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Charged functional domains introduced into a modified pectic homogalacturonan by a mixture of pectin methylesterases isozymes from sweet orange (*Citrus sinensis* L. Osbeck var. Pineapple)☆

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ABSTRACT

A mixture of multiple pectin methylesterase (PME) isozymes present in *Citrus sinensis* L. Osbeck were applied to demethylesterify a model homogalacturonan (HG) with 91% degree of methylesterification (DM) to 50% or 70% at pH 4.5 and 7.0, respectively. Introduced demethylesterified blocks (DMBs) were released by a limited endo polygalacturonase (EPG) digestion, separated and quantified by HPAEC. The average DMB size ($\overline{BS}$) and number of such blocks per molecule ($\overline{BN}$) differed depending on the degree of methylesterification and reaction pH ($P < 0.05$). A significantly larger $\overline{BS}$ was observed in HGs of 50% DM compared to 70% DMs. HG demethylesterified to 50% DM at pH 4.5 showed significantly larger $\overline{BS}$ compared to the 1 at pH 7.0 ($P < 0.05$). Degree of blockiness (DB) and absolute degree of blockiness (DB_{abs}), obtained using exhaustive EPG digestions, were the highest in 50% DM pH 4.5 and the lowest in 70% DM pH 7.0 displaying a similar trend with $\overline{BS}$. However, significant difference in DB/DB_{abs} between the pHs was only observed in 70 DM samples. The distribution of DMBs released by the limited EPG digest was predicted by mathematical modeling and compared with the experimental results. The *in silico* modeling of the distributions of blocks is best explained by a processive multiple attack mode of action. The results thus support a *Citrus sinensis* PME mixture can be applied to introduce demethylesterified blocks of designated size into pectin molecules.

1. Introduction

Pectin is an abundant structural polysaccharide in dicotyledonous plant cell walls that is isolated on commercial scale primarily from citrus peels. Due to its versatile functionalities and also being regarded as a natural ingredient (Cargill, Inc., 2018), consumption of pectin consistently has increased worldwide in the food, medical and personal care industries. The functionality of pectin has been known mostly to depend upon the amount of nonmethylesterified galacturonic acids (GalAs) in the linear homogalacturonan (HG) region. However, recent researches have manifested the importance of distribution and size of contiguous nonmethylesterified GalA blocks rather than its amount (Duvetter et al., 2009; Thibault, Renard, Axelos, Roger, & Crepeau, 1993; Van Buggenhout et al., 2006).

Among numerous functionalities of pectins, the calcium mediated gelling is critically dependent on the ability to form stable junction zones, accordingly, it is regarded critical to introduce blocks successive nonmethylesterified GalA residues long enough for crosslinking via Ca^{2+} ion bridges. The minimum number of successive unmethylesterified GalA residues necessary to make up a junction zone

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has been estimated to range from 6 to 20 (Braccini & Pérez, 2001; Luzio & Cameron, 2008).

To introduce successive unmethylesterified GalAs, pectin methylesterases (PMEs; EC 3.1.1.11) have been efficiently utilized (Denes, Baron, Renard, Pean, & Drilleau, 2000; Hotchkiss et al., 2002). PMEs selectively hydrolyze C6-carboxyl methyl esters in HG regions. PMEs from plants with a basic isoelectric point usually work in a blockwise pattern demethylesterifying substantial parts of a single chain before attacking the next chain (Denes et al., 2000; Fraeye et al., 2007). Several studies have been published on the differences in the mode of action of PMEs according to their origins (Cameron, Luzio, Goodner, & Williams, 2008; Luzio & Cameron, 2008), the isozymes within a single species (Savary, Hotchkiss, & Cameron, 2002), and a single isozyme at pH conditions (Kim et al., 2014). Presence of multiple PME isozymes have been reported from various plants such as citrus fruit tissue, tomato and grapefruit (Thibault et al., 1993; Willats et al., 2001; Hellin, Ralet, Bonnin, & Thibault, 2005; Louvet et al., 2006; Zega & D'Ovidio, 2016). Among them, PME isozymes from *Citrus sinensis* were most extensively explored in relation to the juice cloud destabilization. Loss of juice cloud is the consequence of a series of chemical events initiated by PMEs (Cameron, Baker, & Grohmann, 1998; Cameron, Savary, Hotchkiss, & Fishman, 2005; Savary, Vasu, Nunez, & Cameron, 2010). The most abundant PME present in *C.s sinensis* pulp tissue is a thermolabile (TL) PME with salt-independent character (SI-PME). It readily destabilizes juice cloud at room temperature (Cameron et al., 1998). The second major TL-PME isozyme is a salt-dependent PME (Cameron et al., 2003) which has no cloud-destabilizing action (Cameron et al., 1998). Minor TL-PME (NB-PME) identified in orange and grapefruit does not bind to a cation exchange column (Cameron et al., 1998; Cameron & Grohman, 1995) and the last PME with a minor activity is distinguished as its high thermal tolerance (TT-PME). This one can instantly destabilize juice cloud even under refrigerated storage thus must be strictly controlled in juice processing to avoid cloud loss (Baker & Cameron, 1999; Cameron et al., 1998; Versteeg, Rombouts, Spaansen, & Pilnik, 1980). TT-PME is reported in grapefruit as well (Seymour, Preston, Wicker, Lindsay& Marshall, 1991; Cameron & Grohman, 1995).

Because only certain PME forms are responsible for cloud loss of orange juice, a distinct action pattern of each isozyme has been suggested. Previously, three out of four PME isozymes in *C. sinensis* peel, SI-, TT- and NB-PMEs, were characterized and their modes of action were investigated (Cameron et al., 2008; Cameron, Luzio, Vasu, Savary, & Williams, 2011; Kim, Williams, Luzio, & Cameron, 2017). The dominant SI-PME demonstrated a blockwise demethylation pattern at pH 7.5 (Cameron et al., 2008). TT-PME displayed mode of action with a degree of processivity (p) of ~10 at both pH 7.5 and 4.5 following simulated multiple-attack mechanism. NB-PME also followed $p = 10$ mode of action similar with TT-PME, however, it introduced much larger average sized blocks and number of such blocks (Kim, Cameron, Williams & Luzio, 2017). In result, this PME caused the most rapid cloud loss at both 4 and 30 °C even though it contributed only 6% of total PME activity in *C. sinensis* fruit tissue (Cameron et al., 1998).

