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Separation and Characterization of a Salt-Dependent Pectin Methylesterase from *Citrus sinensis* Var. Valencia Fruit Tissue

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A pectin methylesterase (PME) from sweet orange fruit rag tissue, which does not destabilize citrus juice cloud, has been characterized. It is a salt-dependent PME (type II) and exhibits optimal activity between 0.1 and 0.2 M NaCl at pH 7.5. The pH optimum shifted to a more alkaline range as the salt molarity decreased (pH 8.5−9.5 at 50 mM NaCl). It has an apparent molecular mass of 32.4 kDa as determined by gel filtration chromatography, an apparent molecular mass of 33.5 kDa as determined by denaturing electrophoresis, and a pI of 10.1 and exhibits a single activity band after isoelectric focusing (IEF). It has a K_m of 0.0487 mg/mL and a V_{max} of 4.2378 nkat/mg of protein on 59% DE citrus pectin. Deblocking the N-terminus revealed a partial peptide composed of SVTPNV. De-esterification of non-calcium-sensitive pectin by 6.5% increased the calcium-sensitive pectin ratio (CSPR) from 0.045 ± 0.011 to 0.829 ± 0.033 but had little, if any, effect on pectin molecular weight. These properties indicate this enzyme will be useful for studying the PME mode of action as it relates to juice cloud destabilization.

KEYWORDS: Pectin; pectin methylesterase; pectinesterase; citrus; polysaccharide; homogalacturonan

INTRODUCTION

Pectin is a heterogeneous polysaccharide composed, in large part, of polygalacturonic acid, which comprises the homogalacturonan region (*1*, *2*). A variable proportion of the galacturonic acid residues in this region are methylated at the C6 position via an ester bond. Pectin methylesterase (PME, EC 3.1.1.11) hydrolyzes this ester bond. The stability of cloud material in citrus juices and the functional properties of commercial citrus pectin (extracted from peel) are related to its degree of methylation (*3*), its molecular weight (*4*), and the intramolecular spatial distribution of the methyl esters within the population of pectin molecules (*5*−*7*). Liners et al. (*8*) concluded that a minimal block size of nine de-esterified residues was necessary for calcium cross-linking and hypothesized that a larger de-esterified block might be necessary for gel formation. Calcium-sensitive pectins (CSPs) are high-methoxy pectins in which de-esterified blocks allow them to gel in the presence of calcium without the addition of sucrose (*4*, *5*). Consequently, PME is a critically important enzyme in the citrus and pectin industries.

Multiple forms of PME have been described from citrus fruit tissue (*9*−*14*). In fresh-squeezed or underpasteurized citrus juices, active PME initiates a sequence of events that results in juice cloud destabilization (*15*). Since citrus juice grading and market acceptability are strongly influenced by juice cloud color intensity and density, a stable cloud is highly desirable. Additionally, juice cloud also provides flavor, aroma, and turbidity (*16*). Cameron et al. (*17*) demonstrated that one of the four PME isozymes (peak 4) separated from sweet orange peel would not destabilize orange juice cloud while the other three isozymes destabilized the juice cloud at varying rates. These results suggested that the mode of action and/or physical and kinetic parameters of the different orange peel PME isozymes varied. A few studies have been published on the differences in the mode of action of PME from different origins (*18*−*20*), for different isozymes within a single species (*21*), and even for a single isozyme at different pHs (*22*). The dominant PME (peak 2 PME; *9*, *17*) from citrus fruit tissue was purified (*11*) and demonstrated to have a blockwise demethylation pattern at pH 7.0 (*4*, *23*). Additionally, it is salt-independent and appears to be similar to the fruit specific type I PME from tomato (*23*−*25*). The two groups of PMEs described from tomato fruit have been denoted type I and type II (*23*). Type I PMEs are specific for fruit tissues and do not require cations for activity, while type II PMEs are ubiquitously

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expressed and do require cations for activity. The demethylation pattern for the peak 4 PME has not been determined, and its relationships to other PMEs are unclear. To define peak 4 PME's mode of action, to determine its effect on pectin methyl ester distribution, and to investigate its inability to destabilize citrus juice cloud, it is first necessary to isolate it as a monocomponent PME, establish its physical and kinetic parameters, and determine its structural relationship to PME forms from citrus and other plants.

