Clarification of Juice by Thermolabile Valencia Pectinmethylesterase Is Accelerated by Cations

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Pectinmethylesterase (PME) was isolated from Valencia orange pulp and added to reconstituted juice at 1.2 units/mL of juice in the presence or absence of cations at 4.2 or 16.7 mM. The percent transmittance (%T) of control juices with no added PME or cation did not clarify. The %T of juices with added PME and added cation was 45−55% by the second day. Increases in the average particle size was observed with PME- or cation-added juices and preceded increases in %T. Most likely, cations displaced PME from an inactive pectin substrate complex and increased clarification. PME, in the absence of cations, increased particle size but did not affect %T, suggesting a direct interaction of PME with cloud particles.

KEYWORDS: Pectin; particle size; colloidal dispersion; strontium; calcium; spermidine

INTRODUCTION

Clarification of citrus juices is attributed to pectinmethylesterase (PME) activity, which results in de-esterification of pectin by PME, zones of negative charge, and chelation of calcium. Formation of large, insoluble calcium pectates intiates the precipitation of other colloidal cloud constituents (*1*). Different isozymes of PME have been isolated and described, some of which are thermostable (TS-PME) and resist inactivation at temperatures >70 °C. The TS-PME is most likely to cause clarification (*2*) and to maintain activity during processing treatments (*3−6*). Recently, it was shown that thermolabile PME (TL-PME) also clarifies juices (*7*). PME (TS- or TL-) activity in juices is variable (*8*), and the amount that is TS increases during some purification preparations (*9*). The presence of settling pulp, PME−pectin complexes, inadequate detection methods, non-PME protein, and other factors were credited with variation in the detection of PME activity and thermostability and the effect on clarification potential of juices (*8, 9*).

PME is a cell wall bound enzyme that is optimally solubilized from the cell wall matrix using high pH and/or high salt concentrations in the extraction buffers. Once solubilized, PME can form an inactive enzyme−substrate complex, and activity is influenced by the competitive displacement of PME by monovalent or divalent cations (*10−12*). Cations influence not only the activity of PME but also separation of PME from pectin complexes by ultrafiltration (*5, 8*), ion exchange (*13*), and heat stability of solubilized PME (*14*). The PME complexes are tightly coupled and not easily separated even in electric fields (*15*) and inhibit PME separation on ion exchange membranes (*13*). It is proposed that many if not all of the reported PME isozymes are actually PME−pectin complexes (*16*). On the basis

of unique permeation and activity through ultrafiltration membranes at the same ionic strength, a specific cation effect was suggested (*17*). Calcium, strontium, and spermidine uniquely affected the $\Delta S^{\#}$ and Gibbs free energy for dissolution of grapefruit TS-PME−pectin complexes. The $\Delta S^{\#}$ was positive and independent of concentration in calcium-added reactions (*18*). PME−pectin complexes were postulated to result in unique de-esterification patterns and pectin gelation (*16*).

Despite the ostensible role of PME in juice clarification, a direct correlation of total or TS-PME activity with clarification has not been demonstrated. Bound, potentially active PME may influence the potential for juice clarification. At low levels, cations displace PME from pectin substrate and increase the apparent activity of PME, so cations may increase the potential of clarification of citrus juices. Juices have a wide range of mineral content depending on the growing region, cultivar, production practices, etc. The amount of calcium or strontium naturally present in juice can range from 1.6 to 2.5 mM or from 1 to 11 μM, respectively (*19, 20*). Furthermore, water used to reconstitute juice will vary in the natural mineral content. The cations spermidine, spermine, and putrescine occur naturally in plant cells, modulate PE activity, stabilize membranes, and retard senescence (*21*). Oranges contain 61−67 nmol of spermidine/mL (*22*). The objective of this study was to evaluate clarification of orange juice by PME that was solubilized from a PME−pectin complex in the presence and absence of activating levels of cations.

MATERIALS AND METHODS

Materials. Unheated, frozen Valencia pulp and frozen concentrated orange juice (FCOJ) were donated by Citrus World (Lake Wales, FL). The FCOJ was reconstituted to 16 °Brix with distilled water and centrifuged at 1500*g* for 10 min at 4 °C to remove most of the settling pulp. The colloidal cloud suspension was separated from the pellet,

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and the supernatant was filtered through MiraCloth (Calbiochem, La Jolla, CA) and used for further analysis.

