Effect of Pectin Methylesterase on Carrot (Daucus carota) Juice Cloud Stability

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ABSTRACT: To determine the effect of residual enzyme activity on carrot juice cloud, 0 to 1 U/g pectin methylesterase (PME) was added to pasteurized carrot juice. Cloud stability and particle diameters were measured to quantify juice cloud stability and clarification for 56 days of storage. All levels of PME addition resulted in clarification; higher amounts had a modest effect in causing more rapid clarification, due to a faster increase in particle size. The cloud initially exhibited a tridomal distribution of particle sizes. For enzyme-containing samples, particles in the smallest-sized mode initially aggregated to merge with the second peak over 5–10 days. This larger population then continued to aggregate more slowly over longer times. This observation of a more rapid destabilization process initially, followed by slower subsequent changes in the cloud, was also manifested in measurements of sedimentation extent and in turbidity tests. Optical microscopy showed that aggregation created elongated, fractal particle structures over time.

KEYWORDS: pectin methylesterase, Daucus carota, carrot juice, particle size, relative sediment height, relative turbidity

INTRODUCTION

Many of the sensory characteristics associated with fruit and vegetable juices, such as color, flavor, texture, and appearance, are attributable to the presence of cloud particles suspended in the liquid matrix of the juice.1,2 The destabilization, or “clarification,” of cloud is a serious problem for the juice industry. When cloud becomes unstable, the particles flocculate and settle out of the continuous phase, resulting in a clear, flavorless serum and a potentially unappealing precipitate at the bottom of the container. It is commonly thought that cloud destabilization occurs through the action of either the enzyme pectin methylesterase (PME) or by acidification of juices below their natural pH.

The purpose of this study is to examine the effects of different amounts of PME on cloud stability and how such additions affect particle size as well as the rate and amount of clarification within the juice. From these results, mechanisms of clarification can be proposed. This study seeks to improve our overall understanding of how PME affects cloud stability, a goal that is of broad interest to scientific and commercial communities. To prevent cloud destabilization, the common industrial method is to inactivate PME entirely.3 During juice processing, carrots undergo enzyme inactivation within one day of peeling by several common blanching methods, including steaming or immersion in hot water or acid.3 If inactivation is not accomplished within one day, loss of cloud stability and clarification will result.5 The attempt to completely inactivate PME using heat results in deleterious effects on juice color, flavor, nutrients and viscosity. The present study looks at residual enzyme levels instead of complete inactivation, and could be used to determine whether blanching operations could be reduced, saving energy and maintaining quality.

There is considerable interest in minimally processed juices, and so in order to improve retention of initial fruit and vegetable quality, advanced processing methods such as high pressure and electric field processing are being explored. These processes achieve microbial inactivation using very little heat, and therefore, quality is superior; however, residual enzyme activity of pectin methylesterase in particular has been shown to limit shelf life. Very few studies have addressed the effects of a known amount of residual enzyme activity on refrigerated juices.

The orange juice industry sets a goal of inactivating PME to below 10% of its initial level,4,5 because it is assumed that even a small amount of residual PME can be deleterious to cloud stability. However, in pineapple juice residual PME levels of 4.1 × 10−4 U/mL resulted in a product with acceptable cloud stability, while 7.1 × 10−3 U/mL caused complete clarification.6 For citrus fruit juices, it has been established that when PME activity levels are kept below 10−4 U/mL cloud loss is prevented.7 This residual level for citrus fruits has been assumed to be the same for tomato products,5,9 however, this has not been investigated. Some level of residual enzyme activity may be acceptable in juice products, depending on the shelf life of the product.

