# CLARIFICATION OF VALENCIA ORANGE JUICE IS INFLUENCED BY SPECIFIC ACTIVITY OF THERMOLABILE PECTINMETHYLESTERASE, INACTIVE PME-

## PECTIN COMPLEXES AND THE CHANGES IN SERUM SOLUBLE

#### COMPONENTS

by

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#### (Under the direction of Dr. Louise Wicker)

#### ABSTRACT

Clarification of orange juice is a quality defect attributed to pectinmethylesterase (PME). Thermolabile PMEs with different specific activities were added to stabilized juice. Particle size distribution, % transmittance, uronic acid analysis and SDS-PAGE were used to monitor clarification overtime. Juices with the highest specific activity clarified at the slowest rate and contained 36 and 13 kDa peptides. Juices with a 36 kDa and 27 kDa peptide clarified juices fastest. In fresh juice with cloud insoluble solids (PFJ) and juice with only cloud soluble solids (UCS), UCS formed a floc concurrently with clarification of PFJ. The clarification of juices adjusted to pH 7 was retarded due to electrostatic repulsion while juices at natural pH clarified more rapidly. These results show that serum-soluble factors, total charge and electrostatic interactions influence cloud destabilization.

INDEX WORDS: Pectinmethylesterase, Pectin, Cloud, Orange Juice, Clarification, Floc,

Specific Activity, Degree of Esterification

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# DEDICATION

I want to dedicate this thesis to my family and friends for all of their love and support.

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CHAPTER 1

INTRODUCTION

Citrus clouds have complex requirements for stabilization and clarification. Although cloud has been studied at length, there is still much more to learn about the interaction of specific cloud components and the enzyme systems affecting them. The role of each orange PME isozyme in clarification has yet to be determined or even if they are real isozymes (Macdonald and others 1993). A definite understanding of the clarification process has still not been achieved due to the structural complexities of orange juice pectin, the non-random action of PME and the uncertainties of the exact nature of calcium pectate and cloud interaction. Understanding the parameters involved in orange juice clarification will enable the use of novel technologies to stabilize cloud without producing adverse sensory qualities or violating standards of identity for citrus juice.

The objective of the following experiments was to further investigate the mechanism of orange juice clarification. The ability of partially purified TL-PMEs from Valencia pulp with different specific activities and hence different amounts of non-PME protein, to clarify orange juice was examined. Previous studies suggested that only TS PME could rapidly clarify juice (Versteeg 1979) however other evidence was presented which suggests TL-PME could also be a factor in clarification (Cameron and others 1998). The effect on cloud particle size distribution during clarification was simultaneously examined. Floc formation and changes in serum soluble components in fresh Valencia orange juice were also investigated. The floc, which was first described by Baker and Bruemmer (1969), was further analyzed in our studies for onset of appearance and protein content while the serum was examined for enzyme activity and other qualities over time.

The results of the studies presented in this manuscript support the theory that TL-PME is a factor in orange juice clarification (Cameron and others 1998). Furthermore, it was shown that PME with the highest specific activity (units of enzyme per mg of protein) does not necessarily clarify juice at the fastest rate. Juices that clarified the fastest all contained a 36 kDa and a 27 kDa peptide and juices that did not clarify contained a 36 kDa and a 13 kDa peptide. This suggests that PME complexes with pectin and/or low-molecular weight protein influence the ability of PME to induce clarification. Cloud particle size distribution during clarification increased in agreement with previous studies (Corredig and others 2001). This suggests that the cloud particles themselves change during clarification are not just removed from a stable dispersion by Ca-pectate. Floc formation in ultracentrifuged juice serum was observed slightly before or at the same time as the gross onset of clarification in pulp free juice as measured by % transmittance at 650 nm at all pH values when clarification occurred. This supports Baker and Bruemmer's theory (1969) that certain serum-soluble factors are required to destabilize the cloud colloidal system. The floc was also found to contain the same major peptides (13, 20, 27, and 36 kDa) as in pulp free juice for all pH values examined. Clarification of unstabilized, pulp free juice adjusted to pH 7 was retarded or did not occur in 18 days of storage. Clarification of unstabilized pulp free juice adjusted to pH 7 for one hour and then readjusted to natural pH underwent rapid clarification as compared to the control juice, which remained at natural pH. These results mirror what is seen with apple juice (Yamasaki and other 1964,1967) and support the role of electrostatic destabilization of cloud.

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# CHAPTER 2

## LITERATURE REVIEW

#### Orange Juice Cloud

Cloud gives the characteristic color, aroma, taste and texture to orange juice. Without cloud, the resulting serum is visually unappealing, essentially flavorless and has no particular value. Cloud is considered "definitely" broken or lost in orange juice when light transmittance reaches 36% (Redd and others 1986).

Cloud density and color varies between cultivars (Huggart and others 1975). Season of production, maturity and processing methods can also influence the quality of cloud (Barron and others 1967). Valencia, the late-season cultivar, has the highest cloud density and color followed by Pineapple (mid-season) and by Hamlin (early season). Juices of these cultivars are usually blended to avoid variations in product appearance and to meet minimum color standards. Valencia is also sold unblended as a premium product.

#### Cloud composition

Scott and others (1965) reported on the chemical composition of the suspended material in orange juice. It was suggested that the fraction containing the finest particles (the cloud) has a composition quite different from that of the other fractions. They recommended that the cloud particles should be treated as a distinct anatomical component of the fruit and not as tiny fragments of pulp.

About half of the total cloud by weight is comprised of high molecular weight polymeric materials including protein, pectin, hemicellulose and cellulose (Sinclair 1984). Orange juice cloud contains approximately 52% protein (Klavons and others 1991), 4.5% pectin (Klavons and others 1994), 25% lipid, 5.7% nitrogen, 2% ash

(Crandall and others 1983), 2% hemicellulose, and 1.5% cellulose. Flavonoid crystals (hesperidin) incorporated in the orange juice cloud normally form after the juice has been extracted and stored, but may form in freeze-damaged oranges before extraction (Rouseff 1980). Hesperidin is a relatively minor component of orange juice cloud with the notable exception of Shamouti (Jaffa) oranges, which is saturated with the bioflavonoid (Rothschild and Karsenty 1974; Mizrahi and Berk 1970). The color of orange juice cloud is due to carotenoids. These are contained in the plastids, which constitute a portion of the cloud particulate matter.

In a study of the physico-chemical characteristics of orange juice cloud (Mizrahi and Berk 1970) used electron micrographs to study cloud particles. They discovered four different particles in cloud: 1) regular, intensely colored, smooth-surface particles, approximately 1  $\mu$ m in diameter, which were thought to be chromoplasts; 2) irregular, light colored, rough surface, rag-like particles 2-10  $\mu$ m in diameter, which were thought to be fragments of pulp; 3) spherical droplets of oil, found almost exclusively attached to the surface of rag-like particles, approximately 1  $\mu$ m in diameter; 4) needle-like particles 0.5-3.0  $\mu$ m long, 0.05-0.2  $\mu$ m thick. The needle-like particles were crystallized flavonones (probably hesperidin). The precipitation of bioflavonoids is an important factor in overall cloudiness of orange juice (Mizrahi and Berk 1970). Oil droplets attached to the cloud particles were found to have a stabilizing effect on the suspension by decreasing the average density of the particles bringing it closer to that of the serum (Mizrahi and Berk 1970).

Commercial orange juice cloud pectin is a heterogeneous material. A study by Klavons and others (1992, 1994) reported that soluble pectin bound to protein particles

during formation and suggested that up to 20% of cloud pectin is present as a pectinprotein complex. Binding of orange cloud to amino paramagnetic latex particles demonstrates a clear association of cloud pectin with cloud protein. By simulating orange juice processing conditions (Klavons and others 1994) indicated that some of the cloud pectin arises from the pulp during processing. Overall they found that approximately 60% of the cloud pectin exists as previously soluble pectin that is associated with insoluble protein, 25-30% as calcium pectate and 15% as protopectin (Klavons and others 1994).

#### Apple Juice Cloud and Mechanism of Clarification

Unlike orange juice, removal of apple juice cloud is well received among consumers. Although this review is mainly concerned about cloud stabilization in orange juice, the mechanism of clarification in apple juice is still of interest. Since the early thirties, pectic enzymes have been added to apple juice to achieve desirable clarification and increase yield. The apple juice clarification mechanism has been studied extensively by Yamasaki and others (1964, 1967). They discovered that the suspended material in apple juice is composed of positively charged protein-carbohydrate complexes coated with negatively charged pectin. When pectic enzymes degrade this protective colloid, the positive charge of the protein-carbohydrate complex is exposed. At this point, nearby pectin coated protein-carbohydrate complexes with negative charged exteriors are electrostatically attracted to the partially exposed positive protein resulting in flocculation and clarification. At pH values above the isoelectric point of the cloud protein (above pH 5), the protein is negatively charged and flocculation does not occur even though the pectic enzymes are still active. If pH is reduced back to the natural pH

of juice (pH 3.5), where the proteins are positively charged, flocculation occurs. Increasing the amount of pectin in re-suspension systems retarded clarification, as there was more pectin available to coat and protect the protein. Adding small amounts of negatively charged colloids (sodium alginate or carboxymethyl cellulose) completely inhibited clarification by blocking (or neutralizing) the exposed positive protein from neighboring negatively charged pectin coated protein complexes. Increasing the amount of protein (heated protease partially purified from *Streptomyces griseus* or casein) in the system when pectinase was present accelerated clarification, as there was not enough pectin to coat the added protein and stop flocculation. More information about apple juice cloud can be found in a review by Beveridge (1997).

#### Hesperidin-Pectin Interaction

Hesperidin is located in a soluble form in the vacuole in the intact cell and crystallizes out of the cell during membrane impairment (Bennett and Albach 1981). Hesperidin crystal formation is correlated with increased turbidity of fresh orange juice serum (Mizrahi and Berk 1970; Rothschild and Karsenty 1974). Ben-Shalom and others (1984) studied the effects of enzymatic and chemical degradation of the pectin polymer on its interaction with hesperidin. Stabilization of the flavonoid in aqueous solution seemed to be the result of specific interaction with the pectin polymer. Other polymers (alginate, guar gum, CMC, carrageenan) did not form stable cloud (Kanner and others 1982). Microscopic analysis demonstrated that smaller hesperidin crystals were formed when pectin was present (Ben-Shalom and others 1984). Adamson (1976) reports that the more rapid the nucleation, the larger the number of nuclei formed before relief of the supersaturation occurs and the smaller the final crystal size. Based on this, the authors

hypothesized that hesperidin recognizes the pectin molecule and uses it as a nucleation site (Ben-Shalom and others 1984). In the same study, polygalacturonase was used to reduce the size of the pectin polymer. The authors proposed that there was a critical molecular weight of pectin that was required to stabilize hesperidin crystals. The amount of hesperidin crystals, which are stabilized by the pectin polymer, seems to be pHdependent. For example a smaller amount of hesperidin could be stabilized by the same amount of pectin at pH 2 compared to the normal pH of juice. Changing the degree of esterification of the pectin from 73 to 10% did not affect its interaction with hesperidin or the intensity of colloidal particle formation. The chemical degradation of pectin by heat treatment at pH 3.8 determined that when heat was used, a combination of factors besides the splitting of the pectin polymer by  $\beta$ -elimination was involved.