Preparation of Valencia PME for Clarification. PME was extracted from Valencia pulp using 4 volumes of 0.1 M NaCl and 0.25 M Tris-Cl⁻, pH 8.0. The slurry was homogenized for 1 min at 4 °C (Pro300A, Proscientific Inc., Monroe, CT). The homogenate was maintained at pH 8.0 with NaOH, and the slurry was centrifuged at 4 °C and 8000*g* for 20 min (Sorvall RC-5B, DuPont Instruments, Doraville, GA). The supernatant was filtered through MiraCloth (Calbiochem). The precipitate of a 30−75% ammonium sulfate cut of the filtrate was collected, resuspended in, and dialyzed against 50 mM sodium phosphate, pH 7.0. To minimize loss of PME, dialysis tubing was pretreated by boiling in 10% acetic acid and rinsed with water prior to use. After dialysis, the PME extract was filtered through Whatman no. 1 paper (Fisher Scientific, Atlanta, GA). The PME was loaded onto a 5 mL cationic exchange column (Hi-Trap SP, Amersham-Pharmacia Biotech, Piscataway, NJ) at 5 mL/min. Buffers were degassed and filtered through 0.45 μm filters (Gelman Scientific, Ann Arbor, MI) at room temperature and chilled to 4 °C. Chromatography was performed at 4 °C using FPLC P-500 pumps and a GP-250 gradient programmer (Amersham Pharmacia Biotech). The portion of PME that did not bind Hi-Trap SP (*13*) from three separate fractionations was pooled and reloaded onto a Hi-Trap SP column. Upon reapplication, PME bound Hi-Trap SP and was eluted with a 0−1 M NaCl gradient in 50 mM sodium phosphate, pH 7.0. Positive PME fractions were identified qualitatively using a color-sensitive dye in 1% pectin and 0.1 M NaCl. Aliquots of 10 μL of the PME fraction were added to 1 mL of 1% pectin and 0.1 M NaCl, containing 0.01% bromthymol blue in 10% ethanol, pH 7.5. Tubes that changed color within 30 min at room temperature were quantified for PME activity by titration. Fractions 9 and 10 were combined to yield a PME extract with 323 units/mg of protein and used for clarification studies.

Analytical Methodology. The activity of PME was determined by pH-stat titration (Brinkman, Westbury, NY) in 1% pectin (Citrus Colloids Ltd., Hereford, U.K.) and 0.1 M NaCl, 30 °C, pH 7.5. PME units are expressed as microequivalents of ester hydrolyzed per minute. TS-PME was defined as activity that survived heating at 70 °C for 5 min in 0.1 M NaCl and 50 mM sodium phosphate, pH 7.0. Aliquots of 0.5 mL of PME were added to 2 mL of preheated buffer, vortexed, and heated for 5 min. Protein was determined according to the dye-binding method (*23*) assay using IgG as standard using a microplate reader (Bio-Rad, Hercules, CA).

Cation and PME Addition to Juice. Stock solutions of calcium chloride, strontium chloride, and spermidine were prepared in deionized water and added to juice to achieve a final concentration of 4.2 or 16.7 mM as Ca^{2+} or Sr^{2+} or 10 mM spermidine, respectively. Juices were prepared with and without 1.2 units of PE/mL of juice, with and without cation addition. Deionized water was added to juice treatments and controls to maintain a final 13 °Brix. The spiked juices, positive controls, and negative controls were stored in 15 mL graduated, conical centrifuge tubes at 4 °C.

Particle Size and Percent Transmittance Analysis. At the indicated time, tubes of juice were randomly selected and stored on ice until analysis. Particle size distributions (volume fraction against particle size) were calculated, and the weight-average sizes were expressed as $D_{3,2} = \Sigma_i n_i d_i^3 / \Sigma_i n_i d_i^2$ and $D_{4,3} = \Sigma_i n_i d_i^4 / \Sigma_i n_i d_i^3$, where n_i is the number of particles of diameter d_i as described earlier (*24*) using a Mastersizer S (Malvern Instruments, model MSS, Worcestershire, MA), with a dispersion setting of 2000 rpm (dispersion unit controller, DIF 2023) and an obscuration value of 20%. The values 1.73 and 1.33 were used as the refractive indices of cloud and dispersed phase, respectively, and 0.1 was used as the absorption index for cloud particles (*24*). After particle size analysis, the remainder of the juice was centrifuged at 3000*g* and 4 °C for 10 min. The supernatant was used to measure the percent transmittance (%T) at 650 nm using a Spectronic 20 (Fisherbrand, Atlanta, GA). Duplicate juices were prepared by centrifugation, and cations, PME, and/or water were added in replicate experiments.

