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# Mode of De-esterification of Alkaline and Acidic Pectin Methyl Esterases at Different pH Conditions

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Highly esterified citrus pectin was de-esterified at pH 4.5 and 8.0 by a fungal pectin methyl esterase (PME) that was shown to have an acidic isoelectric pH (p*I*) and an acidic pH optimum and by a plant PME that was characterized by an alkaline p*I* and an alkaline pH optimum. Interchain and intrachain de-esterification patterns were studied by digestion of the pectin products with endo-polygalacturonase and subsequent analysis using size exclusion and anion-exchange chromatography. No effect of pH was observed on the de-esterification mode of either of the two enzymes. Acidic, fungal PME converted pectin according to a multiple-chain mechanism, with a limited degree of multiple attack at the intrachain level, both at pH 4.5 and at pH 8.0. A multiple-attack mechanism, with a high degree of multiple attack, was more appropriate to describe the action mode of alkaline, plant PME, both at pH 4.5 and at pH 8.0.

**KEYWORDS: Pectin methyl esterase; mode of action; effect of pH; effect of p*I***

## INTRODUCTION

Pectin is an important component of the primary cell wall of dicotyledonous plants. The structure of this complex polysaccharide is not yet completely elucidated, but one of its main structural elements is homogalacturonan, a polymer of 1,4-linked α-D-galacturonic acid that can be methyl-esterified (*1*, *2*). Pectin methyl esterase (PME, EC 3.1.1.11) catalyzes the hydrolysis of the methyl-ester bonds, altering the degree and the pattern of methyl esterification. Pectin conversion by PME plays a key role during several stages of plant development and fruit ripening and in the propagation of plant phytopathogens (*1*, *3*). The amount and distribution of methyl esters in pectin determine to a large extent its functional properties, which consequently affect important quality parameters of various fruit- and vegetable-based foods (*4*, *5*).

Three action patterns are generally proposed for enzymatic conversion of polysaccharides (*6*): (a) a single-chain mechanism, whereby the binding of the enzyme is followed by the conversion of all contiguous substrate sites on the polymer chain; (b) a multiple-chain mechanism, whereby the enzyme−substrate complex dissociates after each reaction, resulting in the conversion of a single residue for each attack; and (c) a multiple-attack mechanism, whereby the enzyme catalyzes the transformation of a limited average number of residues for every active enzyme−substrate complex formed. The average number of processed residues has been defined as the degree of multiple attack. The action of alkaline, plant PMEs with pH optima in the neutral to alkaline region is believed to lead to pectins with a blockwise arrangement of free carboxyl groups; single-chain (*7*−*12*) and multiple-attack mechanisms (*13*−*15*) have been proposed to explain the results. Acidic, fungal PMEs from different *Aspergillus* species with acidic pH optima were reported to act following a multiple-chain mechanism generating pectin with a random distribution of de-esterified galacturonic residues, comparable to chemically de-esterified pectin (*7*, *16*, *17*). However, analysis with more developed techniques indicates that the action of *Aspergillus niger* PME results in pectin with a more "ordered" random distribution of de-esterified residues arranged in little blocks, contrary to a "complete" random pattern observed for chemically de-esterified pectin (*8*−*10*). The observation that alkaline, fungal PME from *Trichoderma reesei* (*18*) and alkaline, microbial PME from *Erwinia chrysanthemi* (*19*) with alkaline pH optima acted in a blockwise mode initiated the idea that the de-esterification mechanism of PME could depend not only on the source of the enzyme but rather on its p*I* and pH optimum (*18*). The finding that randomly de-esterified pectin is widespread in plants (*20*) supported the assumption that factors other than the PME source determine the mode of action. Most of the reported studies on pectin de-esterification by PME were conducted, if specified, at pH conditions close to the respective pH optimum. Some studies, however, suggest an influence of pH conditions on the PME action pattern (*13*, *21*). Denes et al. (*22*) proposed a new classification for the pattern of pectin de-esterification distinguishing between the intrachain level (single attack or multiple attack) and the interchain level (single chain or multiple chain).

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Different interchain distributions of free carboxyl groups were observed for pectin de-esterified by apple PME at pH 4.5 and 7.0 (*22*).

In the present work the effect of pH on the mode of action of an acidic, fungal PME with an acidic pH optimum (recombinant *Aspergillus aculeatus* PME) and an alkaline, plant PME with an alkaline pH optimum (*Lycopersicon esculentum* PME) was investigated. Highly esterified pectin was de-esterified using each of the PMEs at pH 4.5 and 8.0. The de-esterified pectin products were digested by endo-polygalacturonase and subsequently analyzed by high-performance size exclusion chromatography with refractive index detection (HPSEC-RI) and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

## MATERIALS AND METHODS

**Materials.** Highly esterified citrus pectin [degree of methyl-esterification (DM) 94%, galacturonic acid (w/w) 83.5%], digalacturonic acid, and trigalacturonic acid were purchased from Sigma. Monogalacturonic acid and polygalacturonic acid were bought from Fluka.