MATERIALS AND METHODS

Tissue Extracts. Fresh Valencia oranges were used to prepare fruit tissue extracts as previously described (*17*).

Chromatography. Anion exchange (DEAE-Sephacel) and heparin CL-6B chromatography were carried out as previously described (*17*). After heparin chromatography, the fractions containing peak 4 PME activity (see below for activity determinations) were pooled and concentrated, and the buffer was changed to 10 mM $NaPO_4$ (pH 8.0) with Centricon-30 microconcentrators (Amicon, 30 kDa cutoff membranes). This material was then loaded onto a CM-Sepharose column (1 cm × 19.5 cm) equilibrated with 10 mM $NaPO_4$ buffer (pH 8.0) containing 0.1 M NaCl. Bound material was eluted with a NaCl gradient between 0.1 and 0.3 M. The column was run at 1 mL/min, and 1 mL fractions were collected. Fractions containing PME activity were pooled; the buffer was changed to 10 mM $NaPO_4$ buffer (pH 6.0) containing 0.2 M NaCl, and after the column had been equilibrated with the same buffer, the pooled fractions were loaded again onto the CM-Sepharose column. Bound material was eluted with a NaCl gradient between 0.20 and 0.22 M. The column was run at 1 mL/min, and 1 mL fractions were collected. After a buffer change to 10 mM Tris (pH 7.5) and 0.2 M NaCl, the pooled and concentrated active fractions were loaded onto a Superdex 200 gel filtration column (1.6 cm × 59 cm) and run with the equilibration buffer at 1 mL/min. Fractions (1 mL) were collected.

Activity Estimates. Column fractions were screened for relative activity using a colorimetric assay as previously described (*17*). Kinetics and the effects of NaCl on enzyme activity were determined with a colorimetric, kinetic microplate assay (*26*). The activity of pooled column fractions (assayed at pH 7.5 and 200 mM NaCl) and the pH curves (at 10, 50, and 200 mM NaCl) were determined titrimetrically on 0.5% citrus pectin (59% DE) with a Radiometer PHM290 pH-stat controller using 10 mM NaOH as the titrant. All activity estimates are the means of three replicates or percentages calculated from the means of three replicates. Protein concentrations were estimated with the Bio-Rad protein assay according to the manufacturer's directions using IgG as the standard. All estimates are based on the means ± the standard error of three replicates.

Electrophoresis. Sodium dodecyl sulfate−PAGE was conducted in a Mini-Protean II dual-slab cell electrophoresis unit (Bio-Rad) on 13.5% acrylamide gels under reducing conditions (*11*, *24*). Approximately 5 μg of protein was loaded per lane. The gels were stained with Coomassie Brilliant Blue R-250. The denatured molecular weight was estimated using broad range molecular weight standards (Bio-Rad). Isoelectric focusing PAGE was carried out on a Bio-Rad mini-IEF gel apparatus (*11*, *24*).

Amino acid sequencing was performed directly from proteins blotted onto a PVDF membrane (*24*). Proteins were separated on a 12% SDS−PAGE gel, electroblotted onto a Bio-Rad Trans-Blot PVDF membrane (*26*), and then briefly stained with Coomassie Brilliant Blue R-250 in 50% MeOH (no acetic acid). Because peak 4 PME was initially found to be blocked, the deblocking treatment of PME followed the method of Wellner et al. (*27*). Sequencing from the PVDF membrane with an Applied Biosystems Procise 491 protein sequenator was then performed following the manufacturer's instructions.