C. sinensis PMEs can be effectively utilized in the pectin industry to endow desirable functional properties. More readily obtained PME isozyme mixture or *in-situ* modification of citrus pectin using endogenous PMEs would be the best in terms of industrial application. In this study, the nanostructural features of HGs demethylesterified by the mixture of multiple PME isozymes from *Citrus sinensis* L. Osbeck fruit tissue were investigated and their combined mode of actions at pH 4.5 and 7.0 were also examined. Non-methylesterified block distribution results from both *limited* and *exhaustive* EPG digests were obtained to characterize the pattern of charge distribution introduced by an enzyme mixture containing multiple PMEs and compared to the results previously obtained using mono-component, individual purified PME isozymes.

2. Materials and methods

2.1. Chemicals and reagents

Most of the chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) otherwise the manufacturer was indicated. Aqueous buffer for the anion exchange chromatography mobile phase was composed of ammonium formate (no. 09735, > 99% purity, Fluka BioChemika, Steinheim, Switzerland) in high purity deionized-distilled water. Endo-polygalacturonase (EPG) M1 was purchased from Megazyme International Limited (Bray, Ireland; M1, lot 00802, M2, lot 00304).

2.2. Preparation of enriched enzyme mixture

Citrus sinensis L. Osbeck var. Pineapple fruit pulp cells were provided by a local orange juice processor (Louis-Dreyfus Citrus Inc., Indiantown, FL). Pectin methylesterases present in the pulp cells were isolated as a mixture as described in Galant, Luzio, Widmer, and Cameron (2014). Briefly, 4 L of orange fruit pulp cells were diluted into two volumes of Tris buffer (20 mM Tris, pH 8.0; 50 mM NaCl) and mixed for 1 h at 4 °C (Savary, Vasu, Cameron, McCollum, & Nuñez, 2013). The resulting slurry was filtered and centrifuged at 12,000 × g, 4 °C for 30 min as previously described (Galant et al., 2014). The recovered solids (containing PME activity) were washed, filtered and centrifuged at 12,000 × g, 4 °C for 30 min and diluted with buffer (20 mM Tris, pH 8.0; 0.5 M NaCl) and stirred over night at 4 °C. The resulting slurry was filtered and centrifuged at 12,000 × g, 4 °C for 30 min and the supernatant with PME activity was concentrated using a tangential flow filtration system (Pall, Port Washington, NY) containing a 30 kDa MWCO cartridge until the volume was reduced by 50%. The retentate was then diluted into 2.5 vol of 20 mM Tris, pH 7.5; 0.2 M NaCl. Diethylaminoethanol (DEAE, 320 g) was added to the PME containing retentate to bind and remove any pectic fragments in the mixture (Savary et al., 2013). This slurry was stirred overnight at 4 °C and decanted into a sintered glass funnel, and the flow-through was collected using gravity filtration. The DEAE-Sephacel (Sigma-Aldrich)was washed twice with 20 mM Tris, pH 7.5; 0.2 M NaCl, and the flow-through was pooled and concentrated to 1.25 L using tangential flow filtration. The concentrated PME solution was made into aliquots and stored at −80 °C. PME activity in the concentrated solution was determined quantitatively by titrating 25 μL of retentate solution against 0.02M LiOH in the presence of 2% of 94% DM pectin (Cameron et al., 2005).

2.3. Demethylesterification

Parent homogalacturonan (P9561 from citrus fruit, Sigma-Aldrich) was the same one used in Kim et al. (2017). Briefly, it was composed of 90.1% GalA and 9% galactose as analyzed by HPAEC-PAD. The weight average and number average (M_n) molecular weight for the parent pectin were 52,060 Da and 30,660 Da, respectively, as analyzed by HPSEC-MALLS. Using the value for M_n, an average degree of polymerization (DP) of 162 was found and a DM of 90.8% was used for calculation of average block size and number of demethylesterified pectin. For detailed analysis methods refer to Kim, Williams, Luzio & Cameron. (2017).

Parent pectin was made to a solution of 1% in 0.2 M LiCl. The PME mixture was added to 250 mL of the pectin solution at the level of 17.5 unit/g pectin at pH 7.0 or 35 unit/g pectin at pH 4.5, and the pH was maintained using 1 M LiOH as the titrant at 30 °C. A controlled demethylesterification series of 50% and 70% DM was produced at both pH values. The reaction was quenched by rapidly (~5 s) draining the contents into a vessel containing two volumes of acidified 95% ethanol (pH 3.8). The precipitated pectin was centrifuged (23,400 × g, 30 min, 4 °C) and the supernatant was discarded. The pellet was lyophilized and

stored at −80 °C until further analysis.

2.4. Limited endo-polygalacturonase digest

Demethylesterified pectins were dissolved at 0.25% (w/v) in 10 mL of 50 mM lithium acetate (pH 5.5), equilibrated at 30 °C and digested with 50 μL of 1000 times diluted EPG M2 for 30 min. One milliliter of the sample was taken and EPG activity was quenched by pipetting 7.5 μL of concentrated HCl, then microwaving for 4 s, and boiling for 10 min. The pH of samples was adjusted to ~5.5 with ammonium hydroxide. For quantification, EPG digested samples were injected onto a CarboPac PA1 column (Cameron et al., 2008; Cameron et al., 2011; Kim et al., 2013) and eluted with an ammonium formate gradient (0–60 min, increasing from 0 to 60%, 60–180 min, from 61 to 80%, 180.1–195 min 100%) in deionized water with a flow rate of 1 mL/min. Masses for each oligomer ($GalA_n$; where n is the number of demethylesterified GalA residues in the oligomer) were estimated using a pooled calibration curve constructed using $GalA_3$ (Sigma-Aldrich, St. Louis, MO, USA) and $GalA_8$ (purified from a partial EPG digest of polygalacturonic acid on a semi-preparative Carbo-Pac PA1 column). Oligomer mass estimates were converted to molar concentration as previously described (Cameron et al., 2008; Cameron et al., 2011; Kim et al., 2013). Briefly, mass concentrations for each $GalA_n$ were used to calculate $GalA_n$ molar concentration per mL (C_n). The M_n for the parent pectin obtained from MALLS-SEC was used to estimate the molar concentration of pectin per mL (C_p). Eqs. (1)–(3) were used to estimate the average number of demethylesterified blocks released of length *n* from a molecule, the average sum of blocks per molecule ($\bar{B}$) and the average DMB size released ($\overline{BS}$), respectively, where *z* is the average degree of polymerization of the pectin population as estimated using the number average molecular weight of the pectin population and the average molecular weight of a GalA unit within the polymer based on the estimated degree of methylesterification for the pectin population. The number of average sized blocks per molecule was estimated according to Eq. (4) which provides information on the proportion of molecules in a population that have been modified by PME. If $\overline{BN}$ is greater than 1, all molecules could be considered to contain at least one average sized, or larger demethylesterified block. If it is less than 1, then all molecules may not have an average sized demethylesterified block and suggests that not all molecules have been modified by the PME.