Endo-polygalacturonase from *Kluyveromyces fragilis* was kindly provided by the Laboratory of Food Chemistry, Wageningen University.

Deionized water (organic free, 18 MΩ resistance) was supplied by a Simplicity Millipore water purification system.

**Extraction and Purification of PME.** PME was extracted from tomatoes (*L. esculentum*) and purified according to the procedure described for polygalacturonase by Verlent et al. (*23*). Extraction was performed at pH 6.0 using a high ionic strength solution (1.2 M NaCl). The extract was partially purified by precipitation with $(NH_4)_2SO_4$ and subsequently dialyzed exhaustively against deionized water. Next, the crude extract was purified by cation-exchange chromatography (Hi-Prep 16/10 SP/XL, GE Healthcare). Fractions containing PME activity were pooled and concentrated by ultrafiltration before application to a gel filtration column (Hi Load 16/60 Superdex 75 Prep Grade, GE Healthcare). Elution was performed with sodium acetate buffer (40 mM, pH 4.4) containing 0.2 M NaCl. Fractions containing PME activity were pooled.

Recombinant *A. aculeatus* PME was purified by gel filtration from a commercial preparation (Novoshape, Novozymes) as described by Duvetter et al. (*24*).

**Protein Determination.** The protein content of the purified PME samples was determined using Sigma procedure TRPO-562. This method of protein quantification is based on the reduction of $Cu^{2+}$ by protein in an alkaline environment (*25*). Bicinchoninic acid (BCA) forms a colored complex with the resulting $Cu^{+}$. The absorbance of this complex was measured at 562 nm. The protein content was estimated using a standard curve obtained from bovine serum albumin.

**Gel Electrophoresis.** For both sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) experiments, a Phastsystem (GE Healthcare) was used. SDS-PAGE was performed with Phastgel homogeneous 20% and Phastgel SDS buffer strips. Samples were boiled at 100 °C for 5 min in a buffer containing SDS (2.5%) and $\beta$-mercaptoethanol (5%). For IEF, Phastgel IEF media with a pH range of 3−9 were used. Gel staining was performed using the silver-staining protocol according to Heukeshoven and Dernick (*26*).

**PME Activity Assay.** PME activity was measured by an automatic pH-stat titration (Titrino 718, Metrohm) (0.01 M NaOH) of the release of carboxyl groups in a mixture of 30 mL of pectin (4 g/L, 0.3 M NaCl) and PME (injected volume depending on pH and PME source) at constant temperature (22 °C). The PME activity is proportional to the rate of base consumption. In the alkaline region (pH ≥9), the PME activity was corrected for pectin autohydrolysis by measuring and subtracting the acid production in a pectin solution containing no PME. In the acidic region, the PME activity was corrected for the incomplete dissociation of the de-esterified carboxyl groups by multiplying the activity with the correction factor $[1 + 10^{(pK_a - pH)}]$, with p$K_a$ equal to 3.5, the p$K_a$ of galacturonic acid (*27*). One unit (U) of PME activity is defined as the amount of enzyme capable of catalyzing the de-esterification of 1 $\mu$mol of methyl ester bonds per minute at 22 °C and at the pH of assay.

The specific activity (U per gram of proteins) of PME was estimated by dividing the activity of the sample (expressed in units) by its protein content (expressed in grams).

**Preparation of De-esterified Pectin Samples.** Highly esterified citrus pectin (4 g/L) was de-esterified for a prolonged time by either recombinant *A. aculeatus* PME or tomato PME at constant pH (pH 4.5 and 8.0) using the pH-stat procedure described for PME activity assay. Salt (0.3 M NaCl) was added to the substrate solution to enhance the PME activity (*28*). The amount of enzyme applied was adjusted to allow extensive de-esterification of pectin within 6 h. Using preset time intervals, 1.1 mL of the reaction mixture was withdrawn and immediately heated at 80 °C for 5 min to inactivate PME. The samples were then cooled in ice water, frozen in liquid nitrogen, and stored at −40 °C for subsequent analysis. The residual DM of the respective samples could be calculated from the initial mass percentage of galacturonic acid and methoxy groups of citrus pectin and the amount of methoxy groups released, which was proportional to the amount of base that was titrated to keep the pH constant. At pH 4.5, the latter was corrected for incomplete dissociation of the carboxyl groups by multiplying with the correction factor $[1 + 10^{(pK_a - pH)}]$, with p$K_a$ equal to 3.5, the p$K_a$ of galacturonic acid (*27*). The estimated, residual DM was the average DM of the whole population of pectins in the sample. Depending on the mode of de-esterification, the DM of the individual pectin chains can differ.

