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Analysis of pectic epitopes recognised by hybridoma and phage display monoclonal antibodies using defined oligosaccharides, polysaccharides, and enzymatic degradation☆

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Abstract

The structure of epitopes recognised by anti-pectin monoclonal antibodies (mAbs) has been investigated using a series of model lime-pectin samples with defined degrees and patterns of methyl esterification, a range of defined oligogalacturonides and enzymatic degradation of pectic polysaccharides. In immuno-dot-assays, the anti-homogalacturonan (HG) mAbs JIM5 and JIM7 both bound to samples with a wide range of degrees of methyl esterification in preference to fully de-esterified samples. In contrast, the anti-HG phage display mAb PAM1 bound most effectively to fully de-esterified pectin. In competitive inhibition ELISAs using fully methyl-esterified or fully de-esterified oligogalacturonides with 3–9 galacturonic acid residues, JIM5 bound weakly to a fully de-esterified nonagalacturonide but JIM7 did not bind to any of the oligogalacturonides tested. Therefore, optimal JIM5 and JIM7 binding occurs where specific but undefined methyl-esterification patterns are present on HG domains, although fully de-esterified HG samples contain sub-optimal JIM5 epitopes. The persistence of mAb binding to epitopes in pectic antigens, with 41% blockwise esterification (P41) and 43% random esterification (F43) subject to fragmentation by endo-polygalacturonase II (PG II) and endo-pectin lyase (PL), was also studied. Time course analysis of PG II digestion of P41 revealed that JIM5 epitopes were rapidly degraded, but a low level of PAM1 and JIM7 epitopes existed even after extensive digestion, indicating that some HG domains were more resistant to cleavage by PG II. The chromatographic separation of fragments produced by the complete digestion of P41 by

☆ Part II. Analysis of pectin structure. For Part I, see Ref. [1].

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pectin lyase indicated that a very restricted population of fragments contained the PAM1 epitope while a (1→4)-β-D-galactan epitope occurring on the side chains of pectic polysaccharides was recovered in a broad range of fractions.

Keywords: Pectin; Pectic epitopes; Monoclonal antibodies; JIM5; JIM7; PAM1; LM5

1. Introduction

The plant primary cell wall consists of load bearing fibrous components (principally the cellulosic and hemicellulosic networks) embedded in a matrix of pectic polysaccharides that accounts for approximately 30% of the cell wall in dicotyledonous species. Pectic polysaccharides are complex, typically highly heterogeneous and composed of a number of structurally distinct domains. Homogalacturonan (HG) consists of contiguous stretches of (1→4)-α-D-galacturonic acid (GalA) residues that may be methyl esterified and/or acetylated and may be substituted with other sugars, such as xylose [2]. HG is believed to be synthesised in a highly esterified form but may be subsequently de-esterified in muro by the action of plant or fungal pectin methyl esterases (PMEs) which can remove methyl groups in a blockwise, or random fashion, respectively. The degree and pattern of methyl esterification has important commercial and biological implications since it is believed to be important in regulating the formation of cross-linked HG multimers and influences the gelling characteristics of the pectic network [3–6]. In rhamnogalacturonan I (RG I) domains, GalA residues alternate with rhamnose residues which are the point of attachment for side chains that predominantly contain galactose and arabinose. Rhamnogalacturonan II (RG II) consists of a relatively short galacturonan backbone with side chains and is extremely complex with respect to sugar composition and glycosyl linkages and may be covalently attached to other pectic domains [7,8].

The pectic network undoubtedly has important structural roles in the primary cell wall and the complexity and heterogeneity of the pectic polysaccharides probably reflects their multifunctionality. Pectic polysaccharides also appear to have roles in the control of cell wall ionic status and porosity, cell-to-cell adhesion, cell expansion, and signalling [5,9–12].

The structural analysis of pectin by chemical methods often requires the pooling of cell wall material from different cell types and extraction methods that may alter the extracted pectin. In contrast, immunochemical techniques typically require small amounts of material and monoclonal antibodies (mAbs) may be used to localise defined pectic domains in muro within a single cell wall. Several mAbs to HG exist. Two of these, JIM5 and JIM7, are widely used and are thought to bind to low-ester and high-ester pectins, respectively. However, the epitopes of JIM5 and JIM7 are not fully defined with respect to size or degrees and patterns of methyl esterification [13–15]. Other more defined anti-HG mAbs have also been generated. These include a phage display mAb (PAM1) which recognises large (containing about 30 contiguous de-esterified GalA residues) de-esterified blocks of HG [16], and an mAb (2F4) which recognises calcium-linked dimers of HG [17,18]. Two mAbs recognising precisely defined epitopes occurring on pectic side chains have been produced using neo-glycoproteins. The monoclonal antibody LM5 recognises four residues of (1→4)-β-linked galactose [19] while LM6 recognises five resides of (1→5)-α-linked arabinose [20]. Momoclonal antibodies have also been prepared that bind to RG I [21] and RG II [22].

We now report the use of a series of model pectins with defined degrees of methyl esterification (DE) and a range of pectic oligosaccharides with defined DE and defined degrees of polymerisation (DP) to further extend our understanding of the structure of the antigens and epitopes recognised by JIM5, JIM7, PAM1 and LM5. We have also combined analytical and immunochemical procedures in order to study the effects of the enzymatic fragmentation of HG domains on JIM5, JIM7 and PAM1 binding and to investigate the structural relationships between epitopes within pectic domains and macromolecules.