The specific interaction between hesperidin and pectin is via the sugar moieties in the hesperidin molecule and in the polyuronide polymer (Ben-Shalom and Pinto 1999). When the sugar moiety (rhamnose and glucose) was removed from hesperidin by acid hydrolysis, the resulting effect was a complete loss of hesperidin's ability to interact with pectin. Differences in the stabilization of hesperidin by various types of pectin is thought to be due to the specific interaction of the neutral sugars of the pectin with the sugar moiety of the hesperidin by hydrogen bonding. Presumably, a polymer with a high content of NS branches should interact with hesperidin much more tightly and strongly than one with a low amount of NS (Ben-Shalom and Pinto 1999).

Ben-Shalom and Pinto (1999) identified two different types of pectin isolated from a model system of orange juice. One pectin forms part of the stable colloidal particles in the juice by interacting with hesperidin. It contains 80% neutral sugars with

more than 10% rhamnose. The second pectin interacts with hesperidin but does not form stable particles so it eventually flocculates out from the stable dispersion. This pectin contains 40% neutral sugars with approximately 2% rhamnose.

#### Cloud Particle Size and Relationship to Cloud Stability

Citrus juice clouds must be of an appropriate particle size, specific gravity and uniformity to remain suspended indefinitely as a result of Brownian motion. These particles range in size from 0.4-5.0 µm (Klavons and others 1994) with those under 2 µm constituting stable cloud (Mizrahi and Berk 1970). Pulp fragments and large particles tend to settle by gravity and are usually denoted as "settling pulp" not cloud. Valencia juice has the highest percentage of particle volume in the critical stable size ranging from 1-2 µm, followed by Pineapple and Hamlin (Buslig and Carter 1974). Polydispersity of distribution of cloud particles in juice confound studies that investigate the mechanism of cloud loss. Corredig and others (2001) reported the changes in cloud particle sizes in orange juice after addition of sensitized pectins. Clarification of juice occurred when stable cloud showed aggregation by shifting the particle size distribution to larger diameters  $(0.9 - 5.0 \,\mu\text{m})$ . For all pectins added to juice, analysis of variance within one pectin type revealed that the average particle size was dependent on pectin concentration. Baker (1976) described that pectins sensitized with the endogenous enzyme pectinmethylesterase (PME) have a greater propensity to clarify juice compared to alkalisensitized pectin. The results from Corredig and others (2001) study supported Baker's study showing that PME-sensitized pectins result in larger cloud particle sizes when compared to alkali-sensitized pectins. Analysis of changes in cloud size, using integrated light scattering, demonstrated that interactions exists between charged pectin particles

and other cloud constituents. The authors suggest that for cloud loss to occur, the cloud particles must aggregate as shown by their increase in particle size distribution and furthermore, aggregation is most likely caused by bridging of cloud particles to charged pectin. Wicker and others (2002) also showed that cloud particle diameter increases before the gross onset of juice clarification.

Loeffler (1941) was the first to suggest using high pressure (homogenization) to stabilize juice cloud before pasteurization. Takahashi and others (1993) reported that the percent distribution of larger pulp particles (180-200  $\mu$ m), which are high in pectin, was higher for juices pressurized at 600 Mpa for 30 min. Crandall and others (1988) reported a viscosity decrease by 13% when 65°Brix juice concentrate was homogenized at 24.7 Mpa. Photomicrographs suggested that breakdown of long, filamentous fibers into smaller less linear particles was responsible for the decrease in viscosity. Conversely, homogenization at a higher pressure (55.2 Mpa) increased viscosity by 3.5% indicating that too high a shear rate may be counter productive.

#### Serum Pectin and Cloud Stability

Pectin molecules maintain colloidal stability of orange juice cloud through a complicated and not well understood mechanism. Previous theories suggesting that serum pectin stabilized cloud were disproved when Baker and Bruemmer (1969) separated cloud and serum by ultracentrifugation (78,000 x g for 30 min) and resuspended the cloud in water. This cloud in water suspension was quite stable and was not harmed by addition of pectin, calcium, sugar or citric acid. Adding KCl clarified the suspension, but not as fast as the suspension of cloud in the original serum. KCl is thought to possibly solubilize PME absorbed to the cloud particles. Different

combinations of heated and unheated serum were combined with heated and unheated cloud. Rapid clarification occurred when both fractions were unheated, however when both fractions were heated, the suspension was stable. Unheated cloud combined with heated serum was less stable than when they were both heated, but more stable compared to adding heated cloud to unheated serum. Adding commercial tomato pectinesterase to the serum resulted in immediate clarification after cloud was reintroduced to the serum. This experiment suggests that while serum pectin is not a cloud stabilizer it may act as a cloud destructing agent when saponifed by PME.

Baker and Bruemmer (1972a) additionally studied the interaction between orange juice cloud and the floc that developed in ultracentrifuged serum overtime. This floc appeared after 6 days, at about the same time as fresh orange juice clarification. The floc consisted of calcium pectate and hesperidin whose relative amounts varied with age. The authors resuspended cloud in floc free serum and also resuspended cloud and floc in fresh, heated and enzyme treated serum to study the influence on cloud stability. They discovered that certain serum-soluble factors were required to destabilize the cloud colloidal system and that soluble pectin appears to inhibit the coacervation of cloud and floc.

#### Non-PME Proteins Role in Cloud Instability

The role of non-PME proteins in juice cloud stability has been studied as complexes with other constituents such as phenols, hesperidin crystals, pectin and tannic acid in apple juice (Van Buren and Robinson 1969) and with other unidentified components in citrus juice (Shomer and others 1985). Non-PME proteins has also been studied in relation to heat coagulation of soluble proteins, which are able to encapsulate

and associate with cloud components as has been shown for emulsified oily droplets, pigment constituents (Shomer 1988), pectin and neutral sugars (Shomer 1991). Shomer and others (1999) suggested that clarification of orange juice was a result of cloud protein coagulation/flocculation and that PME activity increased the association between the pectin and the cloud proteins, leading to protein-pectin flocculation. They showed that insoluble cloud material formed clumps in conditions where proteins tend to coagulate and flocculate (above 70°C and at pH 3-4). Cloud flocculation was more pronounced at pH 3.5 and was enhanced by enzymatic pectin degradation and heating (from ~50 to 75°C). Under these conditions PME is less active and pectin is more soluble.

### PME and Cloud Instability

PME (3.1.1.1) of the International Enzyme Commission is an endogenous enzyme that is typically credited with the destabilization of orange juice cloud. PME initiates a sequence of events by partially de-esterifying (demethylating) the C6 methoxyl ester groups of soluble pectin ( $\alpha$  1,4 – polygalacturonic acid) contained in the juice serum (Stevens and others 1950). The polygalacturonic acid chains of citrus pectins are 70 – 80% methoxyl esterified (Voragen and others 1995). PME cleaves these methoxyl esters, yielding methanol and the carboxylic acid. This action eventually turns high methoxyl (HM) pectin into calcium sensitive low methoxyl (LM) pectin. Once a critical degree of esterification (DE) is obtained, divalent cations such as calcium can cross-link these free acid units to free acid units on adjacent pectin molecules, forming insoluble calcium pectates. Cross-linking increases the pectin apparent molecular weight, which reduces solubility, thereby leading to flocculation. On the basis of the steric configuration of galacturonic acid units within the pectic polymers, it is thought that the structure of calcium pectate gel appears as an "eggbox" model (Grant and others 1973). Precipitation of pectins in this manner was presumed to occlude cloud particles and remove them from suspension (Stevens and others 1950; Joslyn and Pilnik 1961). However, it is not clear how insoluble cloud constituents and particles become involved with the pectate gel complex in relation to the clarification process. Evidence for the important contribution of enzyme deesterification of the pectin to cloud loss was discovered by studies on the changes in juice pectin during clarification (Rouse 1953).

Knowing the critical DE or block of DE groups in which the pectin becomes susceptible to cation precipitation would be useful in predicting juice cloud stability. Unfortunately this is difficult to determine due to the complex nature of juice pectin and molecular weight distribution and the presence of neutral sugar side chains attached to the galacturonic acid backbone. Thus it contains varying amounts of "hairy" regions, in which the "smooth" galacturonic acid backbone is interspersed with rhamnose and short side chains consisting of arabinans, galactans, xylose, and fructose (Voragen and others 1995). These side chains interfere with the action of PME, and thus determine the extent to which de-esterification of the pectin can occur. Furthermore, the extent to which juice pectin is esterified with methoxyl varies from molecule to molecule, exhibiting a distribution centered around the average measured DE (Baker 1979). Endogenous PME will de-esterify in a non-random blockwise manner, de-esterifying some molecules more extensively than others (Krop 1974). Pectins also vary in the total charge and charge distribution (Baker 1979; Kravtchenko and others 1992). A few researchers have attempted to determine the critical DE value in which juice will clarify yielding numbers

ranging from 27% (Krop 1974) to 36% (Ben-Shalom and others 1985). Baker (1979) determined the DE required for a pectate to precipitate was between 14 to 21% which indicates that clarification occurs when the ratio of free acid units to esterified units on the pectin molecule approaches six. Corredig and others (2001) added sensitized pectins with different Des to stabilized (PME-negative) orange juice. Juices with pectins having Des of 21% or greater did not clarify, however the juice with <5% DE pectin did clarify. A study by Ackerley and others (2002) reported that pectins extracted from stable juices had a DE of 19% and pectins from clarified juices had a DE close to 13%.