$$\bar{B}_n = \frac{C_n}{C_p} \tag{1}$$

$$\bar{B} = \sum_{n=3}^{z} \bar{B}_n \tag{2}$$

$$\overline{BS} = \frac{\sum_{n=3}^{z} n\bar{B}_n}{\bar{B}} \tag{3}$$

$$\overline{BN} = \frac{\bar{B}}{\overline{BS}} \tag{4}$$

2.5. Mathematical modeling

For a detailed description related to the mathematical modeling refer to Cameron et al., 2008. Briefly, a set of chains is modeled as a set of simple one dimensional arrays of varying length. And with the elements of each array representing nonmethylesterified GalA or methylesterified GalA in order to represent a hypothetical population of molecules. The starting substrate for modeling was initiated as polygalacturonic acid and a random methylesterification algorithm was used to obtain the final DM. A number average DM of 94% and DP of 140 were used for all simulations and up to 10^5 individual chains comprised the HG. Subsequently a demethylesterification algorithm was run on the starting substrate until specified endpoints were achieved and the sample average DMs corresponded to those measured under the studied experimental conditions. By selecting the degree of multiple attack in the algorithm both random and processive modes of action could be investigated. Following the *in-silico* substrate modification the new intramolecular distribution of methylesters was queried. The relative number (*N*) of different GalA block lengths M-$(G)_n$-M found in substrates demethylesterified to 30%, 50% or 70% assuming variable *p* values was returned by the simulation. Assuming that the endo-PG enzyme that then excises blocks from these M-$(G)_n$-M regions acts randomly within a DMB the relative probability of removing a *k*-mer from a block *n* long is given by:

$$P_{kn} = \frac{(n-2)-k+1}{((n-2)^2+(n-2))/2} \tag{5}$$

In Equation (5), $n-2$ comes from the assumption that two nonmethylesterified residues are needed in the EPG active site so whatever cuts are made a nonmethylesterified residue is always left on either side (i.e. from a block of eight nonmethylesterified residues an oligomer of six would be the largest extracted). With these approximations the relative probabilities (and consequent relative number of molecules) of the different DP oligomers predicted to be released by a limited EPG digest from substrates modified using PME models with various *p* values can be calculated. The simulation described above gives the relative numbers, *N*, of different GalA blocks of varied length, M-$(G)_n$-M, generated in the homogalacturonan and equation (5) is then used to predict the relative number amount of different DP oligomers that would be released from contiguous blocks of a certain length. Merging the approaches and summing the number of generated oligomers from each DMB weighted by the probability of occurrence of each initial contiguous stretch in the HG region the relative total number of GalA *k*-mers released (the experimentally determined quantity) is given by:

$$P_{oligo} = \sum_{n=k+2}^{n=chainlength} \frac{N(M-(G)_n-M)[(n-2)-k+1]}{\{[(n-2)^2+(n-2)]/2\}} \tag{6}$$

2.6. Degree of blockiness and absolute degree of blockiness

Detailed procedure for measuring DB and DBabs refer to Kim et al. (2013). Briefly, a combination of two EPGs, EPG M1 from *Aspergillus niger* (5000 U/mL, Megazyme, Ireland) and M2 from *Aspergillus aculeatus* (5000 U/mL, Megazyme, Ireland) were used for an exhaustive digestion of the nonmethylesterified GalA blocks within the demethylesterified HGs. Twenty microliters of each EPG M1 and M2 were added to 1 mL of 1% w/w pectin solution prepared in 50 mM lithium acetate buffer pH 5.5 and hydrolysis was performed for 24 h at 30 °C. Hydrolysates were heated to quench the EPG activity and then centrifuged at 12,000 × *g* for 2 min. The concentrations of the mono-, di-, and tri-GalA released were analyzed by HPAEC-ELSD. Hydrolysates were injected and eluted with a linear 0–0.6 M ammonium formate gradient (0–60 min) in deionized water with a flow rate of 1 ml/min. DB was calculated by measuring the amount of $GalA_1$ – $GalA_3$ released and dividing by the amount of nonmethylesterified GalA present in the sample (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Daas, Voragen, & Schols, 2000; Lofgren, Guillotin, Evenbratt, Schols, & Hermansson, 2005) while DB_{abs} is the amount of released oligomers expressed as a percentage of total GalA (Guillotin et al., 2005; Yapo, Lerouge, Thibault, & Ralet, 2007). The DB and DB_{abs} were calculated as follows:

$$\mathrm{DB} = \left(\frac{M+D+T}{Free\ GalA}\right) \times 100$$

$$\mathrm{DB_{abs}} = \left(\frac{M+D+T}{Total\ GalA}\right) \times 100$$

$$\text{where (M + D + T)} = \frac{ug^M}{MW^M} + \frac{ug^D}{MW^D} + \frac{ug^T}{MW^T}$$

$$\text{Free GalA} = \frac{(Q - Q(D))(G)(1 - DM)}{Avg\ MW}$$

$$\text{Total GalA} = \frac{(Q - Q(D))(G)}{Avg\ MW}$$

$$\text{Average MW} = (176.14(1-\text{DM})) + (190.18\text{DM})$$

Q = μg of material injected; D = % water content; G = % GalA content; DM = degree of methylesterification; Avg MW = average molecular weight; M = monomer, D = dimer, T = trimer

2.7. Statistical analysis

ANOVA and t-tests were carried out to examine the significance of DM and pH on the DMB parameters and DB obtained from limited and exhaustive digests. Multiple comparison tests were carried out using Tukey's HSD. Pearson's correlation coefficients were calculated to examine relationships between DB or DMB parameter and the rheology data. Regression analyses were carried out between DM, DB, DB_{abs}, and the concentrations of mono-, di- and tri-GalA released from complete EPG digests, using curve-fitting procedures available in SPSS 21.0. Analysis of Covariance (ANCOVA) was carried out with Graphpad Prism (version 4.03) to examine the difference of calibration curves derived with $GalA_3$ and $GalA_8$. All statistical tests were carried out at a significance level of 0.05 (95% confidence interval).

