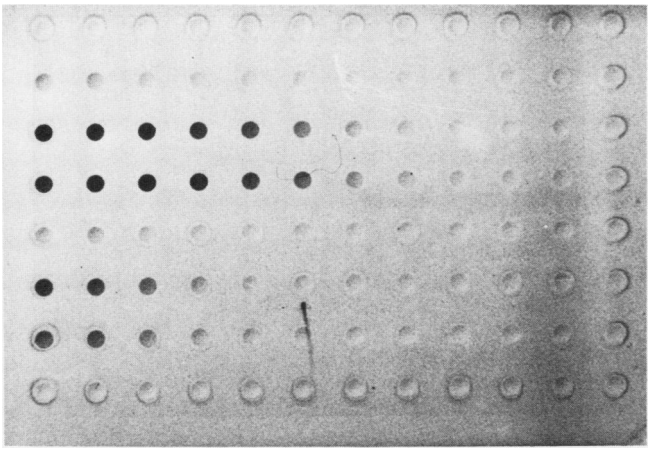

Figure 2. Dot blot assay of the sera from rabbits. Pectin has been adsorbed onto the paper. *Top and bottom rows:* the sera have been replaced by TBS. *Second row:* serum from preimmune animal. *Third and fourth:* sera from two rabbits immunized with PGA-methylated BSA. *Fifth:* serum from PGA-injected rabbit. *Sixth and Seventh:* sera from rabbits injected with OVA-coupled PGA.

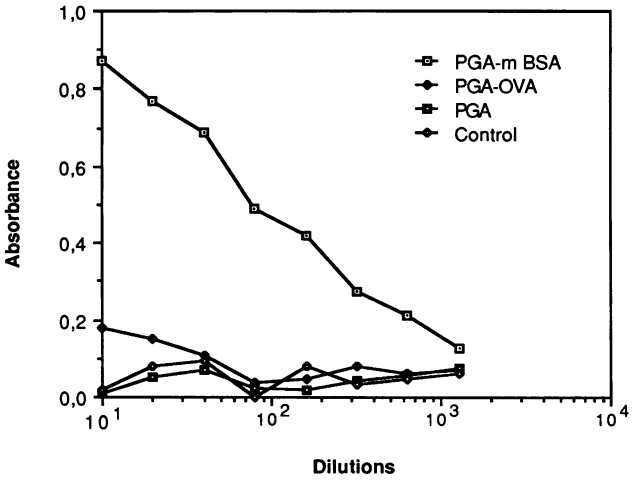

Figure 3. Assay of the mouse sera against pectin in an ELISA test. The only serum to bind specifically to the PLL-immobilized pectin was from mice immunized with mBSA-coupled pectin.

these polysaccharides (6). Mice sera tested by ELISA responded similarly (Fig. 3). The sera from methylated BSA-pectin injected mice recognized pure pectin down to a 1/640 dilution.

MoAbs

The fusion produced eight hybridomas secreting antibodies against pectin: two of them belonged to the murine IgG1 isotype, the six others were of the IgM type. Two IgM (1C7 and 7F7) and one IgG1 (2F4) were retained for further characterization. The supernatants from these three clones were titrated by 1/(2″) dilutions under optimal conditions (Fig. 4). The IgG1 (2F4) gave the best results at a 1/4 dilution and recognized pectin at least down to a 1/128 concentration of the MoAb. The two IgM responded much more weakly.

The optimal conditions for testing the antibodies were

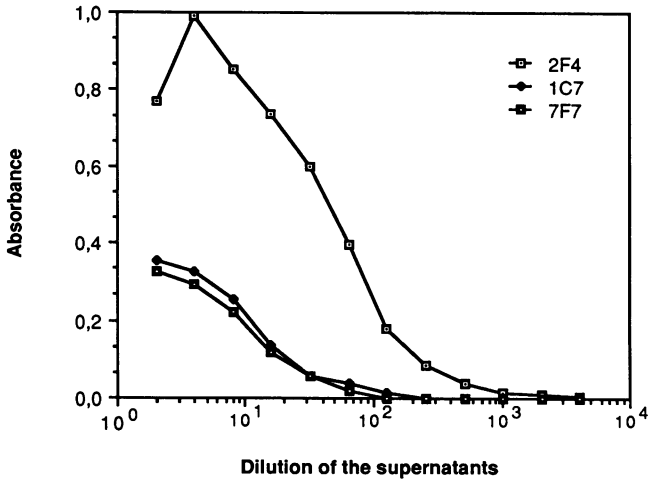

Figure 4. Titration curves of the IgG1 (2F4) and IgM (1C7,7F7) MoAbs.

