Partially esterified oligogalacturonides are the preferred substrates for pectin methylesterase of *Aspergillus niger*

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Investigations on the mode of action of *Aspergillus niger* pectin methylesterase (PME) towards differently C₆- and C₁-substituted oligogalacturonides (oligoGalpA) are described. De-esterification of methyl-esterified (un)saturated oligoGalpA proceeds via a specific pattern, depending on the degree of polymerization. Initially, a first methyl ester of the oligomer is hydrolysed, resulting in one free carboxyl group. Subsequently, this first product is preferred as a substrate and is de-esterified for a second time. This product is then accumulated and hereafter de-esterified further to the final product, i.e. oligoGalpA containing one methyl ester located at the non-reducing end residue for both saturated and unsaturated oligoGalpA, as found by post-source decay matrix-assisted laser-desorption/ionization–time-of-flight MS. The saturated hexamer is an exception to this: three methyl esters are removed very rapidly, instead of two methyl esters. When unsaturated oligoGalpA were used, the formation of the

end product differed slightly, suggesting that the unsaturated bond at the non-reducing end influences the de-esterification process. *In vivo*, PME prefers methyl esters, but the enzyme appeared to be tolerant for other C₆- and C₁-substituents. Changing the type of ester (ethyl esterification) or addition of a methyl glycoside (C₁) only reduced the activity or had no effect respectively. The specific product pattern was identical for all methyl- and ethyl-esterified oligoGalpA and methyl-glycosidated oligoGalpA, which strongly indicates that one or perhaps two non-esterified oligoGalpA are preferred in the active-site cleft.

Key words: *Aspergillus niger*, de-esterification, ethyl esters, matrix-assisted laser-desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF MS), methyl esters, oligogalacturonide, pectin methylesterase.

INTRODUCTION

Pectin is a major constituent of the middle lamella of the primary cell wall of practically all higher-plant tissues [1,2]. It is composed of a homogalacturonan backbone (smooth region) interrupted by heavily branched regions of rhamnogalacturonan (hairy region). Homogalacturonan consists of α 1 $\rightarrow$ 4-linked D-galacturonic acid (GalpA) residues, which can be methyl-esterified [1–4].

Pectin methylesterase (PME; EC 3.1.1.11) is able to hydrolyse the methyl ester, thereby releasing methanol and lowering the degree of methyl esterification (DM). PMEs are found in numerous organisms [5,6]. By far the most are found in plants and often several isoforms are found within one species. These endogenous enzymes are often tissue-specific and contribute to changes in the cell wall during plant development and fruit ripening. By reducing the DM, the gelling properties and calcium reactivity of pectin change and pectin may become susceptible to other endogenous pectinases, such as polygalacturonase.

In micro-organisms, PMEs are mainly found in fungi, such as *Aspergillus* [7,8] or *Botrytis* [9], and bacteria such as *Erwinia* [10], and a few yeast PMEs have also been described [11,12]. However, only a limited number has been purified and characterized in detail.

PMEs are medium-sized enzymes (25–50 kDa). In general, the plant and bacterial PMEs have pH optima between 6 and 8, whereas most fungal PMEs have pH optima between 4 and 6. Another difference is their mode of action. Fungal PMEs attack the methyl groups on the pectin molecules randomly, resulting in a random distribution of the non-methylated GalpA residues,

whereas plant PMEs and the *Erwinia* PME remove blocks of methyl groups on a single chain [13–17]. However, PME action on (highly) methylated pectins does not result in complete de-esterification [6]. Generally, approx. 20–30 % of the methyl esters cannot be removed. In part, this is due to the presence of acetyl groups in certain pectins (e.g. sugar beet pectin).

Pectin is too heterogeneously esterified to study the mode of action of PMEs; therefore we have embarked on a detailed investigation of the structural requirements and chemical nature of substituents at the carboxylic function and at the reducing end for substrate recognition. For this purpose, oligogalacturonides (oligoGalpA) with defined degrees of polymerization (DPs) were purified and derivatized at the C₁- and C₆-positions, as shown for GalpA in Figure 1. Thus oligoGalpA were fully methyl-esterified or ethyl-esterified. In addition, the reducing end (C₁) was methyl-glycosidated [18]. In the present paper, we report on the activity of *Aspergillus niger* PME on the various substrates and the mode of action towards those substrates where significant activity was observed, carried out by state-of-the-art techniques such as high-performance anion-exchange chromatography (HPAEC) and matrix-assisted laser-desorption/ionization–time-of-flight MS (MALDI–TOF–MS) [18,19].

