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# Structural and functional effects of manipulating the degree of methylesterification in a model homogalacturonan with a pseudo-random fungal pectin methylesterase followed by a processive methylesterase

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ABSTRACT

We explored the possibility of controlling charge distribution in the homogalacturonan regions of pectin to produce a population of demethylesterified molecules with desirable functional properties by utilizing consecutive treatments with pectin methylesterases (PME) having different modes of action. A fungal PME from *Aspergillus aculeatus* (*Aa*-PME), with a pseudo-random mode of action, was used to demethylesterify a extremely high methylesterified HG (DM 94%, average degree of polymerization 246) by reducing the degree of methylesterification (DM) from 94% to either 70% or 80%. A second demethylesterification step, to 50% DM, was performed using a processive PME from *Carica papaya* (*CpL*-PME). Introduced demethylesterified blocks were released either by exhaustive or limited endo polygalacturonase (EPG) digestion. Degree of blockiness (DB), absolute degree of blockiness ($DB_{abs}$), average demethylesterified block size ($\overline{BS}$) and number of average sized demethylesterified blocks per molecule ($\overline{BN}$) were estimated. $\overline{BS}$ and $\overline{BN}$ as well as $DB/DB_{abs}$ differed depending on the initial DM reduction by *Aa*PME, the number of activity units of *CpL*PME used and the reaction pH ($P < 0.05$). Consecutive demethylesterification of HG by *AaPME* to 80% DM and then by *CpL*PME to 50% DM at pH 4.5 produced significantly longer oligomer blocks compared to *Aa-PME* demethylesterification to 70% DM followed by *CpL*-PME to 50% DM at pH 7.0. Limited EPG digestion released nearly intact demethylesterified blocks and the released oligomers were coupled with *in silico* modeling. Resulting oligomer distribution corresponded to the *in silico* mode of action representing contiguous demethylesterification of 10 GalA residues rather than that of random or complete block-wise demethylesterification. Calcium-mediated gels of the modified HGs displayed G′ higher than G″ values and both moduli differed significantly according to the amount of *CpL*PME applied even though their final DMs were identical. These results suggest the possibility of controlling $\overline{BS}$ and engineering a population of demethylesterified pectin molecules with specified demethylesterified $\overline{BS}$ and functional properties.



## 1. Introduction

Pectin is a complex polysaccharide abundantly found in the cell wall of fruit and vegetable tissues. Generally, this macromolecule consists of homogalacturonan (HG) and rhamnogalacturonan I and II regions. The major pectic region is HG, a linear polymer composed of 1,4-linked α-D-galacturonic acid which can be methylesterified at C-6 carboxyl and/or acetylated at C-2 or C-3 (Vincken et al., 2003). HG structure is a key determinant of the functional properties of commercial pectins. Specifically, the amount and distribution of contiguous, unmethylesterified galacturonic acid (GalA) residues within HG regions have a direct impact on pectin functionality. However, studies have shown that the pattern of methyl esterification can have more functional significance than the exact amount of methyl ester (Willats et al., 2001).

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HG is the substrate for several pectin remodeling enzymes. Among these enzymes, much attention has been paid to the role of PMEs (EC 3.1.1.11) in the modification of HG structure in developmental processes (Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010; Willats et al., 2001). PMEs with various modes of action have been extensively scrutinized and, in consequence, are successfully being utilized in the food, cosmetic, and pharmaceutical industries (Denes, Baron, Renard, Pean, & Drilleau, 2000; Hotchkiss et al., 2002). Their mode of action is influenced by environmental factors such as pH. Some PMEs are more effective than others on highly methylesterified pectin at a specific pH (Cameron, Luzio, Goodner, & Williams, 2008; Kent et al., 2016; Kim et al., 2013; Kim et al., 2014; Luzio & Cameron, 2008; Mercadante, Melton, Jameson, Williams, & Simone, 2014). At an acidic pH, some PMEs act randomly, thus promoting the formation of calcium bridges only at very low DM, whereas at alkaline pH, some PMEs have a processive action (Kim et al., 2013, 2014; Ngouémazong et al., 2012). Three generalized PME modes of action have been proposed: 1) a single chain mechanism in which the enzyme binds the substrate and hydrolyzes methyl esters on contiguous GalA residues until it reaches the end of the molecule or a blocking residue, 2) a multiple chain mechanism in which only a single methyl ester is hydrolyzed for each enzyme-substrate interaction, and 3) a multiple attack mode of action which is intermediate between the other two and in which some number of reactions are catalyzed by the enzyme between formation of the enzyme–substrate complex and dissociation from the polymer. These three models describe a continuum of enzyme processivity, from dissociation following a single catalytic event to dissociation following tens, hundreds or thousands of catalytic events (Campa et al., 2004; Greenwood & Milne, 1968; Robyt & French, 1970). Both the single chain and multiple attack modes will produce contiguous blocks of demethylesterified GalA and are described as blockwise modes of action.