**Digestion of Pectin by Endo-polygalacturonase (Endo-PG) from *K. fragilis*.** Thawed pectin samples were adjusted to pH 5 using a 0.05 M acetate buffer by applying the following steps: centrifugation (12900*g*, 20 °C, 40 min) of 500 $\mu$L of pectin sample on centrifugal filters (10 kDa, Millipore Corp.), addition of 300 $\mu$L of acetate buffer, centrifugation, and adjustment to the initial sample volume with 0.05 M sodium acetate buffer at pH 5.0.

Subsequently, the pectin samples were digested by endo-PG from *K. fragilis* as described by Daas et al. (*29*). It is believed that this enzyme requires a block of at least four unesterified galacturonic acid residues to bind and depolymerize pectin (*30*). Pectin samples were incubated with 0.0225 units of endo-PG for 24 h at 30 °C. One unit (U) of PG activity is defined as the amount of enzyme capable of catalyzing the release of 1 $\mu$mol of reducing groups per minute. Afterward, PG was inactivated by heating at 100 °C for 5 min.

**High-Performance Size Exclusion Chromatography (HPSEC).** The molar mass (MM) distribution of pectin polymers and large oligomers was investigated by HPSEC using a Dionex system (Dionex Bio-LC system) equipped with a mixed bed column of Bio-Gel TSK GMPWxl (Tosoh Corp.) in combination with a TSK guard column. Twenty-five microliters of pectin sample (diluted to 2 g/L with 0.1 M $NaNO_3$, pH 6.9) was injected. Elution was executed at 35 °C with 0.05 M $NaNO_3$ at pH 6.9 at a flow rate of 0.7 mL/min for 20 min. The eluent was monitored using a Shodex R101 refractive index detector (Showa Denko K.K.). Monogalacturonic acid (100 ppm) was injected before and after each set of samples to verify the reproducibility (elution time = 13.9 min).

**High-Performance Anion-Exchange Chromatography (HPAEC).** The degree of polymerization (DP) of the short galacturonic acid oligomers obtained after digestion of pectin by endo-PG was analyzed by HPAEC (Dionex Bio-LC system) at pH 12 as described by Verlent et al. (*23*), using a CarboPac PA1 column with guard. Detection was achieved using pulsed amperometric detection (PAD) with a gold working electrode. Prior to injection, PG-treated pectins were diluted to 2 g/L with 0.2 M NaOH. Due to the high pH, all methyl ester groups were de-esterified instantaneously when the samples were diluted in NaOH. Consequently, separation of the pectin fragments was based only on their DP and not on their residual degree or pattern of methyl esterification. The elution times of the mono-, di-, and trimer of galacturonic acid were identified by running standard solutions. The elution times of other galacturonic acid oligomers were determined by comparison of the elution profile of the digested pectin with the profile of polygalacturonic acid digested by endo-PG. It should be noted that the PAD could not provide analysis beyond a DP of 10 and that the sensitivity of the detector varies with the oligomers according to their

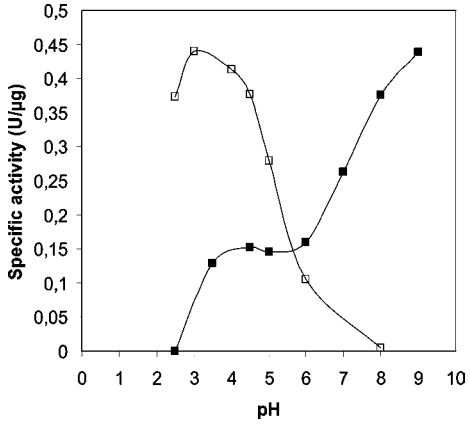


**Figure 1.** Specific activity (U/$\mu$g of protein) of recombinant *A. aculeatus* PME (□) and tomato PME (■) as a function of pH.

DP. Monogalacturonic acid (40 ppm) was injected before and after each set of samples to verify the reproducibility.

## RESULTS AND DISCUSSION

**Purification and Characterization of PME.** Recombinant *A. aculeatus* PME and tomato PME resulted in sharp, single peaks of proteins and PME activity after gel filtration. Fractions containing PME activity were pooled. No PG activity was detected in the tomato PME preparation. A single band on SDS-PAGE was obtained for both tomato PME and recombinant *A. aculeatus* PME, representing MM of 36 and 39 kDa, respectively. This is in agreement with earlier results (*24*, *27*, *28*, *31*, *32*). Analysis by IEF showed a single protein band at the alkaline limit of the gel (pH 9.3) for tomato PME. This is in line with the available literature data for tomato PME (p*I* > 9) (*28*, *31*−*33*). Contrarily, recombinant *A. aculeatus* PME yielded a single protein band at the acidic limit of the gel (pH 3.5). This observation is in agreement with the finding of Christgau et al. (*27*), who estimated the p*I* of the fungal enzyme at 3.8.