MATERIALS AND METHODS

Materials

Polygalacturonic acid was purchased from ICN Biomedicals BV (Zoetermeer, The Netherlands). Apple pectin (70 % DM)

Abbreviations used: DM, degree of methyl esterification; DP, degree of polymerization; GalpA, D-galacturonic acid; HPAEC, high-performance anion-exchange chromatography; MALDI–TOF–MS, matrix-assisted laser-desorption/ionization–time-of-flight MS; oligoGalpA, oligogalacturonide(s); PAD, pulsed amperometric detection; PME, pectin methylesterase; PSD, post-source decay; THAP, 2,4,6-trihydroxyacetophenone.

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Figure 1 Structures of (un)derivatized GalpA

(A) GalpA, (B) 6-O-CH₃-GalpA, (C) 6-O-C₂H₅-GalpA and (D) 1-O-CH₃-6-O-CH₃-GalpA.

was a gift from Obipectin (Bischofszell, Switzerland). Mono- and di-galacturonic acid were from Sigma. Pectin lyase and polygalacturonase 1 for oligoGalpA production were a gift from Novo-Nordisk A/S (Bagsværd, Denmark). PME from *A. niger* and *Citrus sinensis* L. (navel oranges) were purified as described in [7,20]. H⁺-Dowex 50W ×8 50–100 mesh was acquired from Fluka. H⁺-Dowex AG 50W ×8 100–200 mesh (biotechnological grade) was from Bio-Rad and was used for the preparation of ammonium-loaded Dowex [21]. 2,4,6-Trihydroxyacetophenone (THAP) was bought from Acros Organics (Geel, Belgium). Pure nitrocellulose membrane was purchased from Bio-Rad.

Preparation of (derivatized) oligoGalpA

Saturated oligoGalpA (DP 3–10) were purified from a polygalacturonase 1 digest of polygalacturonic acid. Δ4,5-Unsaturated oligoGalpA were purified from a saponified pectin lyase digest of a 1% (w/v) 70% DM pectin solution. The oligoGalpA were separated on a source Q column and isolated as described in [18,19].

The purity of the oligoGalpA (DP 2–10) was determined using HPAEC at pH 12 equipped with pulsed amperometric detection (PAD) [18] and was found to be in the range of 90–99%, based on area percentages.

Methyl glycosidation and/or methyl esterification of the (un)saturated oligoGalpA was performed with acidic methanol as described in [18]. Ethyl esterification was performed identically with methyl esterification, except acidic ethanol was used. Ethyl esterification could be performed with oligoGalpA up to DP 6. OligoGalpA with higher DP were not ethyl-esterified, due to the solubility being too low.

Enzyme incubations

PME activity towards the polymeric substrate (70% DM apple pectin) at pH 4.5 was determined by titration, as described in [20].

PME activity towards oligomeric substrates was determined in a different manner. A typical reaction mixture (200 μl) contained 10 mM (substituted) oligoGalpA in 25 mM sodium acetate buffer (pH 4.5). The reaction was started by the addition of an appropriate amount of enzyme and incubated at 30 °C. Samples (25 μl) were taken at different time intervals (0–113 h) and the enzyme was inactivated by boiling for 5 min. Upon cooling, the samples were frozen at –20 °C, until analysis by HPAEC and/or MALDI-TOF-MS.

Chromatographic analysis

HPAEC at pH 5 was performed essentially as described in [22]. Partially methyl-esterified oligoGalpA were separated on

a CarboPac PA-1 column (Dionex Corp., Sunnyvale, CA, U.S.A.) using a 10 min isocratic elution with 0.05 M sodium acetate (pH 5) at a flow rate of 0.5 ml/min, followed by a 60 min gradient of 0.05–0.5 M and a 20 min gradient to 1 M sodium acetate. After 5 min of washing with 1 M sodium acetate, the column was equilibrated in 15 min with 0.05 M sodium acetate. Δ4,5-Unsaturated oligoGalpA were selectively detected at 235 nm (UV1000, Thermo Separation Products), whereas detection of both saturated and Δ4,5-unsaturated oligoGalpA was accomplished by post-column addition of NaOH to raise the pH (>12) before it entered the pulsed amperometric detector (Electrochemical Detector ED40, Dionex Sunnyvale, CA, U.S.A.).

Sample preparation for MALDI-TOF-MS

In general, one desalting step of the sample using H⁺-Dowex AG 50W ×8 was sufficient for good MS analysis. Whenever the salt concentration appeared too high, an additional treatment with NH₄⁺-loaded Dowex AG 50W ×8 was carried out [21]. Subsequently, the samples were freeze-dried and dissolved in approx. 25 μl of water. For determination of the distribution/location of methyl esters, freeze-dried samples were labelled at the reducing end by dissolving in H₂¹⁸O containing 0.5% formic acid. After 48–72 h of incubation at room temperature (20 °C), the acid-catalysed hydroxide exchange was more than 90% complete and samples were ready for analysis [23].

