Characterization of Nanostructural Modifications Introduced into a Model Pectic Homogalacturonan by Esterases or Chemical Saponification and Modeling of Enzyme Mode of Action

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ABSTRACT

Pectin functionality is largely dependent on the ratio of methylesterified/demethylesterified galacturonic acid (GalA) residues present in its polymeric homogalacturonan region (40 – 60 nm in length, 100 – 150 GalA residues) and how the two types of GalAs are spatially distributed within that linear backbone. These distributions translate into an ordered vs. random distribution of ionic (demethylesterified) and neutral (methylesterified) regions. Enzymatic modification of pectin nanostructure by pectin methylesterase (PME) is the only known method to introduce ionic blocks into pectin having a relatively high degree of methylesterification (> 20%). We have been characterizing the statistical parameters of average demethylesterified block size (1 – 4 nm) and number of demethylesterified blocks per molecule (0.1 – 2.0) following enzymatic or base demethylation of a model 94% DM homogalacturonan by excising demethylated blocks using limited endo polygalacturonase digestion. Additionally, for enzymatic demethylation we have modeled enzyme mode of action. We will report on results obtained by demethylation with several pectin methylesterase isozymes as well as by random acting fungal PME and chemical saponification. Additionally we will present results for rheological properties related to nanostructural modifications.

Keywords: pectin, galacturonic acid, pectin methylesterase, homogalacturonan

1 INTRODUCTION

Our goal is to create an enzyme-based technology for precise engineering of pectin nanostructure, a valuable plant heteropolysaccharide. Pectin, an integral component of land plants' biopolymeric cell wall scaffolding, is composed predominately of a linear homogalacturonan (HG) containing methylesterified and unmethylesterified galacturonic acid (GalA). The average pectic HG is ~ 40 – 60 nm in length and comprises ~ 100 – 150 GalA residues. Multiple HG regions exist within individual molecules interspersed sparsely by regions known as rhamnogalacturonan I (RGI). RGI regions have a repeating disaccharide backbone of rhamnose and GalA with neutral sugar side branches of arabinans and galactans attached to rhamnose, mainly at O-4. [1] The relative amount of methylesterified GalA residues (degree of methylesterification) and distribution of unmethylesterified GalA in the HG, both among chains, and along individual polymer backbones, is a key determinant of pectin molecular functionality. Demethylesterified GalAs may be distributed either randomly or ordered into blocks of contiguous demethylesterified GalAs (DEMBs). Multiple DEMBs may be distributed within a single linear HG region. Development of a technology to predictably introduce ordered DEMB nanostructure into HG regions, creating "ionic islands" of a desired size (number of GalAs), on the level of an individual molecule and population of molecules is necessary to rationally engineer pectin functionality and expand pectin's use as either a formulating agent with optimum and predictable physical properties, or as a foundational molecular scaffold upon which secondary and tertiary structure can be constructed.

Using the plant enzyme pectin methylesterase (PME) ionic islands of contiguous demethylesterified GalAs can be introduced into the HG regions. Multiple PME isoforms are present in plants and their differing modes of action likely produce statistically distinguishable pectin populations with measurable differences in DEMB sizes. In controlled demethylesterifications with a salt-independent PME [2] experimental data conformed to theoretical models describing a "Single Chain" mode of action, where contiguous methylesterified GalAs are progressively demethylesterified until a stop point is reached. The populations mean number of GalAs for each

ionic island introduced (average DEMB size) was estimated to range between 4 and 10 residues (1.6 and 4.1 nm) depending on the final degree of population methylesterification. Only 0.1 – 0.3 ionic islands of these average sizes were estimated to be present per molecule, indicating not all molecules in the population were involved in the enzymatic demethylesterification and that most DEMBs introduced were actually much larger. In contrast a thermally tolerant PME [3] introduced an average ionic island composed of 3 – 7 GalAs (1 – 3 nm) and there were between 1 and 2 such DEMBs per molecule. Experimental data for distribution of DEMB sizes introduced by this PME isozyme was consistent with a modeled "Multiple Attack" mode of action with a degree of processivity equal to the processive demethylation of only 10 GalA residues.