#### **Different Isozymes of PME**

Research by Stevens and others (1950) was the first to suggest the presence of multiple isozymes in citrus PME. Work by Versteeg (1979) confirmed this theory by isolating three isozymes from orange PME. Since then, isozymes of PME from various citrus fruits have been isolated and described (Seymour and others 1991; MacDonald and others 1993; Cameron and others 1994; Cameron and Grohmann 1995, 1996). The major difference between the different isozymes is their tolerance to elevated temperatures (Versteeg 1979; Seymour and others 1991; Cameron and others 1994; Cameron and Grohmann 1996; Sun and Wicker 1999) and to pH sensitivity (Sun and Wicker 1996). Two of the orange PME isozymes isolated by Versteeg are thermolabile (TL-PME) and are inactivated at temperatures above 70°C, however one isozyme is thermolabile (TS-PME) and can retain activity when exposed to this elevated temperature. By individually adding different isozymes of orange PME to cloud stabilized reconstituted frozen concentrated orange juice, Versteeg and others (1980) demonstrated that the TS-PME

was capable of rapidly clarifying the juice (3-4 days) while the two TL-PMEs either destabilized the cloud very slowly (5-6 weeks) or not at all.

A total of seven PME isozymes have been found in Valencia juice sac derived tissue culture cells that could be distinguished by their chromatographic behavior on DEAE-Sephacel and heparin (Cameron and others 1994). Two of the partially purified PMEs retained activity at 90°C and one was still active at 95°C. Results from native gel filtration and denaturing electrophoresis revealed that most TS-PME forms had a molecular weight of 40.5 or 37.5 kDa. None of the partially purified PME isozymes had a molecular weight greater than 50 Kda. In another study (Cameron and Grohmann 1996) purified a thermostable form of PME (40.1 kDa) from commercial Valencia fresh frozen orange juice, which retained 49.2% of its relative activity after 1 min incubation at 95°C.

Han and others (2000) found seven putative isozymes of PME from a commercial Valencia orange peel pectinesterase by separation on a heparin column. Three isozymes were thermolabile and had molecular weights of 70, 60, and 27 kDa. Four were thermostable and all were of the same molecular weight (35 kDa). This study looked at the different time-temperature levels used to distinguish between TL and TS-PMEs in order to recommend/establish a standard heat treatment. Results using 70°C for 5 min to determine TS were quite different from those using 90°C for 1 min. The 70°C-5 min treatment yielded 16.6% TS-PME from the commercial PME while the 90°C-1 min treatment yielded 1.5% TS-PME. Snir and others (1996) determined that 5.6-14% of the total PME activity present in Valencia orange juice was TS as defined by 70°C-5 min. According to the study by Han and others (2000) on the thermostability of PME fractions

separated by affinity chromatography, 90°C-1 min heat treatment is a better test to distinguish between TS-PME and TL-PME in Valencia orange juice.

PME extracted from Valencia orange peel tissue destabilizes juice cloud of pasteurized, reconstituted orange juice more rapidly than PME extracts from rag (intersegmental septa, squeezed juice sacs, and fruit core tissue) or hand expressed juice (Cameron and others 1997). At least four isozymes of PME from peel tissue have been identified, two of which (one TL-PME and one TS-PME) rapidly clarified juice (Cameron and others 1998). Incorporating increasing amounts of fruit peel tissue into the juice during extraction increases the rate of clarification in fresh juice (Cameron and others 1999).

Savary and others (2002) used SDS-PAGE and IEF-PAGE to identify three isozymes from a commercial orange peel PME (P5400, Sigma Chemical Co. St. Louis, MO) at 34, 27, and 8 kDa. When they prepared PME from fresh Valencia orange peel they saw the same three peptides with SDS-PAGE but only the 34 kDa peptide was seen with IEF-PAGE.

#### PME Inactivation (Heat)

The clearing enzymes found in Valencia oranges are more heat resistant compared to those in Navel (Joslyn and Sedky 1940), Pineapple, and Hamlin (Eagerman and Rouse 1976) oranges. PME inactivation by heat is non-linear (Guyer and others 1956) due to the multiple isozymes of PME found in citrus juice (Versteeg and others 1980). Eagerman and Rouse (1976) developed heat inactivation time (HIT) curves for selected varieties of oranges (Pineapple, Hamlin, Valencia). Since commercial juices are often blends of different varieties, the authors recommended using pasteurization

conditions equal to those necessary to stabilize Valencia orange juice (1 min at 194°F with z = 12.2) (Eagerman and Rouse 1976). Cloud stabilization is routinely accomplished by heating the juice to 90-95°C for 15-60 sec to inactivate all isozymes of PME. This temperature is about 20-25°C higher than required for microbial safety. Unfortunately this method has a negative effect on the "fresh" taste of juice by imparting a cooked off-flavor (Kew and Veldhuis 1961). This method also causes losses in vitamins and volatile compounds and promotes non-enzymatic browning reactions, which increase in magnitude with the intensity of heat treatment. An important factor in the heat resistance of some isozymes of PME is pectin. Even small concentrations of pectin (0.01%) in citrate buffer containing the main components of soluble solids of orange juice produce a 17-fold additional increase in the thermal resistance of TS-PME (Vercet and others 1999). Protection of PME by pectin is one of the reasons why such high pasteurization temperatures are required for cloud stabilization in orange juice.

PME inactivation by thermal treatment is by far the most common and inexpensive mean to stabilize cloud, however several other methods have been proposed that hope to stabilize cloud without the negative effect on taste. These non-thermal or reduced thermal methods prevent clarification by inactivation or inhibition of PME or by blocking the sequence of events leading to the flocculation of pectates.

#### PME Inactivation (Pressure)

Pressure treatment utilizing high pressures has been used to process thermosensitive products for microbial destruction and significantly retards the rate of enzyme reaction (Morlid 1981; Knorr and others 1992). High pressure, also known as "cold sterilization", affects noncovalent bonds and does not accelerate most chemical

changes so flavor and appearance are usually superior to those products preserved by heat (Aleman and others 1994). Studies using high pressure to stabilize cloud discovered that inactivation of PME was dependent on pressure level, pressure hold-time, pH and total soluble solids (Basak and Ramaswamy 1996). Instantaneous pressure kill was directly proportional to the pressure level, which is consistent with other studies (Ogawa and others 1990; Balny and Masson 1993), and was higher at lower pH. Joslyn and Sedky (1940) and Rouse and Atkins (1952) also found that heat pasteurization was more effective at lower pH. The pressure-hold inactivation of PME in orange juice followed first-order rate kinetics. Increasing amounts of total soluble solids decreased the inactivation rate, suggesting some protective action at high solid content.

Goodner and others (1999) studied the effect of high pressure processing in decreasing cloud loss by partial inactivation of PME in orange juice. Holding juice for 1 min at 700 Mpa, stabilized cloud for 90 days at 4°C (as measured by % transmittance at 660 nm after centrifugation at 10,000 x g for 10 min). After 90 days flavor deterioration would be a factor in commercial packaged juice quality. Higher pressures were much more effective in preserving cloud at short processing times compared to lower pressures. Treatment at 800 Mpa for 1 sec was enough to stabilize cloud for up to 80 days compared to only 10 days for treatment at 700 Mpa for 1 sec. Parish (1998) used high pressure (~400 Mpa) for 10 min in order to stabilize cloud in juice stored for 2-3 months at room temperature. Shorter treatment times would be more favorable in the juice possessing industry, for faster through times.

Some enzymes (Seyderhelm and others 1996; Quaglia and others 1996) are very pressure stable, so high pressure treatments will most likely be more effective if

combined with other treatments such as mild heating (Farr 1990). High pressure combined with moderate temperature elevation can ensure microbial safety (Gould 1973) and increase the cloud stability in orange juice (Broeck and others 2000). Drawbacks to pasteurization by high pressure are the high cost of the equipment needed to apply this method and concerns of ensuring microbial inactivation (Hoover 1993). More in-depth information about the effect of pressure on enzymes in food can be found in a review article by Hendrickx and others (1998).

#### <u>PME Inactivation (pH)</u>

The predominant isozymes of orange PME have a pH optima above 8 (Versteeg and others 1978, 1980) and decrease in activity as pH decreases. Low pH can inhibit the action of PME (Owusu-Yaw and others 1988). Treating orange juice with active PME with a cation-exchange resin or HCl to reduce the pH to 2, suppressed the activity of PME. However, when the juice pH was readjusted to its original level (pH 3.9), the juice clarified. They said that PME is not inactivated by low pH, but instead inhibited. However, it is possible that cation-exchange resin affected the charge of the cloud preventing flocculation by repulsion as is seen with apple juice (Yamasaki and others 1967). Storing juice in an acidified state provides good cloud stability, however results in the loss of ascorbic acid (Owusu-Yaw and others 1988).

Juice pH can also be reduced by treatment with supercritical  $CO_2$ , but returns to its original pH when  $CO_2$  is evaporated during depressurization. Balaban and others (1991) inhibited PME and stabilized cloud by treating orange juice with supercritical  $CO_2$ for 4 h at 29 MPa at 50°C. Although some PME reactivation occurred during storage, the juice cloud remained stable, possibly as a result of substrate modifications caused by

extreme shear during pressure cell venting. This procedure inhibited PME by the combination of increased acidity, prolonged elevated temperatures and pressure however it had no negative effect on juice cloud, color of sensory quality.

PME can also be competitively inhibited by its end product pectic acid (polygalacturonic acid). However, pectic acid is itself an extremely active juice clarifier (Krop and Pilnik 1974a; Baker 1976). Below a certain degree of polymerization, oligomers of galacturonic acid are too small to precipitate in acid environments, but are still able to inhibit PME. Hydrolysates of pectic acid with an average degree of polymerization from 8 to 15 could inhibit PME without contributing to clarification (Termote and others 1977) because they were long enough to function as end product inhibitors, but were too short to precipitate as insoluble pectates. Adding 1,000 ppm of a hydrolysate with a degree of polymerization of 12 extended juice cloud stability for several weeks at 3°C, however since pectic acid inhibition of PME is competitive and difficult to control, long-term cloud stability was not achievable with this procedure. <u>Enzyme Treatment to Stabilize Cloud</u>

For clarification to proceed, PME, sufficient pectin substrate and divalent cations must be present to permit production and flocculation of LM pectins. This sequence can be interrupted by disrupting the divalent cation-LM pectin reaction by either removing LM pectin as it is formed or by chelation of the cations. LM pectins can be hydrolyzed in unpastuerized juice by the addition of yeast polygalacturonase and pectinases containing high fungal polygalacturonase activity, resulting is cloud stable juice in the presence of active PME (Baker and Bruemmer 1972b, 1973; Krop and Pilnik 1974b).

Wobben and Tan (1983) patented a process to stabilize cloud in citrus beverages by subjecting pasteurized single strength juice or concentrate to one or more enzymes with protease activity. They claimed that cloud stability depended on the degree of protein hydrolysis in the juice.