**Effect of pH on the Rate of PME-Catalyzed Pectin De-esterification.** The activity (initial rate of de-esterification) of recombinant *A. aculeatus* PME and tomato PME was measured by automatic titration in the pH range 2.5−9 (**Figure 1**). The substrate solution contained 0.4% (w/v) citrus pectin (DM 94%) and 0.3 M NaCl. The same substrate solution was used to study the mode of de-esterification. Within the study domain, spontaneous autohydrolysis of pectin was observed only at pH 9.

Tomato PME was more active in alkaline conditions, the specific activity increasing with increasing pH. At pH >9.0 accurate activity measurements were difficult because of extensive autohydrolysis. The increase in tomato PME activity with increasing pH is consistent with published data. Reported pH optima vary from pH 7.5 to 10 (*28*, *31*, *33*, *34*). Purified recombinant *A. aculeatus* PME exhibited high activity in acidic conditions, similar to PME from other *Aspergillus* species (*7*, *16*, *17*). At pH 8.0, very limited activity could be detected. The pH optimum determined here (pH 3.0) differed from the value reported by Christgau et al. (*27*), who measured the activity of recombinant *A. aculeatus* PME at 30 °C with apple pectin (2 g/L, DM 75%) as substrate and obtained a pH optimum of 4.6. Our own experiments with the same substrate solution confirmed this result (not shown).

**HPSEC Analysis of Pectin Samples Not De-esterified by PME.** The enzymatic de-esterification of the pectin samples is quenched by a heat treatment for 5 min at 80 °C to inactivate the enzymes. Kinetic studies of the thermal inactivation of purified recombinant *A. aculeatus* PME and purified tomato PME have been performed previously (*24*, *34*). Additional tests demonstrated that for neither of the PMEs was any residual activity recovered after a heat treatment at 80 °C for 5 min (at pH 4.5 and 8.0) (results not shown). To investigate the potential effects of these heat treatments on the degree and pattern of pectin esterification, the following samples were subjected to HPSEC-RI analysis (**Figure 2**): (a) (DM 94%) not subjected to a heat treatment nor incubated with PG, (b) pectin (DM 94%) that was incubated with PG after a heat treatment at pH 4.5, and (c) pectin (DM 94%) that was incubated with PG after a heat treatment at pH 8.0. The elution patterns were similar for all three samples and were marked by a single peak representing high MM polysaccharides. This indicated that the three samples were homogeneous with respect to MM, proving that PG could not depolymerize the samples with a DM of 94% because of a lack of free carboxyl groups. It also demonstrates that no spontaneous de-esterification occurred during the heat treatment at pH 4.5 or 8.0. Because the elution times of the peaks were similar for the three samples, it can be assumed that the heat treatments did not induce any (nonenzymatic) chemical depolymerization. Hence, the digestion of pectin samples by PG is governed by the degree and pattern of the carboxyl groups de-esterified by recombinant *A. aculeatus* PME and tomato PME, respectively.

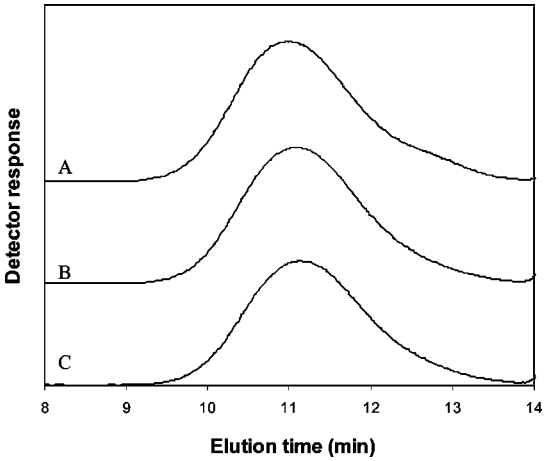


**Figure 2.** HPSEC analysis of pectin (DE 94%) that underwent the following treatments: no heat treatment and no incubation with endo-PG (**A**); heat treatment (80 °C, 5 min) at pH 4.5 and incubation with endo-PG (**B**); heat treatment (80 °C, 5 min) at pH 8.0 and incubation with endo-PG (**C**).

**HPSEC Analysis of Pectin De-esterified by Alkaline, Plant PME.** In the elution patterns of PG digests of pectin converted by tomato PME (DM 94%−31%) at pH 8.0 (**Figure 3A**), two peaks could be observed representing high MM polysaccharides and low MM fragments. At a high DM, PG had a limited digestion effect on the high MM polymers. However, with decreasing DM, the initially dominating peak representing high MM polymers decreased and shifted to a lower MM average, whereas the population of small pectin fragments increased. At a DM of 31% the high MM population was almost completely converted to small oligomers. This pattern is representative for a pectin de-esterification mechanism yielding two different product populations, on the one hand, pectins with large, de-esterified sequences that can be depolymerized by PG into small fragments and, on the other hand, pectins de-esterified to a lower extent that can undergo only limited degradation by PG. This pattern is best explained by a multiple-attack mechanism with a high degree of multiple attack. A single-chain mechanism is less appropriate to describe the observed action pattern of tomato