Pectin De-Esterification. Non-calcium-sensitive pectin (NCSP, 76% DE) was prepared from lemon peel as described by Joye and Luzio (*5*). A 1% solution of NCSP, which also contained 150 mM NaCl and 0.02% NaN_3, was de-esterified by peak 2 PME and peak 4 PME in a water-jacketed reaction beaker at a constant temperature (36 °C, pH 7.0) with NaOH (5.0 M) delivery controlled by a Radiometer autotitration system as described previously (*11*). Following treatment, solutions were adjusted to pH ~3.8 with HCl, and 4 volumes of warm EtOH (40 °C) was added to solutions to inactivate the enzyme and precipitate pectin. After being chilled to 4 °C, the pectin was dialyzed (1000 molecular weight cutoff, Spectrum) in deionized water at 4 °C and lyophilized prior to further analysis. Batches of 4 g were prepared with the change in pectin DE being calibrated to the amount NaOH consumed. NCSP hydrolysis was limited to 6.5% of the total saponifiable methyl ester groups. Sufficient enzyme was added to obtain the calculated amount of base consumption in 10−15 min (~20−30 units of enzyme/g of pectin). An untreated NCSP control was also prepared using the same conditions but without any enzyme. The 6.5% demethylated pectins were subsequently analyzed by the calcium-sensitive pectin ratio (CSPR) assay.

Table 1. Purification of Peak 4, Salt-Dependent PME[a]

purification step	μEq min^{-1} μL^{-1}	μg of protein/μL	μEq min^{-1} (μg of protein)$^{-1}$	purification factor
heparin	0.0316 ± 0.0011	0.4780 ± 0.0125	0.0611	1
CM-Sepharose, pH 8.0	0.0621 ± 0.0003	0.0822 ± 0.0061	0.3175	4.8
CM-Sepharose, pH 6.0	0.0110 ± 0.0004	0.0604 ± 0.0035	0.1821	2.7
Superdex 200	0.0173 ± 0.0002	0.0381 ± 0.0013	0.4541	6.9

[a] The crude extract and unbound DEAE material contained multiple PMEs, preventing their inclusion in the purification table.

End point demethylation was performed titrimetrically at 30 °C on 5 mL of 0.5% citrus pectin (59% DE), 200 mM NaCl, and 0.7 unit of peak 4 PME (1 unit = 1 μEq min^{-1} μL^{-1}) with a Radiometer PHM290 pH-stat controller at pH 4.5 and 7.5 using 10 mM NaOH as the titrant. The titration was carried out for 21−47 h at pH 4.5 and for 34−120 min at pH 7.5. The reported values are means of four replicates.

CSPR Assay. Pectin samples (0.2 g) of PME-treated and untreated NCSP were weighed into duplicate tubes to the nearest milligram and dissolved in 10 g of deionized H_2O by heating to 70 °C. The solutions were cooled to 25 °C, and the pH was adjusted to 4.0. The CSPR was calculated as described by Hotchkiss et al. (*4*). The CSPR was determined by dividing the amount of pectin that gels in 30 mM calcium and 8% 2-propanol (IPA) by the total amount of pectin which precipitates in 60% IPA. Therefore, CSPR values close to 1.0 indicate a CSP, while a NCSP has low CSPR values. The assay was repeated two more times, and average values of triplicate analysis were determined.

Pectin Molecular Properties. High-performance size exclusion chromatography (HPSEC) of pectin samples was performed according to the method of Fishman et al. (*28*) with minor modifications noted here. Samples (2 mg/mL) were dissolved in 0.05 M $NaNO_3$ and then filtered with a 0.22 μm membrane filter prior to injection of a 200 μL sample. HPSEC separation utilized two PL-Aquagel OH-60 columns and one OH-40 column (Polymer Labs) in series and a 0.05 M $NaNO_3$ mobile phase at 45 °C and 0.7 mL/min. Detection was with a Dawn DSP multiangle laser light scattering (MALLS) photometer (Wyatt Technologies), a model 100 differential pressure viscometer (Viscotek Corp.), and an Optilab DSP interferometric refractometer (Wyatt). A value of 0.132 mL/g in 0.05 M $NaNO_3$ was used for the concentration dependence of the differential refractive index (d*n*/d*c*) of pectin. The d*n*/d*c* was determined off-line using the HPLC pump, the autoinjector, and the Optilab refractometer as suggested by the manufacturer of the refractometer. The weight average molecular weight (M_w) and intrinsic viscosity (η_w, weight average intrinsic viscosity for a polydisperse collection of macromolecules) and the *z*-average radius of gyration (R_{gz}, the effective radius of a polydisperse collection of macromolecules rotating in solution under Brownian motion) were determined for triplicate HPSEC runs for each pectin sample.