3. Results and discussion

3.1. Average demethylesterified block size and average number of blocks per molecule by limited EPG digest

GalA oligomers were released from all the demethylesterified pectins by limited digestions with EPG M2. $\overline{BS}$ of the DMBs released by *limited* EPG digest are regarded as an estimate of the actual DMBs present in the HG molecules. Because secondary fragmentation of the released DMEs were minimized by preliminary adjustment of the amount and time for the EPG digestion thus increase in the frequency of small oligomers were restricted. Block information from *limited* and *exhaustive* EPG digest were presented in Table 1. $\overline{BS}$ and $\overline{BN}$ were significantly different between the samples of different DMs for both pH series ($p < 0.05$). Trends associated with decreasing DM values were similar for both pH series with the length of block ($\overline{BS}$) increasing, and the number of blocks ($\overline{BN}$) decreasing. In other words, a small number of large blocks are present at 50% DM, while a large number of small blocks are present at 70% DM. Sum of demethylesterified blocks ($\bar{B}$) was the highest in the 50% DM at pH 7.0 and followed by 50% DM at pH 4.5 and both 70% DM samples. $\bar{B}$ could be explained as the number of blocks within a molecule regardless of the size of blocks, in this regard, $\bar{B}$ addresses active demethylesterification of multiple isozymes at pH 7, which is close to pH optimum of these PMEs. It also reveals that the possibility of connection of small blocks leading to generation of larger blocks in the 50% DM at pH 7.0 sample. Significant differences in $\bar{B}$, $\overline{BS}$ and $\overline{BN}$ between the two pH values were present at 50% DM ($p < 0.05$). A similar trend was observed from the results of separate *C. sinensis* PME isozymes (Cameron et al., 2008; Cameron et al., 2011; Kim, Williams, Luzio & Cameron., 2017). Previously, block structure introduced by three PME isozymes of *Citrus sinensis* were investigated individually (Cameron et al., 2008; Cameron et al., 2011; Kim et al., 2017). When comparing the average block size and number in 50% DM samples with previous block reports of individual PME isozymes from *C. sinensis*, the mixture of orange PMEs produced relatively high numbers of small blocks (Table 2). Number of average sized blocks were the highest in NB-PME at pH 7.0 and SI-PME at pH 4.5 and followed by the PME mixture. TT-PME showed the smallest block size as well as the number of average sized blocks. Values reported here for average block lengths ($\overline{BS}$) are larger than those reported previously for jelly fig *Fa*PME, yet smaller than papaya *Cp*LPME with equivalent decreases in DM (Kim et al., 2013; Kim et al., 2014). Interestingly, the longest oligogalacturonide released from NB-, SI- and TT-PME ranged from a degree of polymerization (DP) of 50–57 at 50% DM but demethylesterified HGs treated with PME cocktail showed much shorter oligomers, DP of 39 and 35 at pH 7.0 and 4.5, respectively. These results suggested the possibility of customizing the block size and in advance, modulating the mode of action of PME via controlling treatment conditions and using *C. sinensis* isozymes as well as other plant PMEs. Detailed comparison of block structure of each PME isozyme and PME cocktail will be discussed in *3.4*.

Table 1
Sum of DMB per molecule ($\bar{B}$), average DMB size ($\overline{BS}$) and number of average size DMB ($\overline{BN}$) per molecule [a].calulated from limited EPG digest and degree of blockiness (DB) and absolute degree of blockiness (DB_{abs}) from exhaustive EPG digest.

	DM (%)	$\bar{B}$	$\overline{BS}$	$\overline{BN}$	DB (%)	DB_{abs} (%)
Parent	91	–	–	–	–	–
pH4.5	50	6.11^{b}	7.80^{a}	0.78^{c}	35.22^{a}	17.61^{a}
	70	5.82^{b}	5.31^{c}	1.09^{ab}	32.39^{b}	9.71^{b}
pH7.0	50	7.09^{a}	7.29^{b}	0.97^{b}	33.80^{ab}	16.90^{a}
	70	5.70^{b}	4.80^{d}	1.19^{a}	25.95^{c}	7.78^{c}

[a] Abbreviations: DM, degree of methylation; $\overline{BS}$, average demethylesterified block size; $\overline{BN}$, number of average size demethylesterified block per molecule; GalA, galacturonic acid; DB, degrees of blockiness; DB_{abs} absolute degree of blockiness.

Table 2
Comparison of block structure of 50% DM HGs treated with individual isoforms of PME from Valencia orange[a] and multiple PME isoforms.[b]

50% DM	pH	$\overline{BS}$	$\overline{BN}$	Longest oligomer detected	DB (%)	DB_{abs} (%)
NB-PME	4.5	9.29	0.49	51	37.62	19.83
SI-PME	4.5	8.9	3.6	50	–	–
TT-PME	4.5	6.82	0.09	57	40.05	20.02
Multiple PME	4.5	7.80	0.78	35	35.22	17.61
NB-PME	7.0	6.59	1.45	56	37.03	18.51
SI- PME	7.5	–	–	47	–	–
TT-PME	7.5	4.41	0.16	53	36.28	18.14
Multiple PME	7.0	7.29	0.97	39	33.80	16.90

[a] Block information of NB-, SI- and TT-PME was adopted from Cameron et al. (2008), Cameron et al. (2011), Kim et al. (2017), respectively.
[b] Abbreviations: DM, degree of methylation; $\overline{BS}$, average demethylesterified block size; $\overline{BN}$, number of average size demethylesterified block per molecule; DB, degrees of blockiness; DB_{abs} absolute degree of blockiness.