RESULTS AND DISCUSSION

In the absence of PME or cations, orange juices that have been properly pasteurized are cloud stable at 4 °C for several
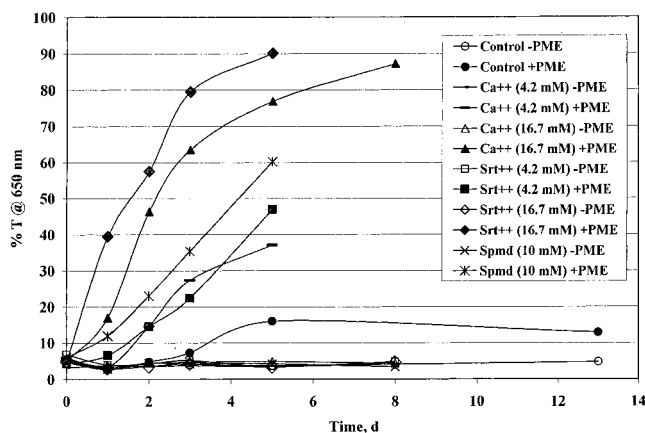

Figure 1. Percent transmittance (%T) at 650 nm of reconstituted juices after storage at 4 °C and centrifugation at 3000*g* for 10 min at 4 °C. Juices had 1.2 units/mL PME, calcium (Ca^{2+}), strontium (Sr^{2+}), or spermidine (Spmd) added according to the figure legend.

months. At long storage times, juices may clarify due to the high acidity and other factors of the juices (*25*), but clarification within a few days or weeks of storage is typically attributed to PME activity. Generally, only TS-PME will rapidly clarify juice at 4 °C and juice pH (*2*). Some forms of TL-PME will clarify juice at 30 °C if added at levels that are higher than typically found in fresh juices (*26*). The PME used in this study was thermolabile, and no activity remained after heating for 5 min at 70 °C. The juice used in this study was PME negative and had 2.3 mM calcium, naturally present. Clarification was observed under some conditions, and the rate of clarification as measured by the change in %T of stored juices is reported in **Figure 1**. In this study, FCOJ diluted to single strength, without added PME or cations, was stable for up to 13 days of storage. With added cations, no change in %T was measured in 8 days of storage at 4 °C. Addition of 1.2 units of PME/mL of juice initiated an increase in %T at 5 days of storage that did not change through 13 days at 4 °C. The %T of control or cation-added juices at 5 and 13 days was <20%. The %T of juices with 1.2 units/mL active PME and cation-added began to increase at the first or second day of storage depending on the type and concentration of cation. The %T of juices with 1.2 units/mL and strontium or calcium at 16.7 mM was 45−55%T by the second day of storage. The %T continued to increase through the fifth to eighth days of storage. The %T of juices with active PME and lower amounts of calcium or strontium (4.2 mM) or 10 mM spermidine also increased, but at a lower rate and to a lesser extent than juices with active PME and higher cation levels. The increase in rate and extent of clarification of PME active juices with cations may result from an increase in apparent activity of PME in juice. Competitive displacement of PME from an inactive pectin complex (*10−12*) increases PME activity, permeation in ultrafiltration (*5*), and ion exchange separation (*13*). Solubilization of PME from pectin increased apparent activity and influenced thermostability (*14*). In this study, cations also increase the apparent PME clarification potential in juices stored at 4 °C.

