Multiple Forms of Pectinmethylesterase from Citrus Peel and Their Effects on Juice Cloud Stability

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ABSTRACT

Heparin chromatography of a dialysis supernatant fraction from total salt extractable proteins of *Citrus sinensis* (L.) Osb. var. Valencia fruit peel (flavedo plus albedo) resolved four peaks of pectinmethylesterase activity (PME 1–4). One of these was thermally tolerant (PME 3). Binding to concanavalin A suggested PME 3 was a glycoprotein. At 30°C and 5 U · mL^{-1} PME 1, 2 and 3 destabilized the cloud of pasteurized FCOJ within 10 days. PME 1 caused the most rapid cloud loss (3d), followed by PME 3 and PME 2. PME 4 had no effect on juice cloud during this time period. At 4°C PME 3 caused the most rapid cloud loss (3d), followed by PME 1 (14d) and PME 2 (>14d). PME 4 had no effect on juice cloud stability after 10 days at 30°C or 14 days at 4°C.

Key Words: pectinmethylesterase, pectinesterase, citrus, juice cloud, Valencia

INTRODUCTION

ENZYMATIC PRECIPITATION OF CLOUD IN CITRUS JUICES IS THE consequence of a series of events initiated by pectinmethylesterase (PME; EC 3.1.1.11) (Joslyn and Sedky, 1940; Stevens et al., 1950; Guyer et al., 1956; Cameron et al., 1996). Sequential cleavage of the C6 methyl esters of galacturonic acid residues in pectin produces a distribution of "blocks" of free acid groups. When of sufficient size such blocks on adjacent pectin molecules can be cross-linked by divalent cations, leading to precipitation of the pectins. In the presence of juice cloud and adequate serum calcium ions, this precipitation entrains and removes the cloud particulates (Stevens et al., 1950; Joslyn and Pilnik, 1961; Baker and Bruemmer, 1969, 1972). The result is an unattractive, largely flavorless clear serum deficient in sensory properties. As juice is concentrated, levels of demethylated pectins may become sufficient to cause gelation. When this occurs, cross-linked pectins form a three-dimensional network immobilizing the aqueous component (Oakenfull, 1987). Gelation in juice concentrates may vary from slight curdiness to firm gels, which are unsightly and can hinder reconstitution. Formation of such gels during bulk storage of refrigerated concentrate can interfere with pumping and blending of the product.

Rapid precipitation (<6 wk) at ≤ 5°C has only been reported when pasteurized orange juice was amended with either a purified, thermally stable PME (TS-PME; Versteeg et al., 1980) or citrus fruit tissue extracts. In the extracts thermally labile PME's (TL-PME) had been inactivated so only TS-PME activity was present (Cameron et al., 1996). Versteeg et al. (1980) suggested that TS-PME was the problematic form of PME that led to juice cloud precipitation and concentrate gelation in non- or under-pasteurized orange juice products. Cameron et al. (1996) demonstrated that TS-PME's present in extracts from various tissues of Valencia oranges destabilized juice cloud at variable rates, dependent upon the tissue source of the extract. These results suggested that different fruit tissues may contain different forms of TL- and TS-PME and that some forms of TL-PME may also cause problems in regards to juice cloud stability.

Previous studies have demonstrated multiple forms of PME in various citrus fruits (Versteeg et al., 1980; Seymour et al., 1991; Macdonald et al., 1993, 1994; Cameron et al., 1994; Cameron and Grohmann, 1995, 1996). Results from Cameron et al. (1996) suggested that the peel (flavedo plus albedo) of orange fruit contained multiple PME forms, both TS- and TL-PME. Our objective was to estimate the number of PME forms present in the dialysis supernatant fraction of Valencia fruit peel tissue extracts and to determine any effects of each PME form on juice cloud stability.