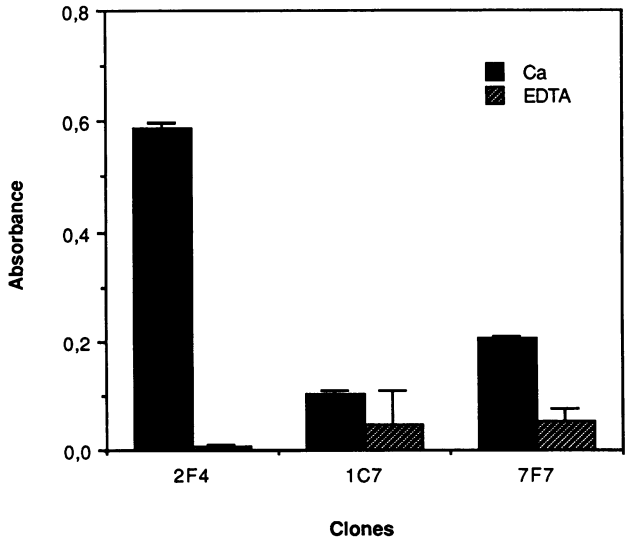

Figure 5. Pectin recognition by the MoAbs in presence of either Ca^{2+} or EDTA in an ELISA test.

established by trial and error. The above-mentioned literature reported that pectin associated through calcium bridges to form multimers, and that the association was limited to dimers formation in concentrated monovalent cations solutions. We have thus tested (Fig. 5) the ability of our antibodies to recognize pectin in presence of either $CaCl_2$ 1 mN or EDTA 5 mM, a well-known chelator of bivalent cations. These two chemicals were present throughout all the steps of the ELISA assay. The 2F4 MoAb has clearly an absolute requirement towards calcium to recognize pectin. The two IgM responded best in presence of Ca^{2+}.

We tested the effect of monovalent cations by keeping the calcium concentration of the cationic solutions constant at 1 mN and varying the sodium content from 1 mN to 900 mN. There is clearly an optimum Ca/Na ratio between 1/100 and 1/200 for the IgG1 (Fig. 6) and between 1/200 and 1/700 for the two IgM (Figs. 7 and 8). In all cases, at both high

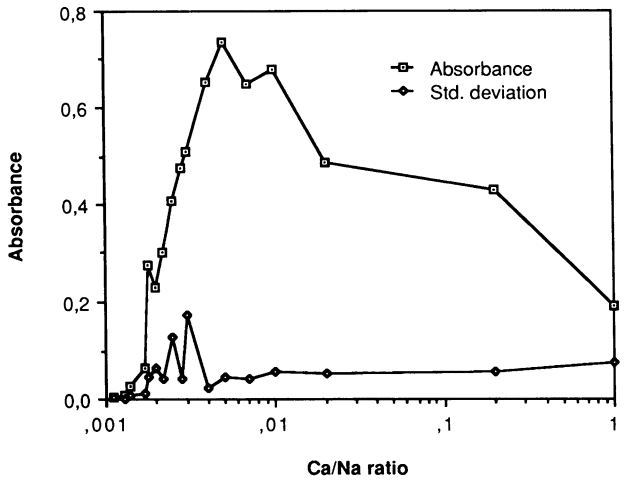

Figure 6. Effect of increasing Ca^{2+}/Na^{+} ratios on the binding of the 2F4 IgG1 to pectin.

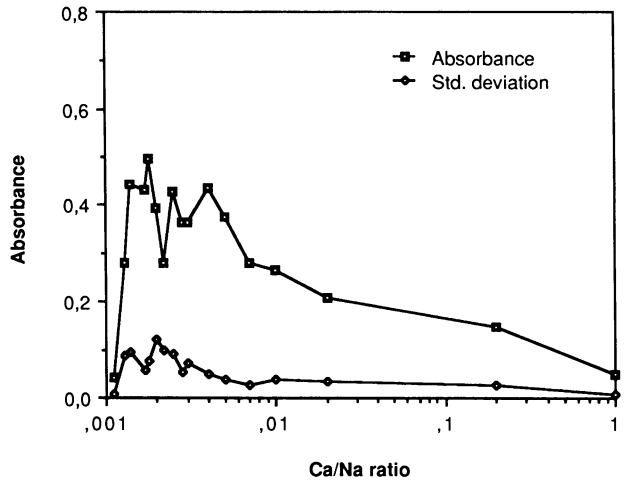

Figure 7. Effect of increasing Ca^{2+}/Na^{+} ratios on the binding of the 7F7 IgM to pectin.