Characterization of PME. In plants, PME is cell wall-bound and mainly found in the middle lamella between the individual cells, where it is important in plant development and fruit ripening.10–12 PME catalyzes the hydrolysis of methyl esters from the O6 position of galacturonic acid monomers of which pectin is composed. This reaction increases the number of free carboxylic acid groups within the pectin chain, and produces methanol and hydrogen ions.8,11,13,14 PME action typically does not result in complete deesterification, as 20–30% of the methyl esters are not removed.15
Hydrolysis of methyl ester groups from the pectin backbone by PME can occur via three different mechanisms. The first mechanism involves a multiple chain reaction, in which PME removes one methyl ester from the pectin chain and then dissociates from the substrate, resulting in random deesterification of the chain. This is typically the mode of action of acidic fungal PME. The second mechanism is single chain, or linear, deesterification, where PME follows along one chain and cleaves all the methoxyl groups, which results in blocks of demethylated pectin. This is generally the mode of deesterification for higher plant PME. This would suggest that carrot PME also employs linear deesterification, although no research to date has confirmed this. The third mechanism involves multiple attacks of the enzyme on methyl esters, where only a limited number of methyl groups are removed from each pectin substrate. The mechanism by which carrot PME catalyzes deesterification of carrot pectin has not yet been determined.

Carrot Juice Cloud Stability. The rate of juice clarification is governed by two mechanisms: flocculation and sedimentation. Flocculation is the phenomenon in which particles cluster together to form aggregates, increasing the effective particle size. Sedimentation is the settling out of flocculated particles from suspension to the bottom of the container. Typically, flocculation leads to faster sedimentation in accordance with Stokes’ Law. Larger particles formed from flocculation increase the rate of sedimentation and, hence, cause more rapid loss of cloud stability.

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Stokes

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If particle size influences the rate of cloud loss and clarification, then it is worthwhile exploring how particle size increases within juices over storage time. In a suspension, particles remain separate from one another due to the presence of an energy barrier between them, which reduces their rate of collision. The energy barrier is influenced by several different contributions, including attractive van der Waals interactions (which reduce the barrier) and repulsive electrostatic forces (which increases it).19–22 Factors that lower the energy barrier make it more likely that cloud particles will closely approach one another, at which point they can “stick” to each other and floc as a result of short-range attraction.

In juices with active enzymes, PME cleaves methoxyl groups from the pectin backbone and additional negatively charged residues are exposed. These like-charged residues would normally enhance the repulsive interparticle energy barrier; however, naturally present divalent cations such as calcium may bind to these sites, creating electrostatic attractive interactions between particles. The calcium can then cross-link pectin chains on different particles together, thereby forming insoluble calcium pectate within aggregates that are large enough to sediment out according to Stokes’ Law.1,17,20,23

Little research has been conducted regarding the effect that residual PME levels have on cloud stability of carrot juice, which is a low-acid juice product. Therefore, the goal of this study was to observe the effects of adding PME to pasteurized carrot juice and suggest a mechanism for cloud flocculation and sedimentation.

MATERIALS AND METHODS

Material Sourcing. Sugarsnax variety carrots (\textit{Daucus carota} L. ssp. sativa cv. ‘Sugarsnax’) were used to make the PME extraction, and were sourced from Bolthouse Farms in Bakersfield, CA. The average PME activity in raw carrot juice made from these carrots was found to be 0.43 U/g (range: 0.218–0.806 U/g), as measured by individually

amount of enzyme activity and, hence, the amount of enzyme present in the sample. Pectin used in the assay was dialyzed overnight in water. Assays were started by adding 5 μL of a 10-fold dilution of the carrot juice to 95 mL of the assay medium, which contained 3 mg/mL pectin and had a pH of 7.5. All samples were placed into a 30 °C water bath, and at four time points (40, 70, 100, and 130 min) after the start of the assay, 0.1 mL aliquots were removed and added to 0.1 mL of a solution containing 5 mg/mL of each ferric ammonium sulfate and sulfamic acid. After 30 min at room temperature, 0.8 mL of water was added and absorbance at 620 nm was determined using a Shimadzu spectrophotometer (Columbia, MD). Methanol concentrations were determined from a separate methanol standard curve. The amount of methanol formed was plotted and the activity was calculated from the slope of the plot.

