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High-throughput microarray analysis of pectic polymers by enzymatic epitope deletion

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Abstract

Pectic polysaccharides are abundant plant cell wall glycan polymers and are also widely used as functional food ingredients. The fine structure of pectins, and especially the degree and pattern of methyl-esterification of the homogalacturonan (HG) backbone, play a major part in determining functional properties. We have developed a high-throughput method of analysing pectic polymers by combining microarray-technology, pectin lyase (PL) and anti-HG monoclonal antibodies (mAbs). The technique demonstrates that pectins immobilised in a microarray format are effective substrates for PL, and that epitope loss or degradation can be tracked by the deletion of mAb binding.

Keywords: Pectin; Microarrays; Antibodies

1. Introduction

Pectic polysaccharides constitute a major proportion of the cell walls of all land plants and have several known biological roles: they are involved in regulating wall porosity and have roles in signaling, defense, development and cell-to-cell adhesion (Ridley, O'Neil, & Mohnen, 2001; Willats, McCartney, Mackie, & Knox, 2001a). Extracted pectin also has numerous applications as a functional food ingredient and is widely used as a gelling agent in the production of jams and jellies, fruit juice, confectionary products, and bakery fillings. Pectin is also used for the stabilisation of acidified milk drinks and yogurts (Thakur, Singh, & Handa, 1997; Willats, Knox, & Mikkelsen, 2006).

Pectins are a family of glycan polymers based on a galacturonan backbone (Carpita & Gibeaut, 1993). Although pectic polymers are extremely diverse in their fine structures, three major domains are generally recognized, HG, rhamnogalacturonan-I (RGI) and rhamnogalacturonan-II (RGII). HG is a linear polymer consisting of α(1-4)-linked galacturonic acid (GalA), whilst RGI consists of the repeating rhamnogalacturonan (RG) disaccharide (→4)-α-D-GalA-(1→2)-α-L-Rha-1→] to which a variety of different glycan chains (typically rich in arabinan and galactan) are attached to the rhamnose (Rha) residues (O'Neill, Albersheim, & Darvill, 1990). Despite its name, RGII has a backbone of HG rather than RG, with complex side chains attached to the GalA residues. GalA is typically methyl-esterified to a greater or lesser extent and this is controlled *in planta* by the activity of a large number of pectin methyl-transferase and pectin methyl-esterase enzymes (Catoire, Pierron, Morvan, Hervé du Penhoat, & Goldberg, 1998). Both the degree of methyl-esterification (DE), and also the distribution pattern (PE) of

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methyl-esters along the HG backbone have important implications with respect to calcium cross-linking and pectin gel formation (Löfgren, Walkenström, & Hermansson, 2002). This in turn impacts upon the functionality of pectic polymers both *in planta* and in industrial applications (Knox, Linstead, King, Cooper, & Roberts, 1990). However, our current understanding of the structure/function relationships within pectic matrixes is limited.

Monoclonal antibodies and pectinolytic enzymes are valuable tools for the analysis of pectin. An extensive library of mAbs with well defined specificities for both HG and the side chains of RGI are available (Liners, Letesson, Didembourg, & Van Cutsem, 1989; Knox, 1997). For anti-HG mAbs, epitope specificity may be defined by both DE, PE and the extent of calcium cross-linking. The epitope structures of the JIM5 and JIM7 anti-HG mAbs used in this study have previously been characterized in detail using synthetic oligogalacturonides with known DEs and PEs (Clausen, Willats, & Knox, 2003). JIM5 binds to un-esterified GalA residues with adjacent or flanking methyl-esterified residues. In contrast, JIM7 binds to methyl-esterified residues with adjacent or flanking un-esterified GalA residues. Similarly, for pectinolytic enzymes that cleave HG, the DE and PE dictate if, and where cleavage occurs along the HG backbone. For example, both endo-polygalacturonases (endoPGs) and pectin lyases (PLs) both cleave HG, but whereas endoPGs preferentially cleave non-esterificed HG, PLs preferentially cleave methyl-esterified HG (Limberg et al., 2000; Sanchez-Torres, Visser, & Benen, 2003; van Pouderoyen, Snijder, Benen, & Dijkstra, 2003).