2 MATERIALS AND METHODS

2.1 Enzyme Purification

Enzymes were purified as previously described [2, 3] or used as present in a commercial papaya extract (Liquipanol T-200, Enzyme Development Corporation).

2.2 Pectin Demethylation

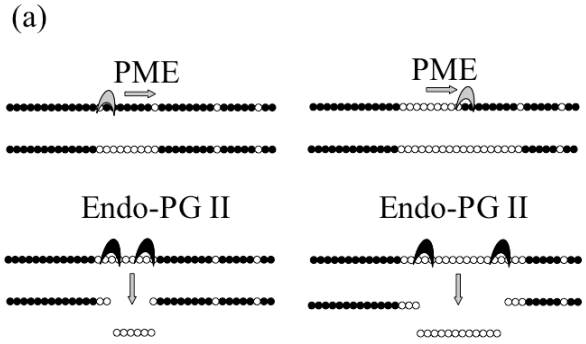
Pectin (86% anhydrous galacturonic acid; AGA, 94% DM, containing minor amounts of galactose) was made to a final solution of 1% in 0.2 M LiCl at pH 4.5 or pH 7.5 (pH adjusted with LiOH). Pectin solution was added to a 5 L water-jacketed stirred bioreactor and equilibrated at 30 °C (18). Sufficient volume of enzyme was added to equal 175 U of PME activity (as measured at 30 °C) per gram pectin and the pH was maintained at either 4.5 or 7.5 with a Radiometer PHM290 pH-stat controller using 1 M LiOH as the titrant. When sufficient titrant was added to indicate the desired DM had been reached the reaction was quenched by rapidly (~ 5 sec) draining the reactor contents into a vessel containing two volumes of acidified 95% ethanol (pH 3.8) at 37 °C, precipitating the pectin and inactivating the enzyme. The precipitated pectin was stored at 4 °C to facilitate further precipitation. The precipitated pectin was centrifuged (23,400 X g, 30 min, 4 °C) and the supernatant discarded. The pellet was placed in liquid nitrogen (forming small spheres), lyophilized and then comminuted in a small kitchen style mill. The comminuted pectin was frozen at -80 °C in a desiccated container.

2.3 Endo Polygalalcturonasse Digests

Demethylesterified pectins at 0.25% (w/v) in 50 mM lithium acetate (pH 5.5) and 0.02% lithium azide (w/v) were equilibrated at 30 °C in a benchtop incubator with stirring and digested with EPG M2 (0.1 U • mL-1) for 10 min. Preliminary results (data not shown) demonstrated these conditions released unmethylated oligomers without significant hydrolysis of the released oligomer products. EPG activity was quenched by pipetting the solution into a beaker containing 160 uL concentrated HCl to lower the pH to ~2, then microwaving the sample for 10-12 sec to boiling and finally immersing the sample in a boiling water bath for 10 min. High pressure anion exchange chromatography coupled to an evaporative light scatering detector was performed as previously described. [2, 3]

2.4 Mathematical Modeling

A set of one-dimensional arrays was used to model a population of polymer molecules. The lengths of the arrays were selected so that the population had a Gaussian distribution of DP with a defined width and mean value. The array elements represented the esterification state of each sugar ring and were assigned so that the population had a Gaussian distribution of the chain-averaged DM between chains with a defined width and mean value. [2, 3] Subsequently various de-esterification algorithms were applied to the starting substrate set until a specified endpoint was achieved, where the DM had been reduced to a value for which experimental data were available. By selecting the degree of multiple attack both random and processive modes of action could be investigated. Following substrate modification the new intramolecular distribution of methylesters (E) could be monitored in-silico. For the different models of PME enzyme action investigated the question as to how the differences in the generated unmethylesterified GalA block lengths in the population would be mirrored in the distribution of DPs of oligogalacturonides released by a limited EPG digestion was addressed by calculation. Predictions can be made for each PME model that can be compared directly with experimental data. With such a simulation methodology in hand then (i) different PME models can be assessed for their ability to describe the results of EPG limited digest experiments of the PME engineered substrates and (ii) for the model that best describes the experimental results, the full unmethylesterified GalA blocklength distribution of the pre-digested PME-generated polymers can be displayed. The strategy is summarized in Figure 1 (a) and (b).