The particle size distribution of a representative juice sample during 13 days of storage at 4 °C, with no added PME or cation, is depicted in **Figure 2A**. A bimodal distribution of particle size is depicted. The major volume distribution is near 1−2 μm ($D_{3,2}$), and a second volume distribution is observed near 30 μm ($D_{4,3}$). The change in volume distribution of the larger particles did not change with time in a recognizable pattern. Most likely, the larger particle size distribution is the result of
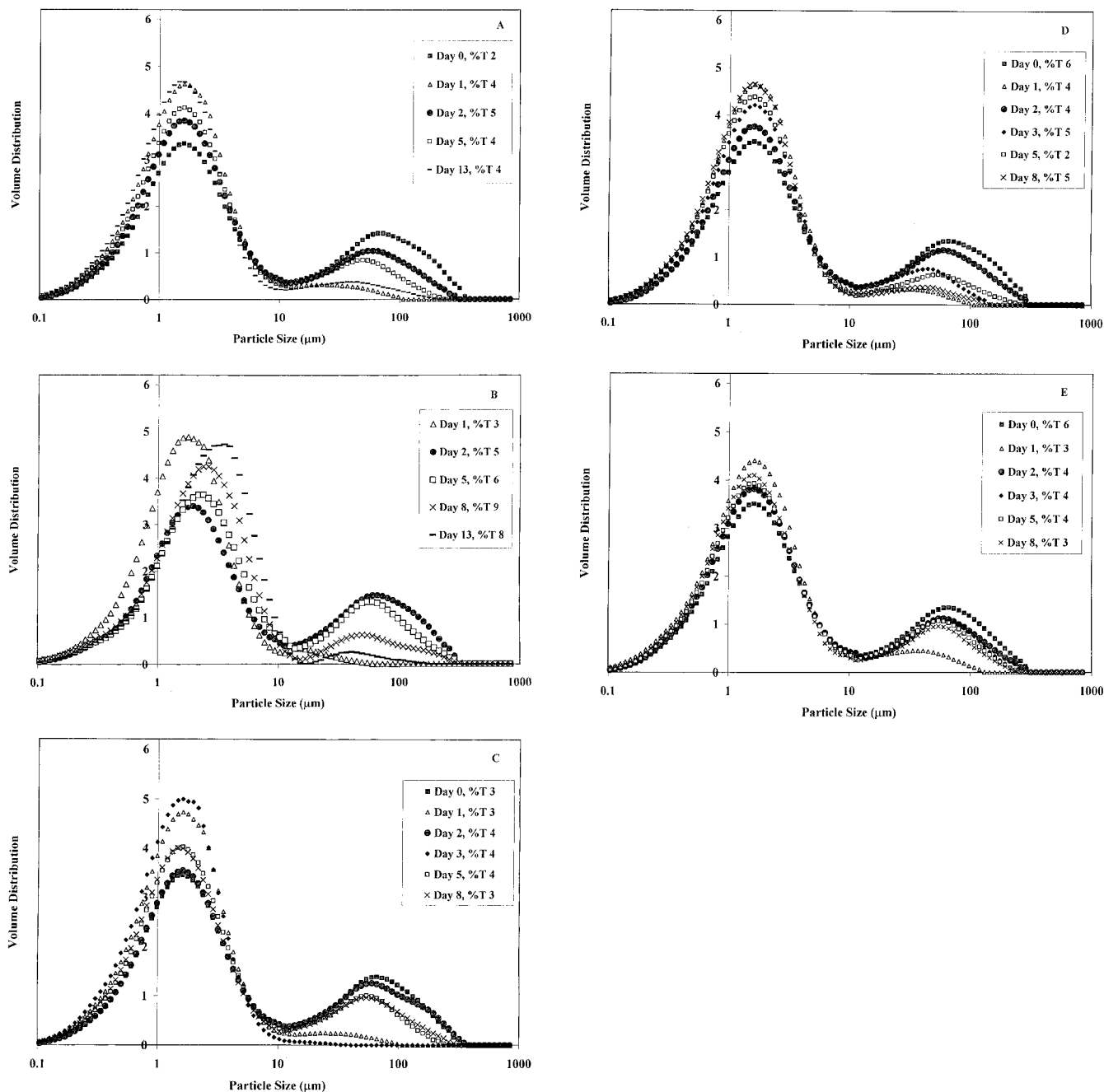
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Figure 2. Change in particle size distribution with time of storage of (A) control juice (no PME or cation added), (B) 1.2 units of PME/mL of juice added, (C) 16.7 mM Ca^{2+} added (no PME), (D) 16.7 mM Sr^{2+} added (no PME), and (E) 10 mM spermidine added (no PME). The %T values at the indicated day of storage are given in the inset.

incomplete separation of settling pulp, and the large particles of pulp distort the particle size results (unpublished observations). Under more rigorous separation of juice cloud after centrifugation, large pulp particles were not observed in juice cloud (*27*).

The particle size distribution of control juice with added PME is depicted in **Figure 2B**. As discussed earlier, the bimodal peak distribution and presence of larger particles near 30 μm is likely to be due to small amounts of settling pulp. More interesting is the increase in particle size of the peak near 1−2 μm. After 1−2 days of incubation, a clear shift to a larger size is observed that progressively increases with longer storage times. At 2 days of storage, when the %T is <5% and there is no apparent clarification, the particle size distribution increases with PME present. A similar increase from 0.7 to 1.0 μm cloud particle

size was reported earlier with Marsh grapefruit PME added to Valencia juice (*27*).