2. Experimental

Monoclonal antibodies.—The anti-HG mAbs JIM5 and JIM7 [13,14] and the anti-(1→4)-β-D-galactan mAb LM5 [19] have been previously described. All were generated in rats using hybridoma technology and were used unpurified from hybridoma supernatants. The anti-HG mAb PAM1 [16] was generated from a naive phage display library and consists of single chain antibody binding fragments (scFvs) expressed at the surface of M13 filamentous bacteriophage. Whole PAM1 phage particles were used throughout this study. A summary of the monoclonal antibodies used in this study is shown in Table 1.

Production of lime pectins with defined degrees of esterification (DE).—A series of lime pectins with defined degrees of methyl esterification were prepared by enzymatic and chemical treatments of a commercial highly methyl-esterified (81%) lime mother pectin (Grindsted™ Pectin URS 1200) as described elsewhere in this issue [1]. Throughout this study, this pectin is referred to as E81. Briefly, one series with a blockwise distribution of de-esterified GalA residues (P-series, DE 41–76%) was prepared using a plant PME while another with a random distribution of de-esterified GalA residues was prepared using a fungal PME (F-series, DE 11–76%) or a fungal PME followed by acid hydrolysis (DE 9%). A further series with a random distribution of de-esterified GalA residues was prepared by base catalysed de-esterification (B-series, DE 15–43%). A sample of completely de-esterified pectin (PGA, DE 0%) was prepared by treatment with fungal PME followed by base catalysed de-esterification [1].

Production of non and fully methyl-esterified oligogalacturonides with defined degrees of polymerisation (DP).—Polygalacturonic acid (PGA, 1% w/v in sodium acetate pH 5, 2 L) was treated with polygalacturonase I from *Aspergillus aculeatus* (80 U) for 45 min at 40 °C. The soln was boiled for 30 min in order to inactivate the enzyme and high molecular weight oligogalacturonides (OGAs) were precipitated with 6 N HCl at pH 2 [23]. The supernatant containing the lower molecular weight OGAs was recovered and adjusted to pH 12 with 5 N NaOH in order to dissolve aggregates [24]. Subsequently, the pH was set to 5 using Dowex-50W X8 (H^+) resin (Fluka). The OGAs were separated on a 1.2 L Source 15Q (Pharmacia) column equilibrated with 50 mM ammonium acetate, pH 5 using a gradient of 50 mM to 200 mM in 2 column volumes (CV). Subsequently, the concentration was increased in 5 CV to 400 mM followed by an 8 CV gradient to 1 M. Fractions (150 mL) were collected and aliquots analysed by high-performance anion-exchange chromatography (HPAEC) at pH 12 for the presence of OGAs. Fractions containing pure OGAs were pooled and concentrated to approximately 100 mL with a rotating evaporator at 50 °C. The OGAs were precipitated by the addition of an equal volume of 100 mM barium chloride and 300 mL 96% EtOH. The precipitate was washed twice with 60% EtOH and dissolved in 50 mL deionised water containing 5 g of H^+ loaded cation-exchanger (Dowex 50W X8) to exchange barium for H^+. This Dowex treatment was repeated once, in order to completely remove barium-ions. The Dowex was

Table 1
Specificities of anti-pectin monoclonal antibodies used in this study

mAb	Antigen	Epitope	Reference
JIM5	HG	unknown	VandenBosch and co-workers, 1989 [14]; Knox and co-workers, 1990 [13]
JIM7	HG	unknown	Knox and co-workers, 1990 [13]
PAM1	de-esterified HG blocks	~30 residues α-(1→4)-linked GalA	Willats and co-workers, 1999 [16]
LM5	pectic galactan	4 residues β-(1→4)-linked Gal	Jones and co-workers, 1997 [19]

removed by filtration and the filtrate freeze-dried. The freeze-dried OGAs were analysed using HPAEC at pH 12 and MALDI–TOF–MS.

Methyl esterification was performed essentially as described [25,26]. Purified OGAs (1% w/v) were incubated in anhyd 0.02 N methanolic H_2SO_4 at 4 °C for at least 14 days. After completion of the esterification reaction, the OGAs were recovered. Since OGAs are not solubilised completely in methanolic acid, the insoluble residue was separated from the soln by centrifugation and washed twice with isopropanol and dried under a stream of air. The soluble fraction was neutralised with solid barium carbonate and then centrifuged. The supernatant was collected and the solid material washed with anhyd MeOH. These supernatants were combined and dried under a stream of air at ambient temperature. Both the soluble and insoluble part was dissolved in water, freeze-dried and stored at −20 °C until used. The products were analysed by MALDI–TOF–MS. The purity of all OGAs (percentage area of PAD response trace) ranged from 63 to 93% apart from methyl-esterified OGAs with DP of 8 and 9 which had purities of 58 and 51%, respectively.

Digestion of lime pectin with PL and PG II.—Lime pectin with a DE of 41% produced by plant PME (P41) was digested with endo-pectin lyase (PL, EC 3.2.1.15) or endo-polygalacturonase II (PG II, EC 4.2.2.10) [27], both from *Aspergillus niger* [1]. P41 was dissolved in 50 mM NaOAc (pH 5.0 for PL and pH 4.2 for PG II) at a concentration of 5 mg mL^{-1} by overnight rocking at room temperature (rt). Endo-pectin lyase (PL, 0.1 U) or PG II (0.2 U) was added to 1 mL of the above pectin soln and in both cases incubated at rt for 20 h. The reaction was stopped by boiling samples for 5 min. For time course analysis of PG II digestion, P41 or lime pectin with a DE of 43% produced by fungal PME (F43) were digested as described above but aliquots were taken at selected time-points and in each case the reaction stopped by boiling. The progression of digestion was followed by determining the production of newly formed reducing ends as described by Nelson and Somogy [28].