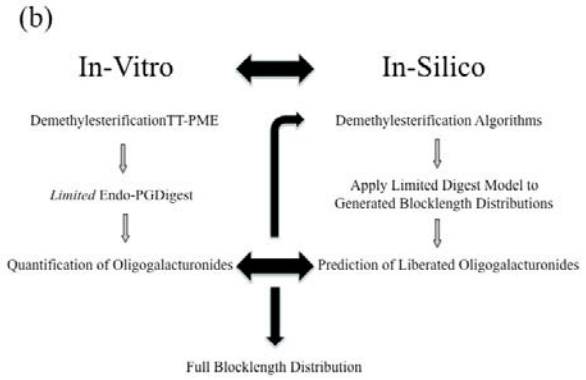


Figure 1. (a) Schematic of the TT-PME and EPG processing of model HG substrates, showing the introduction of demethylesterified blocks by PME and the subsequent random release of oligogalacturonides of different length, whose concentrations are measured experimentally (b) Schematic of the modeling procedure.

3 RESULTS AND DISCUSSION

Oligogalacturonides were released from all of the demethylesterified pectins (Figure 2, Table 1-3) by a limited digestion with EPG. The salt dependent PME (pSD-PME) from papaya introduced more DEMBs per molecule ($\overline{B}$) than the citrus salt-independent (cSI-PME) and the

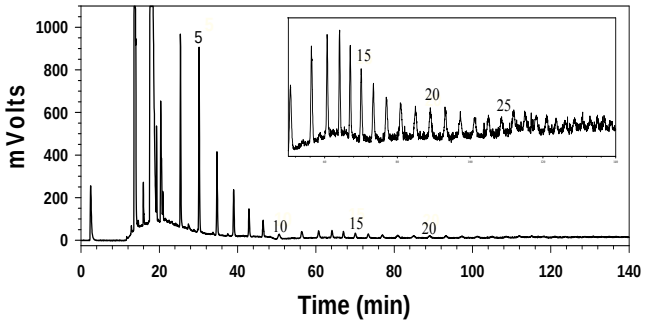


Figure 2. Representative HPAEC–ELSD chromatogram of oligogalacturonides released after an EPG limited digest Numbers indicate oligomer DP of the corresponding peak. The inset is a zoom for part of the chromatogram.

DM	$\overline{B}$					
	cSI-PME		cTT-PME		pSD-PME	
	pH 7.5	pH 4.5	pH 7.5	pH 4.5	pH 7.5	pH 4.5
80	0.8	1.5	0.6	0.7	2.5	2.2
70	1.0	1.8	0.5	1.6	4.2	3.4
60	1.0	1.5	0.7	0.7	5.9	3.3
50		1.9	0.7	0.6	5.5	3.5

Table 1. Average number of demethylated blocks ($\overline{B}$) per molecule for each pectin series.

DM	$\overline{BS}$					
	cSI-PME		cTT-PME		pSD-PME	
	pH 7.5	pH 4.5	pH 7.5	pH 4.5	pH 7.5	pH 4.5
80	5.3	4.1	3.5	3.6	3.5	3.5
70	10.2	5.3	4.1	5.4	4.2	4.1
60	8.4	7.9	4.3	5.8	3.9	4.3
50		8.6	4.4	6.8	4.4	4.5

Table 2. Average size of demethylated blocks ($\overline{BS}$).

DM	$\overline{BN}$					
	cSI-PME		cTT-PME		pSD-PME	
	pH 7.5	pH 4.5	pH 7.5	pH 4.5	pH 7.5	pH 4.5
80	0.1	0.3	0.2	0.2	0.7	0.6
70	0.1	0.3	0.1	0.3	1.0	0.8
60	0.1	0.2	0.1	0.1	1.5	0.8
50		0.2	0.2	0.1	1.2	0.8

Table 3. Number of average sized demethylated blocks per molecule ($\overline{BN}$).

citrus thermally-tolerant PMEs (cTT-PME; Table 1). Enzyme and pH dependent differences were also observed (Table 2) for the average demehtylated block size ($\overline{BS}$). Reaction pH also had a major affect on the number of average sized blocks per moolecule (Table 3, $\overline{BN}$).