Cloud Stability during Juice Storage. PME Addition. The PME extract, made as described above, was added to commercial carrot juice in 5 quantities, 0, 0.15, 0.25, 0.5, and 1 U/g, where a unit of enzyme activity (U) is defined as the number of micromoles of methanol produced in 1 min, and grams refers to the mass of carrot juice. Since different volumes of PME extract (enzyme suspended in 0.05 M citric acid/sodium citrate buffer) were added to the juice to achieve different enzyme levels, it was necessary to compensate for the subsequent changes in juice volume. The largest volume of extract added (for addition of 1 U/g) was used as a baseline, and the other samples had a combination of enzyme extract and citric acid/sodium citrate buffer added so that all samples had the same final volume. This was done to ensure each juice was diluted the same small amount and any effects of the buffer were mitigated. Final juice pH after PME addition was 6.20. To prevent microbial growth during the storage study, 0.2% sodium azide was added to the juice. Juices were stored for up to 56 days at 5 °C.

Relative Sediment Height. Relative sediment height, also known as the sedimentation test, is a measure of the quantity of visible settling that occurs in the juice. 21,27 It is the height \( H_{\text{sediment}} \) of the cloud settled divided by the total height \( H_{\text{total}} \) of the juice in a vial.

Samples used for measuring the relative sediment height of the sediment were placed in 7 mL glass vials with screw caps and allowed to remain undisturbed except for gentle transportation of the vials from the refrigerator to the laboratory bench for measurements. A caliper was used to make height measurements in triplicate.

Relative Turbidity. Relative turbidity is a spectrophotometric method to determine cloud stability by comparing the turbidity in a juice sample before and after centrifugation. 21,22,28–30 For the carrot juices examined here, absorbance at 660 nm was used as the measure of juice turbidity. At this wavelength, absorbance is predominantly determined by the scattering due to the particles, rather than specific chemical absorption. 31 Relative turbidities were determined by measuring the absorbance values for the whole juice and for the supernatants obtained after centrifugation (4200 g, 15 min, 20 °C). When absorbance values measured in standard 1 cm cuvettes were unacceptably high, shorter path length cuvettes (0.5 mm for whole juice and 5.0 mm for juice supernatants) were used. Turbidity \( (\tau) \) was calculated as the absorbance divided by the path length used for measurement. Relative turbidities (expressed as %) were calculated as \( \tau_{\text{serum}}/\tau_{\text{total}} \times 100 \).

Particle Size Determination. Static light scattering was used to measure average particle size. 21,28,30,32–34 A laser is passed through the sample and, upon hitting a particle, diffracts and creates a light pattern against the detector that depends on the particle size. Measurements were performed using a Microtrac S3500 (Montgomeryville, PA). Results were obtained as the volume fraction of particles contained within a discrete size range (bin). The mean diameter was determined as a volume average:

\[
D_{3,4} = \frac{\sum (n_i d_i^3)}{\sum (n_i d_i^2)}
\]

Here \( D_{3,4} \) is the volume mean diameter, \( n_i \) is the number of particles in bin \( i \), and \( d_i \) is their diameter. Carrot juice was inverted gently 10 times to ensure even distribution of particles and then added to the instrument sample port, which was operated at a flow rate of 15% of maximum. Particles were assumed to be irregular in shape and absorbing, and a refractive index value for water of 1.333 was used. Three replicates were measured for each sample.

RESULTS

Effect of Added PME on Cloud Stability. It is generally understood that PME impacts juice cloud stability, but the time frame over which destabilization occurs and the impact of the amount of active enzyme is not known, nor is the mechanism of cloud destabilization by PME well understood. Therefore, a storage study was conducted that examined the effects of different quantities of added enzyme on the cloud stability of commercial carrot juice. Commercial pasteurized carrot juice was preferred as the starting material for these studies, rather than raw unpasteurized juice made in the laboratory, because of the former’s uniform particle content and minimal active PME (see Material Sourcing in Materials and Methods). Starting with juice that lacked any PME, then adding known amounts of a partially purified carrot PME, juices with different known levels of PME activity could be obtained. The levels of PME added (0.15, 0.25, 0.5, and 1.0 U/g) were based on the level of endogenous PME measured in raw Sugarsnax carrot juice made with a benchtop juicer. This level varied widely across the juice from individual carrots, even within carrots planted and harvested at the same time and grown in the same field. For 30 individually measured carrots, the PME activity of the juice ranged from 0.218 to 0.806 U/g, with an average of 0.43 U/g.