At 5 and 13 days of storage, when the %T was <20% T, the particle size of cloud of PME added juices continued to increase. In cation added, no-PME control juices, there was no change in the particle size distribution of the 1−2 μm particles. The particle size distribution for juices with 4.2 mM calcium (**Figure 2C**), 16.7 mM strontium (**Figure 2D**), 10 mM spermidine (**Figure 2E**) is depicted. Juices with calcium (16.7 mM) or strontium (4.2 mM) were similar to the results shown. The addition of cations without added PME did not affect juice cloud particle size.

In juices with added PME and added cation, an increase in particle size of juice cloud is seen even at day 1 of storage (**Figure 3**). A smaller increase in cloud particle size is seen at
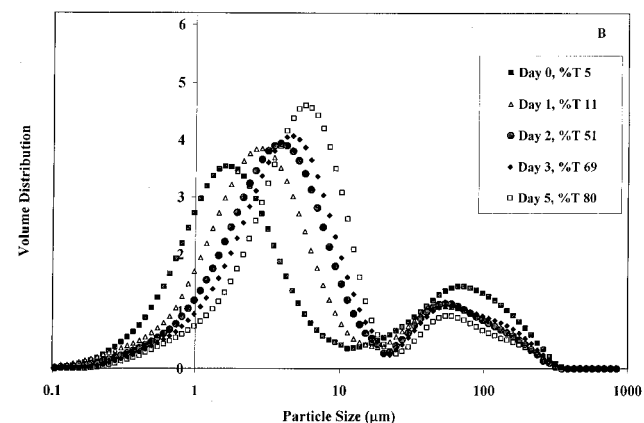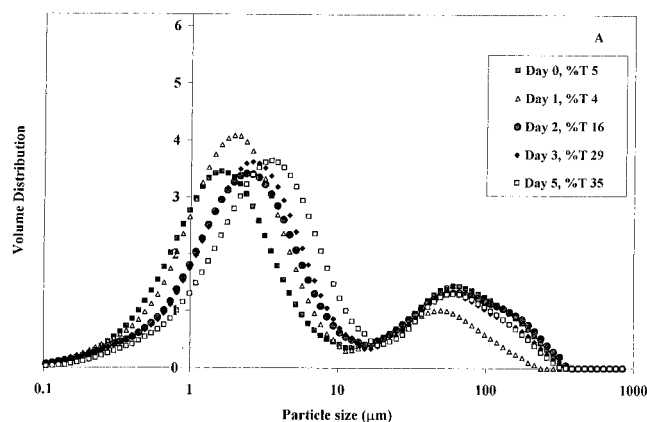
Figure 3. Change in particle size distribution with time of storage of (A) PME added at 1.2 units/mL of juice and 4.2 mM Ca²⁺ and (B) PME added at 1.2 units/mL of juice and 16.7 mM Ca²⁺.

Figure 4. Change in particle size distribution with time of storage of (A) PME added at 1.2 units/mL of juice and 4.2 mM Sr²⁺ and (B) PME added at 1.2 units/mL of juice and 16.7mM Sr²⁺.

Figure 5. Change in particle size distribution with time of storage of PME added at 1.2 units/mL of juice and 10 mM spermidine.

lower levels of strontium (**Figure 4A**) or calcium (**Figure 3A**) than at higher concentrations (**Figure 4B** or **3B**). Changes in cloud particle size of PME/10 mM spermidine added juices (**Figure 5**) were similar to particle size changes of higher concentrations of strontium and calcium. The increase in %T with active PME and increasing cation concentration supports the role of PME as the initiator of cloud loss and subsequent calcium bridging of pectates to form large, unstable high molecular weight aggregates. The increase in particle size can result from several possible causes. Insoluble calcium pectate may entrain cloud particles and lead to gross clarification as measured by %T. Interestingly, increases in particle size were apparent before the onset of gross clarification as measured by %T. The increase in particle size may result from the direct interaction of PME with pectin or from the possible depletion of a protective colloid from the cloud surface that allows the aggregation of cloud particles. The increase in particle size before changes in %T suggests the latter.