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MATERIALS & METHODS

Extract preparation

Source material for chromatographic separation of multiple PME forms was *Citrus sinensis* var. Valencia fruit peel tissue dialysis supernatant (Cameron et al., 1996). Valencia oranges were halved and the juice was expressed with a kitchen-type juicer. Peel tissue flavedo plus albedo was separated from rag, ground with a meat grinder and frozen at −20°C until needed. The ground tissue was mixed with three volumes (w/v) of 0.1M Tris, pH 8.0 at 27°C, 1M NaCl, 0.02% NaN_3 (w/v) (unless indicated all reagents from Sigma Chemical Co., St. Louis, MO) and homogenized (~5 min) in a blender at 4°C. The suspension was stirred overnight at 4°C, filtered through two layers of cheesecloth, and the filtrate stored at 4°C. The ground peel tissue was re-extracted with three volumes of buffer, homogenized with a Polytron (Brinkman) (~3 min), and stirred overnight at 4°C. After cheesecloth filtration, the filtrate was pooled with filtrate from the first extraction and centrifuged at 12,100 × *g* for 30 min at 4°C. Peel oil was aspirated from the top of the centrifuged bottles and discarded. Proteins in the pooled supernatant extracts were precipitated with ammonium sulfate at 75% saturation and stirred overnight at 4°C. Centrifugation at 12,100 × g for 30 min at 4°C pelletized the precipitated proteins. The supernatant (no PME activity) was discarded and the protein pellets were solubilized in 10 mM Tris, pH 7.5 (at 31°C), 20 mM NaCl, 0.02% NaN_3 (w/v) (TBS) and dialyzed (6000 dalton molecular weight cut-off) overnight at 4°C against 4 L TBS with a total of four buffer changes. During dialysis a precipitate formed within the tubing which was separated from the dialysis supernatant (DS) by centrifugation at 12,100 × *g* for 30 min at 4°C. The pooled peel DS was then concentrated with a 10,000 dalton molecular weight cut-off hollow fiber cartridge (A/G Technology). The concentrated peel DS was then frozen and stored at −75°C in 50 mL aliquots.

Chromatographic separation

Anion exchange (DEAE Sephacel, Sigma) and affinity (Heparin Sepharose CL-6B, Pharmacia LKB; Concanavalin A-Agarose, Sigma) chromatography were used to separate the multiple forms of PME present in the peel DS. All columns were attached to an FPLC (Pharmacia LKB) system and the eluents were monitored at 280 nm. Chromatography was carried out at room temperature (≈23°C) and fractions were placed at 4°C immediately after collection.

Anion exchange chromatography. Peel DS (10 mL) was loaded onto the DEAE Sephacel column (2.5 × 10.5 cm), which was washed

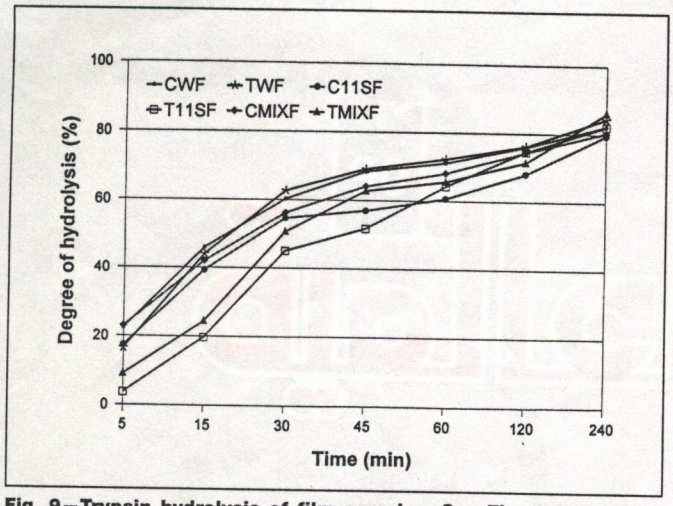

Fig. 9—Trypsin hydrolysis of film samples. See Fig. 1 for sample codes.