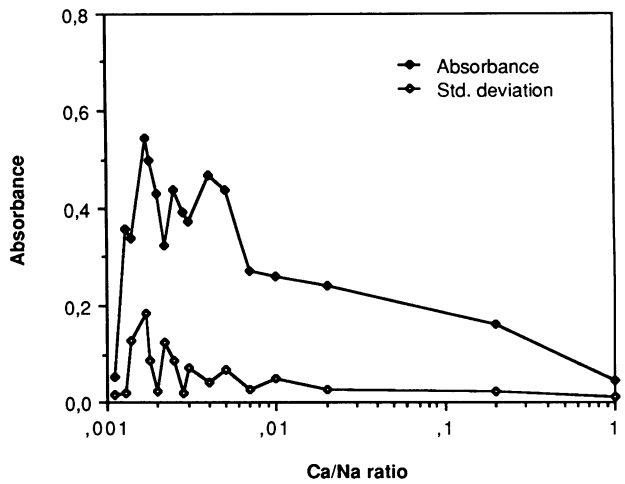

Figure 8. Effect of increasing Ca^{2+}/Na^{+} ratios on the binding of the 1C7 IgM to pectin.

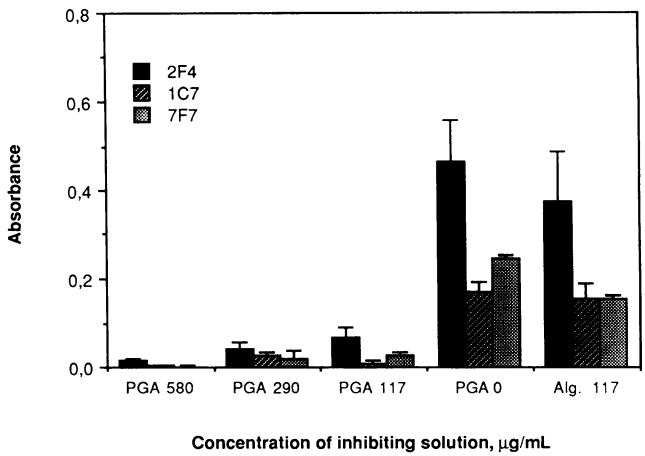

Figure 9. Inhibition of the three MoAbs by different concentrations of pectin or by an irrelevant (but 'egg-box' forming) alginate.

and low sodium concentrations, pectin is not detected by the antibodies.

The three MoAbs have been incubated with different concentrations of pectin and with alginic acid, a polysaccharide containing guluronic acid sequences that can adopt a similar 'egg box' conformation in presence of calcium. Free pectic acid effectively associated with the three MoAbs, inhibiting any further association with pectin immobilized in the wells (Fig. 9). Alginates in the 1/150 cationic solution did not cross-react with the antibodies, as appeared from the large positive reading of the test.

DISCUSSION

The molecular model (the 'egg box model') hypothesized (2) for explaining the large decrease of amplitude of the $n \rightarrow \pi^*$ band in the circular dichroism spectrum of a Ca^{2+} gel of PGA, although widely accepted, has not been up to now, neither confirmed nor ruled out by experimentation. However, the loss of intensity of the $n \rightarrow \pi^*$ dichroic band in presence of divalent ions could not be attributed to the 'cooperativity' of the polymer, because it is also observed with the monomer (P Debongnie, personal communication). The interpretation of the circular dichroism spectra in terms of conformational changes, whether cooperative or not, should therefore be considered with caution.

In this study, we have produced MoAbs against pectin. The MoAbs have been tested in presence of increasing Ca^{2+}/Na^{+} equivalent ratios, the calcium being kept constant at 1 mN. At swamping Na^{+} concentrations, intermolecular associations through Ca^{2+} bridges were probably hindered, and pectin chains were isolated and were not recognized by the antibodies. At intermediate Ca^{2+}/Na^{+} ratios, pectin was best recognized. It is worth noting that the optimum cationic ratio for MoAb recognition was higher than 1/300 and lower than 1/50. This last ratio is precisely the same as the one found by Morris *et al.* (10) working on equilibrium dialysis of pectin chains. They kept pectin in a constant calcium concentration of 12 mN and added progressively tetramethylammonium (TMA^{+}) cations up to 3 M. By increasing the $(CH_3)_4N^{+}$ from

zero, the relative amount of Ca^{2+} bound by polygalacturonate chains dropped to 50% at a 1/50 Ca^{2+}/TMA^{+} ratio and remained constant down to a 1/250 ratio. They did not try lower ratios, but they obtained pectin films containing only those calcium ions resistant to displacement by excess monovalent counterions, by immersing films in the Ca^{2+} salt form in 0.5 M NaCl for ~3 h. They concluded from their experiments that interchain association in hydrated systems with swamping levels of univalent counterions was limited to the formation of dimers of chains in a 2_1 conformation, with specific site-binding of Ca^{2+} along one face of each participating chain. These conclusions have received some support from an electron paramagnetic resonance and potentiometric study (1).