Calcium, but not strontium or spermidine, decreased the temperature optimum of PME activity (*18*), suggesting a specific interaction of calcium with PME. Furthermore, a specific calcium effect on PME and PME−pectin complexes was observed, and a positive entropy of activation ($\Delta S\#$) was reported, independent of calcium concentration (*18*). The resultant decrease in the Gibbs free energy could not be attributed solely to the depletion of PME from an inactive PME−pectin complex. This suggests that the role of PME in cloud stability may not be limited to cloud destabilization by de-esterification of pectin. The growth in pectin molecular weight (*28*) and particle size (*29*) due to de-esterification and transacylation 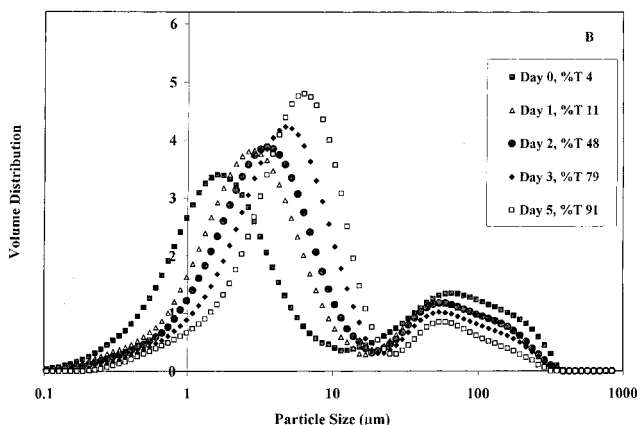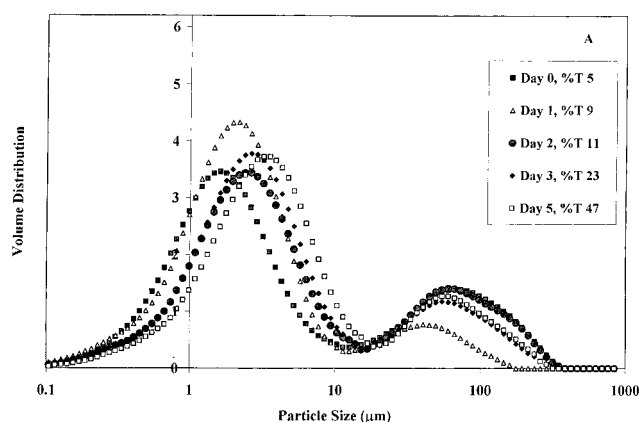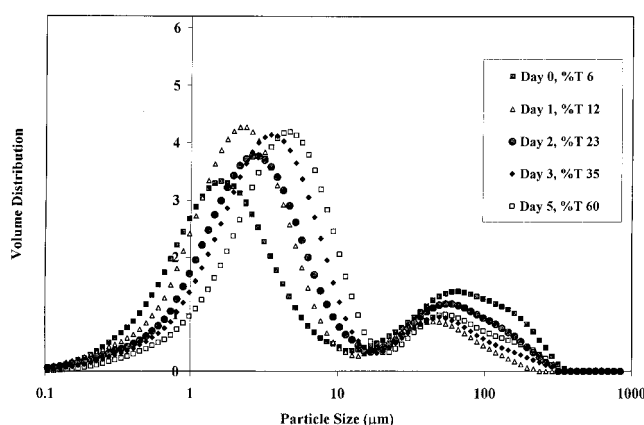by PME has been reported. The depletion of a protective colloid from the cloud particle surface and coalescence of particles as a possible additional factor in juice clarification are supported by this study. The increase in particle size in PME-added juices before the onset of gross clarification as measured by %T indicates the aggregation of cloud particles as a result of PME, perhaps unrelated to PME activity. Under certain conditions of pectin concentration, source, and degree of esterification, gelation of pectin was observed with heat-inactivated PME (2.3 mg/mL) in the presence of NaCl (*30*). Only PME expressed in *Aspergillus oryzae* induced gelation, most likely due to basic charge domain at the C terminus. A similar interaction of PME with pectin in dilute solution may be occurring here.

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CONCLUSIONS

In this study, clarification of citrus juices by TL-PME at 4 °C was observed in the presence of cations within 1−2 days of storage. This is likely related to the displacement of PME from an inactive pectin complex and an increase in apparent PME activity and subsequent clarification. These results have implications for the need of quality assessment of water that is used to reconstitute juices with low levels of active PME or pasteurized juices that were inadvertently exposed to PME before pasteurization.

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