MALDI-TOF-MS

MALDI-TOF-MS was performed with the Voyager DETM-RP MALDI-TOF Mass Spectrometer (PerSeptive Biosystems Inc., Framingham, MA, U.S.A.) as described in [24]. THAP-nitrocellulose was used as a matrix [21]. As soon as the matrix (0.2 μl) was applied to the sample plate, it formed a thin layer. On top of this layer, a 0.2 μl sample (containing 2–8 μmol of oligoGalpA) was applied. After application, the sample plate was dried under a stream of air. Whenever appropriate, an extra 0.2 μl of 10 mM NaCl was added to the sample to improve ionization in the positive mode.

Post-source decay (PSD) MALDI-TOF-MS was performed on a peak that was selected from the mass spectrum (window set at 20 mm) of the compound of interest. The system was calibrated with the selected peak before fragmentation. Hereafter, the laser power was increased to enhance the fragmentation and the PSD spectra were collected at different mass ranges. Finally, the different spectra were stitched together, resulting in a spectrum covering the complete mass range.

RESULTS

The mode of action of PME is influenced by the identity of the carboxyl function of the GalpA residues present in the homogalacturonan backbone. In nature, this carboxyl group can be either free or methyl-esterified (Figures 1A and 1B respectively). In order to determine the specificity of PME in more detail, oligoGalpA were purified and derivatized (Figure 1), and were used to determine substrate specificity.

In addition, PME from *Aspergillus niger* was purified to homogeneity [7] and the specific activity appeared to be 70 units/mg using 70 % DM pectin (0.63 %, w/v) as the substrate. This preparation was used for the determination of the mode of action on differently C₁- and C₆-substituted oligoGalpA.

Mode of action of PME on fully methyl-esterified oligoGalpA

PME de-esterification of fully methyl-esterified saturated and unsaturated oligoGalpA was monitored by HPAEC (pH 5) using a post-column sodium hydroxide delivery system for PAD and UV detection (235 nm). UV specifically detects unsaturated oligoGalpA, whereas PAD detects both saturated and unsaturated oligoGalpA. At pH 5, the methyl esters remain on the oligoGalpA and separation occurs according to the charge, as well as to the charge distribution of the oligoGalpA. A typical set of time-dependent elution patterns of fully esterified unsaturated tetramer de-esterified by PME is presented in Figure 2. The UV elution profiles (Figure 2A) clearly demonstrate a gradual decrease of the fully methyl-esterified unsaturated tetramer (not bound to the column) with time, with a simultaneous appearance of peaks at 8.8, 35.1 and 60.8 min. The same samples were also analysed with MALDI-TOF-MS (Figure 2B), where a clear de-esterification of fully methyl-esterified unsaturated tetramer, 783 *m/z* (Na⁺-adduct), into the tetramer lacking one (769 *m/z*), two (755 *m/z*) or three methyl esters (741 *m/z*) could be seen in time. In addition, K⁺-adducts were seen, which are 16 *m/z* larger than the corresponding Na⁺-adducts. Comparison of the mass spectra with the UV elution profiles led to the identification of the peaks in the HPAEC elution patterns as indicated in Figure 2(A). Based upon the UV patterns, the relative amounts of differently methyl-esterified Δ 4,5-unsaturated oligoGalpA were calculated, resulting in progression profiles as shown in Figure 3. Here, the control samples (not incubated) are set at time -1 h, since de-esterification had already taken place at zero time. For all methyl-esterified unsaturated oligomers tested (DP 3–6), a fast initial de-esterification was seen. The fully methyl-esterified Δ 4,5-unsaturated trimer was rapidly de-esterified to a trimer with two methyl esters, followed by a slow de-esterification to a trimer with one methyl ester. OligoGalpA of higher DP all showed similar patterns, but different from that of the trimer. After removal of the first methyl ester, a second methyl ester was removed almost immediately, although the starting material was still present. This results in an accumulation of oligoGalpA lacking two methyl esters. Hereafter, the other methyl esters were removed one by one at a decreasing rate until the final product, i.e. oligoGalpA containing one methyl-ester. In all cases, the final product is formed almost immediately from the start at a comparable linear rate, except for the tetramer. In a similar experiment, the fully methyl-esterified saturated oligoGalpA (DP 3–6) were de-esterified with PME as well. De-esterification was monitored by both MALDI-TOF-MS and HPAEC (pH 5) PAD. Since response factors (PAD) for partly methyl-esterified oligoGalpA are not available, progression profiles based on peak areas were constructed (results not shown). The MALDI-TOF mass spectra and progression profiles were in good