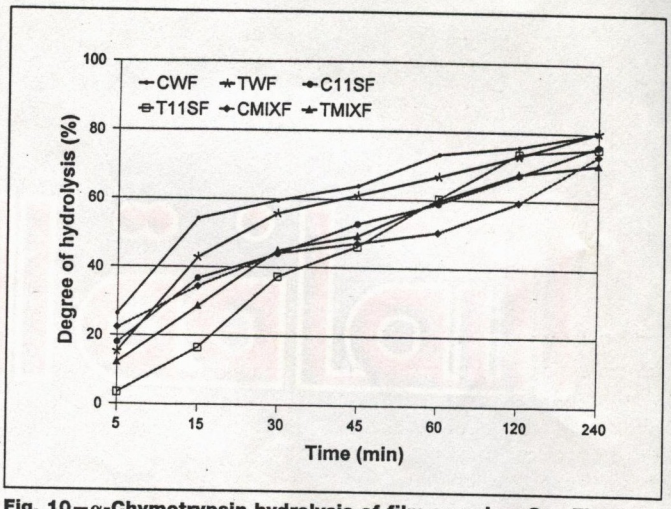

Fig. 10—α-Chymotrypsin hydrolysis of film samples. See Fig. 1 for sample codes.

molecules around each other could produce additional pores or cause enlargement of existing pores.

Mechanical properties

Mechanical properties of protein films provide an indication of expected film integrity under conditions of stress that would occur during processing, handling, and storage. When tensile strengths (TS) were compared (Table 1), no significant differences were found among control films ($P>0.05$). However, tensile strength of TG cross-linked samples were greater than those of controls ($P<0.05$). Tensile strength of T11SF (16.41 MPa) was similar to that of TMIXF (17.86 MPa), but the two samples had higher TS values than TWF (12.53 MPa) ($P<0.05$). The average tensile strength values of TG cross-linked films were more than 2 times greater than those of the homologous controls. Introduction of covalent iso-peptide bonds into the protein structure apparently increased the strength of films. Similar results had been reported for α-lactalbumin and β-lactoglobulin by Mahmoud and Savello (1993) and for α_{s1}-casein by Motoki et al. (1987).

The puncture strength (PS) values of film samples showed similar trends to those for tensile strength (Table 1). No differences were found in PS values of control films ($P>0.05$) while PS values of TG cross-linked samples were greater than those for controls ($P<0.05$). Although the highest absolute puncture strength was obtained for TMIXF, there were no differences among PS values for TG cross-linked samples ($P>0.05$).

CONCLUSIONS

THE CROSS-LINKING OF WHEY PROTEINS AND 11S GLOBULIN by transglutaminase can be used to produce films with effective mechanical properties. The protein polymer network of films was resistant to solubilization in aqueous buffers at various pH, but it was protease-digestible. Average tensile strength of TG cross-linked films were more than two times greater than those of homologous controls. Effectiveness of TG cross-linked films as an edible film or food-coating material would depend on the acidity and the proteolytic activity of the coated surface. TG cross-linked films could increase mechanical integrity and handling characteristics of food products. TG cross-linked films could find application as a wrapping to prevent quality changes in products such as meat pies and high-moisture low-sugar cakes, that require films highly permeable to water vapor. Incorporation of colorant, antioxidants and flavoring agents in TG cross-linked films could improve other quality characteristics of food products.

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with TBS (130 mL) followed by 35 mL of TBS plus 5% of the elution buffer containing 10 mM Tris, pH 7.5 (31°C), 1M NaCl (addition of 5% elution buffer gave a concentration of 25 mM NaCl). The remaining bound proteins were eluted with 100% elution buffer. The flow rate was 1 mL · min^{-1} and 2 mL fractions were collected throughout the run. Every third fraction was qualitatively assayed for PME activity (Cameron and Grohmann, 1995). Fractions containing the majority of PME activity were pooled and concentrated (Amicon, Centricon -30, 30 kDa molecular weight cut-off membranes). Seven runs were made of the peel DS (10 mL per run) on the DEAE Sephacel column. Fractions containing the majority of PME activity were pooled for each run, concentrated as described and frozen separately at −75°C.

Heparin chromatography. Pooled material from individual DEAE Sephacel chromatography runs was loaded in TBS (10 mL) onto the Heparin Sepharose CL-6B column (1 × 20.5 cm) at 0.5 mL · min^{-1}, and washed with 30 mL TBS to remove unbound proteins. Bound proteins were eluted with a linear gradient from 0.02 to 0.32M NaCl in TBS (100 mL total volume) followed by an increase to 1M NaCl in TBS over 5 mL and then 10 mL of 1M NaCl in TBS. Fractions (1.5 mL) were collected throughout the run. Every second fraction was assayed for PME activity (Cameron and Grohmann, 1995). Fractions contained within individual activity peaks were pooled, concentrated and frozen at −75°C as described. Each run produced four peaks of PME activity. Identical peaks from separate runs were pooled, concentrated, buffer changed to TBS (Amicon, Centricon 30) and reapplied to the Heparin Sepharose CL-6B column. Run conditions and fraction collection were identical to those described. Every second fraction was qualitatively assayed for PME activity and fractions within the activity peaks were pooled, concentrated, buffer changed to TBS and frozen at −75°C.