Our aim in this work was to combine the use of mAbs and PL in a microarray format for high-throughput pectin analysis. The strategy we employed involved printing a library of pectin samples as microarrays which were then subject to *in situ* enzymatic digestion. The activity of PL on each pectin sample was assessed by probing arrays before and after digestion with HG-specific mAbs. The experimental plan is outlined in Fig. 1.

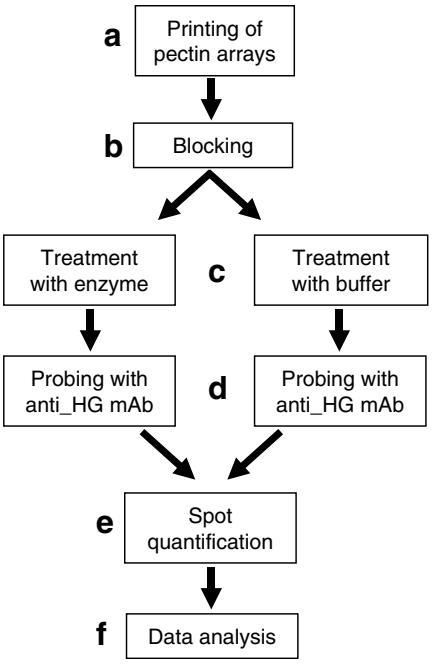

Fig. 1. Overview of the experimental approach. (a) A series of pectin samples with various degrees and patterns of methyl-esterification were printed as microarrays onto nitrocellulose. The samples contained differing levels of epitopes recognised by anti-homogalacturonan (HG) mAbs and/or enzymatic cleavage sites. After blocking (b), some arrays were digested with pectin lyase whilst control arrays were treated with buffer only (c). (d) Arrays were probed with anti-HG mAbs and spot signals were quantified using ImaGene 6.0 spot quantification software (e and f). Analysis and quantification of spot signals revealed changes in mAb binding to the arrayed samples.

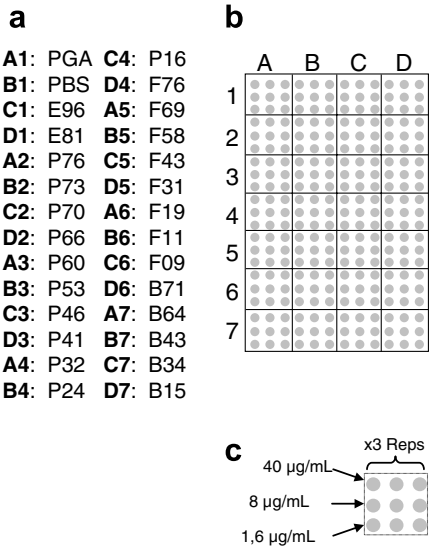

Fig. 2. (a and b) layout of the pectin samples on arrays. (c) Each pectin sample was printed at the three concentrations shown and in triplicate. Pectins were named according to their series (P-, F- or B) and DE (%). E81 and E96 were not enzyme or base treated.

2. Experimental

2.1. Pectin samples

The pectins used in this study (listed in Fig. 2a) had a range of DEs and PEs and were prepared by enzymatic digestion of a common highly methyl-esterified starting sample (E81) as described previously (Limberg et al., 2000). Methyl-esters were removed using either pectin methyl-esterase (PME) from either orange or *Aspergillus niger* to produce the P-(plant) and F-(fungal) series pectins, respectively. A third set of samples were prepared by chemical (base) de-methyl-esterification (B-series). Although essentially the same in all other respects, the differing activities of PMEs from orange and *A. niger* and base treatment yielded pectins with different PEs. The P-series samples contain contiguous stretches or 'blocks' of un-esterified GalA residues. In contrast, in the F- and B-series samples the distribution of un-esterified GalA residues is non-blockwise and methyl-esterified and un-esterified GalA residues are interspersed along the HG backbone.

The pectin samples were named according to their series, and DE (%), for example 'F43'.

2.2. Printing of microarrays

A microarray robot (MicroGrid II, Genomic Solutions, Ann Arbor, MI, USA) equipped with split pins (MicroSpot 2500, Genomic Solutions, Ann Arbor, MI, USA) was used to print pectin samples onto pure nitrocellulose membranes with a pore size of 0.45 μm (Schleicher & Schuell, Dassel, Germany). Pectins were dissolved in water and printed at three concentrations (40, 8, and 1.6 μg/mL) and in triplicate so that each sample was represented by a total of 9 spots. Arrays contained a total of 252 spots in an area 17×30 mm. Pins were washed in two wash cycles of three washes each in deionised water between samples.