Chromatographic separation of PL digestion products.—The fragments produced by PL digestion of P41 were separated using an analytical weak anion-exchange column (Mono P, HR 5/5 Pharmacia). Deionised water and 0.75 M NH_4HCO_3 in deionised water were used as eluents A and B, respectively at a flow rate of 1 mL min^{-1}. Eluents were de-gassed prior to use. Pectin fragments were detected by UV absorption at 235 nm (Aekat Explorer 100, Pharmacia). The digest sample was centrifuged and filtered and 500 μL loaded onto the column which had been equilibrated with 100% A. Fully methyl-esterified oligomers were eluted with 100% A in 5 min. Thereafter, a linear gradient (0–10% B in 20 min, 10–50% B in 30 min and 50–100% B in 5 min) was used for elution of partially methyl-esterified oligomers. After each run the column was washed for 5 min with 100% B and re-equilibrated for 15 min with 100% A. Fractions (1 mL) were collected throughout the separation and freeze-dried to remove excess NH_4HCO_3. Fractions were re-dissolved in 1 mL water prior to detection of pectic epitopes by immuno-dot-assays.

Immuno-dot-assays (IDAs).—Pectic samples were dissolved in water to a concentration of 5 or 10 mg mL^{-1} and applied by hand as 1 μL aliquots to nitrocellulose (Schleicher & Schuell, Dassel, Germany) in a five- or ten-fold dilution series. Nitrocellulose membranes were then air-dried at rt for at least 30 min. All subsequent treatments were at rt. Membranes were blocked with phosphate buffered saline (PBS) containing 5% fat-free milk powder (MPBS) for 1 h prior to incubation with primary antibodies. Hybridoma supernatants of JIM5, JIM7 or LM5 were diluted 1/10 in MPBS while PAM1 was used at a concentration of ca. 10^{11} phage mL^{-1} in MPBS (corresponding to an approximately 1/100 dilution of phage prepared by polyethylene glycol precipitation [16]). In all cases, membranes were incubated in primary antibodies for 1.5 h. After washing extensively under running tap water and for 10 min rocking in PBS containing 0.1% (v/v) Tween 20, membranes were incubated for 1.5 h in a secondary antibody (anti-rat horse radish peroxidase conjugate, Sigma, Poole, UK or anti-M13 horse radish

peroxidase, Pharmacia) diluted 1/1000 in MPBS. Membranes were again washed as described above prior to development in substrate soln (25 mL deionised water, 5 mL MeOH containing 10 mg mL^{-1} 4-chloro-1-naphthol, 30 μL 6% (v/v) H_2O_2).

Competitive inhibition enzyme linked immuno-sorbent assays (ciELISAs).—ciELISAs were used to assess binding of antibodies to potential oligosaccharide and polysaccharide inhibitors in soln. Microtitre plates (Maxisorp, Nunc, Denmark) were coated (100 μL $well^{-1}$) overnight at 4 °C with P41 (50 μg mL^{-1}) in Tris-buffered saline (TBS). After brief washing in tap water, plates were blocked at rt with 3% bovine serum albumin in TBS in (3% BSA/TBS) for 2 h (200 μL $well^{-1}$). Following brief washing with tap water, competitor solns (OGAs or P41) were applied (100 μL $well^{-1}$) as serial dilutions in 3% BSA/TBS while JIM5 or JIM7 were added to all wells as 1/100 dilutions of hybridoma supernatants (corresponding to ca. 90% maximal binding on antibody capture ELISAs). After 2 h, the plates were washed extensively with tap water and secondary antibody (anti-rat horse radish peroxidase conjugate, Sigma, Poole, UK, diluted 1/1000 in 3% BSA/TBS) applied (100 mL $well^{-1}$) and incubated for 2 h at rt. Following extensive washing in tap water, plates were developed with 150 μL $well^{-1}$ of a tetramethyl-benzidine-based substrate. After stopping the reaction with 2 M H_2SO_4 (35 μL $well^{-1}$) absorbances were read at 450 nm. Concentrations of competitors resulting in 50% inhibition (IC_{50}) of antibody binding were determined by plotting competitor concentrations against absorbance. Values from controls with no competitor were taken as 0% inhibition of antibody binding, and values from controls with no antibody represented 100% inhibition of binding.

3. Results and discussion

The effects of the degree and distribution of methyl esterification on antibody binding.—A series of model pectins with defined degrees and patterns of esterification were prepared from a common mother lime pectin by treatment with plant or fungal PMEs and chemical modifications. The effects of the extent and pattern of methyl esterification on antibody binding was assessed by IDAs and ciELISAs.

The binding in IDAs of the anti-HG mAbs JIM5, JIM7 and PAM1 to these series of model pectins is shown in Fig. 1. For the samples de-esterified with plant PME (P-series), fungal PME (F-series) and base catalysis (B-series), there was some correlation between JIM5 binding and DE with binding increasing with decreasing DE down to a DE of 31% (F31) as shown in Fig. 1(a). Below this level of DE, the correlation was reversed with JIM5 binding decreasing with decreasing DE, and JIM5 binding to polygalacturonic acid (PGA, DE 0%) was weak. In contrast, PAM1 bound most strongly to PGA or samples in which large de-esterified blocks were formed either by the blockwise action of plant PME or by very extensive random de-esterification (F11, FA9 and B15) as shown in Fig. 1(c). Compared to JIM5, the binding of JIM7 did not correlate closely with DE as shown in Fig. 1(b). For example, there was no significant difference in JIM7 binding to pectin samples with a DE of 81 and 15% (E81 and B15). However, in common with JIM5, optimal JIM7 binding was clearly dependent on the presence of some methyl ester groups since the binding of JIM7 to PGA, F11 and FA9 was weak. For both JIM5 and JIM7 the degree, rather than the pattern of methyl esterification appeared to be the more important factor influencing binding. For example, for both JIM5 and JIM7, binding to P41 (blockwise de-esterification) and F43 (random de-esterification) was similar (Fig. 1(a) and (b)). In contrast, for PAM1 binding, both pattern and degree of methyl esterification was important in influencing binding and while PAM1 bound relatively strongly to P41, it did not bind to F43 (Fig. 1(c)). These results indicate that JIM5 and JIM7 epitopes are produced by a wide range of degrees and distributions of methyl esterification.