Enzyme treatment is not only used to stabilize citrus cloud by PME inactivation. Mouri and Kayama (1981) have proposed a method referred to as "total liquefaction" in which different degradative enzymes with pectinase, npastuer, and hemicellulase activities are used to achieve a complete breakdown of fruit cell walls. A study by Xu and others (2001) investigated the difference in storage stability of frozen concentrated orange juices (FCOJ) produced by enzymatic and traditional mechanical squeezing methods at different temperatures. The results showed that the enzymatic juice had greater color and cloud stability to the squeezed juice. The enzymatic juice concentrate had a lighter color than that of the squeezed juice concentrate suggesting the concentrates produced by the two techniques could differ in their constituents. The enzymatic juice concentrate also had a higher viscosity. Enzyme treatment is also used to recover sugarcontaining soluble solids (SS) remaining in the finisher pulp after juice extraction (Braddock and Kesterson 1975). The primary sugars present in the pulp wash include glucose, fructose and sucrose. Using the enzyme method, the authors increased the total sugars in the pulp wash by 33%, glucose by 14%, fructose by 28% and sucrose by 37%. The pulp wash can be concentrated and used as a beverage base or a clouding agent in artificial juice drinks. Manufacturers who use this concentrated pulp wash in their products can list it on their ingredient list as concentrated orange juice or solids (Braddock 1999).

#### PME Inactivation (other methods)

Castaldo and others (1991) applied the finding that kiwi fruit contains a glycoprotein proteic inhibitor (Balestrieri and other 1990) to increase cloud stability in orange juice. These researchers partially purified the inhibitor (PMEI) and used it as an additive to "cut back" juice (fresh juice added to pasteurized concentrate). The fresh cut back juices with PMEI were similar to the pasteurized cut back juice in their ability to completely preserve cloud stability over long term storage, whereas fresh cut back juices without PMEI decreased cloud stability when added to pasteurized concentrate. The ability of the kiwi inhibitor to successfully preserve cloud stability in cut back orange juice, even over long-term storage, could allow for lower thermal juice treatment. The results of this study also suggest that this inhibitor could be used in frozen product technology. After long-term storage at 5°C in the presence of PMEI, the juice did not undergo changes characteristic of PME action. Thus addition of PMEI might allow storage of frozen products at higher temperatures.

Monothermosonication (MTS) is an emerging technology that combines the inactivation effect of heat and ultrasonic waves (Burgos 2000). MTS has proved to be an efficient tool to inactivate some other enzymes, such as lipoxygenase, peroxidase, and proteases and lipases from psychrotrophic bacteria (Lopez and others 1994; Sala and others 1995; Vercert and others 1997). Vercet and others (1999) studied the use of MTS to stabilize cloud in orange juice. The ultrasonic waves of MTS are thought to inactivate enzymes and destroy microorganisms by the intensity of the implosion of "internally cavitating" bubbles. This implosion generates microscopical hot spots (temperatures estimated at 5000 K) and local pressures (~0.5 Mpa) (Suslick 1988). Water is

decomposed (sonolysis) under these conditions to generate free radicals. Very high shear forces are generated by bubble implosions, which can break covalent bonds and split polymeric materials. PME is quite sensitive to shear as seem by the loss in activity during ultrafiltration at low pH and high NaCl (Snir and others 1995). MTS was effective in inactivating PME. This technology could be used to reduce the time-temperature of heat treatments to stabilize cloud. Temperatures of ~65-70°C could be used if MTS was used because citrus juice microflora are not very thermoresistant and a few seconds at these temperatures would be enough for its destruction (Kimball 1991). Before this technology can be employed further studies on the effects of nutrients and sensory quality must be undertaken.

Carbon dioxide is a well-known microstatic agent for numerous microorganisms (Daniels and others 1985). Flushing the headspace of orange juice filled jars with carbon dioxide extended the shelf life of npastuerized juices (Shomer and others 1994). Flushing carbon dioxide into a 10% headspace of a 350 ml jar resulted in 6 mM dissolved CO<sub>2</sub> and increased shelf life to 25 days at 4°C and 10 days at 10°C compared to 17 and 5 days without CO<sub>2</sub>, respectively. There was no difference in the triangle sensory evaluation between juices containing 6 mM CO<sub>2</sub> and juices without CO<sub>2</sub>. Juices with up to 10 mM CO<sub>2</sub> were judged unacceptable in hedonic evaluations. The addition of CO<sub>2</sub> to minimally heat processed juices (60°C, 16 s) only extended shelf life by 1-2 days suggesting that the flora of heated juices was different from that of fresh juice.

#### PME Activation by Cations

Monovalent and divalent cations increase activity of PME in alfalfa (Lineweaver and Ballou 1945), orange (MacDonnell and others 1945), soybean (Charnay and others

1992), and Marsh grapefruit (Snir and others 1995). Anions do not produce the same effect (MacDonnell and others 1945). Cations increase the activity of PME, however are not required for activity (Nari and others 1991). Cations increase the catalytic rate by interacting with the substrate rather than with the enzyme (Nari and others 1991). This was concluded after no change in the intrinsic fluorescence spectra of the enzyme, or of the fluorescent probes was observed in the presence or absence of the metal ions (Nari and others 1991). MacDonnell and others (1945) first hypothesized that cations simulate PME activity by competitive displacement of PME from an inactive PME-pectin complex and this was confirmed by Lineweaver-Burk plots (Nari and others 1991). Cations can interact with the negatively charged groups, releasing PME to cleave more ester bonds. High concentrations of cations can inhibit PME by blocking PME binding sites. Carboxyl groups must be adjacent to the ester bond in order for PME to cleave the bond. Sun and wicker (1999) proposed that an isokinetic temperature near the assay temperature for calcium activated reactions was responsible for the broad calcium concentration effect. In addition, cations uniquely influenced the free energy of the PME-pectin complex (Corredig and Wicker 2000). Calcium can be chelated by ammonium oxalate, which will prevent cloud loss due to pectic acid even when PME is present (Krop and Pilnik 1974a). However, this cannot be applied to citrus juice processing.

Polyamines are naturally occurring cations found in the cell walls (Pistocchi and others 1987) and cell membranes (Srivasta and Smith 1982) and include spermidine, spermine and putrescine. Polyamines increase PME activity similarly to inorganic cations like calcium and have a similar mechanism of activation, where the amine group

interacts with the carboxyl group of pectin (Charnay and others 1992). Leiting and Wicker (1997) further evaluated the effects of cations and polyamines on the activity of citrus PME extracts and solubilization of PME from the cell wall. At the concentrations used, the polyamines did not stimulate PME activity, however inhibition was observed at higher levels. Inorganic cations (lead acetate, ferric chloride and calcium chloride) stimulated PME activity at different cation concentrations and to different magnitudes. This suggests that competitive displacement is not the only factor involved in the activation and solubilization of PME. A study by Wicker and others (2002) added 1.2 U/ml TL-PME (as determined by 70°C for 5 min) to stabilized reconstituted juice in the presence and absence of cations (calcium, strontium, spermidine) at 4.2 or 16.7 mM. They reported that higher concentrations of cations clarified juices at a faster rate compared to lower concentrations and that no clarification was observed when cations were added in the absence of PME.

#### Other Cloud Destabilizers

The typical microorganisms present in orange juice are composed of acid tolerant bacteria, yeasts and molds. Molds can clarify orange juice through the production of extracellular enzymes such as pectinesterase (Nussinovitch and Rosen 1989). The major spoilage bacteria are of *Lactobacillus* and *Leuconostoc* genera (Parish and Higgins 1988). Yeasts of Saccharomyces and Candida genera were also found in spoiled orange juice (Parish and Higgins 1989).

#### <u>Summary</u>

Citrus clouds have complex requirements for stabilization and clarification. Although cloud has been studied at length, there is still much more to learn about the

interaction of specific cloud components and the enzyme systems affecting them. The role of each orange PME isozyme in clarification has yet to be determined or even if they are real isozymes (Macdonald and others 1993). A definite understanding of the clarification process has still not been achieved due to the structural complexities of orange juice pectin, the non-random action of PME and the uncertainties of the exact nature of calcium pectate and cloud interaction. Understanding the parameters involved in orange juice clarification will enable the use of novel technologies to stabilize cloud without producing adverse sensory qualities or violating standards of identity for citrus juice.

The objective of the following experiments was to further investigate the mechanism of orange juice clarification. The ability of partially purified TL-PMEs from Valencia pulp with different specific activities and hence different amounts of non-PME protein, to clarify orange juice was examined. Previous studies suggested that only TS PME could rapidly clarify juice (Versteeg 1979) however other evidence was presented which suggests TL-PME could also be a factor in clarification (Cameron and others 1998). The effect on cloud particle size distribution during clarification was simultaneously examined. Floc formation and changes in serum soluble components in fresh Valencia orange juice were also investigated. The flocculent precipitation or "floc", which was first described by Baker and Bruemmer (1969), was further analyzed in our studies for onset of appearance and protein content while the serum was examined for enzyme activity and other qualities over time.
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# CHAPTER 3

# CLARIFICATION OF CITRUS JUICE IS INFLUENCED BY SPECIFIC ACTIVITY OF THERMOLABILE PECTINMETHYLESTERASE AND INACTIVE PME-PECTIN COMPLEXES<sup>1</sup>

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## Abstract

Thermolabile pectinmethylesterase (PME) from Valencia orange pulp was extracted, partially purified by cation exchange chromatography (IEX), and added to reconstituted orange juice at 2 units/mL. Of the juices that clarified, %T increased, cloud particle size increased and % degree of esterification (DE) decreased in the 15-day storage study. The rate of clarification was most rapid for juices with added PME extracts that never bound Hi-Trap SP and contained a 27-kDa peptide, intermediate for crude extracts of PME not applied to IEX, and lowest for PME extracts that bound Hi-Trap SP and contained a 13kDa peptide. These results suggest that PME-pectin complexes and a non-PME protein moderate PME activity and juice clarification.

Keywords: pectinesterase, particle size, colloidal stability, de-esterification, ion exchange chromatography

#### Introduction

Cloud plays an important role in the sensory attributes of juice including color, flavor, aroma and texture. Sporadic separation and sedimentation of juice cloud is a problem for citrus and other juice beverages. Citrus cloud particle sizes range from 0.4 to 5  $\mu$ m in diameter (Klavons and others 1994) with those around 2  $\mu$ m constituting stable cloud (Mizrah and Berk 1970). Pectinmethylesterase (PME)-sensitized pectins increased particle size (D<sub>3,2</sub>) distribution of pasteurized orange juice from 0.9  $\mu$ m to 4.9  $\mu$ m, but did not affect cloud stability (Corredig and others 2001).