Cloud stability was measured via relative turbidity (\( \tau_{\text{serum}}/\tau_{\text{total}} \)), which has been used previously by others as a method sensitive to the extent of flocculation within the cloud. 21,28,29 Flocculation creates larger particles within the suspension, which are removed more effectively by centrifugation, and leave behind a serum with a lower particle concentration and lower turbidity. Thus, smaller values of \( \tau_{\text{serum}}/\tau_{\text{total}} \) indicate that more flocculation has occurred. As shown in Figure 1, at all levels of added PME the cloud was less stable than in the control juice with no added enzyme. The relative turbidity of the control sample remained consistently high throughout storage, while those samples with added PME showed a large degree of clarification after centrifugation (Figure 1). When the amount of added PME was at or above 0.25 U/g, there was an inverse relationship at the 90% confidence level between the level of PME addition and the relative turbidity of the cloud at periods longer than 15 days.

![Figure 1. Relative turbidity (\( \tau_{\text{serum}}/\tau_{\text{total}} \)) of carrot samples with 0 (□), 0.15 (○), 0.25 (△), 0.5 (○), and 1.0 (×) U/g of added PME enzyme over the course of 56 days of storage.](dx.doi.org/10.1021/jf4043979)
Loss of cloud stability and juice clarification was also monitored by measuring relative sediment height ($H_{\text{sediment}}/H_{\text{total}}$). Consistent with the results for relative turbidity, much less clarification occurred in the control than in the enzyme-treated samples (Figure 2). However, the relative sediment height of the control juice increased slowly over the course of the storage period, indicating that there was natural settling occurring within the juice that is not attributable to PME activity.

All samples containing active PME had an initial period (~10 d) of rapid sedimentation, after which the sediment height leveled off (Figure 2). The kinetics of this process can be represented using the empirical formula

$$H_{\text{sediment}} = \frac{t^2H_{\infty}}{t^2 + T_o^2}$$

where $t$ is time, $H_{\infty}$ is the steady-state dimensionless plateau height, and $T_o$ is a characteristic time of the initial, rapid sedimentation period. $H_{\infty}$ and $T_o$ could thus be obtained by a fit of eq 2 to the data in Figure 2; results for these two fit parameters are given in Table 1.

Table 1. Sediment Plateau Height and Time of Initial Height Increase From Eq 2, for the Four Levels of Added PME Enzyme

<table>
<thead>
<tr>
<th>enzyme added (U/g)</th>
<th>$H_{\infty}$ plateau height (%)</th>
<th>$T_o$ (days)</th>
<th>$R^2$</th>
<th>$\chi^2$</th>
</tr>
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<td>0</td>
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<td></td>
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<td>13</td>
<td>2.1</td>
<td>0.92</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Figure 2. Relative sediment height ($H_{\text{sediment}}/H_{\text{total}}$ %) of carrot samples with 0 (□), 0.15 (○), 0.25 (△), 0.5 (◇) and 1.0 (♦) U/g of added PME over the course of 56 days of storage at 5 °C.

Carrot juice with greater amounts of added enzyme became unstable more quickly during storage, as shown by the number of days ($T_o$) it took for the initial rapid sedimentation period to occur (Table 1). With added enzyme, just a few days were needed for the cloud to separate visually into a sediment layer with approximately steady thickness. The thickness of that sediment layer increased slightly but significantly ($p \geq 0.05$) as more enzyme was added into individual samples.

**Effects of Added PME on Particle Size.** Measurements of relative turbidity and sedimentation indicated that PME addition increased both flocculation and clarification rates in the cloud. Rates of flocculation can be estimated more directly by analyzing changes in particle sizes. As discussed above, the particle size distribution of the entire juice sample was measured by gently inverting the juice samples, thus ensuring an even distribution of particle sizes throughout each sample, and then using static light scattering to determine particle diameters. Inspection of the particle size distribution profiles for each level of enzyme added showed that the carrot juice started with a trimodal distribution (Figure 3), i.e., three overlapping populations with different modes of approximately 0.7, 1.7, and 13 μm. In the absence of enzyme, this distribution did not change over time (Figure 3a). For the enzyme-containing systems, (Figure 3b–e), there was a rapid initial change occurring over the first ~9 days, during which the second modes shifted to larger diameters, leaving the size of the other two modes unaffected. As a result, the height of the first peak at smaller sizes shrank over time as that of the larger mode grew.