The model pectin series was also probed with the anti-β-(1→4)-D-galactan mAb LM5 as shown in Fig. 1(d). Monoclonal antibody LM5 bound with approximately equal intensity to E81, P-series samples with DE from 76

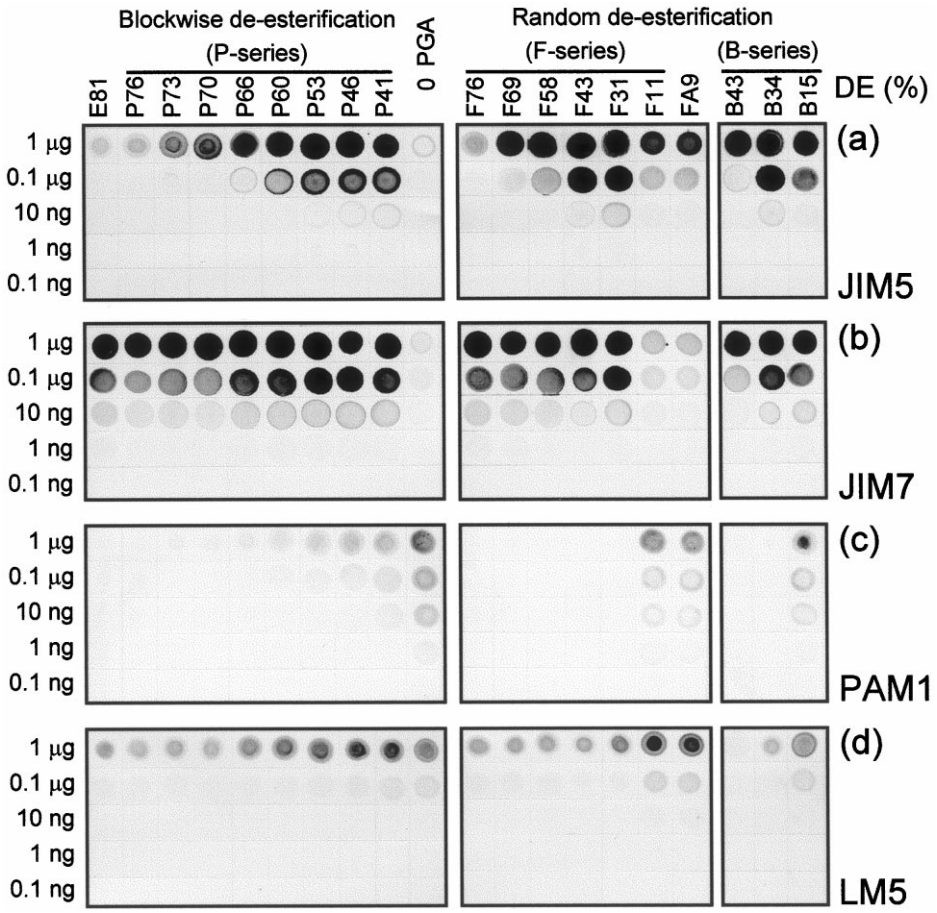

Fig. 1. Immuno-dot-assays of JIM5 (a), JIM7 (b) PAM1 (c) and LM5 (d) binding to lime pectins with defined degrees of esterification (DE). High-ester lime pectin (E81) was de-esterified in a blockwise fashion by treatment with plant pectin methyl esterase (PME, P-series) or in a random fashion by treatment with fungal PME alone (F-series) or by treatment with fungal PME followed by acid hydrolysis (FA9). A further series of randomly de-esterified pectin samples was prepared by base catalysed hydrolysis (B-series). A sample of completely de-esterified pectin (PGA) was prepared by treatment with fungal PME followed by base catalysed de-esterification. All samples were applied in dilution series at the levels indicated.

to 53% (P76 to P53) and F-series samples with DE from 76 to 31% (F76 to F31) as shown in Fig. 1(d). Binding of LM5 was slightly stronger to P46, P41, PGA, F11 and FA9. The greater binding of LM5 to samples with lower DE may be because epitope accessibility at lower DE is increased by conformational hanges in pectin structure that are associated with DE. There was also an increase in LM5 binding with decreasing DE for the B-series samples. However, in contrast to F43, LM5 bound very weakly to B43 and it is possible that base catalysed de-esterification also had a direct effect on the LM5 epitope or caused conformational changes that resulted in reduced LM5 binding.

The binding of JIM5, JIM7, PAM1 and LM5 to acetylated HG (from sugar beet), RG I (from sycamore cell walls) and RG II (from red wine) was also analysed by IDAs. None of these mAbs bound to any of these samples at the highest level tested (1 mg) (data not shown).