2.3. Enzymes and monoclonal antibodies

Pectin lyase (E.C. 4.2.2.10) from *A. niger* was used for *in situ* digestions. The generation and specificities of the JIM5 and JIM7 monoclonal antibodies used in this study have been described extensively elsewhere (Clausen et al., 2003; Knox, 1997).

2.4. In situ enzymatic digestion

Pectin arrays were incubated with PL in 50 mM Na–acetate, pH 4.5 for 18 h at 40 °C unless otherwise stated.

2.5. Antibody probing

Pectin arrays were blocked by incubation for 1 h in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4 and pH. 7.5) containing 5% w/v low fat milk powder (5%MPBS). Arrays were then probed for 2 h with JIM5 or JIM7 diluted 1:10 in 5%MPBS. After washing with PBS, arrays were incubated for 2 h in anti-rat secondary antibody conjugated to alkaline phosphatase (Sigma, Poole, UK) diluted 1:5000 in 5%MPBS. After washing in PBS, arrays was developed using substrate containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) in BCIP/NBT buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM diethanolamine and pH 9.5).

2.6. Quantification of spot signals

Developed arrays were scanned (CanoScan 9950F, Canon Denmark, Copenhagen, Denmark), converted to 16-bit grey-scale TIFFs and uploaded into ImaGene 6.0 microarray analysis software (BioDiscovery, El Segundo, CA, USA). Semi-automatic gridding was used to create an analysis area for each spot, and a five pixel zone around each spot was used for calculation of local background signals. Individual spot signals were defined as the mean pixel value within each spot area minus the median pixel value in the surrounding local background area. For each array, mean spot values were derived from the nine individual spot signals from each sub-array that represented that sample (Fig. 2c). Final mean spot signals were derived from three independent experiments and were converted into heatmaps using heatmapper software (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_heatmapper.cgi). For each complete data set (for example as shown in Fig. 4c) the maximal mean spot signal was set to 100% and all other values within that data set adjusted accordingly. A mean spot signal minimum was set at 5% and this was also imposed when calculating fold changes (Fig. 4e).

3. Results

3.1. Development of pectin microarrays: spotting and analysis

Pectin samples were printed as arrays with a layout as shown in Fig. 2b. In order to avoid saturated spot signals, all samples were spotted at three concentrations. Furthermore, three replicates were also printed of each concentration of each sample so that each sample was represented by a total of 9 spots (Fig. 2c). ImaGene 6.0 software gridding facility enabled each spot to be delineated and numerical values generated for both spots and local backgrounds.

3.2. Development of pectin microarrays: consistency and dynamic range

Visual examination of arrays indicated that the printing and probing of the arrays was highly consistent. However, in order to formally assess consistency, two independent experiments were performed in which two batches containing three arrays each were printed, probed with JIM5 and analysed. The scatter plot in Fig. 3a shows the data from these experiments plotted against each other. The tight clustering of the signals along the 1:1 line (R^2 value of 0.977) indicated a high level of consistency within and between the arrays. In order to test if spot signals were correlated to epitope level, a series of P-series pectins that contained the JIM5 epitope to a greater or lesser extent were printed at three dilutions and probed with JIM5 (Fig. 3b). Quantification of the spot signals obtained indicated that there was a high degree of correlation between pectin concentration and signal. Similarly, in order to test if enzyme concentration was correlated to epitope degradation or loss, arrays containing a range of P-series pectins were digested with PL used at a range of concentrations and the effect on JIM5 binding was assessed. As expected, the different pectins varied in their susceptibility to PL digestion, but in most cases tested PL concentration was correlated to loss of JIM5 binding (Fig. 3c).