Examination of all enzyme levels (Figure 4a) indicated that the extent of this shift at earlier times did not strongly depend on the amount of enzyme; there was a similar increase in particle size for mode 2 at all levels of added enzyme. The first rapid shift was followed by a second, slower change in the distribution, as the second peak diameter gradually increased to merge with the third mode over ~30–40 days. This second growth process occurred more rapidly at higher enzyme concentrations at longer times. Consequently, data at day 5 exhibited little dependence on the amount of enzyme added, as long as some active enzyme was present (Figure 4a). Samples with 0.15, 0.25, and 0.5 U/g added PME also had very similar particle size distributions at 33 days of storage; it was only the sample with 1.0 U/g added PME that had a significantly greater particle size (Figure 4b). Finally, at day 43, the two highest enzyme concentrations both exhibited more significant particle growth than the other samples (Figure 4c).

The increase in particle size is an indication that flocculation was occurring within the cloud. This is qualitatively visible in the light microscopy photos (Figure 5), as one can distinguish the individual particles that make up an aggregate. Clearly, the presence of active enzyme corresponded to the formation of flocs in the mixture, with the size of those flocs larger in the two samples with highest amounts of enzyme. Overall, the data in Figures 1–5 consistently indicates that as particles flocculated and grew in diameter, they began to settle out, causing clarification. Therefore, it can be concluded that the amount of enzyme present had a direct effect on the rate of flocculation and sedimentation. In addition, measurements of turbidity, sedimentation, and particle size distributions all indicate a process that occurred more rapidly over an initial period of ~5–10 days. This period is followed by a time of slower flocculation rates with little change in the observed sediment layer, although changes in the cloud structure were still occurring.

**DISCUSSION**

Adding PME to carrot juice caused juice clarification, as measured by sediment height and relative turbidity. With more added enzyme, the juice clarified more rapidly and to a greater extent. The clarification was accompanied by an increase in particle size. Although no previous research has been found on
the addition of different enzyme levels back into juice, these results are consistent with those reported for native residual enzyme activity in commercial juices. In citrus and tomato juices, clarification occurred above a certain minimum threshold of PME activity. In pineapple juice, residual PME activities of $4.1 \times 10^{-4}$ U/mL did not cause complete clarification, whereas PME values 10 times greater resulted in complete clarification.

Commercial juice was used instead of raw, unpasteurized juice for consistency and relevancy to the industry. However, there are important differences between commercial, pasteurized juice and raw juice that could affect the results. Preliminary work showed that juice made in the laboratory and filtered through cheesecloth had particle sizes 50 times greater than those of commercial carrot juice, with much greater variation in particle size. Additionally, our laboratory juice had 1.25 times more solid matter than commercial juice. Interestingly, when the carrots were blanched before juicing in the laboratory, the amount of solid matter became equal to that of the commercial juice, which would suggest that the heating of the carrot weakens the internal structure and allows particles to be broken down into smaller sizes and removed. This could account in

Figure 3. Particle size distribution curves for commercial carrot juice with no added PME (a) and with the following amounts of enzyme added: 0.15 (b), 0.25 (c), 0.5 (d), and 1.0 U/g (e). Though particle sizes were measured every three days for 56 days, data for only days 1, 5, 9, 26, and 43 were used to represent the size changes that occurred.
part for the smaller particle sizes in pasteurized commercial carrot juice versus raw laboratory juice, and is supported by the work of Reiter et al. who found that pasteurized juice had smaller particle diameters than unpasteurized juice. Processing techniques have a significant impact on cloud particle size; a study by Reiter et al. showed the wide range of particle sizes (0.1–700 μm) for carrot juices produced with decanter technology from five different manufacturers. The commercial juice used in this study had <1% of particles >10 μm. This indicates there is a wide range of processing techniques used in the juice industry that can result in different particle sizes.