3.2. Modeling

Comparison of the experimental and theoretical distributions of released oligomers from limited EPG digestions of demethylesterified HGs suggested a multiple attack mode of action for the PME cocktail (Fig. 1). The dotted, dashed and solid line in Figs. 1 and 2 show *in silico* distributions of oligogalacturonides predicted to be released by limited EPG digestions of HGs of 70% and 50% DM, generated from a 94% DM starting substrate using either random demethylesterification or PME models as described above. The oligogalacturonide distributions produced by the PME cocktail reaction conform to the simulated multiple-attack mechanism but additionally with a processivity (p) value of around 10 giving good agreement of the predicted and experimental oligomers released by the limited digest. The 70% DM from both pH

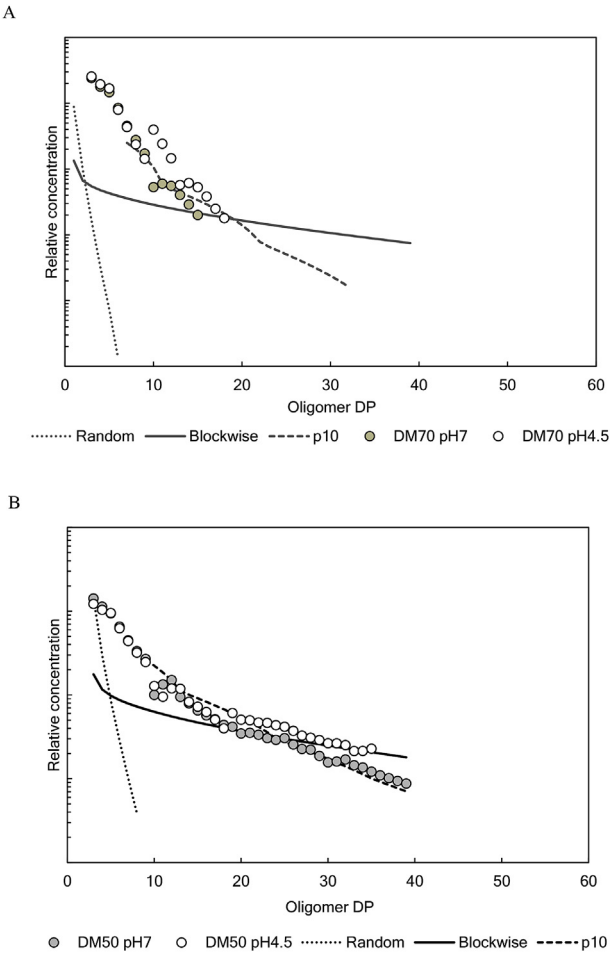

Fig. 1. Experimental vs. theoretical oligomer distributions for sequential demethylesterifications with citrus PME cocktail compared to expectations dependent on PME mode of action (random vs. single chain (blockwise) vs. multiple attack with a degree of processivity = 10). Demethylesterification to DM 70 (A) and DM 50 (B).

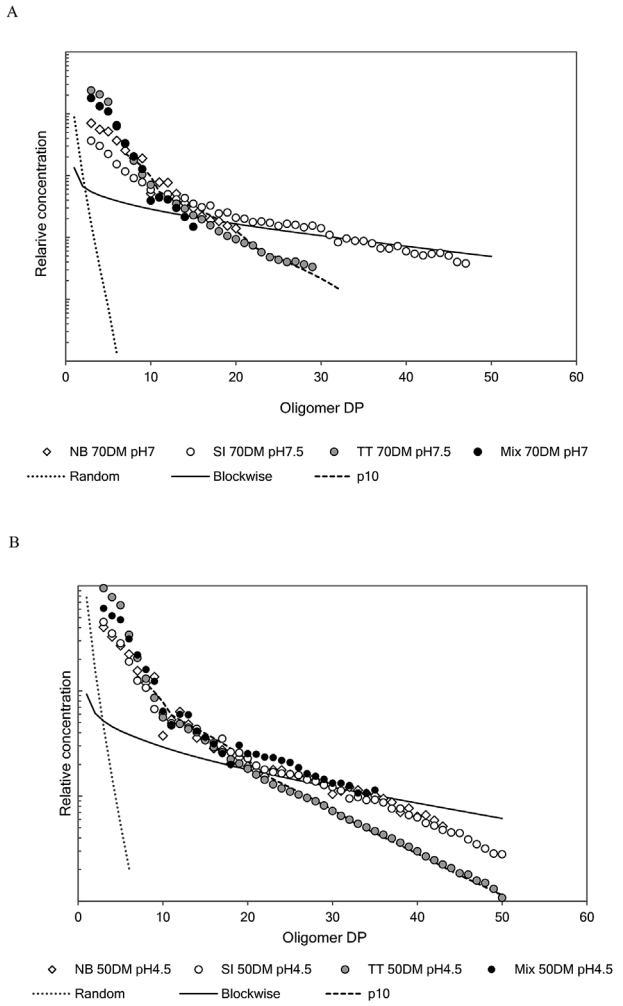

Fig. 2. The comparison of Average $E-(G)_n$-E GalA oligomer distributions obtained limited digestion with EPG from demethylesterified pectins using individual PME isozyme (A) 70DM at pH7.5 (B) 50DM at pH4.5 (three independent experiments). Adopted from Kim, Cameron, Williams & Luzio (2017).

treatments produced DMBs of a very limited size, DP 15 (pH 7.0) and 18 (pH 4.5).

Previous results of experimental curves of SI-PME and papaya PME also showed a change of slope at around DP 20, with longer length fragments over DP 20 being better predicted by a single-chain mechanism, while smaller fragments were fitted with $p = 1$ (Cameron et al., 2008; Kim et al., 2013). Oligomers with a DP over 20 released from 50% DM (pH 4.5) shifted towards a blockwise mode of action as observed from the modeling of NB- and TT-isozymes. Previouly, Versteeg (1979) reported increase in the affinity between the PMEs and the substrate in accordance with a reduction in DM (95–32%) which supports the variable processive mode of action presented in this study (Cameron et al., 2011). Other studies also support the variable processivity of PME by revealing that PME binding groove are strongly influenced by the pattern of methylesterification of the HG chain (Mercadante, Melton, Jameson, & Williams, 2013; Mercadante, Melton, Jameson, Williams, & Simone, 2014). Demethylesterification performed by PME changes the conformation of the substrate HG, then it could affect the subsequent demethylesterification pattern shifting PME's mode of action. This variable mode of action was previously proposed for a separate PME isozyme dependent on the distribution of GalA C6 methyl esters on the HG contained within the active site of enzyme (Cameron et al., 2008; Cameron et al., 2011; Kim et al., 2017). Detailed comparison about mode of actions of each *C. sinensis* isozymes and multiple PME isozyme will be discussed in Section 3.4.