Figure 2 UV (235 nm) elution profiles (A) and MALDI-TOF mass spectra (B) of PME de-esterification of unsaturated (6-O-CH₃-GalpA)₄ monitored in time

UV elution profiles are corrected for baseline increase as the result of the acetate gradient. The masses and the number of methyl groups are indicated (e.g. uG₄², an unsaturated tetraGalpA having two methyl groups). A control (Cont.) is added to illustrate the 'real' starting point of the reaction.

agreement with each other. The trimer, tetramer and pentamer were de-esterified in a similar fashion as the unsaturated counterparts. The hexamer showed a different pattern of de-esterification. After removal of the first methyl ester, both the second and third methyl esters were removed immediately, resulting in the accumulation of hexaGalpA bearing three methyl esters. Subsequently, the fourth and fifth methyl esters were removed, again at a slower rate as shown for the unsaturated hexamer, leaving one methyl ester on the hexamer. However, all saturated oligomers are de-esterified at faster rates compared with the unsaturated counterparts: the end of the reaction was reached earlier by a factor of 2–3. For the saturated oligoGalpA, de-esterification was also performed at lower enzyme concentrations. Based on MALDI-TOF mass spectra, the relative release of methanol was calculated with time. Since both the enzyme and substrate concentrations are known, the specific activity can be estimated. For the trimer, a specific activity of 0.3 unit/mg was found. The specific activity for the tetramer strongly increased to 6.96 units/mg. For larger oligoGalpA, the increase was somewhat less, but still considerable, since the

Figure 3 Progression profiles of PME de-esterification on unsaturated (6-O-CH₃-GalpA)₃ (A), unsaturated (6-O-CH₃-GalpA)₄ (B), unsaturated (6-O-CH₃-GalpA)₅ (C) and unsaturated (6-O-CH₃-GalpA)₆ (D)

Profiles are calculated on the basis of the UV-elution profiles as shown in Figure 2(A) for the unsaturated (6-O-CH₃-GalpA)₄. The differently esterified oligoGalpA are indicated according to the number of methyl esters lost: ○, none; □, one; △, two; ▽, three; ●, four; ■, five. At -1 h, the control situation is shown to illustrate the 'real' starting conditions of the reaction.

Figure 4 PSD fragmentation pattern (positive mode) of the final product mono-methyl unsaturated (GalpA)₃ as a result of PME de-esterification

The peaks are coded with their mass, corresponding ion and the amount of methyl esters. Inset, the structure of the isomer.

specific activities for the pentamer and hexamer were found to be 10.5 and 27 units/mg respectively.

End-product determination of methyl-esterified oligoGalpA after PME incubation

For all the substrates tested so far, both saturated and unsaturated oligoGalpA, the same final product always resulted: an oligoGalpA containing one methyl ester. Since this is a very specific product, an experiment was performed to establish the precise position of this methyl ester using PSD MALDI-TOF-MS as described in [19]. Saturated oligoGalpA were ^{18}O -labelled at the reducing end, to discriminate between reducing and non-reducing end fragments. Because unsaturated oligoGalpA give rise to very specific fragmentation products, these were not labelled [19].

A typical fragmentation pattern for unsaturated trimer containing one methyl ester is shown in Figure 4. Besides the expected B-, C- and Y-fragments, a typical ring-cleavage product at the non-reducing end, $^{0,2}\text{X}_2$, was found. No Z-fragments were observed. The methyl ester was only found on the B- and C-fragments, corresponding to the non-reducing end, but never on the Y-fragments, which correspond to the reducing end. The $^{0,2}\text{X}_2$ ring cleavage product, 435 m/z , arose after a loss of 130 m/z , which proves that a methyl ester is on the non-reducing end of the GalpA residue. Also, the presence of the double-fragmented products B_2 -130 and C_2 -130 m/z confirm this. The absence of other fragmentation products and especially the $^{0,2}\text{X}_2$ ring-cleavage product, 449 m/z , indicates that no other isomers were present. The final products for unsaturated tetramer and pentamer were also determined and appeared to have the methyl ester at the non-reducing end as well. The final products of saturated oligoGalpA (DP 3–5) de-esterified by PME also showed the presence of a methyl ester at the non-reducing end (results not shown).