The changes in sediment height and relative turbidity observed here were similar to one another in that both indicated an initially rapid destabilization process, followed by slower changes over time. Both also showed small but significant differences between juices with the three highest amounts of PME added at the later stages of the storage time course. Changes in particle size showed a similar trend. Over the first 5–10 days of storage, there was a relatively rapid increase in diameter of the population of smallest particles in the mixture. At longer times, sizes in the population of larger particles grew slowly, with the rate of this growth showing a modest dependence on enzyme concentration especially at the highest levels of added PME. By the end of the storage time course, all three measures of juice clarification, sediment height, relative turbidity, and particle size distribution, showed similar small differences between the juices with different levels of added PME. If PME induced clarification is due to enzymatic de-esterification of the juice pectins, then one might expect that clarification would occur more rapidly with more added enzyme but that all the juices would eventually reach the same end point.

Why the final states of the juices differed with differing amounts of added enzyme is not clear. One possibility is that the de-esterification of pectin by PME was only partial and that

Figure 4. Comparison of particle size distribution of carrot juices with different amounts of added PME after 5 (a), 33 (b), and 43 days.

Figure 5. Commercial carrot juice with added enzyme viewed with 20X light microscopy at 56 days of storage at 5 °C. From left to right, enzyme levels are 0, 0.15, 0.25, 0.5, and 1.0 U/g.
the final degree of pectin esterification achieved depended on the amount of enzyme added. It is known that in the absence of high salt concentrations, PME binds strongly to de-esterified stretches of pectin, resulting in the formation of inactive PME–pectin complexes. As a result, PME typically only partially de-esterifies a pectin chain before becoming inactive. It is possible that at higher levels of added PME, a higher final level of de-esterification is obtained because more pectin can be de-esterified before all the PME enzyme forms inactive PME–pectin complexes and de-esterification stops. The differences in particle size, aggregation, flocculation, and sedimentation between samples with different levels of added PME may all result from this difference in this final degree of esterification. It is even possible that all the PME catalyzed de-esterification in the juice occurred very rapidly, within the first day or two of the experiment. In that case, not only the different extents but also the different time courses for the particle sizes and sedimentation over the next 56 days could be due to the differences in final pectin esterification between the different levels of added PME, rather than from different rates of de-esterification.

A second possibility is that particle aggregation and clarification is partially due to direct protein binding effects. Proteins (including PME) present in the high salt extract of cell wall material that was used as the source of the added PME may bind to the particulate matter in the juice, possibly affecting particle size aggregation. This is supported by the observations of Schmelter et al. who found that even thermally inactivated PME was able to cause pectin gelation in the presence of salts. This ability of PME to create gels even when inactivated was taken as an indication that, in addition to its catalytic mechanism, the PME protein could participate in direct electrostatic and hydrophobic interactions with the pectin. Proteins also have the capability of interacting with each other in high acid environments (pH 4.4), and it has been argued that it is protein–protein interactions that influence cloud stability more than protein–pectin interactions.

Our micrograph data and the overall kinetics in the system may indicate that particle growth is in the diffusion limited aggregation (DLA) regime. In such a regime, once a nucleation site is formed, additional particles may be added one at a time via the “hit and stick,” describing a rapid, irreversible flocculation, without time for rearrangement. Loose, fractal flocs are the result of such a mechanism, consistent with what is observed in Figure 5. As more PME is added, flocculation occurs at a greater rate, creating even looser and therefore larger flocs. Such flocs have a greater effective diameter, and so sediment out more with centrifugation (Figure 1), yet they do not pack as tightly on the bottom because of their loose structure (Figure 2). Initially, in juice with a high level of added PME, it may be that particles do not have to diffuse far to encounter another particle with which they are able to flocculate. High amounts of PME create more particles of pectin that have a higher lower degree of esterification, and the PME itself can participate in flocculation. This would account for the relatively rapid increase in particle size at the beginning of storage.

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Notes
The authors declare no competing financial interest.

ABBREVIATIONS
DLA, diffusion limited aggregation; PME, pectin methylesterase; U, unit of enzyme activity

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