The binding of JIM5 and JIM7 to oligogalacturonide haptens.—In order to fully define the nature of an epitope occurring in a complex heteropolymer such as pectin, the capacity of defined oligosaccharides to inhibit antibody binding to a known immobilised antigen must be determined. To further explore the epitope structures of JIM5 and JIM7, a series of fully de-esterified OGAs and a series of fully methylesterified OGAs (MeOGAs) were tested for their capacity to inhibit JIM5 and JIM7 binding to immobilised P41 in ciELISAs as shown in Fig. 2 and Table 2. Immobilised antigen P41 was used, because JIM5 and JIM7 both bind

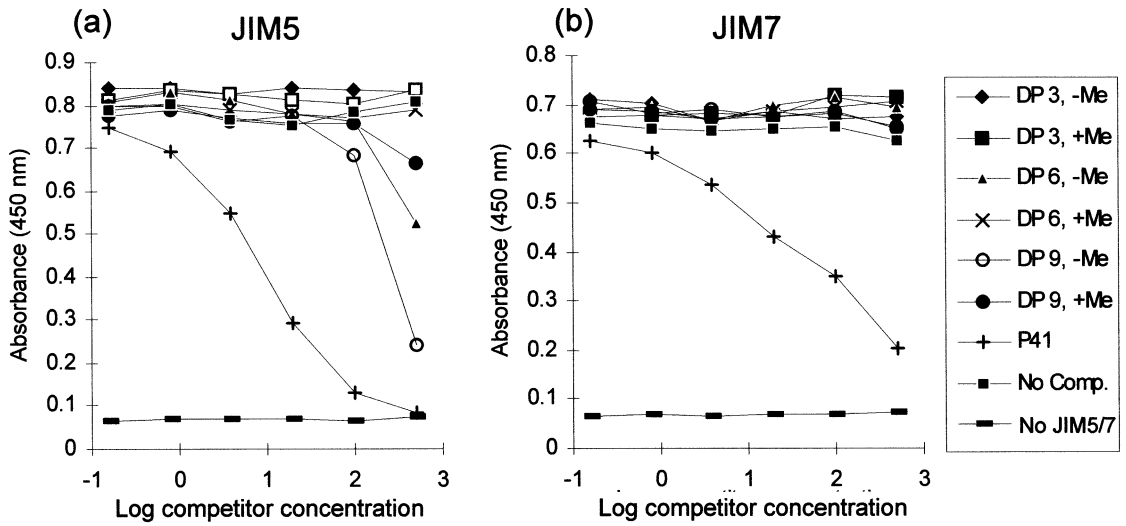

Fig. 2. Competitive inhibition ELISA analysis of JIM5 (a) and JIM7 (b) binding to oligogalacturonides (OGAs) with defined degrees of polymerisation (DP). OGAs were either fully methyl-esterified (+Me) or completely de-esterified (−Me). Lime pectin with a degree of esterification of 41% (P41) was used as the immobilised antigen and as a soluble inhibitor.

strongly to P41 in antibody capture ELISAs (data not shown). No OGAs or MeOGAs up to a DP of 9 (the highest DP for which samples of high purity were available) inhibited JIM7 binding at the highest level tested (0.5 mg mL^{-1}) (Fig. 2(b) and Table 2). For JIM5, no samples with DP less than 7 produced 50% inhibition at the highest level used (0.5 mg mL^{-1}). However, OGAs with a DP of 7 did weakly inhibit JIM5 binding (Fig. 2(a) and Table 2) and while OGAs with a DP of 9 (OGA9) were the most effective inhibitor of JIM5 binding of the samples tested, MeOGAs with a DP of 9 failed to produce 50% inhibition of JIM5 binding at the highest level tested (0.5 mg mL^{-1}) (Fig. 2(a) and Table 2). The IC_{50} of OGA9 required for inhibition of JIM5 binding was over 30-fold greater than the IC_{50} of P41 (used as a soluble inhibitor) required for inhibition of JIM5 binding (Table 2) indicating that OGA9 did not contain an optimal JIM5 epitope structure. These results indicate that there is likely to be a range of sub-optimal binding sites to which some JIM5 binding occurs. In order to fully characterise the epitopes of JIM5 and JIM7, a series of OGAs with precisely defined patterns of methyl esterification will be required.

The effects of enzymatic degradation of P41 on epitope occurrence.—In order to further explore the structural features and spatial relationships of the epitopes of JIM5, JIM7, PAM1 and LM5 in the context of pectic polysaccharide macromolecules, we examined epitope occurrence after complete digestion of P41 by both endo-pectin lyase (PL) and endo-polygalacturonase II (PG II) [1]. Endo-pectin lyase cleaves HG preferentially, at regions which are completely methyl-esterified while PG II cleaves HG preferentially at regions which are completely de-esterified [1]. Antibody binding to digested samples was assessed by IDAs as shown in Fig. 3.

Anti-HG monoclonal antibody JIM5 binding to P41 was totally abolished by PG II digestion, but binding was less affected by PL digestion as shown in Fig. 3(a). This indicates that JIM5 binding occurs in regions of HG in

Table 2
Binding of JIM5 and JIM7 in competitive inhibition ELISA to de-esterifed (−Me) and fully methyl-esterified (+Me) oligogalacturonides with degrees of polymerisation (DP) from 6–9

	JIM5	JIM7
	IC_{50} (μg mL^{-1})	IC_{50} (μg mL^{-1})
DP 6, −Me	>500	>500
DP 6, +Me	>500	>500
DP 7, −Me	326	>500
DP 8, −Me	324	>500
DP 9, −Me	260	>500
DP 9, +Me	>500	>500
P41	8.5	73