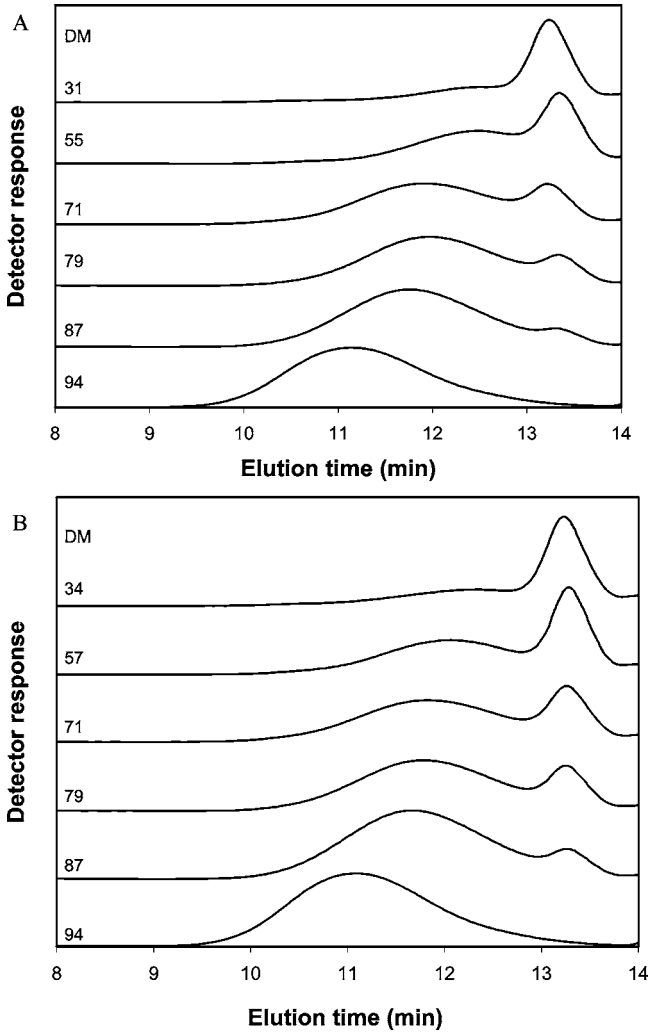


**Figure 3.** HPSEC analysis of PG digests of pectin de-esterified by tomato PME at pH 8.0 (**A**) and pH 4.5 (**B**).

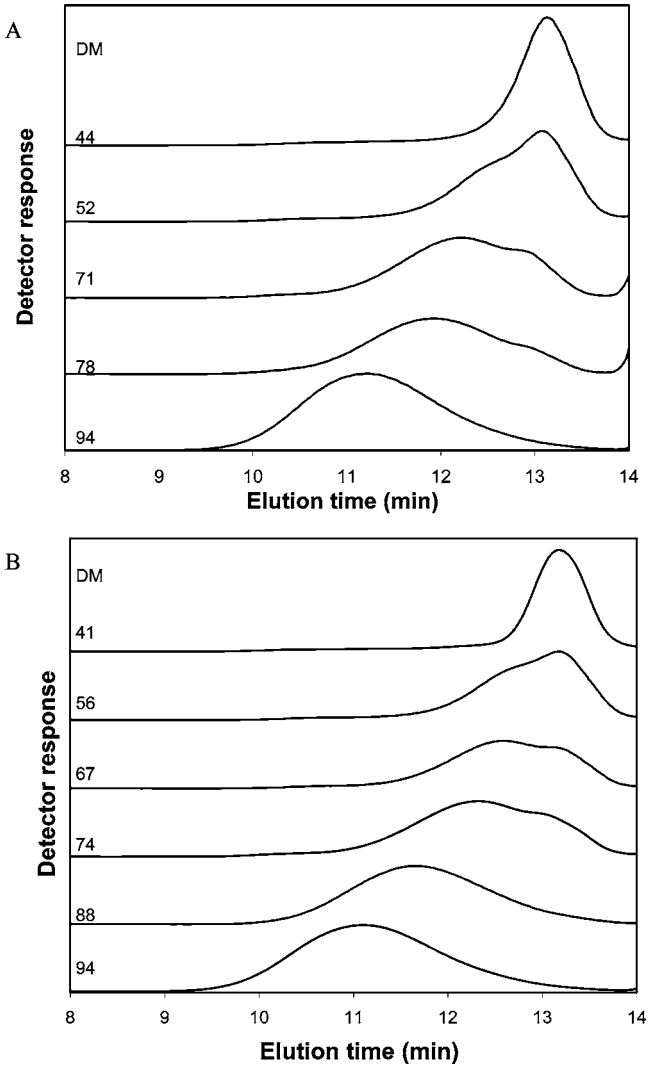


**Figure 4.** HPSEC analysis of PG digests of pectin de-esterified by recombinant *A. aculeatus* PME at pH 8.0 (**A**) and pH 4.5 (**B**).