Juice clarification originates from PME de-esterification of native high-methoxyl pectin. The negatively charged sites of the pectic polymer react with endogenous divalent cations forming insoluble calcium pectate, which destabilizes cloud particles (Stevens and others 1950). This mechanism was proposed because heat treatment, which denatures enzymes, decreases the probability of sporadic clarification and the percent of de-esterification (% DE) of juice pectin decreases during clarification (Josyln and Pilnik 1961).

In spite of the purported role of PME, cloud loss has not been directly correlated with PME activity. Clarification was attributed to a small amount of thermostable PME (TS-PME) (Versteeg and others 1980). However, some forms of thermolabile PME (TL-PME) also decrease juice cloud stability (Cameron and others 1997; Ackerley and others 2001). PME activity of fresh juices did not have a high correlation to % pulp or % Brix (Snir and others 1996). Since, no significant difference was found in total PME and TS-PME between different citrus cultivars, other factors may be involved in detection of PME (Snir and others 1996).

Residual effects of PME in pasteurized juices or other moderators of PME activity may play a more important role than previously recognized. Pectin modification by PME in fresh juice contributed to the rapid decline in cloud stability of subsequentially pasteurized juice (Chandler and Robertson 1983). PME-pectin complexes influence apparent PME activity (Charney and others 1992), TS-PME activity (Leiting and Wicker 1997), separation on ion exchange (Chen and others 1998), and ultrafiltration (Snir and others 1995). Some PME isoenzymes are probably PME-pectin complexes (Macdonald and others 1993) and pectin co-chromagraphs with PME during purification (Corredig and others 2000). Shomer and others (1999) proposed that clarification results from cloud protein-pectin flocculation, and PME increased association between cloud proteins and pectin. An apparent increase in activity of TS-PME by ion exchange chromatography was attributed to the separation of non-PME protein from the PME fraction (Corredig and others 2000). It was reported that non-enzyme protein moderates polygalacturonase enzyme activity, either by increasing thermostability and activity (Pressey 1984) or by inhibition of activity (Johnston and others 1993; Cook and others 1999). Since complexes of PME, pectin and/or protein influence apparent PME activity and thermostability, then it is likely that differences in the ability of PME-complexes to bind cation exchange resins will show different clarification behaviors. During purification an increase in specific activity, defined as units of PME per mg of protein, indicates removal of non-PME protein. In this research, PME fractions of varying affinity for cation exchange resin and varying amounts of non-PME protein and specific activities were prepared. The objective was to evaluate the interrelationship of the

affinity of PME for cation exchange and presence of non-PME protein on the degree of de-esterification and clarification of orange juice.

#### **Materials and Methods**

# Enzyme extraction and chromatography

Crude extract was prepared as described by Wicker and others (1988) by combining Valencia orange pulp (donated by Citrus World, Lake Wales, FL) with 4 parts (w/v) 0.1 M NaCl, 0.25 M Tris buffer, pH 8. The extract was homogenized (Pro 300A; Proscientific Inc., Monroe, CT) for 1 min at 4°C. The crude extract was concentrated by making a sequential 30% to 75% ammonium sulfate precipitation. The pellet, collected by centrifugation at 1,550 X g and 4°C for 20 minutes (Sorvall RC-5B centrifuge, Dupont Instruments, Doraville, GA), was resuspended and dialyzed overnight against buffer (50 mM sodium phosphate, pH 7). All buffers were degassed and filtered through a 0.45-µm filter (Gelman Scientific, Ann Arbor, MI) at room temperature before use in chromatography. Chromatography separations were performed at 4°C using an FPLC system (P-500 pumps and GP-250 gradient programmer; Amersham Pharmacia Biotech, Piscataway, NJ). The dialysis tubing (Spectrapor, MWCO 6000, Fisher Scientific, Atlanta, GA) was boiled in 10% acetic acid and rinsed in deionized water to minimize loss of PME activity. After dialysis, the enzyme extract was filtered through a No. 1 Whatman filter (Fisher Scientific, Atlanta, GA). The PME was loaded onto a 5-mL cation exchange column (Hi-Trap SP; Amersham Pharmacia Biotech) at 5 mL/min and eluted with 0 to 1 M NaCl gradient in 15 column volumes. Fractions were collected in 2 mL volumes. PME activity in fractions was qualitatively identified using a pH-sensitive dye (Corredig and others 2000) and positive fractions were quantified by titration.

Positive fractions were quantified for PME and protein and pooled for the clarification study.

The PME that bound a Hi-Trap SP cationic column on the first application was denoted BP++. PME that did not bind Hi-Trap SP in 3 individual chromatographic separations was pooled, reapplied to another SP column and eluted with NaCl. The PME activity that bound upon re-chromatography and was eluted by NaCl was denoted BP+. The PME that did not bind upon re-chromatography was denoted UBP-. PME activity of the crude extract was initially measured immediately after re-suspension of the ammonium sulfate pellet and was denoted as CE-I. The activity was re-measured just before juice clarification studies were begun and was denoted CE-F.

#### Juice preparation

Juice was reconstituted from frozen concentrated Valencia orange juice (Citrus World, Lake Wales, FL) to 16°Brix with distilled water. The reconstituted juice was centrifuged at 1,500 X g for 10 min (Sorvall RC-5B centrifuge; Dupont Instruments, Doraville, GA) and filtered through Miracloth (Calbiochem, La Jolla, CA). Control juices had buffer (50 mM sodium phosphate, pH 7) only added. Volumes were adjusted with PME and buffer so that the final units of PME were 2 units per mL of juice and the final brix was 13.3°. Juices were stored in 15-mL graduated, conical centrifuge tubes at 4°C. At selected times duplicate tubes were pulled for analysis.

### **Analytical methods**

PME activity was quantified using a pH stat titrator (Brinkmann, Westbury, NY) at pH 7.5, 30°C in 1% high-methoxyl pectin, 0.1 M NaCl (Citrus Colloids Ltd., Hereford, U.K.). PME units were expressed in microequivalents of ester hydrolyzed per minute. Thermostable PME activity was defined as activity that survived heating 0.5 mL of PME in 2 mL of preheated buffer (50 mM sodium phosphate, 0.1 M NaCl, pH 7) at 70°C for 5 min. Residual activity was determined by comparison to unheated controls diluted in the same buffer. Activity measurements were performed within 2 h of heating.

Protein was determined using the Bradford (1976) method using bovine immunoglobulin (IgG) as a standard (Biorad, Hercules, CA) and quantified using a Biorad microplate reader and software (Model 550). SDS-PAGE, using Phastgel Gradient<sup>TM</sup> 8-25% gels, was run on selected enzyme fractions using a PhastSystem (Amersham Pharmacia Biotech, Piscataway, NJ) and stained with Coomassie blue according to the manufacturer's specifications. The enzyme fractions were diluted to the same concentration of protein before being combined with sample buffer and loaded onto the gel.

Particle size was determined using a Malvern Mastersizer (Model MSS; Malvern Instruments Limited, Worcestershire, U.K.) with a dispersion unit controller (Model DIF2023, Malvern Instruments Limited) using 1.73 and 1.33 as the refractive indices and dispersed phase, respectively, and 0.1 as absorption index for cloud particles (Corredig and others 2000). Size distributions (volume fractions against particle size) were calculated and the weight-average sizes were expressed as  $D_{3,2} = \sum_i n_i d_i^3 / \sum_i n_i d_i^2$ , where  $n_i$ is the number of particles of diameter  $d_i$ . After centrifugation of stored juice at 1,500 X g, 10 min, the % transmittance at 650 nm of the supernatant was measured (Spectronic 20D, Milton Roy Company, Ivyland, PA).

# **Degree of esterification**

Alcohol-insoluble solids (AIS) were made from control and CE-F juices on the first day of the study and from all juices at the last sampling period of the study. Juices were boiled in 4 volumes of 95% ethanol, cooled, filtered through a sintered glass funnel, then sequentially washed with 6 parts ethanol and 4 parts acetone. The AIS was dried at room temperature overnight and stored at  $-20^{\circ}$ C.

The % de-esterification (DE) of pectin extracted from AIS was estimated from the mole ratio of methanol and uronic acid (Voragen and others 1986). Approximately 60-90 mg of each sample was saponifed in 2 mL of isopropanol and water 1:1 (v/v) with 0.4 M NaOH by mixing on a vortex and shaking on a Red-Rotor (Model PR70; Hoeffer Scientific Instruments, San Francisco, CA) at an angle for 2 h at ambient temperature. The samples were centrifuged (International Clinical Centrifuge Model CL; International Equipment Co., Needham Heights, MA) for 5 min and then placed on ice for 30 min. Methanol in the supernatant was quantified using a SpectraSYSTEM P2000 pump and a SpectraSYSTEM AS1000 autosampler (Thermo Separation Products, San Jose, CA) equipped with an Aminex HPX-87H column (300 x 7.8 mm; Bio-Rad Labs, Richmond, CA) in combination with a guard column (Hi-Pore guard cartridge, 30-4.6mm; Bio-Rad Labs) and a SpectraSYSTEM RI-150 refractive index detector at ambient temperature. The column was operated at ambient temperature at a flow rate of 0.6 mL/min with 5 mM sulfuric acid as eluent. After saponification and centrifugation, the pellets were frozen and stored at -20°C until galacturonic acid analysis. Total pectin (Hudson and Buescher 1984) and uronic acid was determined by *m*-hydroxydiphenol (Blumenkratz and Asboe-Hanson 1973).