3.3. In situ enzymatic digestion of pectin microarrays

Pre-treatment of pectin arrays with PL prior to mAb probing had a significant effect on JIM5 and JIM7 binding

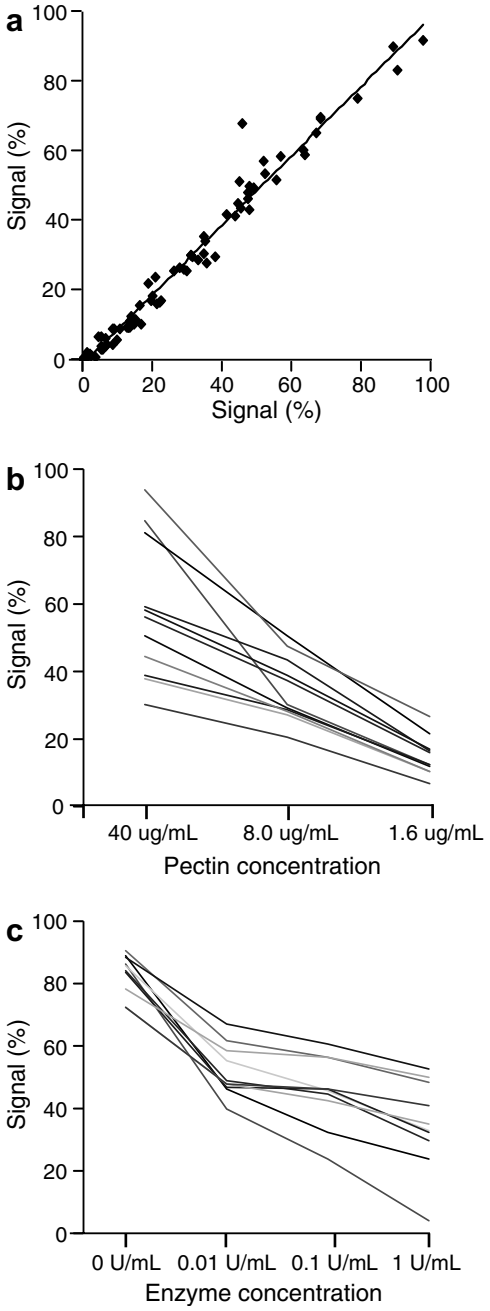

Fig. 3. (a) The reproducibility of the pectin arrays was tested by probing a series of arrays with JIM5 in three independent experiments. The scatter plot shows the spot signals obtained for two of these experiments with a R^2 value of 0.977 indicating a high level of consistency. (b) Pectin concentration was correlated to mean spot signals obtained. The data shown is for JIM5 binding to a subset of pectin samples printed at the concentrations shown. (c) Reduction in mAb binding was correlated to PL concentration as shown by the decrease in JIM7 binding to a sub-set of pectin samples digested with PL at the concentrations shown. The pectins used in (b) and (c) were: P76, P73, P70, P66, P60, P53, P46, P41, P32, P24, and P16.