3.3. Demethylesterified block distribution by degree of blockiness and relationship between block parameters from exhaustive and limited EPG digestion of demethylesterified pectins

The degree of blockiness and absolute degree of blockiness values were higher in 50% DMs than 70% DMs (Table 1). DB/DB_{abs} difference between pH pairs was only observed at 70 DM samples displaying pH 4.5 higher in those values compared to pH 7.0. The range of values for DB 25–35 and DB_{abs} 7–17 were obtained within DM range of 50–70% which were similar to DB 23–37 and DB_{abs} 7–19 obtained from NB-PME isozyme from Valencia orange (Tables 1 and 2). The DB/DB_{abs} values were also less than those reported from pectins treated with papaya PME (Kim et al., 2013) and jelly fig PME (Kim et al., 2014) within a similar DM range and rather similar to those obtained from base saponified or fungal PME demethylesterified pectins (Ngouémazong, Jolie, Cardinaels, Fraeye, Van Loey, Moldenaers, 2012; Ström et al., 2007; Tanhatan-Nasseri, Crepeau, Thibault, & Ralet, 2011).

Highly significant coefficients (r) between DM, DB, DB_{abs}, $\bar{B}$, $\overline{BS}$ and $\overline{BN}$ were demonstrated by Pearson's correlation from pooled data of both pH series (Table 3). Positive correlations appeared between DB, DB_{abs}, mono-, di-, and tri-GalA and $\overline{BS}$ ($P < 0.001$), accordingly, negative correlations were observed between DB, DB_{abs} and $\overline{BN}$

Table 3
Pearson's correlation coefficient (r) representing the relationship between block parameters [a] from demethylesterified pectin with PME from *Citrus sinensis* L. Osbeck. [b]

	$\overline{BS}$	$\overline{BN}$	Mono-GalA	Di-GalA	Tri-GalA	DB	DB_{abs}	DM
	0.609*		0.645*	0.732**			0.642*	−0.695**
		−0.911***	0.986***	0.964***	0.862*	0.823***	0.990***	−0.978***
			−0.887***	−0.789**	−0.952***	−0.822**	−0.887***	0.836***
Mono-GalA				0.956**	0.950***	0.885***	0.996***	−0.965***
Di-GalA					0.889***	0.738**	0.973***	−0.987***
Tri-GalA						0.813***	0.959***	−0.933***
DB							0.845***	−0.732**
DB_{abs}								−0.982***

[a] Abbreviations: $\overline{BS}$, average demethylesterified block size; $\overline{BN}$, number of average size demethylesterified block per molecule; GalA, galacturonic acid; DB, degrees of blockiness; DB_{abs} absolute degree of blockiness; DM, degree of methylation.
[b] Coefficients were calculated using pooled data of pH4.5 and pH 7.0. *, ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

($P < 0.01$). Negative correlation between $\overline{BS}$ and $\overline{BN}$ was based on the nature of these parameters since $\overline{BN}$ was calculated from $\bar{B}$ divided by $\overline{BS}$ (see equations 1- 4 presented in methods). Both DB and DB_{abs} also displayed a highly significant correlation coefficient with DM ($P < 0.001$). This verified that the parameters from both complete and limited EPG digest gave consistent information in relation to the DM of pectins. High correlation between the block parameters demonstrated the effectiveness of the techniques for the assessment of the fine structure of nonmethylesterified GalA blocks introduced by multiple PME isozymes. Notably, previous studies revealed high positive correlations between $\overline{BS}$, DB_{abs} and the storage modulus (G′) of the calcium mediated gel, verifying that the block size and distribution pattern is critical to determining the gelling properties (Kim et al 2013, 2014; Kim, Cameron, Williams, & Luzio, 2018).

3.4. Comparison of block pattern introduced by separate PME isozyme and multiple PME isozymes from orange and their mode of actions

The demethylesterified block information of 50% DM samples estimated by both *exhaustive* and *limited* EPG digest of three PME isozymes, SI-PME, TT-PME and NB-PME from *Citrus sinensis* were presented together with those of multiple PME isozymes in Table 2. Each three isozyme showed significant difference in $\overline{BS}$ and $\overline{BN}$ between pH conditions introducing a lower number of larger blocks at pH 4.5 compared to at pH 7.0 or 7.5. Accordingly, a similar trend was observed from HGs treated with PME mixture. The longest oligogalacturonide observed from individual PMEs ranged from around DP 50–57 while the PME mixture introduced much shorter oligomers presenting the longest oligomer less than DP 40. $\overline{BS}$ and $\overline{BN}$ from *limited* EPG digest displayed a difference among the PME isozymes as predicted from their distinct influence on the juice cloud. Unlike the results from *limited* EPG digest, values for DB 36–40 and DB_{abs} 18–20 from *exhaustive* EPG digest showed no significant differences among the individual PMEs. This demonstrated the incapability of DB/DB_{abs} to separate the block pattern introduced by each or multiple PME isozymes.

Fig. 2A and B shows the predictions for liberated GalA oligomers, as calculated by the summation method for 70 and 50% DM pectins demethylesterified assuming PME has a multiple-attack degree given by 1 (random), 10 or single-chain blockwise compared with released oligomer profiles from each NB-, SI-, TT- PME isozyme and PME mixture after *limited* EPG digestion. Previously, it was reported that the experimental curves from SI-PME showed a change of slope or inflection at ~ DP 20 (Cameron et al., 2008). The amount of released oligomers followed degree of multiple attack of around 1 at pH 4.5 or 10 at pH 7.5 until DP 20, then longer length fragments over DP 20 were much better predicted by a single-chain block-wise mechanism. For TT-PME treated samples, the experimental data for DP over 7 were best described by the curve simulated assuming the degree of multiple attack with processivity of ~10 (Cameron et al., 2011). For NB-PME, oligogalacturonide distributions also followed simulated multiple-attack mechanism with a processivity of ~10 then shifted towards a blockwise mode of action for oligomers with a DP over 20, with some difference between DMs and pH condition (Kim et al., 2017).