#### **Results and Discussion**

Of the PME that bound Hi-Trap SP on the first application (BP++), a single peak was observed over 4 fractions. The pooled PME activity and specific activity of these fractions were 281 U/mL and 312 U/mg protein, respectively, as reported in Table 2.1. The PME that bound SP after re-application (BP+) was eluted in 2 fractions with an activity of 290 U/mL and specific activity of 323 U/mg protein, similar to BP++. The activity of PME that never bound SP was 1557 U/mL with a specific activity of 484 U/mg. The most striking result is the extraordinary activity and specific activity of the resuspended ammonium sulfate pellet and subsequent decrease after dialysis and 11 days of storage. The difference in CE-I and CE-F activity may be due to the activation of CE-I by ammonium ions (McDonnell and others 1945), loss of activity in CE-F by binding onto dialysis tubing (Versteeg and others 1978), formation of inactive PME-pectin complexes (Macdonald and others 1993; Chen and others 1998) or loss of activity of CE-F during storage (Sun and Wicker 1996). The lower specific activity of BP++ or BP+ compared to CE-F suggests that a cationic protein competes with PME or that anionic pectin masks the PME charge and influences ion exchange separation. The higher specific activity of UBP- compared to BP+ or BP++ suggests the removal of cationic protein by Hi-Trap SP chromatography. In addition to differences in specific activity, the protein profile was markedly affected. The SDS-PAGE profile of CE-F, UBP-, BP++ are depicted in Figure 2.1. A 36-kDa protein is seen in all lanes and is presumably PME based on MW. An additional protein at approximately 27 kDa is seen in UBP- (lanes 3 and 4) and CE-F (lanes 6 and 7) this 27 kDa protein may also be PME (Han and others 2000; Savary and others 2002). This band constituted 13% and 25% of the selected area

for UBP- and CE-F, respectively. A lower MW band at approximately 13-kDa is seen in the BP++ fraction and accounted for 18% of the selected area. As a standard, heparinpurified PME with a specific activity of 429 U/mg is depicted in lane 2. It contains the 36-kDa and 13-kDa bands and is missing the 27-kDa band.

The total and specific activities of the 5 PME extracts and the day of onset of clarification are reported in Table 2.1. Of the PME fractions evaluated for clarification, the juice with PME that never bound Hi-Trap SP had the earliest onset of a change in %T, beginning at day 3. The juice with PME (BP+) that bound Hi-Trap SP upon rechromatography began to increase in %T at 15 days, but %T remained less than 20%. The %T of other juices with BP++ or CE-I did not change in the course of this study. Depending on the chromatographic separation, the specific activity after chromatography ranged from 311 U/mg protein to 483 U/mg protein. The activity in the crude extract between the time of dialysis and completion of the chromatographic separations decreased from 9,453 U/mL to 3,770 U/mL. Complete loss of PME activity was reported by others (Sun and Wicker 1996).

The rate of clarification of PME-added juices as measured by %T, varied according to the ability to bind Hi-Trap SP and specific activity of PME (Figure 2.2). All PME extracts used in this study were thermolabile, defined as loss of activity after heating at 70°C for 5 min. The onset of clarification as measured by an increase in %T was not observed in the control juices with no added PME in the 15 days of storage. The fastest rate of change in %T occurred in juices with UBP- added, the fraction of PME that never bound the Hi-Trap SP column. The %T of juices with UBP- was the highest initially and the rate of change increased markedly after 3 days of storage. Onset of

clarification was observed by day 11 in juices with CE-F PME and the %T was 26.2 by day 15. Juices with BP+ showed little evidence of clarification at day 15 (15.4 %T). In juices with BP++ PME added, no evidence of clarification was observed in the 15-day storage study. Of the column fractionated PMEs, the order of the rate of clarification was fastest for the juices with added PME that never bound the SP column, intermediate for the PME that bound SP only after re-chromatography and slowest for the PME that bound SP on the initial application.

The %DE of pectins extracted from juice at the end of storage decreased from about 27 %  $\pm$  6.6 DE in the control, no PME added juice to 13%  $\pm$  1.0 DE in pectins extracted from clarified juice (UBP-) and 13%  $\pm$  3.7 extracted from clarified juice (CE-F). Pectins from juices with added PME that did not clarify within 15 days, also decreased to about 19%  $\pm$  4.0 and 23%  $\pm$  3.7 in BP++ and CE-I added juice pectins, respectively. These values are similar to the %DE of clarified juices reported by Krop and others (1974) and Baker (1979). A critical limit of about 12 to 15% DE seems necessary for clarification based on data of this study. The decrease in %DE supports the theory of calcium pectate initiation of clarification, but a direct relationship was not observed.

The particle size data for the juices shows a bimodal distribution of size (Figure 2.3a). The first peak represents stable cloud particles (Klavons and others 1994) and range in size from 0.4 to 5  $\mu$ m. The second peak of larger particle sizes near 100  $\mu$ m represents settling pulp (Ackerley and others 2001) and did not change in a discernable trend with storage time. In control, no PME added juices at day 0, the volume distribution of cloud particle size is approximately 1 to 2  $\mu$ m (Figure 2.3a). During

storage, the smaller particle sizes remain stable at 2 to 4  $\mu$ m and the juices are not clarified according to %T values.

The cloud particle size of PME-treated juices show slight migration towards larger particle sizes after the initial sampling on day 0. In juices that did not clarify according to %T measurements (Figure 2.3b and 2.3c), the volume distribution of particle size remained below 5 µm. Interestingly, the addition of PME caused a shift to larger particle sizes for all column separated PMEs (Figure 2.3b, 2.3d, 2.3e) as reported by Ackerley and others 2001. In juices that showed clarification by day 3 of storage, there was an earlier shift to larger particle sizes towards 10 µm by day 1 (Figure 2.3d). The volume distribution of the cloud particle sizes increased with storage time with an increase in %T. In juices that clarified at a slower rate, the volume distribution of cloud particles increased at a slower rate (Figure 2.3e and 2.3f). The particle size data of the juices in this study are similar to previous studies (Ackerley and others 2001; Corredig and others 2000; Mizrahi and Berk 1970). The average diameter of the juice cloud particles  $(D_{3,2})$  was approximately 1-2  $\mu$ m on day 0, with the exception of the CE-I added juice, which was closer to 6  $\mu$ m. By day 1, all D<sub>3,2</sub> values increased from 4 to 6  $\mu$ m. Throughout the rest of the study the  $D_{3,2}$  values increased slightly to 4 to 8  $\mu$ m. After day 0, the UBP- PME juice had the highest  $D_{3,2}$  values on all days.

Clarification of citrus juice can be initiated by thermolabile PME and the clarification potential of TL-PME extracts was greatest for the PME fraction that had the least cationic character and ability to bind Hi-Trap SP. Poor column affinity and clarification potential may be due to masking of the native positive charge of PME or by steric exclusion of column interaction by a pectin complex. PME binding and

clarification potential may also be affected by a non-PME protein. PME extracts (UBPand CE-F) that induced rapid clarification in juices, also contained a 27-kDa peptide in addition to the 36 kDa peptide. PME extracts (BP++) that did not cause clarification contained a low-MW 13-kDa peptide and the 36-kDa peptide, but did not contain the 27kDa peptide. Competitive displacement of PME from an inactive PME-pectin complex enhances activity (Leiting and Wicker 1997) and clarification (Ackerley and others 2001). This study also suggests that PME affinity for a cation exchange column, ability to de-esterify pectin and induce clarification of juices, may also be moderated by the presence of naturally occurring low-MW, non-PME proteins. Other factor(s) besides the amount and thermostability of PME activity in the juice are likely involved in the rate of clarification.

#### Conclusion

Thermolabile PME, partially purified by cationic exchange chromatography, can clarify juices as measured by %T or particle size. Clarification was more likely for PME extracts with the least cationic character and/or the presence of a 27-kDa peptide. Extracts that were least likely to induce clarification were more likely to bind a cation exchange column and/or contained a 13-kDa peptide. The results suggest that PME complexes with pectin and/or low-molecular-weight protein influence the ability of PME to induce clarification.

rreatment	Total Activity	Specific Activity	%DE	%DE	Unset of Clarification
	(U/ml)	(U/mg)	(Day 1)	(Day 15)	% T @ 650 >20 <sup>d</sup>
Control	0	0	24 ± 2.3	27 ± 6.6	NC
CE-I	9453	1673	24 ± 2.7	23 ± 3.7	NC
CE-F	3771	667		13 ± 3.7	Day 11
BP++	281	312		19 ± 4.0	NC
BP+	290	323		16 ± 1.8	NC
UBP-	1557	483		13 ± 1.0	Day 3

Table 2.1 – Clarification of juice and % degree of esterification of juice pectin after addition of PME of varying specific activity. <sup>a</sup> Control = no treatment, CE-I = crude enzyme initial activity, CE-F = crude enzyme final activity after 11 days, BP++ = PME that bound SP on first application pooled fractions 6-10, BP+ = PME that bound SP on second application pooled fractions 9-10, UBP- = PME that did not bind SP on second application. Two units of enzyme per mL of juice were added to each sample. <sup>b</sup> Total activity = units/mL of enzyme, Specific activity = units/mg of protein. <sup>c</sup> Percent degree of esterification. <sup>d</sup> NC = no clarification, %T was less than 20% in 15 days at 4°C.



Figure 2.1: SDS-PAGE of selected enzyme fractions. Lane 1 and 8, molecular weight standards (Amersham Pharmacia Biotech); lanes 2-7 contained 1.8 µg protein; lane 2, heparin-purified Valencia PME standard; lane 3 and 4, UBP-; lane 5, BP++; lane 6 and 7, CE-F.



Figure 2.2: Average percent transmittance at 650 nm of stored juices after addition of 2U PME / mL juice of varying specific activity. Standard deviation between juices <10%.



Figure 2.3A: Particle size distribution of control orange juice with no PME added.



Figure 2.3B: Particle size distribution of orange juice with BP++ PME added.



Figure 2.3C: Particle size distribution of orange juice with CE-I added.



Figure 2.3D: Particle size distribution of orange juice with UBP- PME added.



Figure 2.3E: Particle size distribution of orange juice with BP+ PME added.



Figure 2.3F: Particle size distribution of orange juice with CE-F added.

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# CHAPTER 4

# FLOC FORMATION AND CHANGES IN SERUM SOLUBLE CLOUD COMPONENTS OF FRESH VALENCIA ORANGE JUICE<sup>1</sup>

<sup>1</sup>Ackerley, Jennifer L. and Louise Wicker. 2002. To be submitted to the Journal of Food Science.

#### Abstract

Juice was extracted from Valencia oranges and centrifuged to remove settling pulp (PFJ). The pH was adjusted and some PFJ was ultracentrifuged to remove suspended cloud materials producing ultracentrifuged serum (UCS) and pellet (UCP). The UCS and the PFJ were stored at 4°C for 11 days. The UCS and PFJ had no measurable pectinmethylesterase (PME) activity. Floc (UCF) in the UCS appeared approximately the same time as the %T increase in PFJ. The UCP, UCF and UCS were analyzed by SDS-PAGE. Predominant bands at 13, 27 and 36 kDa were found in both UCF and UCP. The UCS had no detectable protein. The 36 and 27 kDa bands are presumptively PME. Soluble (in UCS) and insoluble (in PFJ) PME are involved in clarification. Proteins at 13, 27 and 36 kDa are presumptively involved in clarification and precipitate during floc formation.