RESULTS

The final purification factor for the heparin peak 4 PME (salt-dependent, SD-PME) was 6.9 (**Table 1**), but this did not take

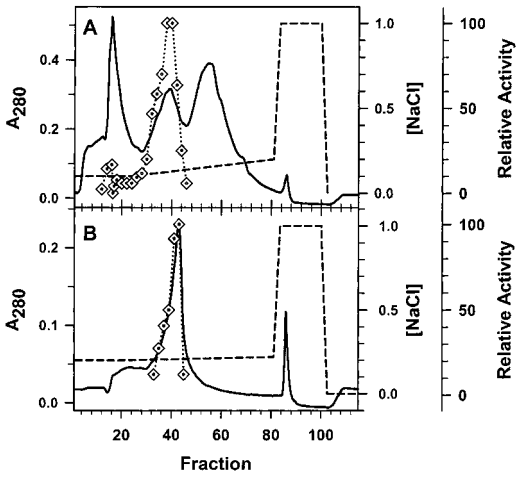

Figure 1. (A) CM-Sepharose chromatography of heparin peak 4 PME in 10 mM $NaPO_4$ at pH 8.0. Bound proteins were eluted with a 100 to 200 mM NaCl gradient. (B) CM-Sepharose rechromatography at pH 6.0 of CM-Sepharose-bound PME activity at pH 8.0. Bound proteins were eluted with a 200 to 220 mM NaCl gradient: A_{280} (—), PME activity (•••), and NaCl gradient (– – –).

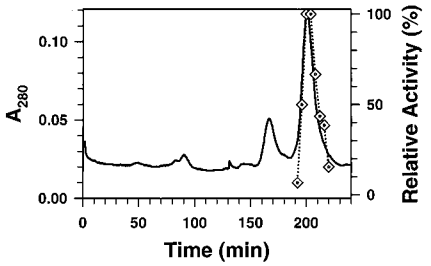

Figure 2. Superdex 200 gel filtration chromatography of CM-Sepharose-bound PME activity at pH 6.0. See the legend of **Figure 1**.

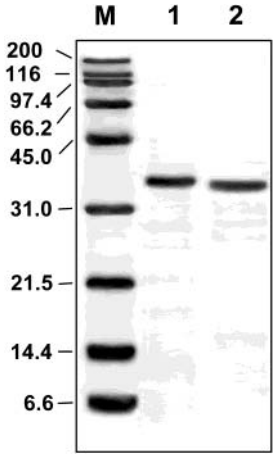

Figure 3. SDS–PAGE of monocomponent heparin peak 2 and peak 4 PME in which 5 μg of protein per lane was loaded onto a 13.5% gel: lane 1, peak 2 PME; lane 2, peak 4 PME; and lane M, Bio-Rad broad range molecular weight markers.

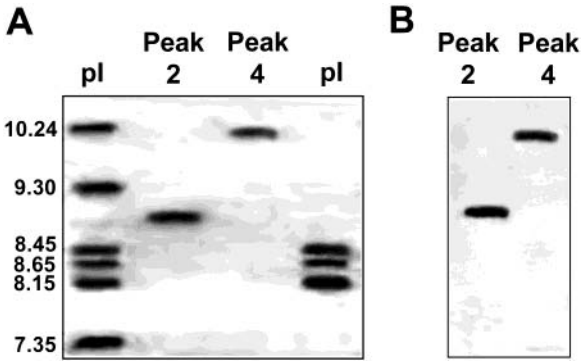

Figure 4. (A) IEF of monocomponent heparin peak 2 and peak 4 PME stained with Coomassie Brilliant Blue for total proteins. (B) Activity gel overlay of the IEF gel in panel A.

into account the large amount of protein that was removed during DEAE chromatography (data not shown). The DEAE flow-through material (with PME activity) run on the heparin–Sepaharose CL-6B column resolved four peaks of PME activity (*9*, *17*). The SD-PME was most strongly bound, requiring approximately 0.3 M NaCl to elute. After a buffer change, the sample was loaded onto the CM-Sepharose column and bound proteins were eluted (**Figure 1A**). The major activity peak eluted between 0.11 and 0.17 M NaCl. This sample was rechromatographed on CM-Sepharose at pH 6.0 and eluted at the midpoint of a shallow gradient (**Figure 1B**). The activity peak coincided with the major protein peak. The activity peak was finally resolved by a Superdex 200 gel filtration column. It eluted as a single activity peak corresponding to a molecular mass of 32.4 kDa (**Figure 2**).