Small oligomers are the numerically most abundant oligomers released from the demethylated pectin by EPG (Figure 3). Although the possibility of releasing oligomers

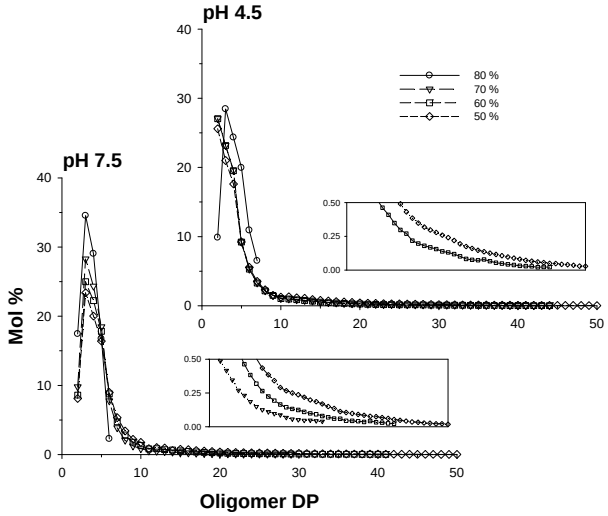


Figure 3. Experimentally measured average unmethylesterified oligogalacturonide distributions obtained following limited digestion of TT-PME generated substrates with EPG.

by secondary digestion cannot be discounted, the frequency of small oligomers did not increase with a decrease in DM as might be anticipated if high levels of secondary fragmentation occurred.

Figure 5 illustrates the a multiple attack mode of action for the cTT-PME modelled with data from a limited EPG digest of a 50% DM pectin. Similar results were obtained at 60% and 70% DM for this enzyme at both pH 4.5 and 7.5. A similar degree of processivity was observed with the pSD-PME but not the cSI-PME, which possessed a single chain mode of action.

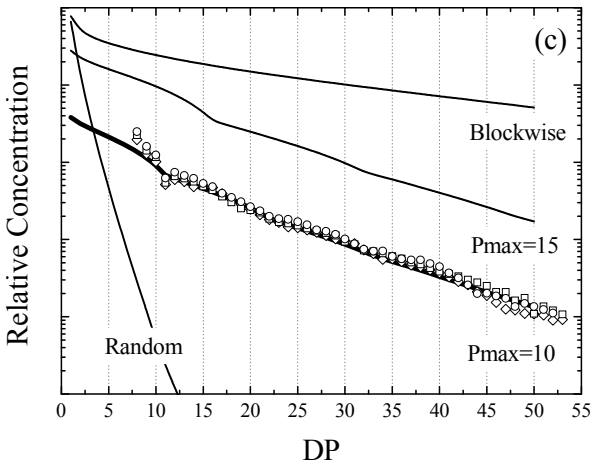


Figure 5. Distributions of oligogalacturonides predicted to be released by limited EPG digestions from homogalacturonans, generated from a 94% DM starting substrate using either random demethylesterification or PME models as described above, with maximum processivities of either greater than the DP of the chains ("completely blockwise"), or 15 and 10 residues compared with experimental data from the cTT-PME (three independent experiments at pH 7.5) for final sample averaged DMs of 50%.

Results presented here and for plant PMEs [2-7] suggest that pectin nanostructure is amenable to enzymatic engineering to produce tailored structural/functional properties. These results also indicate that PME mode of action is different for two PME isoforms purified from the same species and also for a PME present in a crude enzyme extract from papaya. Previous results also have shown that reaction parameters can also influence the mode of action [2, 4, 6, 7]. Future work is needed to demonstrate how the frequency distribution of can be controlled and also can be manipulated.

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