The minimum number of successive non-methylesterified GalA residues required for forming a junction zone to chelate Ca^{2+} ions previously estimated to 6–13 (Luzio & Cameron, 2008), 9 (Liners, Thibault, & Vancutsem, 1992), 14 (Powell, Morris, Gidley, & Rees, 1982) or up to 20 (Braccini & Pérez, 2001). The calculated average block size manifested that the blocks from individual PME or a PME mixture treatment fall within the suggested ranges. This revealed their feasibility in industrial utilization for efficient modifying functionality of pectins. In summary, the mixture of four *C. sinensis* PME isozymes introduced demethylesterified block sizes and numbers distinguishing from previous results with individual isozymes in a model homogalacturonan. These differences in block pattern were clearly manifested by parameters from *limited* EPG digest results ($\overline{BS}$, $\overline{BN}$) but not by those from the *exhaustive* EPG digest (DB and DB_{abs}) in some cases. Even though the high correlation between both parameters were observed within the *C. sinensis* PME mixture treated HGs, additional modification or enhancement of the DB parameters should be required to best explain the demethylesterified blocks within pectin molecules. From the results, the demethylesterified GalA blocks introduced by *C. sinensis* PME mixture are distinguishable to those from previously characterized PME isozymes. This study shows is may be industrially feasible to directly use the *Citrus* PME mixture rather then individual isozymes, which would require a complicated separation process with attendant high cost. The three well-characterized *C. sinensis* PMEs, SI-PME, TT-PME, NB-PME as well as the PME mixture with distinctive demethylesterification properties might help improve the production of customized pectins for specific usage and expand pectin's use for industrial and medical applications.

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Appendix A. Supplementary data

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References

Baker, R. A., & Cameron, R. G. (1999). Clouds of citrus juices and juice drinks. *Food Technology, 53*, 64–69.

Braccini, I., & Pérez, S. (2001). Molecular basis of Ca (2+)-induced gelation in alginates and pectins: The egg-box model revisited. *Biomacromolecules, 2*(4), 1089–1096.

Cameron, R. G., Baker, R. A., & Grohmann, K. (1998). Multiple forms of pectin methylesterase from citrus peel and their effects on juice cloud stability. *Journal of Food Science, 63*, 253–256.

Cameron, R. G., & Grohman, K. (1995). Partial purification and thermal characterization of pectin methylesterase from red grapefruit finisher pulp. *Journal of Food Science, 60*, 821–825.

Cameron, R. G., Luzio, G. A., Goodner, K., & Williams, M. A. K. (2008). Demethylation of a model homogalacturonan with a salt-independent pectin methylesterase from citrus: I. Effect of pH on demethylated block size, block number and enzyme mode of action. *Carbohydrate Polymers, 71*(2), 287–299.

Cameron, R. G., Luzio, G. A., Vasu, P., Savary, B. J., & Williams, M. A. K. (2011). Enzymatic modification of a model homogalacturonan with the thermally tolerant pectin methylesterase from citrus: 1. Nanostructural characterization, enzyme mode of action, and effect of pH. *Journal of Agricultural and Food Chemistry, 59*(6), 2717–2724.

Cameron, R. G., Savary, B. J., Hotchkiss, A. T., & Fishman, M. L. (2005). Isolation, characterization, and pectin-modifying properties of a thermally tolerant pectin methylesterase from *Citrus sinensis* var. Valencia. *Journal of Agricultural and Food Chemistry, 53*, 2255–2260.

Cameron, R. G., Savary, B. J., Hotchkiss, A. T., Jr., Fishman, M. L., Chau, H.-K., Baker, R. A., et al. (2003). Separation and characterization of a salt-dependent pectin methylesterase from *Citrus sinensis* var. Valencia fruit tissue. *Journal of Agricultural and Food Chemistry, 51*, 2070–2075.

Cargill, Inc (2018). *Label-friendly sweeteners and texturizing solutions.* https://www.cargill.com/food-beverage/na/label-friendly-sweeteners-and-texturizing-solutions, Accessed date: 27 September 2018.

Daas, P. J. H., Meyer-Hansen, K., Schols, H. A., De Ruiter, G. A., & Voragen, A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research, 318*(1–4), 135–145.

Daas, P. J. H., Voragen, A. G., & Schols, H. A. (2000). Characterization of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. *Carbohydrate Research, 326*(2), 120–129.

Denes, J. M., Baron, A., Renard, C. M., Pean, C., & Drilleau, J. F. (2000). Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. *Carbohydrate Research, 327*(4), 385–393.

Duvetter, T., Sila, D. N., Van Buggenhout, S., Jolie, R., Van Loey, A., & Hendrickx, M. (2009). Pectins in processed fruit and vegetables: Part I—stability and catalytic activity of pectinases. *Comp. Rev. Food Sci. Safety, 8*(2), 75–85.

Fraeye, I., De Roeck, A., Duvetter, T., Verlent, I., Hendrickx, M., & Van Loey, A. (2007). Influence of pectin properties and processing conditions on thermal pectin degradation. *Food Chemistry, 105*, 555–563.

Galant, A. L., Luzio, G. A., Widmer, W. W., & Cameron, R. G. (2014). Characterization of molecular structural changes in pectin during juice cloud destabilization in frozen concentrated orange juice. *Food Hydrocolloids, 41*, 10–18.

Guillotin, S. E., Bakx, E. J., Boulenguer, P., Mazoyer, J., Schols, H. A., & Voragen, A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers, 60*(3), 391–398.

Hellin, P., Ralet, M.-C., Bonnin, E., & Thibault, J.-F. (2005). Homogalacturonans from lime pectins exhibit homogeneous charge density and molar mass distributions. *Carbohydrate Polymers, 60*, 307–317.

Hotchkiss, A. T., Savary, B. J., Cameron, R. G., Chau, H. K., Brouillette, J., Luzio, G. A., et al. (2002). Enzymatic modification of pectin to increase its calcium sensitivity while preserving its molecular weight. *Journal of Agricultural and Food Chemistry, 50*(10), 2931–2937.

Kim, Y., Cameron, R. G., Williams, M. A. K., & Luzio, G. A. (2018). 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. *Food Hydrocolloids, 77*, 879–886.

Kim, Y., Williams, M. A. K., Galant, A. L., Luzio, G. A., Savary, B. J., Vasu, P., et al. (2013). Nanostructural modification of a model homogalacturonan with a novel pectin methylesterase: Effects of pH on nanostructure, enzyme mode of action and substrate functionality. *Food Hydrocolloids, 33*(1), 132–141.