PME because the latter mechanism produces an increasing population of completely de-esterified pectins next to a decreasing fraction of non-de-esterified pectin (not degradable by PG). Hence, with decreasing DM, the population of initial pectin polymers would decrease but its MM average would not shift to lower values, contrary to what is observed here. Previously, a multiple-chain mechanism differing from a single-chain mode was suggested for tomato PME action at pH 6.5, on the basis of NMR studies (*14*, *35*). A multiple-attack mechanism was also suggested for the action of orange PME at pH 7.0 (*15*) and mung bean PME isoforms at pH 7.6 (*13*). Similar elution patterns could be observed for PG digests of pectin de-esterified to different DMs by tomato PME at pH 4.5 (**Figure 3B**). With decreasing DM, an initially predominant population of high MM polymers decreased and shifted to a lower MM average, whereas the concentration of small pectin fragments increased. The resemblance between the elution patterns of pectin de-esterified to an equivalent DM by tomato PME at pH 8.0 and 4.5 is striking. Consequently, it can be assumed that tomato PME acts according to same interchain mechanism at both pH values.

**HPSEC Analysis of Pectin De-esterified by Acidic, Fungal PME.** A different elution profile was obtained for digests of pectin de-esterified by recombinant *A. aculeatus* PME to varying DM at pH 4.5 (**Figure 4B**) compared to the samples converted by tomato PME. At different DMs, only a single peak could be observed, which progressively shifted to a lower MM average with decreasing DM. No distinct peak of low MM fragments was detected, contrary to what was observed for tomato PME. This pattern is in agreement with a multiple-chain mechanism, which yields a more homogeneous population of de-esterified pectin products. A multiple-chain mechanism at the interchain level has already been suggested for *A. niger* PME action at pH 4.5 (*8*, *22*). Here it was shown that recombinant *A. aculeatus* PME follows a similar action pattern at pH 4.5. The elution pattern of digests of pectin de-esterified by recombinant *A. aculeatus* PME at pH 8.0 is presented in **Figure 4A**. Again, a single peak representing a homogeneous population of polysaccharides with respect to MM could be observed, and the average MM shifted to lower values with decreasing DM. No significant differences could be noted between the elution behaviors of the pectins de-esterified by recombinant *A. aculeatus* PME at acidic and alkaline pH. Apparently, acidic, fungal PME acts following a multiple-chain mechanism in acidic and alkaline conditions. To our knowledge, no studies about the mode of action of acidic PME at alkaline conditions have been published before.

**HPAEC Analysis of PG Digests of De-esterified Pectin.** The PG digests of pectin de-esterified by tomato PME (**Figure 5**) and recombinant *A. aculeatus* PME (**Figure 6**) were analyzed by HPAEC with PAD at pH 12. In the elution profiles of the initial, highly esterified pectin (DM 94%) that was heated (80 °C, 5 min) at pH 4.5 or 8 and subsequently incubated with PG