Concanavalin A chromatography. The one TS-PME peak from Heparin Chromatography was applied to a concanavalin A-agarose column (1 × 19 cm) at 0.5 mL · min^{-1} after the buffer had been changed to 20 mM Tris (pH 7.5 at 31°C), 0.5M NaCl, 1 mM $MgCl_2$, 0.02% NaN_3 (w/v) (Buffer A). The column was washed with 25 mL Buffer A and bound proteins were eluted with a linear gradient of 0-50 mM methyl-α-D-glucopyranoside in buffer A over 62.5 mL. Fractions (1 mL) were collected and assayed for TS-PME activity. The fractions contained within the activity peak were pooled, concentrated, buffer changed to TBS and frozen at −75°C.

Enzyme assays

Enzyme activity in column fractions was qualitatively screened for PME and TS-PME activity and quantitative assays were conducted according to Cameron and Grohmann (1996) using a kinetic microplate reader (Cameron et al., 1992). Each reported value is the mean of a minimum of three replicates. One unit of activity equals one micro equivalent of acid released/min/μL.

Juice cloud stability

Pasteurized frozen concentrated orange juice (FCOJ) was obtained from a local processor (Citrus World, Lake Wales, FL) and reconstituted to 11 °Brix with deionized water plus partially purified PME (5 U · mL^{-1}). The reconstituted juice also contained 0.02% sodium azide and 4.35 g · L^{-1} potassium metabisulphite.

After PME addition, juice samples were placed in glass bottles and incubated at either 30°C or 4°C. At selected times triplicate samples (10 mL · replicate^{-1}) were pipetted into 15 mL graduated, conical centrifuge tubes from each treatment after inverting the glass bottle three times. The replicates were centrifuged for 10 min at 360 × *g*, supernatant (1 mL of each replicate) was transferred to a cuvette and absorbance at 660 nm was recorded (Krop, 1974). Settling pulp was estimated by measuring the volume of pelleted solids. Means ± standard errors (P=0.05) are reported for three replicates. TBS and pasteurized FCOJ controls were analyzed for cloud stability at room temperature. At 4°C only the pasteurized FCOJ control was analyzed.

RESULTS

Anion exchange chromatography

The majority of PME activity in the peel DS did not bind to the DEAE column under our conditions (data not shown). A single large peak of PME activity eluted between 8 and 40 min, with an activity tail between 42 and 80 min. A slight increase in eluted activity occurred with 5% elution buffer but was likely the tail of the unbound activity peak.

Heparin chromatography

Four peaks of activity were observed when the DEAE unbound PME was eluted from the heparin column (Fig. 1). Peak 1 (= PME 1; Fig. 1) did not bind to the heparin matrix, even after its fractions were pooled and reapplied to the same column. Peak 2 (= PME 2; Fig. 1) eluted between 1.5%-6.5% elution buffer (≅17-67 mM NaCl). Peak 3 (= PME 3 = TS-PME; Fig. 1) eluted between 8.4%-15.8% elution buffer (≅86-160 mM NaCl) and peak 4 (= PME 4; Fig. 1) eluted between 15.8%-22% elution buffer (160-222 mM NaCl). Only PME 3 showed TS-PME activity. Fractions from multiple runs contained within peaks associated with PME 1, 2 or 4 were rechromatographed on the heparin column and the peak of activity corresponding to each