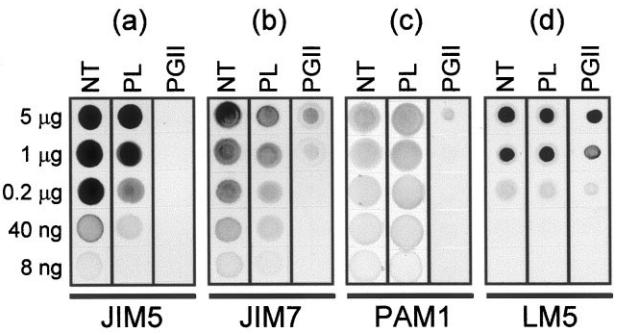

Fig. 3. The effects of endo-pectin lyase (PL) and endo-polygalacturonase (PG II) digestion on anti-pectin mAb binding. Lime pectin with a DE of 41% (P41) was probed by immunodot-assays with JIM5 (a), JIM7 (b) PAM1 (c) and LM5 (d) following complete digestion with PL or PG II. Control samples were untreated (NT). Samples were applied in dilution series at the loading levels indicated.

which there are sufficient contiguous de-esterified GalA residues to form PG II cleavage sites and that the methyl-esterified regions of HG, that are cleavage sites for PL, do not contain optimal JIM5 epitopes. Binding of JIM7 to P41 was also slightly reduced by PL digestion and was significantly reduced by PG II digestion (Fig. 3(b)), but in contrast to JIM5, there was some residual binding of JIM7 to P41 following complete PG II digestion. The post-PG II treatment JIM7-reactive component was resolved predominantly on the nitrocellulose as a smaller diameter dot compared with the untreated P41. The appearance or immunoprofile of heterogeneous pectic samples when applied to nitrocellulose and probed with mAbs is related to the relative mobilities of different pectic components within the sample away from the point of application on the nitrocellulose before drying and dot size relates to the degree of branching and size of the components [29]. Unbranched HG regions migrate further than branched domains producing a dot or ring of greater diameter than less mobile larger and/or branched components. The immunoprofile of the post-PG II JIM7-reactive component suggests that a relatively large and/or branched (and therefore relatively immobile) JIM7-reactive component of P41 is more resistant to PG II digestion than a more mobile component which may be relatively unbranched and HG-rich. Endo-pectin lyase (PL) and PG II digestion did not have a significant effect on LM5 binding since the β-(1→4)-D-galactan epitope recognised by this mAb is not a substrate for PL or PG II. However, the very faint outer rings resolved by probing untreated P41 and PL treated P41 with LM5 were absent following PG II digestion. This suggests that a low level of the LM5 epitope is located on relatively mobile components that contained PG II cleavage sites.

We also investigated the temporal profile of the loss of the JIM5, JIM7, and PAM1 epitopes during the fragmentation of P41 and F43 by PG II digestion over time as shown in Fig. 4. The production of PG II cleavage products was assessed by measuring the rate of formation of newly formed reducing ends as shown in Fig. 4(a). The fragmentation of P41 by PG II was faster than the fragmentation of F43, presumably because the regions of contiguous de-esterified GalA residues that constitute optimal PG II cleavage sites are more abundant in P41 than in F43. This accords with the greater binding of PAM1 to P41 compared to F43 shown in Fig. 1(c). Abolition of PG II activity by boiling was not instantaneous because the temperature optimum of the enzyme was passed through during boiling and some decrease in JIM5, JIM7 and PAM1 binding was observed at 0 min as shown in Fig. 4(b–f). As a control, the P41 digest fragments were also probed with LM5, the binding of which was essentially unaffected by PG II digestion, as shown in Fig. 4(g).

Binding of JIM5, JIM7 and PAM1 to PG II treated P41 was greatly reduced by 5 min as shown in Fig. 4(b), (c) and (f). In contrast, some JIM5 binding to F43 persisted up to 30 min digestion (Fig. 4(d)), reflecting the slower fragmentation of F43 compared to P41 (Fig. 4(a)). However, despite this slower fragmentation of F43 compared to P41, JIM7 binding to F43 was reduced compared to P41 (Fig. 4(e)), and binding was abolished in 45 min. This suggests that although fragmentation of F43 was slower, the PG II digestion products of F43 contained less JIM7-reactive fragments compared to the products produced by the digestion of P41. Although most PAM1 reactivity was lost by 5 min digestion of P41, a low level of PAM1 reactivity was present even