Keywords: pectinmethylesterase, cloud, clarification, de-esterification, electrostatic

#### Introduction

Citrus juices are rich in vitamin C and folic acid, which are essential to maintaining health. The American Cancer Society, March of Dimes and the American Heart Association have recognized the important role of a balanced diet, including citrus fruit and juices, in helping to reduce the risk of certain cancers, neural tube birth defects and heart disease. The acceptability of orange juice is affected by its appearance. Orange juice will clarify, as a result of the enzyme pectinmethylesterase (PME), if not pasteurized at high temperatures above which is needed for microbial safety. Pasteurizing at high temperatures has a negative effect on the "fresh taste" of juice. Understanding the factors that are involved in clarification, will allow pasteurization at lower temperatures, and production of premium quality juices.

Juice clarification originates from the action of PME, which de-esterifies the methyl ester groups of pectin ( $\alpha$  1,4 – polygalacturonic acid) (Stevens and others 1950). Subsequent formation of insoluble calcium pectate destabilizes cloud particles from suspension. PME is a cell bound enzyme and forms complexes of variable activity with pectin (McDonnell 1945). More recently, calcium and other inorganic cations and non-PME proteins have been implicated in displacement of a protective colloid from the pectin surface and accelerating clarification (Wicker and others 2002). When PME is released from a PME-pectin complex, it can react with other methoxyl ester groups, decreasing the degree of esterification (DE). At higher levels, divalent cations act as competitive inhibitors (Charnay and others 1992).

Cloud consists of a fine suspension of particles, which gives the characteristic turbidity, color, flavor and aroma to orange juice (Mizrahi and Berk 1970). Cloud

particles range in size from  $0.4 - 5.0 \mu m$  (Klavons 1994) with those around 2  $\mu m$  constituting stable cloud (Mizrahi and Berk 1970). About half of the total cloud by weight is composed of high molecular weight polymeric materials such as protein, pectin, hemicellulose and cellulose (Sinclar 1984). Cloud also contains several colloidal fractions including membranes, hesperidin crystals, oil microdroplets and complexes of these colloidal bodies with proteins (Shomer 1988; Shomer and others 1985). Previous studies (Baker and Bruemmer 1969, 1972) examined cloud stability in the absence of various insoluble components and described a flocculate precipitation in juice serum that coincided with the onset of clarification in juice. The possible role of soluble cloud constituents and PME on juice clarification has not been considered in subsequent research.

Unlike orange juice, removal of apple juice cloud is well received among consumers. Since the early thirties, pectic enzymes have been added to apple juice to achieve the desirable clarification. The apple juice clarification mechanism was initially studied by Yamasaki and others (1964, 1967). They discovered that the suspended material in apple juice is composed of positively charged protein-carbohydrate complexes coated with negatively charged pectin. When pectic enzymes degrade this protective colloid, the positive charge of the protein-carbohydrate complex is exposed. Pectin coated protein-carbohydrate complex with negative charged exteriors are electrostatically attracted to the partially exposed positive protein resulting in flocculation and clarification. At pH values above the isoelectric point of the cloud protein (above pH 5), the protein is negatively charged and flocculation does not occur even though the pectic

enzymes are still active. If pH is reduced back to the natural pH of juice (pH 3.5), where the proteins are positively charged, flocculation rapidly occurs.

The objective of this research was to evaluate the contribution of serum soluble factors in clarification of juices and the possible role of electrostatic interactions in cloud stability of soluble and insoluble juice components.

# **Materials and Methods**

# Juice preparation

Fresh juice was extracted at room temperature from Valencia oranges using a commercial juicer (Waring Model 31JC33, New Hartford, CT) and filtered through 4 layers of cheesecloth to remove large pulp and seeds. The juice was stored on ice prior to centrifugation (Sorvall RC-5B centrifuge, Dupont Instruments, Doraville, GA) at 1500 g for 10 min at 4°C to remove settling pulp. The supernatant was collected and denoted as "pulp free juice" (PFJ). Another portion was ultracentrifuged at 150,000 x g for 30 min at 4°C using a Sorvall OTDB ultracentrifuge (Dupont, Newton, CT). This force was sufficient to remove virtually all suspended cloud materials (Baker and Bruemmer 1969). The supernatant was collected and denoted as "ultracentrifuged serum" (UCS). The pellet (UCP) was saved and washed 3 times with water and freeze dried for later analysis. The PFJ and UCS juices were stored at 4°C. Samples were pulled daily and analyzed in triplicate for pH, % transmittance at 650 nm (%T), PME, particle size (PFJ only) and °Brix. Alcohol insoluble solids were made at selected intervals.

In a subsequent study, fresh juice was prepared in the same way as stated above, however after the settling pulp was removed, some of the juice was adjusted to pH 7 while mixing at a low speed at 4°C with a Proscientific homogenizer (Pro 300A,

Proscientific Inc., Monroe, CT) for an hour and then readjusted to its initial pH (PFJ474), a second portion of PFJ was adjusted and kept at pH 7 (PFJ7), and a third portion was kept at its initial pH (PFJ4) however it was also mixed. A portion of each of these three batches was ultracentrifuged as above and denoted as UCS4, UCS7, UCS474. The pellet produced from ultracentrifugation was denoted as UCP4, UCP7, UCP474. The °Brix, PME, PS (PFJ only), %T at 650nm were measured for all juices on Day 0 and then particle size and %T at 650 nm were measured daily in duplicate throughout the study. After juices clarified, the floc (UCF) was collected from UCS4, UCS7, and UCS474 by centrifugation and protein was analyzed for all UCF and UCP by SDS-PAGE.

## **Analytical determinations**

The pH was determined on all juice samples using an Accumet pH meter model 825MP (Fisher Scientific, Pittsburgh, PA). <sup>o</sup>Brix was determined using a refractometer (Milton Roy Company, Ivyland, PA). PME activity was quantified using a pH stat titrator (Brinkmann, Westbury, NY) at pH 7.5, 30°C in 1% high methoxyl pectin, 0.1 M NaCl (Citrus Colloids Ltd., Hereford, U.K.). PME units were expressed in microequivalents of ester hydrolyzed per minute. Thermostable PME activity was defined as activity that survived heating 0.5 ml of PME in 2 mL of preheated buffer at 70°C for 5 min. Residual activity was determined by comparison to unheated controls diluted in the same buffer. Activity measurements were performed within two hours of heating.

The floc that formed in the UCS juices was collected by centrifugation at 10,000g for 20 min at 4°C (Sorvall RC-5B centrifuge, Dupont Instruments, Doraville, GA). The pellet (UCF) and supernatant were freeze dried for later protein analysis.

Protein was determined for UCP, UCF and UCS (with floc removed) by the Bradford (1976) method using bovine immunoglobulin (IgG) as a standard (Biorad, Hercules, CA) and quantified using a Biorad microplate reader and software (Model 550). SDS-PAGE, using Phastgel Gradient<sup>TM</sup> 8-25% gels, was run on selected enzyme fractions using a PhastSystem (Amersham Pharmacia Biotech, Piscataway, NJ) and stained with silver stain according to the manufacturers specifications.

Particle size was determined on PFJ juices only using a Malvern Mastersizer (Model MSS, Malvern Instruments Limited, Worcestershire, U.K.) (Dispersion Unit Controller DIF2023, Malvern Instruments Limited, Worcestershire, U.K.) using 1.73 and 1.33 as the refractive indices and dispersed phase respectively, and 0.1 as absorption index for cloud particles (Corredig and others 2000). Size distributions (volume fractions against particle size) were calculated and the weight-average sizes were expressed as  $D_{3,2}$  $= \sum_i n_i d_i^3 / \sum_i n_i d_i^2$ , where  $n_i$  is the number of particles of diameter  $d_i$ . The %T at 650 nm of PFJ and UCS juices were measured (Spectronic 20D, Milton Roy Company, Ivyland, PA).

## **Degree of esterification**

Alcohol insoluble solids (AIS) were made from PFJ and UCS juices on the first 3 days of the study, after the %T > 60 for PFJ and at the last sampling period of the study (PFJ only). Juices were boiled in 4 volumes of 95% ethanol, cooled, filtered through a sintered glass funnel, sequentially washed with 6 parts ethanol and 4 parts acetone. The AIS was dried at room temperature overnight and stored at -20°C.

The % de-esterification (DE) of pectin extracted from AIS was estimated from the mole ratio of methanol and uronic acid (Voragen and others 1986). Approximately 100

mg of each sample was saponifed in 2 ml of isopropanol and water 1:1 (v/v) with 0.4 M NaOH by mixing on a vortex and shaking on a Red-Rotor (Model PR70, Hoeffer Scientific Instruments, San Francisco) at an angle, for 2 hr at ambient temperature. The samples were centrifuged at 1200 g (Marathon 3200, Fisher Scientific, Pittsburgh, PA) for five minutes at ambient temperature and then placed on ice for 30 min. Methanol in the supernatant was quantified using a SpectraSYSTEM P2000 pump and a SpectraSYSTEM AS1000 autosampler (Thermo Separation Products, San Jose, CA) equipped with an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad Labs, Richmond, CA) in combination with a guard column (Hi-Pore guard cartridge, 30-4.6mm, Bio-Rad Labs) and a SpectraSYSTEM RI-150 refractive index detector at ambient temperature. The column was operated at ambient temperature at a flow rate of 0.6 ml/min with 5 mM sulfuric acid as eluent. After saponification and centrifugation, the pellets were frozen and stored at -20°C until galacturonic acid analysis. Total pectin (Hudson and others 1984) and uronic acid was determined by *m*-hydroxydiphenol (Blumenkrantz and Asboe-Hanson 1973).

# **Results and Discussion**

An initial clarification study at the natural pH of juice was conducted to verify that floc formation occurred in juice serum. Contrary to previous clarification studies that used heat stabilized frozen concentrated orange juice (FCOJ) with added PME, this study used fresh squeezed juice. PFJ and UCS floc at day 15 of the study are depicted in Figure 3.1. The °Brix and pH of UCS and PFJ were approximately 11.6 and 3.7, respectively, and did not change during storage. The UCS and PFJ had no detectable enzyme activity. The PFJ had an initial %T of 20%, and increased significantly by day 4

to 40-60% indicating clarification (Figure 3.2). The %T of the UCS was initially high >70% and increased slightly throughout the study. Floc (UCF) in the UCS appeared approximately the same time the %T increase in PFJ. The particle size distribution  $(D_{3,2})$ for PFJ increased overtime in agreement with previous studies (Ackerley and others 2002). At day 4, the particle size  $(D_{3,2})$  of the PFJ increased from 2.61 to 3.17 (Figure 3.3). After the onset of gross clarification as measured by %T, there was an increase in particle size from 3.17 to 4.34 µm that remained constant after day 10. This break in particle size between day 4 and 5, after the onset of gross clarification had not been seen in previous studies with FCOJ spiked with PME (Ackerley and others 2002). With increasing time the particle size distribution became narrower and shifted towards larger particle sizes. This suggests that a larger number of cloud particles were participating in aggregation after gross clarification compared to before the juice clarified. Aggregation of cloud constituents and increase in particle size of PFJ precede clarification. Laser diffraction light scattering could not detect particles in the UCS, which supports that all insoluble particles were removed through ultracentrifugation. The initial DE for both PFJ and UCS was approximately 20 to 30% and decreased to a final DE < 10% by day 6 (Table 3.1).