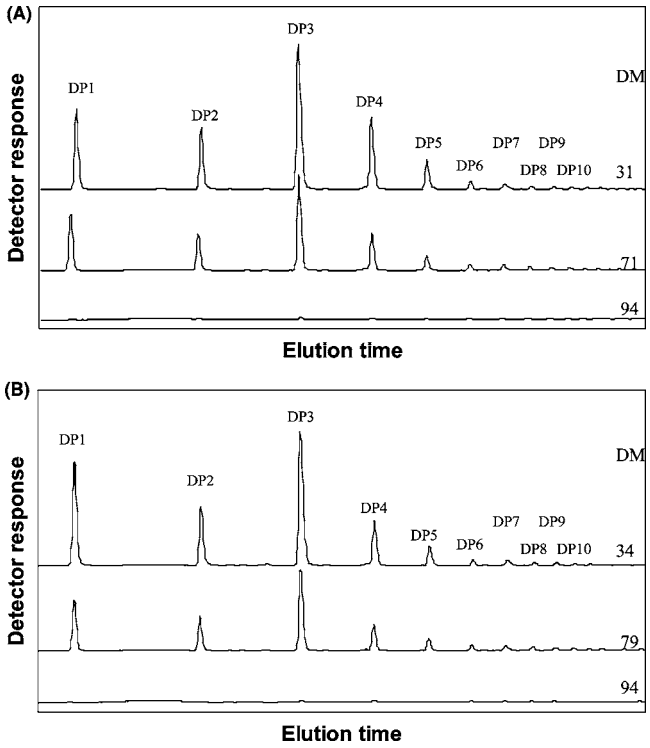


**Figure 5.** HPAEC analysis of PG digests of pectin de-esterified by tomato PME at pH 8.0 (**A**) and pH 4.5 (**B**).

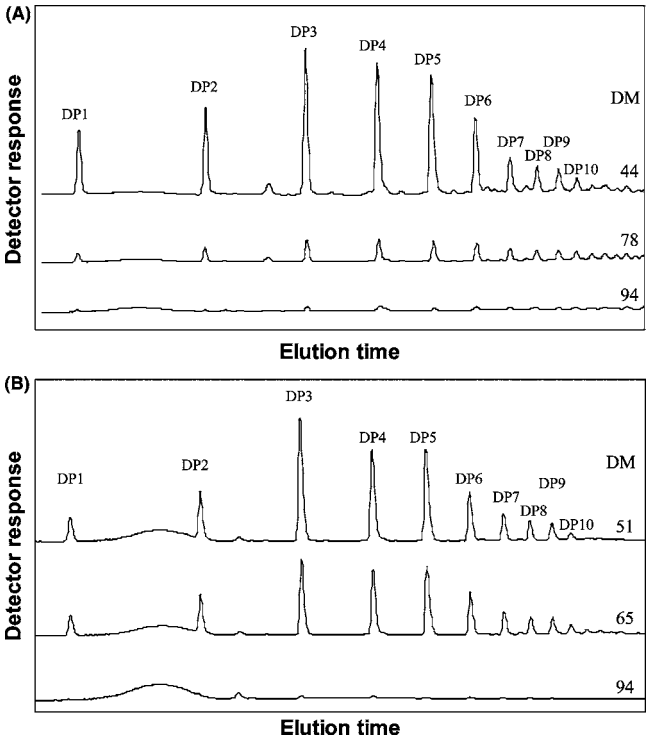


**Figure 6.** HPAEC analysis of PG digests of pectin de-esterified by recombinant *A. aculeatus* PME at pH 8.0 (**A**) and pH 4.5 (**B**).

(**Figures 5** and **6**) and pectin (DM 94%) not subjected to heat or PG treatment (not shown), hardly any monomer or oligomer peaks could be detected. This confirmed the observation that the initial pectin did not contain binding sites suited for PG activity and that a heat treatment at 80 °C at pH 4.5 or 8.0 did not lead to autohydrolysis.

De-esterification by tomato PME at pH 8.0 (**Figure 5A**) led to an increase in peak area of the galacturonic acid monomer and short-chain oligomers (dimer through pentamer), the most pronounced change being observed in the peak area of trimers. No remarkable changes in the peak areas of oligomers larger than the pentamer were noted. A nearly identical chromatogram could be recognized for pectin de-esterified by tomato PME at pH 4.5 (**Figure 5B**), that is, an increase of the peak area of the monomer and oligomers up to DP5. Hence, no effect of pH on the intrachain mechanism of de-esterification of tomato PME was detected.

As was the case for tomato PME, the elution profiles of the pectin de-esterified by recombinant *A. aculeatus* PME to different DMs at pH 8.0 (**Figure 6A**) and pH 4.5 (**Figure 6B**) did not differ significantly. In both cases, with decreasing DM, an increase in the peak area of particularly the trimer, the tetramer, and the pentamer could be observed, next to increasing peak areas for the monomer and dimer. Contrarily to pectin converted by tomato PME, the increase in peak area of larger oligomers (DP 6−10) was clear for pectin de-esterified by fungal PME, both at pH 4.5 and at pH 8.0. Daas et al. (*36*) also observed more larger oligomers in PG (from *K. fragilis*) fingerprints of chemically de-esterifed pectin compared to tomato PME converted pectin (pH 7.5). PG (from *A. niger*) digests of pectin incubated with orange PME at pH 7.0 showed an increase of mono-, di-, and trimers and only a minor increase of tetramers and pentamers, in contrast to pectin de-esterifed by *A. niger* PME that was degraded by PG into larger oligomers with residual methyl esterification (*9*).