Previous studies revealed that plant/bacteria PMEs generally differ from fungal PMEs in their mode of action as well as their isoelectric point and optimum pH. PMEs from plant and most bacterial sources with a neutral to alkaline *p*I have a processive mode of action, generating blocks of galacturonic acid residues with free carboxyl groups on the methyl-esterified homogalacturonan back-bone (Cameron et al., 2008; Cameron, Luzio, Vasu, Savary, & Williams, 2011; Duvetter et al., 2006; Kim et al., 2013; Willats et al., 2001). In contrast, fungal enzymes have been reported to act randomly (Willats et al., 2001). Tanhatan-Nasseri, Crepeau, Thibault, and Ralet (2011) previously reported that HGs generated by the sequential demethylesterification by alkaline treatment followed by plant PME exhibited intermediate DB, $DB_{abs}$ and calcium binding properties, in agreement with the presence of numerous but short demethylesterified blocks of GalA, when compared to HGs generated from plant PME demethylesterification only. Accordingly they suggested the possibility of manipulating the initial DM of randomly methylesterified parent HGs prior to enzymatic blockwise demethylesterification, to allow the recovery of various HG types with respect to the length and number of demethylesterified GalA stretches. In this study we applied sequentially two types of PMEs with different modes of action to a model HG with high DM. An *Aspergillus aculeatus* PME with pseudo-random mode of action (Willats et al., 2001) was used first to lower an initial DM of 94% down to 70% or 80% producing an initial pseudo-random demethylesterified pattern. Subsequently a *Carica papaya* PME having a processive mode of action was used to further demethylesterify this pectin at pH 4.5 and 7.0 to a DM of 50% (Kim et al., 2013). We hypothesized that a novel series of demethylesterified HGs could be generated with a block distribution distinguishable from the mode of action of each separate PME. We achieved different DM levels under different reaction pHs to endow variations in the charge distribution and the pattern of block distribution in the HG as supported by previous researches (Cameron et al., 2008, 2011; Catoire, Pierron, Morvan, Herve du Penhoat, & Goldberg, 1998; Kim, Williams, Luzio, & Cameron, 2017; Kim et al., 2013, 2014). The demethylesterified block distribution was characterized using an exhaustive (degree of blockiness and absolute degree of blockiness; Daas, Voragen, & Schols, 2000) and a limited EPG digest (average block size and block number; Cameron et al., 2008, 2011). The impact of this coupled enzyme use on the resulting calcium mediated gel was also investigated.

## 2. Materials and methods

### 2.1. Chemicals, reagents, parent pectin and commercial pectin methyl esterase

To elucidate the demethylesterified pattern via consecutive PMEs' reaction, extremely high methyl esterified pectin from citrus fruit (94% methylesterified, P9561) was purchased from Sigma-Aldrich (St. Louis, MO, USA) which has only 6 demthylesterified GalA per 100 GalA residues. DM, sugar composition and molecular weight of parent homogalacturonan are described in Cameron et al. (2011). In brief, the number average molecular weight of the parent pectin was estimated to be 27,613 Da by size exclusion chromatography and GalA (93%) and galactose (7%) were the only sugars detected in the parent pectin. The lack of detectable rhamnose suggests that the parent pectin is a homogalacturonan. All chemicals were purchased from Sigma–Aldrich unless otherwise indicated. Aqueous buffer for the mobile phase was composed of ammonium formate (no. 09735, >99% purity, Fluka BioChemika, Steinheim, Switzerland) in high purity deionized-distilled water. Endo-polygalacturonase (EPG-M2) was purchased from Megazyme International Limited (Bray, Ireland; Lot 00801). A commercial preparation of pectin methylesterase (EC. 3.2.1.11) from *Aspergillus aculeatus* (NovoShape™) was purchased from, Novo Nordisk (Bagsvaerd, Denmark).

### 2.2. Isolation and purification of pectin methyl esterase from Carica papaya

Detailed information related to enzyme isolation and purification was reported by Vasu, Savary, and Cameron (2012). Briefly, a commercial papain, Liquipanol T-200® (Enzyme Development Corporation, New York, NY) was obtained and dialyzed using a 6–8 kDa nominal molecular weight limit membrane against 0.01M $Na_3PO_4$, pH 6.5 for two days with exchange of buffer. White precipitate formed in the dialysis bags and the dialysate was centrifuged at 4 °C for 35 min at 12,100×*g*. The supernatant, which contained the bulk of PME activity, was collected and stirred overnight at 4 °C in 30% ammonium sulfate followed by centrifugation at 25,700×*g* at 4 °C. The supernatant was collected and brought up to 80% ammonium sulfate and stirred at 4 °C for 4 h. It was centrifuged again at 25,700×*g* for 35 min at 4 °C. The ammonium sulfate pellet was then solubilized into 10 mM $Na_3PO_4$ buffer, pH 6.5, and dialyzed overnight at 4 °C with slow stirring. The dialysate was collected and stored at −80 °C.

### 2.3. Demethylesterification

Pectin was prepared at 1% (w/v) in 200 mM LiCl. *Aa*-PME (17.5 unit/g pectin) was added to the pectin solution and the pH was maintained with a pH-stat controller (TIM856, Radiometer, France) using 1 M LiOH as the titrant at 30 °C until the DM was reduced to either 80% or 70%. After reaching the desired DM, the reaction