Kim, Y., Williams, M. A. K., Luzio, G. A., & Cameron, R. G. (2017). Introduction and characterization of charged functional domains into an esterified pectic homogalacturonan by a citrus pectin methylesterase and comparison of its modes of action to other pectin methylesterase isozymes. *Food Hydrocolloids, 69*, 422–431.

Kim, Y., Williams, M. A. K., Tzen, J. T. C., Luzio, G. A., Galant, A. L., & Cameron, R. G. (2014). Characterization of charged functional domains introduced into a modified pectic homogalacturonan by an acidic plant pectin methylesterase (Ficus awkeotsang Makino) and modeling of enzyme mode of action. *Food Hydrocolloids, 39*, 319–329.

Liners, F., Thibault, J. F., & Van Cutsem, P. (1992). Influence of the degree of polymerization of oligogalacturonates and of esterification pattern of pectin on their recognition by monoclonal antibodies. *Plant Physiology, 99*(3), 1099–1104.

Lofgren, C., Guillotin, S., Evenbratt, H., Schols, H., & Hermansson, A. M. (2005). Effects of calcium, pH, and blockiness on kinetic rheological behavior and microstructure of HM pectin gels. *Biomacromolecules, 6*(2), 646–652.

Louvet, R., Cavel, E., Gutierrez, L., Guenin, S., Roger, D., Gillet, F., et al. (2006). Comprehensive expression profiling of the pectin methylesterase gene family during silique development in *Arabidopsis thaliana*. *Planta, 224*, 782–791.

Luzio, G. A., & Cameron, R. G. (2008). Demethylation of a model homogalacturonan with the salt-independent pectin methylesterase from citrus: II. Structure function analysis. *Carbohydrate Polymers, 71*, 300–309.

Mercadante, D., Melton, L. D., Jameson, G. B., & Williams, M. A. K. (2014). Processive pectin methylesterases: The role of electrostatic potential, breathing motions and bond cleavage in the rectification of Brownian motions. *PLoS One, 9*, e87581.

Mercadante, D., Melton, L. D., Jameson, G. B., Williams, M. A. K., & Simone, A. D. (2013). Substrate dynamics in enzyme action: Rotations of monosaccharide subunits in the binding groove are essential for pectin methylesterase processivity. *Biophysical Journal, 104*, 1731–1739.

Ngouémazong, D. E., Jolie, R. P., Cardinaels, R., Fraeye, I., Van Loey, A., Moldenaers, P., et al. (2012). Stiffness of Ca2+-pectin gels: Combined effects of degree and pattern of methylesterification for various Ca2+ concentrations. *Carbohydrate Research, 348*, 69–76.

Powell, D. A., Morris, E. R., Gidley, M. J., & Rees, D. A. (1982). Conformations and interactions of pectins. II. Influences of residue sequence on chain association in calcium pectate gels. *Journal of Molecular Biology, 155*(4), 517–531.

Savary, B. J., Hotchkiss, A. T., & Cameron, R. G. (2002). Characterization of a salt-independent pectin methylesterase purified from Valencia orange peel. *Journal of Agricultural and Food Chemistry, 50*, 3553–3558.

Savary, B. J., Vasu, P., Cameron, R. G., McCollum, T. G., & Nuñez, A. (2013). Structural characterization of the thermally tolerant pectin methylesterase purified from *Citrus sinensis* fruit and its gene sequence. *Journal of Agricultural and Food Chemistry, 61*(51), 12711–12719.

Savary, B. J., Vasu, P., Nunez, A., & Cameron, R. G. (2010). Identification of thermolabile pectin methylesterases from sweet orange fruit by peptide mass fingerprinting. *Journal of Agricultural and Food Chemistry, 58*, 12462–12468.

Seymour, T. A., Preston, J. F., Wicker, L., Lindsay, J. A., & Marshall, M. R. (1991). Purification and properties of pectinesterases of Marsh White grapefruit. *Journal of Agricultural and Food Chemistry, 39*, 1080–1085.

Ström, A., Ribelles, P., Lundin, L., Norton, I. T., Morris, E. R., & Williams, M. A. K. (2007). Influence of pectin fine structure on the mechanical properties of calcium−pectin and acid−pectin gels. *Biomacromolecules, 8*(9), 2668–2674.

Tanhatan-Nasseri, A., Crepeau, M. J., Thibault, J. F., & Ralet, M. C. (2011). Isolation and characterization of model homogalacturonans of tailored methylesterification patterns. *Carbohydrate Polymers, 86*(3), 1236–1243.

Thibault, J. F., Renard, C. M. G. C., Axelos, M. A. V., Roger, P., & Crepeau, M. J. (1993). Studies of the length of homogalacturonic regions in pectins by acid hydrolysis. *Carbohydrate Research, 238*(4), 271–286.

Van Buggenhout, S., Messagie, I., Maes, V., Duvetter, T., Van Loey, A., & Hendrickx, M. (2006). Minimizing texture loss of frozen strawberries: Effect of infusion with pectin methylesterase and calcium combined with different freezing conditions and effect of subsequent storage/thawing conditions. *European Food Research and Technology, 223*(3), 395–404.

Versteeg, C. (1979). *Pectin esterases from the orange: Their purification, general characteristics and juice cloud destabilizing properties.* Wageningen, The Netherlands: Agricultural University Ph.D.

Versteeg, C., Rombouts, F. M., Spaansen, C. H., & Pilnik, W. (1980). Thermostability and orange juice cloud destabilizing properties of multiple pectinesterases from orange. *Journal of Food Science, 45*, 969–971 998.

Willats, W. G., Orfila, C., Limberg, G., Buchholt, H. C., van Alebeek, G. J., Voragen, A. G., et al. (2001). Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. *Journal of Biological Chemistry, 276*, 19404–19413.

Yapo, B. M., Lerouge, P., Thibault, J.-F., & Ralet, M.-C. (2007). Pectins from citrus peel cell walls contain homogalacturonans homogeneous with respect to molar mass, rhamnogalacturonan I and rhamnogalacturonan II. *Carbohydrate Polymers, 69*, 426–435.

Zega, A., & D'Ovidio, R. (2016). Genome-wide characterization of pectin methyl esterase genes reveals members differentially expressed in tolerant and susceptible wheats in response to *Fusarium graminearum*. *Plant Physiology and Biochemistry, 108*, 1–11.