The distribution pattern of the short galacturonic acid oligomers, determined by HPAEC-PAD, delivers information on the (intrachain) degree of blockiness of the de-esterified sequences on the pectin chains. Because both tomato PME and recombinant *A. aculeatus* PME generated pectin degradable by endo-PG from *K. fragilis* after only limited de-esterification, both PMEs must liberate at least four consecutive carboxyl groups, the condition suitable for PG to bind its substrate (*30*). This converts with the multiple-attack mechanism already postulated for tomato PME on the basis of the HPSEC data (see above) and NMR studies (*14*). It has already been observed previously that *A. niger* PME, although acting according to a multiple-chain (interchain) mechanism, created blocks of de-esterified units (*8*−*10*), suggesting a certain degree of multiple attack at the intrachain level (*8*, *22*), distinghuishing the de-esterification by fungal PME from the complete random (single-attack) chemical hydrolysis. On the basis of the HPAEC results, it can be assumed that the action mechanism of recombinant *A. aculeaus* PME is similarly characterized by a certain degree of multiple attack. From the results presented here, it is clear that tomato PME creates larger sequences of completely unesterified residues, as compared to recombinant *A. aculeatus* PME. The low DP (≤5) of the oligomers present in the digests of pectin converted by tomato PME indicates that PG action was mostly inhibited by the length of the substrate and was hardly hindered by residual methyl esters. It has been observed that all nonesterified oligomers larger than the tetramer can be hydrolyzed by PG from *K. fragilis* (*29*, *30*). Hence, the relatively higher occurrence of larger oligomers (DP ≥ 4) in the digests of pectin de-esterified by recombinant *A. aculeatus* PME can be explained by the presence of more residual methyl esters on the pectin chains hydrolyzed by fungal PME interrupting the PG action. Identification by matrix-assisted laser desorption ionization time-of-flight of the peaks obtained by HPAEC at pH 5 (in the absence of postcolumn alkali addition) of endo-PG degraded pectin that was de-esterified chemically to DM 30% demonstrated that solely the monomer and dimer existed

completely unesterified and that all oligomers larger than the tetramer contained at least one methyl ester (*36*).

In this study no effect of pH was observed on the action mechanism of tomato PME (an alkaline, plant PME with alkaline pH optimum) and recombinant *A. aculeatus* PME (an acidic, fungal PME with acidic pH optimum) as analyzed by enzymatic fingerprinting combined with HPSEC-RI to investigate the MM distribution of polymers and large oligomers and with HPAEC-PAD to analyze the DP of small oligomers. These findings differ partly from the results obtained by Denes et al. (*22*), who investigated the action mode of purified apple PME at pH 7.0 and 4.5. Using $^{1}$H NMR spectroscopy the same intrachain distribution of de-esterified galacturonic acid residues, suggesting a multiple-attack mechanism, was observed for both pH values, agreeing with our results. However, conversely to our data, an effect of pH on the interchain action mechanism was detected: analysis of the interchain distribution of free carboxyl groups by high-performance ion exchange chromatography (HPIEC) indicated a typical single-chain mechanism at pH 7.0, but a mechanism differing from the single-chain and multiple-chain ones at pH 4.5. Catoire et al. (*13*) used $^{13}$C NMR spectroscopy to compare the mode of de-esterification by different isoforms of PME from mung bean hypocotyls at pH 5.6 and 7.6. In the case of the $\alpha$ and $\gamma$ isoforms, a single-chain mechanism could be postulated at pH 5.6, whereas some multiple-attack character was required to reproduce the data at pH 7.6. Several mechanisms differing from the preceding ones were compatible with the data for the $\beta$ isoform at the two pH values.

Thus, different studies deliver different conclusions on the effect of pH on the PME mode of action. It must be emphasized that different analyzing techniques have been used in the respective studies (enzymatic fingerprinting, IEC, NMR spectroscopy). To our knowledge, a comparative study of the different analytical methods to estimate the pattern and degree of methyl esterification has not yet been conducted. Besides, also different sources (and isoforms) of PME have been investigated. As the action mechanism can differ between different PME isoforms (*13*), it is reasonable to assume that the action mechanism and the effect of pH on it can vary between different sources. The recent progress to unravel the crystal structure of different PMEs (*37*−*39*) may help to explain why different PMEs act according to different action mechanisms and what the influencing factors are. On the basis of the available crystal structures a hypothesis has already been formulated to explain the different affinities of certain PMEs for PME inhibitor (*40*). To explain the different action mechanisms, the crystal structure of a multiple-chain-acting PME (as, for example, PME from *Aspergillus*) is still lacking.

Insight and control of the factors determining the mode of PME-catalyzed pectin de-esterification can enable an efficient application of the enzyme in producing tailor-made pectins and altering plant product qualities and can also lead to a better understanding of the role PME and of pectin in regulating plant development processes. In this context, however, it needs to be investigated if the observations on the mode of PME action in vitro can be translated to the mode of enzymatic pectin de-esterification in planta. The latter can be studied using recently developed antibodies with affinity for pectins with specific degrees and patterns of esterification (*20*).

## ABBREVIATIONS USED

DM, degree of methyl esterification; DP, degree of polymerization; HPAEC, high-performance anion-exchange chromatography; HPIEC, high-performance ion-exchange chromatography; HPSEC, high-performance size exclusion chromatography; IEF, isoelectric focusing; MM, molar mass; NMR, nuclear magnetic resonance; PAD, pulsed amperometric detection; PG, polygalacturonase; p*I*, isoelectric pH; PME, pectin methyl esterase; RI, refractive index (detection); SDS(-PAGE), sodium dodecyl sulfate(−polyacrylamide gel electrophoresis); U, unit of enzyme activity.

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