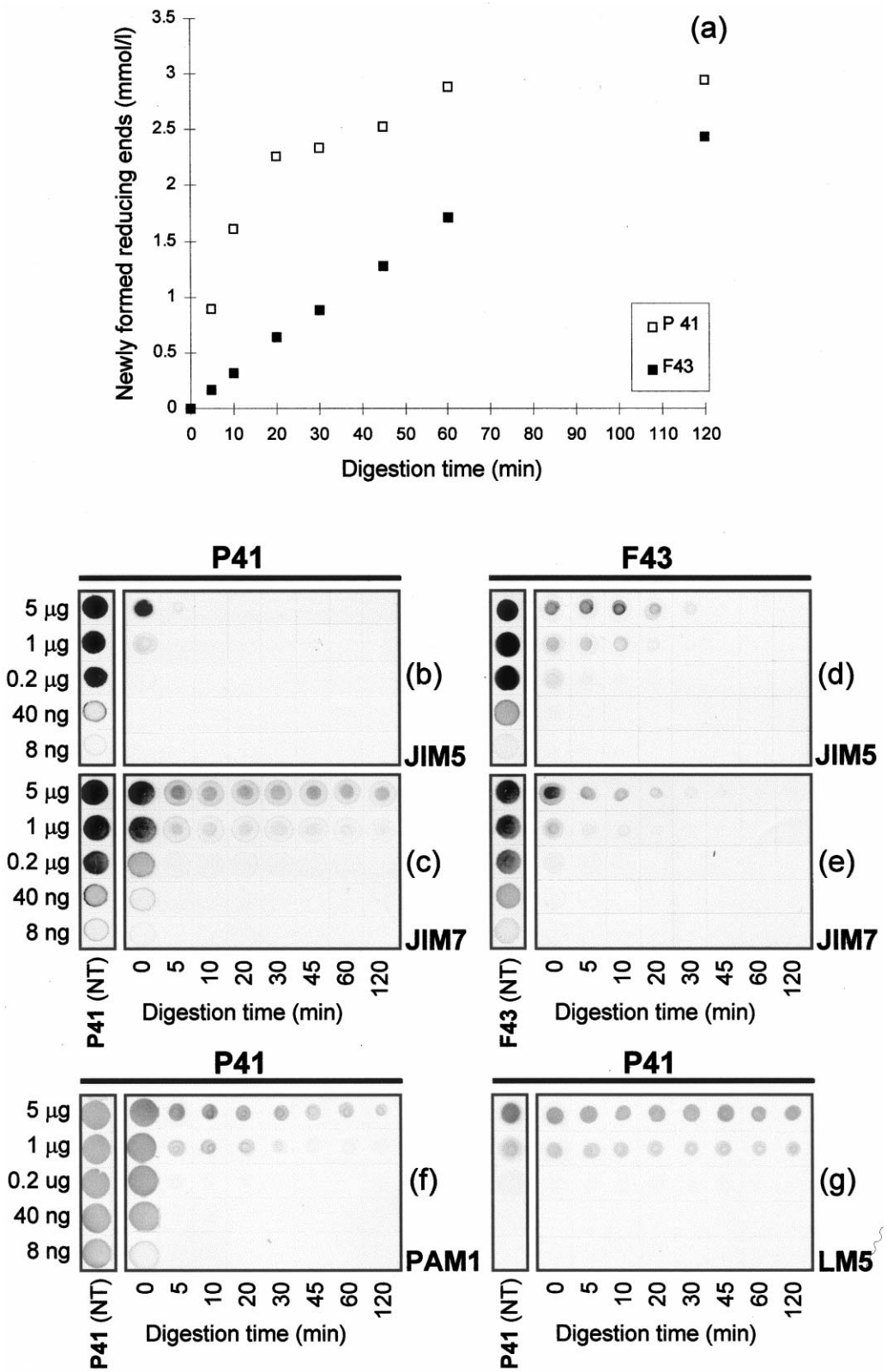

Fig. 4. Time-course analysis of the degradation of pectin epitopes by PG II. The rate of degradation of P41 and F43 was assessed by the generation of newly formed reducing ends as detected by Nelson and Somogy assays (a). The binding of JIM5 (b), JIM7 (c), PAM1 (f) and LM5 (g) to digest fragments of P41 or binding of JIM5 (d) and JIM7 (e) to digest fragments of F43 was assessed by immuno-dot-assays. Samples were applied in dilution series at the loading levels indicated.

after 120 min digestion (Fig. 4(f)) indicating that some of de-esterified stretches of HG in P41 were resistant to even extended periods of PG II digestion. It is significant that the dot size in all the P41 samples probed with LM5 (Fig. 4(g)) and for samples taken at 0 min onwards and probed with JIM5 (Fig. 4(b)) or from 5 min onwards and probed with PAM1 (Fig. 4(f)), were approximately half the diameter of those produced by undigested P41 and probed with JIM5 and PAM1 (Fig. 4(b) and (f)). On the basis of the immunoprofiling observations described above, this suggests that most of the de-esterified stretches of GalA that comprise the PAM1 epitope are within unbranched (and therefore relatively mobile) regions of pectic backbone that are rapidly digested by PG II. However, there may also be low levels of de-esterified GalA blocks that are embedded within branched (and therefore relatively immobile) regions that are more PG II resistant. Some regions of HG may be protected from PG II activity by the proximity of side chains that may limit the access of PG II to the backbone. It has previously been shown that endo-PG digestion of lemon and apple pectin results in the accumulation of a polymeric component, rich in neutral sugars [30]. In contrast to PAM1, no residual JIM5 binding was observed after 5 min digestion (Fig. 4(b)). This may have been due to a difference in detection limits of the two mAbs but may also indicate that the relatively PG II resistant components recognised by PAM1 are completely de-esterified, and therefore bound only weakly by JIM5 (see also Fig. 1(a)).

Analysis of pectic fragments produced by digestion with pectin lyase and separated by anion-exchange chromatography.—In order to further explore the complex structure of pectic polysaccharides and their fragmentation by PL, fractions obtained from anion-exchange chromatographic separation of a complete PL digest of P41 were probed with PAM1 and LM5 using IDAs as shown in Fig. 5. The epitopes of PAM1 and LM5 are unaffected by PL digestion as shown in Fig. 3, see also [1]. Following PL digestion, only a very limited population of the fragments produced (Fractions 61–71) contained the PAM1 epitope. Fractions 67 and 68 eluting between approximately 63 and 64 min, were identified as containing the highest levels of PAM1 epitope by probing a dilution series of selected PAM1-reactive fractions as shown in Fig. 5(c). The late elution of fragments containing the PAM1 epitope is consistent with the high charge density associated with the large number of de-esterified residues required for PAM1 binding. Monoclonal antibody LM5 bound most strongly to material eluting almost immediately (Fractions 2 and 3), indicating that an essentially uncharged β-(1→4)-galactan-rich component that was excised by PL digestion was not retained on the column. Most fractions eluting after approximately 21 min (Fractions 23 onwards) contained some LM5 reactivity indicating that β-(1→4)-galactan side chains were also attached to a series of pectic fragments with a range of charge densities.