Influence of the type of carboxyl ester on the mode of action of PME

To investigate the specificity of PME for methyl-esterified oligoGalpA, ethyl-esterified saturated oligoGalpA (DP 4–6) were subjected to PME de-esterification. The reactions were monitored over time using MALDI-TOF-MS as shown for the hexamer (Figure 5). The ethyl-esterified hexamer (1265 m/z) is gradually de-esterified ($-28\text{ }m/z$) into the hexamer lacking one (1237 m/z), two (1209 m/z), three (1181 m/z) and four (1153 m/z) ethyl esters. The mass spectra monitoring the de-esterification of methyl-esterified hexaGalpA and ethyl-esterified hexaGalpA demonstrate a striking resemblance. In this experiment, the reaction has not reached an end-point, because the hexamer lacking five esters was not formed yet. For the ethyl-esterified tetramer and pentamer, similar patterns were found as for their methyl-esterified counterparts. However, in all cases, the rate of de-esterification for ethyl-esterified oligosaccharides calculated from the mass spectra as described above decreases by a factor of 2 for the hexamer and by a factor of 8 for the tetramer, compared with methyl-esterified oligosaccharides. So it seems that the size of the ester does not influence the mode of de-esterification, but only influences the rate of de-esterification.

Effect of the reducing end of fully methyl-esterified oligoGalpA on the mode of action of PME

To investigate whether the reducing end of fully methyl-esterified saturated oligoGalpA influences PME de-esterification, both methyl-glycosidated and non-glycosidated methyl-esterified oligoGalpA were incubated with PME. The de-esterification

Figure 5 MALDI-TOF mass spectra (positive mode) showing the de-esterification by fungal PME of (6-O-CH₃-GalpA)₆ (A) and (6-O-C₂H₅-GalpA)₆ (B) over time

The signal intensity is plotted against the mass-to-charge ratio (m/z). A control is added to illustrate the 'real' starting point of the reaction. The masses and the number of methyl or ethyl groups are indicated (e.g. $\text{G}_6^{2\text{CH}_3}$, a saturated hexaGalpA having two methyl groups).

was followed over time using MALDI-TOF-MS, and typical sets of mass spectra are shown in Figure 6 for the methyl-glycosidated and/or methyl-esterified saturated tetramer. The methyl-glycosidated and methyl-esterified tetramer (815 m/z) were de-esterified in the same manner as that of the non-glycosidated tetramer. After the first methyl ester has been removed, the second is removed very rapidly. Finally, a third methyl ester is removed, leaving two methyl groups (one esterified and one glycosidated). Only a tiny amount of tetramer with one methyl ester was formed, which originated from some contamination of the substrate with non-glycosidated tetramer. The methyl-glycosidated and methyl-esterified tetramer seem to be less susceptible for de-esterification, since they are visible over a longer time frame. However, this was less obvious for larger oligoGalpA and did not influence the rate of de-esterification.

DISCUSSION

The sequence of de-esterification of differently C₆- and C₁-substituted oligoGalpA by PME from *A. niger* is described. In contrast with pectin lyase A, which is very specific for methyl

Figure 6 MALDI-TOF mass spectra (positive mode) showing the de-esterification by fungal PME of (6-O-CH₃-GalpA)₄ (A) and 1-O-CH₃-(6-O-CH₃-GalpA)₄ (B) over time

The signal intensity is plotted against the mass-to-charge ratio (*m/z*). A control (Cont.) is added to illustrate the 'real' starting point of the reaction. The masses and the number of methyl groups are indicated (see Figure 5).

esters [25], PME appeared to be tolerant to ethyl esters as well as for methyl-glycosidation at the reducing end. Only the rate of conversion was decreased by a factor of 2 for the hexamer and by a factor of 8 for the tetramer when ethyl esters were present

instead of methyl esters. PME activities that were determined on the basis of MALDI-TOF mass spectra towards saturated (6-O-CH₃-GalpA)₃ and (6-O-CH₃-GalpA)₆ showed an increase by a factor of 100, similar to the results found previously using HPAEC (pH 5) [15]. This indicates that (at least six) multiple subsites are present in the substrate-binding cleft of the PME of *A. niger*.