Denaturing electrophoresis of the final PME peak from gel filtration chromatography demonstrated the PME exists as a monomeric protein with a relative molecular mass of 33.5 kDa (**Figure 3**). Side-by-side electrophoresis of this heparin peak 4 SD-PME and the heparin peak 2 PME, a salt-independent PME that was characterized by Savary et al. (*11*), indicates the mass is slightly smaller than the mass of 34 kDa reported for the peak 2 PME. A partial amino acid sequence of the densely staining band from the peak 4 SD-PME sample was obtained after deblocking the N-terminus with a trifluoroacetic acid treatment (preliminary attempts indicated the N-terminus was blocked). The peptide sequence that was determined was SVTPNV. Isoelectric focusing of both the peak 2 and peak 4 PMEs (**Figure 4A**) indicates there also is a difference in pI. Peak 2 PME has a pI of 9.2, while peak 4 PME is more basic with a pI of 10.1. The enzyme activity overlay of this IEF gel (**Figure 4B**) indicated there was only a single band of activity in each sample and that it corresponded with the intensely stained polypeptide band visualized with Coomassie Brilliant Blue.

The PME isozyme is readily distinguished by the total lack of activity in the absence of NaCl in the assay mixture. The optimal NaCl concentration at pH 7.5 and 0.5% citrus pectin was 200 mM, although NaCl concentrations higher than this were not tested (**Figure 5A**). Only approximately 6% of the maximal activity was observed at 5 mM NaCl. At 200 mM NaCl, the pH optimum was observed at pH 7.5 (**Figure 5B**). Reducing the NaCl concentration to 50 mM shifted the pH optimum to pH 8.5. At 50 mM NaCl, no activity was observed below pH 6.0. Only minimal levels of activity were observed when the enzyme was assayed in the presence of 10 mM NaCl between pH 7.5 and 9.5, and no activity was observed below pH 7.5. At pH 7.5, 200 mM NaCl and 59% DE pectin peak 4 SD-PME had a K_m of 0.0487 mg/mL and a V_{max} of 4.2378 nkat/mg of protein. De-esterification of citrus pectin to its end point resulted in final DE values of 39.9% DE at pH 7.5 and 43.7% DE at pH 4.5. Following limited de-esterification of a non-calcium-sensitive pectin with peak 4 SD-PME, the CSPR increased from 0.045 ± 0.011 to 0.829 ± 0.033. No difference

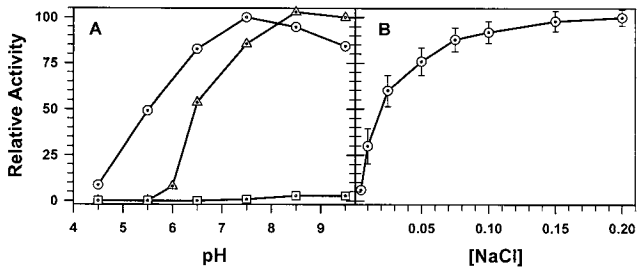

Figure 5. (A) Effects of pH on peak 4 PME activity at 200 (○), 50 (△), and 10 mM NaCl (□) and (B) effects of NaCl concentration on peak 4 PME activity.

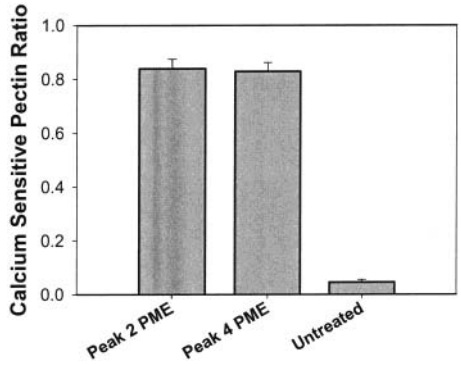

Figure 6. Calcium-sensitive pectin ratio of a non-calcium-sensitive pectin after treatment with peak 2 and peak 4 PME.