4. Conclusions

The binding requirements of PAM1 and LM5 are well defined, but the optimal binding requirements of JIM5 and JIM7 are not fully characterised and from the work reported here, it is clear that both antibodies can bind to a range of undefined sub-optimal epitope structures, involving methyl-esterified HG. This is significant because both JIM5 and JIM7 have been widely used as probes for HG in immunolocalisation studies. The interpretation of some of this work may now need to be modified and the use of these antibodies in future work should be approached with the findings of this study in mind. JIM5 bound weakly to completely de-esterified pectin but binding was greatly increased by the presence of methyl-esterified GalA residues up to level of about 40%. At DE greater than this JIM5 binding was reduced. Differences in DE did not significantly influence JIM7 binding over a range of DE from about 15 to 80%. However, the presence of some methyl groups is clearly required for optimal JIM7 binding since JIM7 binding was weaker to model pectins that had DE less than about 15%. In contrast to PAM1, the pattern (blockwise or random) of methyl-esterification did not appear to signifi-

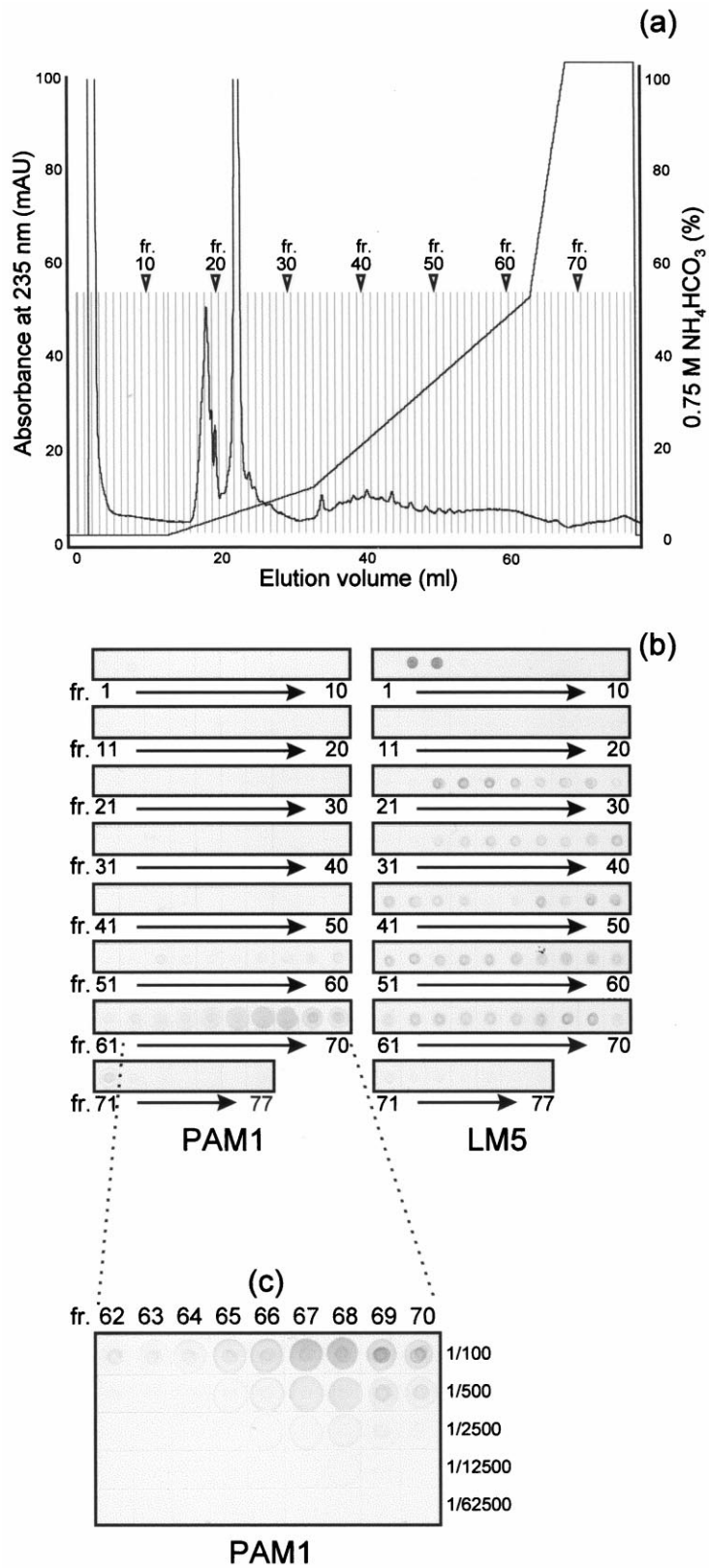

Fig. 5. Analysis of the occurrence of the epitopes of PAM1 and LM5 following complete digestion of P41 by PL. Digest fragments were fractionated by anion-exchange chromatography and oligomers detected by their absorbance at 235 nm (a). Fraction numbers are indicated (fr.). The distribution of PAM1 and LM5 epitopes within the fraction series was analysed by immuno-dot-assays (b). The binding of PAM1 to selected Fractions (62–70) was further assessed by probing dilution series of these fractions (c). Dilution factors in (c) refer to the level of dilution of column fractions.

cantly influence JIM5 and JIM7 binding to the model pectin series used in this study. To define the optimal binding requirements for JIM5 and JIM7, a range of pure and fully characterised oligogalacturonides with known degrees and patterns of methyl esterification will be required. The use of enzymatic degradation in this study has emphasised the complexity of pectic polysaccharides and indicated that epitopes may occur on a range of structurally distinct domains within the polymers. Used in combination with degradative and chromatography procedures, monoclonal antibodies with defined epitopes such as PAM1 and LM5 are powerful tools for the dissection of the molecular structure of complex pectic polysaccharides.

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