Kester et al. [15] reported the de-esterification of saturated oligoGalpA (DP 2–6) by fungal PME. The reaction products were identified by tandem MS and the order of de-esterification was determined by comparing 'early' and 'late' reaction products. However, quantification of the intermediate products was not possible using tandem MS. Therefore, we have focused our investigation on the detailed sequence of de-esterification and used the known intermediates as determined by Kester et al. [15] to identify the intermediates in our experiments as well. Fully methyl-esterified saturated and unsaturated oligoGalpA (DP 3–6) were subjected individually to PME. Both the progression profiles (based on HPAEC, pH 5) and MALDI-TOF mass spectra showed similar patterns of de-esterification. After the first removal of a methyl ester from one of the inner residues, a second methyl ester was removed almost instantly from either an inner residue or the reducing end, except for the trimer. Here, only the first methyl ester was removed quickly. From the saturated hexamer, even a third methyl ester was removed from either an inner residue or the reducing end before accumulation of a product occurred. Hereafter de-esterification occurred at a lower rate to end at the final product, oligoGalpA with one methyl ester at the non-reducing end for both the saturated and the unsaturated oligoGalpA. In addition, we found that it appeared to be more difficult for PME to remove the penultimate methyl ester from unsaturated oligoGalpA compared with the saturated oligoGalpA, suggesting that the unsaturated bond at the non-reducing end reduces the catalytic efficiency of PME. One might expect that this methyl ester is located closest to the non-reducing end. Also the fact that the unsaturated trimer, pentamer and hexamer show the same linear increase in the final product formation could be an indication that this methyl ester is located at an identical position that is next to the non-reducing sugar. This would imply that the unsaturated oligoGalpA are de-esterified slightly differently compared with their saturated counterparts. The unsaturated trimer would be de-esterified starting from the reducing end, followed by the middle residue, whereas the saturated trimer does not show this specific order of de-esterification (Figure 7).

Figure 7 Schematic presentation of the proposed optimal binding mode of (un)saturated trimer and pentamer in the active-site cleft of *A. niger* PME during first and second attack

The reducing end is on the positive side. The positions of de-esterification (↑), saturated GalpA (○), unsaturated GalpA (●) and methyl esters (◐) are indicated.

The above findings clearly indicate that a substrate with at least one or two free carboxyl groups is preferred in the active-site cleft, except when the substrate is of a limiting size, such as the trimer. Apparently, at least four subsites are preferably occupied, one of which prefers a carboxyl group. Assuming that de-esterification occurs at subsite +1 and the reducing end is on the positive side, subsite +2 would be the best candidate to prefer a de-esterified GalpA residue. Both the saturated and unsaturated (6-O-CH₃-GalpA)₄ demonstrate the fast second de-esterification. It was argued above that the unsaturated tetramer is probably de-esterified at the reducing-end residue or neighbouring residue. The resulting products can never bind with the free carboxyl group to subsite -3 and -2 and, at the same time, cover subsite +1 to form a productive enzyme-substrate complex. Therefore these subsites can be excluded and subsites -1, +2 and +3 are left. Subsite -1 can be ruled out as well, since it is covered at all times and an effect on the trimer would be expected. A close look at the intermediate products identified by Kester et al. [15] shows small stretches of two or three carboxyl groups for almost all products, suggesting that PME preferably de-esterifies next to a free carboxyl group. This is only possible when subsite +2 has a preference for a free carboxyl group. So the second de-esterification occurs next to the first de-esterified GalpA towards the non-reducing side (Figure 7). There might also be a second subsite present preferring a carboxyl group, since both the methyl-esterified and ethyl-esterified saturated hexamers are de-esterified in two fast steps. Since this was not that obvious for the unsaturated hexamer, the difference in binding mode could be the reason, which would imply that a second subsite could be located at either subsite +4 or +5.

The ethyl-esterified oligoGalpA showed similar patterns of de-esterification, indicating that the presence of an ester is more important than its size. The methyl-glycosidated oligoGalpA gave similar patterns of de-esterification as well. So the reducing end is probably not involved in the binding of the substrate to PME.

Fungal PME action on pectin is thought to be according to a random mechanism. However, in the present study, it has been demonstrated that fungal PME action on oligoGalpA is not completely in accordance with a random mechanism, in contrast with chemical de-esterification of oligoGalpA, which is acting in a really random manner, as shown in Figure 8(A). Here, increasing amounts of sodium hydroxide clearly result in a gradual decrease in the number of methyl esters. Fungal PME differs significantly from the blockwise-acting navel orange PME, which performs not only a blockwise de-esterification of pectin, but a blockwise de-esterification of oligoGalpA as well (Figure 8B). Each hexamer is de-esterified almost completely before the next molecule is attacked, which results in the accumulation of (GalpA)₆ with one methyl ester, while there is still an excess of initial substrate. So the navel orange PME also prefers partly methyl-esterified oligoGalpA, but, in contrast with fungal PME, it is able to remove the next methyl esters of a single oligomer at a similar rate.