When lowering further the Na^{+} concentration (increasing Ca/Na ratios), the pectin was less and less recognized by the antibodies. It is well known that in calcium solutions, pectin forms strong tridimensional gels in which both faces of the zig-zag chains associate intermolecularly through calcium.

Clearly, our antibodies recognize a conformation of pectins that is most stable at a Ca^{2+}/Na^{+} ratio of about 1/150, and that does not exist to any large extent in concentrated calcium or sodium solutions. We believe that these antibodies are specific for a dimeric association of pectin chains such as the one described by the 'egg-box' model (10), and not for isolated chains or multimeric associations. Indeed, the loss of recognition of pectins by our antibodies in solutions of increasing calcium-sodium ratios suggests that the epitope identified by the MoAbs includes the external, unbound faces of the dimers. This specificity of the antibodies for the dimeric form could originate from the fact that the blood of the mice used for immunization has that calcium-sodium ratio. The pectic antigen is thus bound by the lymphocytes under the dimeric conformation, triggering production of antibodies specific mostly to that conformational epitope.

Human protein C, a member of the vitamin K-dependent plasma zymogens, undergoes Ca^{2+}-induced conformational changes required for activation by the thrombin-thrombomodulin complex. A Ca^{2+}-dependent monoclonal antibody that blocks protein C activation has been produced and used to study conformational changes near the activation site in protein C (13). Ca^{2+} not only bound to the antigen resulting in a conformational change exposing the epitope on the antigen, but it also bound to a calcium-specific site of the antibody, inducing a high affinity for the antigen and stabilizing the antigen-antibody complex.

We do not know whether calcium interacts to stabilize the pectin-antibody association, but it is clear from Figure 10 that the dimeric conformation of pectin is stabilized in high sodium solutions by the IgM antibodies. Such an antibody-antigen interaction has a generally short half-life and results in a continuous association-dissociation process during which antibody and antigen may become separated. In case of a multivalent antibody like the pentameric, decavalent IgM, the multiple bonds do not separate synchronously, making it less likely that the complex becomes separated. It is common that the multivalent IgM has an avidity of 10^2 to 10^4 times higher than the affinity of the isolated Fab fragments (14). In our case, the higher avidity of the IgM for the dimers of pectin

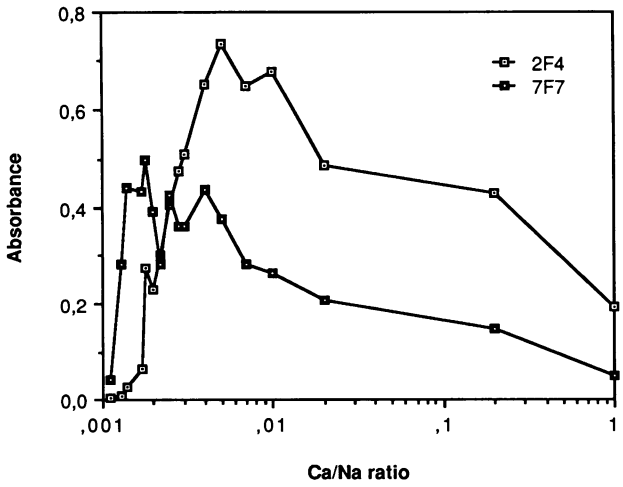

Figure 10. Comparison at increasing Ca^{2+}/Na^{+} ratios of the recognition of pectin by either an IgG1 (2F4) or an IgM (7F7) MoAb.

probably stabilized the calcium-pectin chelate to such an extent that calcium was not completely removed by EDTA treatment (Fig. 4).

In conclusion, we have produced MoAbs that probably recognize a particular supramolecular association of pectic acid chains: the dimeric form provided a suitable calcium/monovalent cation ratio is maintained during the test. The potential use of these antibodies to recognize homopolygalacturonic sequences in native pectins, to label pectins *in situ* by visible-electron microscopy or flow cytometry, and to realize immunoaffinity purifications is under study.

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