Table 2. Pectin Molecular Properties

pectin treatment	M_w ($\times 10^5$)[a]	$R_g z$ (nm)[a]	η_w (dL/g)[b]
PME I	2.32 ± 0.01	45.0 ± 0.6	5.30 ± 0.2
PME II	2.36 ± 0.05	47.5 ± 2.0	5.24 ± 0.02
untreated NCSP[c]	2.44 ± 0.06	49.5 ± 2.0	5.88 ± 0.03

[a] Determined by multiangle laser light scattering. [b] Determined by a combination of light scattering at 90° and viscometry. [c] Non-calcium-sensitive pectin.

was observed between peak 2 PME (type I, salt-independent) and peak 4 SD-PME (type II, salt-dependent) for the increase in the CSPR (**Figure 6**).

De-esterification of non-calcium-sensitive pectin with peak 2 PME and peak 4 SD-PME isozymes by 6.5% had little if any effect on the molecular properties of pectin (**Table 2**). These results are consistent with our previous report using the salt-independent orange peak 2 PME isozyme (*4*), which did not alter the weight-average molecular weight, the *z*-average radius of gyration, or the Mark−Houwink exponent over the de-esterification range of 69−26% for BB Rapid Set citrus pectin (Hercules). The peak 2 PME enzyme did reduce the intrinsic viscosity of pectin by 16% after its degree of esterification dropped below 47% (*4*). The results in **Table 2** demonstrate that both salt-independent and salt-dependent orange PME isozymes (peak 2 PME and peak 4 SD-PME) generally preserved these pectin molecular properties following conversion of non-calcium-sensitive pectin to calcium-sensitive pectin. Subtle differences may exist between the pectins treated with the two different PME isozymes. For example, the molecular weight properties of peak 2 PME- and peak 4 SD-PME-treated pectins were not significantly different. However, the molecular weight properties of the peak 2 PME-treated pectin were consistently lower than those for the untreated NCSP, while only the intrinsic viscosity of the peak 4 SD-PME-treated pectin was lower than that of the untreated NCSP.

DISCUSSION

The variability observed in citrus juice cloud destabilizing properties of the multiple PME forms present in fruit tissue (*17*) likely indicates differences in the biochemical properties and suggests differences in the mode of action. Partial amino acid sequencing of the peak 4 SD-PME strongly suggests it is identical to the salt-dependent (type II) PME previously isolated from Navelina fruit peel (*14*) and Valencia juice vesicles (*29*). Both papers reported that the N-terminus was blocked, as was ours, and the short amino acid sequence we determined for the peak 4 PME (SVTPNV) is identical to that reported by Christensen et al. (*14*) and varied by only one amino acid from the sequence reported by Nairn et al. (SVTPNAV; *29*). A denatured M_r of 36 kDa was reported by both Christensen et al. (*14*) and Nairn et al. (*29*), while it migrated as a 33.5 kDa polypeptide in our electrophoretic system. Nairn et al. (*29*) reported a pI between 8.5 and 10.5, and Christensen et al. (*14*) reported an isoelectric point of >9. We demonstrate the enzyme has a pI of 10.2 (**Figure 4A**) and is clearly distinguishable from the salt-independent peak 2 PME (Type I) based on chromatographic behavior, IEF, and denaturing electrophoresis (**Figures 3** and **4**). Kinetic data and the effects of pH differed from those reported by Christensen et al. (*14*). We reported a K_m of 0.0487 mg/mL and a V_{max} of 4.2378 nkat/mg of protein (0.61 μEq min^{-1} mL^{-1}) with 59% DE citrus pectin, while Christensen et al. (*14*) reported a much higher K_m (0.7 mg/mL for citrus pectin with 70% DE and 17 mg/mL for citrus pectin with 25% DE) and V_{max} (387 μEq min^{-1} mL^{-1}; pectin DE not reported). These differences could be a result of the different pectins used to estimate these parameters. Christensen et al. (*14*) reported a pH optimum of pH 7 with a 50% decrease at pH 6 with 150 mM NaCl. We observed a pH optimum at 7.5 with 200 mM NaCl and an approximately 15% decrease at pH 6.5. At 50 mM NaCl, there was a 50% decrease at pH 6.5 and a nearly 90% decrease at pH 6.0 (**Figure 5A**). In agreement with Limberg et al. (*18*), who used the same PME characterized by Christensen et al. (*14*), we observed a blockwise de-esterification pattern following a 6.5% decrease in DE with a NCSP of 76% DE, based on an increase in the CSPR (**Figure 6**).