At pH 7, apple PME acts according to a single-chain mechanism, whereas at pH 4.5, it behaves as a multiple-chain, multiple-attack enzyme. An inter-chain and an intra-chain mechanism can also be distinguished [26]. The intra-chain mechanism of action of apple PME is the same at both pH 7.0 and 4.5. So whatever the pH, the same size of blocks are formed, the only difference is that at pH 4.5, all chains are de-esterified, whereas at pH 7, part of the chains remains unaffected. So it seems that the binding of the enzyme is less strong at lower pH and the enzyme-substrate complex dissociates faster. The free enzyme can then attack a new chain. Although the apple PME behaves differently at pH 7.0 compared with pH 4.5, in both cases, methyl esters are removed in a blockwise fashion and are equal in size. The only thing that

Figure 8 MALDI-TOF mass spectra (negative mode) showing the chemical de-esterification of (6-O-CH₃-GalpA)₄ by different amounts of sodium hydroxide (equivalent to 25, 50, 75 and 100% of the methyl-ester concentration) (A) and the enzymic de-esterification of (6-O-CH₃-GalpA)₆ by navel orange PME over time (B)

The signal intensity is plotted against the mass-to-charge ratio (*m/z*). The masses and the number of methyl groups are indicated (see Figure 5).

seems to change is that the mode changes from single to multiple attack. It is clear that the change from an intra-chain mechanism to an inter-chain mechanism can only be measured with polymeric substrate. Oligosaccharides, as used in our study, are too short to be able to distinguish between these two mechanisms.

What are the implications for the mode of action of fungal PME on pectin? If larger oligoGalpA (DP > 7) or a polymer is used as a substrate, all subsites of the enzyme will be occupied and the proposed second subsite for a carboxyl group would gain in importance. This might result in de-esterification patterns, which are different from the de-esterification patterns as shown above for the smaller oligoGalpA. The attack of the enzyme on a polymer will still be random, but PME will try to find an optimal binding mode, which is dependent on the DM and the distribution of free carboxyl groups on the pectin molecule. PME action on pectin will probably result in single GalpA residues and short blocks of two or three GalpA residues randomly distributed over the pectin molecule, which is still in contrast with the large blocks formed by plant PME and *Erwinia* PME.