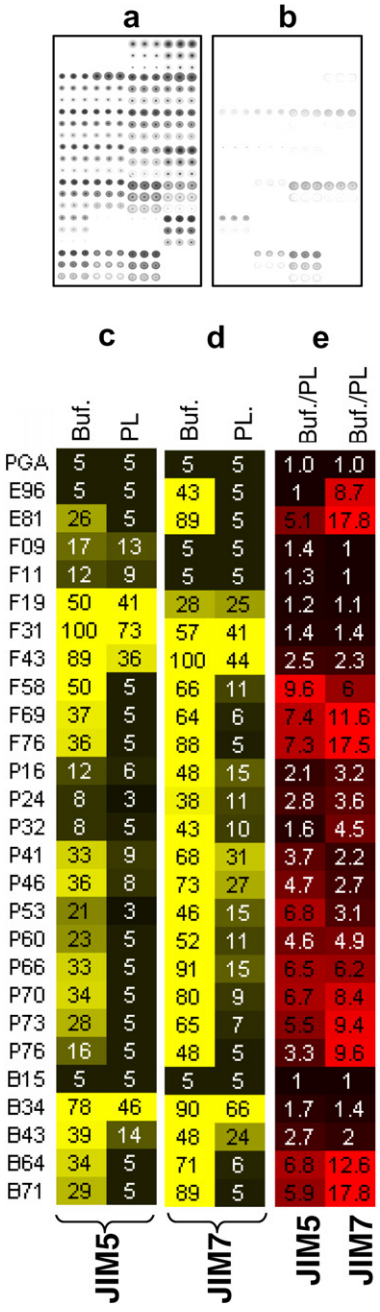

Fig. 4. (a and b) Representative examples of pectin arrays probed with JIM7 that were treated with buffer only (a) or with PL at 1 U/mL (b). (c and d) Heatmaps showing mean spot signals obtained for arrays treated with PL or buffer and probed with JIM5 or JIM7. (e) Heatmap showing fold changes (buffer signal/post-PL digestion signal) in mean spot signals obtained for JIM5 and JIM7 binding to pectins following PL digestion. The values in the heatmaps are mean spot signals obtained from three independent experiments. For both the JIM5 and JIM7 data sets, the maximal value was set to 100 and all other values adjusted accordingly. Buf, buffer.

to pectin samples, indicating that the pectins remained effective substrates for these enzymes when immobilised in an array context. A representative example of epitope deletion is shown in Fig. 4a and b. Quantified mean spot signals (derived from three independent experiments) are presented as heatmaps in which colour intensity is proportional to mean spot signal (Fig. 4c and d). Previous analyses have shown that JIM5 binds with greatest avidity to pectins of intermediate DE that contain short stretches of partially methyl-esterified HG (Clausen et al., 2003; Willats et al., 2001b). In agreement with this, JIM5 binding was greatest to F-series pectins F31, F43 and B-series pectin B34 that all have a non-blockwise PE created by the

fungal PME or base treatment (Fig. 4c). JIM5 binding was considerably less to P-series pectins with similar DEs (for example P32 and P41) reflecting the fact that these samples contain relatively long stretches of un-esterified HG, created by the blockwise activity of orange PME. Like JIM5, JIM7 bound to some extent to most of the arrayed pectins, but in contrast to JIM5, the ability of JIM7 to recognise samples with a high DE was demonstrated by its binding to samples E96 and E81 (Fig. 4d). The effect of PL digestion of the arrayed pectins was detected by the reduction of JIM5 and JIM7 binding and changes in mean spot signals that resulted from PL digestion are presented as fold changes (buffer treated values/PL treated values) shown in Fig. 4e. For many samples, both JIM5 and JIM7 binding was reduced to background levels by digestion with PL at 1 U/mL for 18 h, indicating that these samples contained JIM5 and JIM7 epitopes that incorporated the PL cleavage sites, or that cleavage sites were close enough to disrupt the binding of mAbs to the epitopes. For samples that JIM5 or JIM7 bound to strongly ($\geqslant$50% maximal signal), there were significant differences in their susceptibility to PL digestion. For example, there was a 9.6-fold reduction in JIM5 binding to F58, but only a 1.2- and 1.7-fold reduction in JIM5 binding to F19 and B34, respectively. This is likely to be due to the fact that F19 is relatively resistant to PL digestion because of its low DE but still contained enough appropriately distributed methyl-esters to allow JIM5 binding. Similarly, it is of note that JIM7 bound with high avidity to both E81 and F43 (with mean signal values of 89% and 100%, respectively), but whilst PL digestion reduced JIM7 binding to E81 by 17.8-fold, digestion of F43 with PL reduced binding by only 2.3-fold. This presumably reflects the fact that E81, with its higher DE, contains more PL cleavage sites than F43.

4. Discussion

The work described here demonstrates the feasibility of performing *in situ* enzymatic digestion of immobilised polysaccharides in a microarray format. We used pectins, a pectinolytic enzyme and anti-pectin mAbs in this study, but it is likely that a similar approach could also be applied to the analysis of other polysaccharides, other enzymes and other mAbs, lectins or carbohydrate binding modules. We have previously described the generation of glycan microarrays based on oxidised polystyrene 'MaxiSorp' slides (Willats, Rasmussen, Kristensen, Mikkelsen, & Knox, 2002). These slides were a suitable substrate for the immobilisation and subsequent antibody probing of diverse glyans, including pectins. However, we found that the MaxiSorp surface, unlike nitrocellulose, was not suitable for *in situ* enzymatic analysis because of limited dynamic range (data not shown). This is probably due to the fact that whilst the surface of plastic and glass slides is essentially two dimensional, nitrocellulose has a three dimensional 'flattened sponge' matrix structure that allows a higher loading of sample per spot.

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