Three other properties of the peak 4 SD-PME described here that might account for its failure to destabilize citrus juice cloud are the relatively high end point pectin DE obtained at pH 4.5, its low levels of activity at acidic pH values, and its requirement for cations, especially at lower pH values (**Figure 5A**). The end point DE value of 43.7% obtained after exhaustive demethylation of the initial 59% DE pectin at pH 4.5 is far from the critical value (27−36%) necessary for destabilization of orange juice cloud reported by others (*30*, *31*). It must be remembered, however, that the reported DE value is an average for a population of pectin molecules. Baker (*7*) was able to demonstrate that the actual DE required for a more homogeneous pectate to precipitate cloud was between 14 and 21%. While it is possible that some individual pectin molecules in the 45% DE pectin would have a DE within the range reported by Baker (*7*) necessary for cloud destabilization, their overall proportion was likely to be too low to initiate cloud precipitation as illustrated by the juice sample incubated with this PME (*17*). Further structural characterization of the pectin population exhaustively demethylated at pH 4.5 would be required to determine if any of it had been demethylated below the critical value reported by Baker (*7*). Since molecular characterization of pectin de-esterfied with this peak 4 PME indicated no loss in molecular weight following a 6.5% reduction in citrus pectin DE, its inability to destabilize juice cloud was probably not

related to the reduction of the degree of polymerization to a value below that which allows precipitation (*32*). Other reports of pectin demethylation with the salt-dependent PME from navel oranges (*14*, *18*, *19*) also indicate it has a limited ability to decrease the end point DE. A final DE of 41% was the lowest value achieved by this PME (*18*, *19*) following pectin demethylation at pH 7.0. Versteeg (*15*) also demonstrated that his PME II, the PME isozyme which bound most strongly to a cation exchange matrix as our peak 4 PME did, was unable to reduce the DE from an initial value of approximately 68% to much below 40%.

Cation effects on salt-dependent PMEs from several sources (*33*−*35*) have been shown to vary depending on the cation used, with divalent cations having an effect at lower concentrations than monovalent cations. Cations are believed to affect PME activity by competitively displacing PME from unproductive binding to ionized carboxylic acid groups of galacturonic acid in the homogalacturonan chain (*36*). Five cations (Na, K, Ca, Mg, and Fe) are present in orange juice (*37*), ranging in concentration from approximately 1 mM for Na, 4 mM for Ca, and 13 mM for Mg to 100 mM for K (*30*), although these concentrations are known to vary (*38*). The concentration of Ca present in orange juice (4 mM) was sufficient to increase the total PME activity extracted from grapefruit pulp approximately 10−30% (*38*) at pH 7.5. Cameron and Grohmann (*9*) have shown that the total PME activity present in red grapefruit finisher pulp contains the same four peaks of PME activity after heparin chromatography that have been observed in orange fruit peel (*17*), rag tissue (*11*), and hand-expressed juice (unpublished results). The dominant PME form present in these PME extracts is peak 2 PME (*11*) which is a type I, salt-independent PME. Lineweaver and Ballou (*39*) and data presented here (**Figure 6**) demonstrate that the positive effects of cations on peak 4 SD-PME, a salt-dependent type II PME, are diminished at lower pHs, requiring higher cation concentrations that still result in activity levels lower than that observed at higher pH values. These results support the hypothesis that a combination of a limited ability to demethylate pectin, relatively low levels of cations, and acidic pH values that diminish peak 4 SD-PME activity act in concert to prevent juice cloud destabilization by this enzyme.

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