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REFERENCES

- O'Neill, M. A., Albersheim, P. and Darvill, A. G. (1990) The pectic polysaccharides of primary cell walls. In *Methods in Plant Biochemistry*, vol. 2 (Dey, P. M., ed.), pp. 415–441, Academic Press, London.
- Voragen, A. G. J., Pilnik, W., Thibault, J.-F., Axelos, M. A. V. and Renard, C. M. G. C. (1995) Pectins. In *Food Polysaccharides and their Applications* (Stephen, A. M., ed.), pp. 287–339, Marcel Dekker, New York.
- McNeil, M., Darvill, A. G., Fry, S. C. and Albersheim, P. (1984) Structure and function of the primary cell walls of plants. *Annu. Rev. Biochem.* **53**, 625–663.
- Schols, H. A. and Voragen, A. G. J. (1996) Complex pectins: structure elucidation using enzymes. In *Progress in Biotechnology, Pectins and Pectinases*, vol. 14 (Visser, J. and Voragen, A. G. J., eds.), pp. 3–19, Elsevier, Amsterdam.
- Pilnik, W. and Voragen, A. G. J. (1993) Pectic enzymes in fruit and vegetable juice manufacture. In *Enzymes in Food Processing*, 3rd edn (Nagodawithana, T. and Reed, G., eds.), pp. 363–399, Academic Press, London.
- Versteeg, C. (1979) Pectinesterases from the orange fruit – their purification, general characteristics and juice cloud destabilizing properties. Ph.D. thesis, Wageningen Agricultural University, Wageningen, The Netherlands.
- Baron, A., Rombouts, F. M., Drilleau, J. F. and Pilnik, W. (1980) Purification et propriétés de la pectinesterase produite par *Aspergillus niger*. *Lebensm. Wiss. Technol.* **13**, 330–333.
- Christgau, S., Kofod, L. V., Halkier, T., Andersen, L. N., Hockauf, M., Dörreich, K., Dalbøge, H. and Kauppinen, S. (1996) Pectin methyl esterase from *Aspergillus aculeatus*: expression cloning in yeast and characterization of the recombinant enzyme. *Biochem. J.* **319**, 705–712.
- Reignault, P., Mercier, M., Bompeix, G. and Boccara, M. (1994) Pectin methylesterase from *Botrytis cinerea*: physiological, biochemical and immunochemical studies. *Microbiology* **140**, 3249–3255.
- Plastow, G. S. (1988) Molecular cloning and nucleotide sequence of the pectin methyl esterase gene of *Erwinia chrysanthemi* B374. *Mol. Microbiol.* **2**, 247–254.
- Gainvors, A., Frezier, V., Lemaire, H., Lequart, C., Aigle, M. and Belarbi, A. (1994) Detection of polygalacturonase, pectin-lyase and pectin-esterase activities in a *Saccharomyces cerevisiae* strain. *Yeast* **10**, 1311–1319.
- Nakagawa, T., Miyaji, T., Yurimoto, H., Sakai, Y., Kato, N. and Tomizuka, N. (2000) A methylotrophic pathway participates in pectin utilization of *Candida boidinii*. *Appl. Environ. Microbiol.* **66**, 4253–4257.
- Dongowski, G. and Bock, W. (1984) Effects of molecular parameters of pectin substrates on the activities of pectin esterases from *Aspergillus niger* and from higher plants (oranges, tomatoes, white cabbage). *Nahrung* **28**, 507–516.
- Grasdalen, H., Andersen, A. K. and Larsen, B. (1996) NMR-spectroscopy studies of the action pattern of tomato pectinesterase – generation of block structure in pectin by a multiple attack mechanism. *Carbohydr. Res.* **289**, 105–114.
- Kester, H. C. M., Benen, J. A. E., Visser, J., Warren, M. E., Orlando, R., Bergmann, C., Magaud, D., Anker, D. and Doutheau, A. (2000) Tandem mass spectrometric analysis of *Aspergillus niger* pectin methylesterase: mode of action on fully methyl-esterified oligogalacturonates. *Biochem. J.* **346**, 469–474.
- Kohn, R., Dongowski, G. and Bock, W. (1985) Distribution of free and esterified carboxyl groups within the pectin molecule after influence of pectinesterase from *Aspergillus niger* and oranges. *Nahrung* **29**, 75–85.
- Benen, J. A. E., Vincken, J.-P. and van Alebeek, G.-J. W. M. (2002) Microbial pectinases. In *Pectins and Their Manipulation* (Seymour, G. and Knox, P., eds.), pp. 174–221, Sheffield Academic Press, Sheffield.
- van Alebeek, G.-J. W. M., Zabotina, O., Beldman, G., Schols, H. A. and Voragen, A. G. J. (2000) Esterification and glycosylation of oligogalacturonides: examination of the reaction products using MALDI-TOF MS and HPAEC. *Carbohydr. Polym.* **43**, 39–46.
- van Alebeek, G.-J. W. M., Zabotina, O., Beldman, G., Schols, H. A. and Voragen, A. G. J. (2000) Structural analysis of (methyl-esterified) oligogalacturonides using post-source decay matrix-assisted laser desorption/ionization time-of flight mass spectrometry. *J. Mass Spectrom.* **35**, 831–840.
- Versteeg, C., Rombouts, F. M. and Pilnik, W. (1978) Purification and some characteristics of two pectinesterase isoenzymes from orange. *Lebensm. Wiss. Technol.* **11**, 267–274.
- Körner, R., Limberg, G., Mikkelsen, J. D. and Roepstorff, P. (1998) Characterization of enzymatic pectin digests by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass. Spectrom.* **33**, 836–842.
- Daas, P. J. H., Arisz, P. W., Schols, H. A., De Ruiter, G. A. and Voragen, A. G. J. (1998) Analysis of partly methyl-esterified galacturonic acid oligomers by high-performance anion-exchange chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Biochem.* **257**, 195–202.
- Körner, R., Limberg, G., Christensen, T. M. I. E., Mikkelsen, J. D. and Roepstorff, P. (1999) Sequencing of partially methyl-esterified oligogalacturonates by tandem mass spectrometry and its use to determine pectinase specificities. *Anal. Chem.* **71**, 1421–1427.
- van Alebeek, G.-J. W. M., Schols, H. A. and Voragen, A. G. J. (2001) Amidation of methyl-esterified oligogalacturonides: examination of the reaction products using MALDI-TOF MS. *Carbohydr. Polym.* **46**, 311–321.
- van Alebeek, G.-J. W. M., Christensen, T. M. I. E., Schols, H. A., Mikkelsen, J. D. and Voragen, A. G. J. (2002) Mode of action of pectin lyase A of *Aspergillus niger* on differently C₆-substituted oligogalacturonides. *J. Biol. Chem.* **277**, 25929–25936.
- Denès, J.-M., Baron, A., Renard, C. M. G. C., Péan, C. and Drilleau, J.-F. (2000) Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. *Carbohydr. Res.* **327**, 385–393.

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