The proteins from UCP, UCF and UCS were analyzed by SDS-PAGE. Predominant bands at 13, 20, 27 and 36 kDa were found in both UCF and UCP (Figure 3.4). The UCS had no detectable protein by Bradford assay. The UCP also had some higher molecular weight peptides that were present in too low of a concentration to quantify. The 36 and 27 kDa bands are presumptively PME. In a previous study, PME extracts containing both 36 kDa and 27 kDa peptides clarified juice rapidly and juices

that contained 36 kDa and 13 kDa peptides did not clarify (Ackerley and others 2002). Those results suggested that 36 kDa peptide complexes with low molecular weight proteins (27 kDa or 13 kDa) and it influences the ability of PME to induce clarification in soluble and insoluble cloud constituents. In this study, even in the absence of detectable PME activity, the same proteins are observed in PFJ and floc as reported earlier.

In a subsequent study, electrostatic interactions in soluble and insoluble juice components was considered. The °Brix of UCS and PFJ at pH 4, pH 7 and pH 474 was approximately 11, 11.8 and 11.8, respectively, and did not change during storage. The lack of change (or slight increase) in °Brix would argue against a significant volume change in juice by pH adjustment. At all pH values, the UCS and PFJ had no detectable PME activity. The %T of the PFJ4 was initially 11% and increased to over 36% between 9 and 11 days (Figure 3.5). The %T of the PFJ7 was initially 20% and did not change markedly until day 13 and increased to 50% by day 18. In a second replication, the %T of PFJ7 did not change from ~20%T by day 18, the end of the study. The %T of PFJ474 was initially 12% to 15% and had increased to over 36% between day 2 and 3. Floc appeared in the UCS4 between day 8 and 9 and between day 2 and 3 in UCS474. In UCS7, even in the absence of clarification, floc appeared in both replications by day 11.

The particle size distribution  $(D_{3,2})$  for PFJ at pHs 4 and 474 also increased with time of storage (Figure 3.6-3.8). This was more apparent for the PFJ474, which had an initial  $D_{3,2}$  value between 1.93 and 1.98 and increased to between 3.02 and 3.42 by day 3 compared to PFJ4 which had an initial  $D_{3,2}$  value of 1.57 to 1.69 and increased to 2.21 to 3.8 by day 11. The particle size distribution of PFJ7 decreased overtime, having an initial  $D_{3,2}$  value of 2.46 to 2.99 and a final value of 1.11 to 1.40 at the end of the study.

The proteins from UCP4, UCP7, UCP474, UCF4, UCF7 and UCF474 were analyzed by SDS-PAGE. Predominant bands at 13, 20, 27 and 36 kDa were found in all (Figure 3.9). Due to the hydroscopic nature of the freeze dried flocs the amount of protein loaded onto the gels is uncertain, thus the lanes can not be quantitatively compared for protein content. Since all juices have all proteins of interest, then clarification or stable cloud is related to other factors such as electrostatic interactions i.e. charge repulsion at pH 7 and charge attraction at pH 4.

These results are consistent with Yamasaki and others (1964, 1967) studies with apple juice where adjusting the juice to pH 7 retards clarification even though the enzyme is at its optimal pH and bringing the pH back to its natural pH after being at pH 7 speeds the process of clarification. This suggests that PME is active at pH 7 and is deesterifying the methoxyl ester groups along the pectin chain, but 1) clarification is slowed due to charge repulsion of pectin and proteins, which presumably both have net negative charges at this pH and/or 2) the net negative charge of pectin is masked preventing crosslinking with cations. However, when the pH is reduced back to its natural pH the negative charge on the pectin is exposed and/or the net charge of the proteins become positive allowing cross-linking to occur forming Ca-pectate.

#### Conclusion

Floc in the UCS forms at the same time as clarification in juice as measured by particle size and transmittance. Thus, soluble factors, probably PME, are involved in clarification. Proteins at 13, 27 and 36 kDa presumptively influence not only clarification of juice, but also floc formation of soluble cloud components. In addition to clarification by precipitation of colloidal cloud constituents with insoluble calcium

pectate, these results support the role of non-PME protein and clarification by another mechanism. These results mirror the electrostatic phenomenon seen in apple juice during pH adjustment. The results are consistent with the displacement of a protective colloid and aggregation of soluble constituents.

Day	PFJ	UCS
0	$22.8 \pm 2.0$	$27.8 \pm 2.2$
1	17.9 ± 1.7	20.1 ± 2.1
2	$13.9 \pm 4.5$	14.5 ±1.5
6	7.1 ± 3.3	5.7 ± 2.3

Table 3.1: Change in %DE of ultracentrifuged serum (UCS) and pulp free juice (PFJ) during storage at 4°C.



Figure 3.1: Pulp free juice, PFJ (Left) and Ultracentrifuged serum, UCS (Right) at natural pH 4.0, day 15.



Figure 3.2: Average (three replications) % transmittance at 650 nm overtime of pulp free juice (PFJ) and ultracentrifuged serum (UCS) at natural pH 4.0. Standard deviation between replications <10%.



Figure 3.3: Pulp free juice (PFJ) particle size distribution during storage at 4°C. Percent transmittance at 650 nm given in legend.



Figure 3.4: SDS-PAGE of ultracentrifuged pellet (UCP) and ultracentrifuged floc (UCF). Lanes 1 and 8, molecular weight standards (Amersham Pharmacia Biotech), lanes 2-4 UCF, lanes 5-7 UCP. Protein not loaded at the same concentration.



Figure 3.5: Average (two replications) % Transmittance at 650 nm overtime of pulp free juice at pH 4 (PFJ4), pH 7 (PFJ7), and pH 474 (PFJ474), and ultracentrifuged serum at pH 4 (UCS4), ph 7 (UCS7) and pH 474 (UCS474). Standard deviation between replications <10% except for PFJ7, which had a standard deviation of 16%.



Figure 3.6: Particle size distribution of pulp free juice at natural pH 4.0 (PFJ4) during storage at 4°C. Percent transmittance at 650 nm values given in the legend.



Figure 3.7: Particle size distribution of pulp free juice at pH 7 (PFJ7) during storage at 4°C. Percent transmittance at 650 nm values given in legend.



Figure 3.8: Particle size distribution of pulp free juice at pH 474 (PFJ474) during storage at 4°C. Percent transmittance at 650 nm values given in legend.



Figure 3.9: SDS-PAGE of ultracentrifuged floc (UCF) at pH 4, 7 and 474 [UCF4 (lane 2), UCF7 (lane 3), UCF474 (lane 4)] and ultracentrifuged pellet (UCP) at pH 4, 7 and 474 [UCP4 (lane 5), UCP7 (lane 6), and UCP474 (lane 7)]. Lanes 1 and 8, molecular weight standards (Amersham Pharmacia Biotech). Proteins not loaded at same concentration.

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CHAPTER 5

CONCLUSION

Citrus cloud has complex requirements for stabilization and clarification. A definite understanding of the clarification process has not been achieved due to the structural complexities of orange juice pectin, the non-random action of PME, PME isozymes and the uncertainties of the exact nature of calcium pectate and cloud interaction. Understanding the parameters involved in orange juice clarification will enable the use of novel technologies to stabilize cloud without producing adverse sensory qualities or violating standards of identity for citrus juice.

The objective of this thesis was to investigate the mechanism of orange juice clarification. Pectinmethylesterase (PME) is typically credited with the destabilization of orange juice cloud. PME initiates a sequence of events by partially de-esterifying (demethylating) the C6 methoxyl ester groups of soluble pectin ( $\alpha$  1,4 – polygalacturonic acid) contained in the juice serum (Stevens and others 1950). PME cleaves these methoxyl esters, yielding methanol and the carboxylic acid, eventually turning high methoxyl (HM) pectin into calcium sensitive low methoxyl (LM) pectin. Once a critical degree of esterification (DE) is obtained, divalent cations such as calcium can cross-link these free acid units to free acid units on adjacent pectin molecules, forming insoluble calcium pectates. Cross-linking increases the pectin apparent molecular weight, which reduces solubility, thereby leading to flocculation. Precipitation of pectins in this manner was presumed to occlude cloud particles and remove them from suspension (Stevens and others 1950), however, it is not clear how insoluble cloud constituents and particles become involved with the pectate gel complex in relation to the clarification process.

The results of the studies presented in this manuscript support the theory that TL-PME is a factor in orange juice clarification (Cameron and others 1998). Furthermore, it

was shown that PME with the highest specific activity (units of enzyme per mg of protein) does not necessarily clarify juice at the fastest rate. Juices that clarified the fastest all contained a 36 kDa and a 27 kDa peptide and juices that did not clarify contained a 36 kDa and a 13 kDa peptide. This suggests that PME complexes with pectin and/or low-molecular weight protein influence the ability of PME to induce clarification. Cloud particle size distribution during clarification increased in agreement with previous studies (Corredig and others 2001). This suggests that the cloud particles themselves change during clarification are not just removed from a stable dispersion by Ca-pectate. Floc formation in ultracentrifuged juice serum was observed slightly before or at the same time as the gross onset of clarification in pulp free juice as measured by % transmittance at 650 nm at all pH values when clarification occurred. This supports Baker and Bruemmer's theory (1969) that certain serum-soluble factors are required to destabilize the cloud colloidal system. The floc was also found to contain the same major peptides (13, 20, 27, and 36 kDa) as in pulp free juice for all pHs examined. Clarification of unstabilized, pulp free juice adjusted to pH 7 was retarded or did not occur in 18 days of storage. Clarification of unstabilized pulp free juice adjusted to pH 7 for one hour and then readjusted to natural pH underwent rapid clarification as compared to the control juice, which remained at natural pH. These results mirror the electrostatic phenomenon seen in apple juice when pH is adjusted (Yamasaki and other 1964,1967) and support the role of electrostatic destabilization of cloud.

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