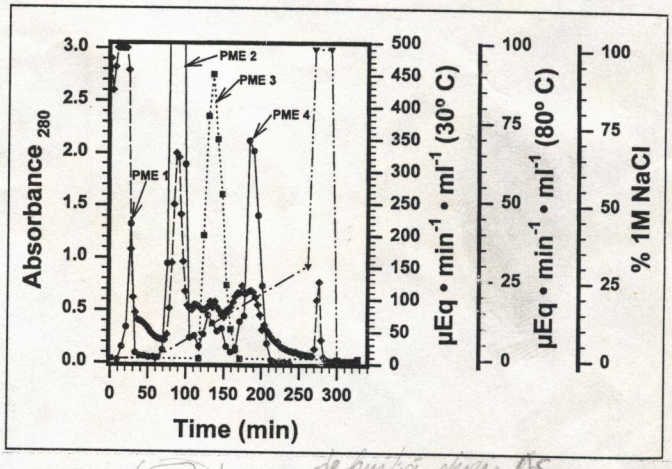

Fig. 1—Heparin chromatogram of Valencia fruit peel DS. ◇ = Absorbance at 280 nm; ∇ = % 1M NaCl; ○ = μEq · min^{-1} · mL^{-1} at 30 °C; □ = Eq · min^{-1} · mL^{-1} after a 2 min incubation in an 80°C water bath and then assayed at 30°C.

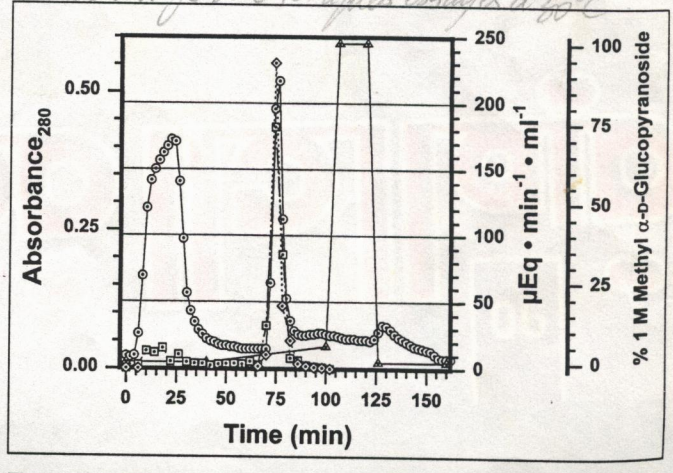

Fig. 2—Concanavalin A chromatogram of PME 3 from heparin chromatography. ○ = Absorbance at 280 nm; △ = % 1M Methyl-α-D-Glucopyranoside; □ = μEq · min^{-1} · mL^{-1} at 30°C; ◇ = μEq · min^{-1} · mL^{-1} after a 2 min incubation in an 80°C water bath and then assayed at 30°C.

PME form was concentrated and added to pasteurized FCOJ for determination of effects on juice cloud stability. Based on the weight of cut-out activity peaks (Fig. 1, 30°C activity axis was expanded to contain the entire PME activity peak) the contribution to total PME activity was: PME 1=6.0%, PME 2=63.1%, PME 3=7.9% and PME 4=22.9%.

Concanavalin A chromatography

Elution of PME 3 from a concanavalin A column resulted in two peaks of activity (Fig. 2). One did not bind to the matrix, contained no TS-PME activity and was not included in subsequent studies on cloud stability. The other eluted between 1.4%-2.4% elution buffer (≅14-24 mM methyl-α-D-glucopyranoside), contained TS-PME activity and was used to amend pasteurized FCOJ for determination of its effect on juice cloud stability.

Effects on juice cloud stability

At 30°C PME 1 caused the most rapid cloud loss (Fig. 3). At the Day 3 sampling PME 1 (TL-PME) had completely precipitated the juice cloud. PME 2 (TL-PME) and PME 3 (TS-PME) had nearly equivalent effects on juice cloud, but acted more slowly than PME 1 (Fig. 3). At Day 5 PME 3 had reduced the A_{660} to 0.41±0.03 while PME 2 reduced the A_{660} to 0.64±0.0. At Day 10, the A_{660} of juice treated with PME 3 was 0.11±0.01, while that of juice treated with PME 2 was 0.25±0.003. Both controls and juice treated with PME 4 had maintained an A_{660} of 2.28±0.00 throughout the sampling period. Changes in settling pulp coincided with decreases in A_{660} for juices treated with PME 1, 2 and 3.

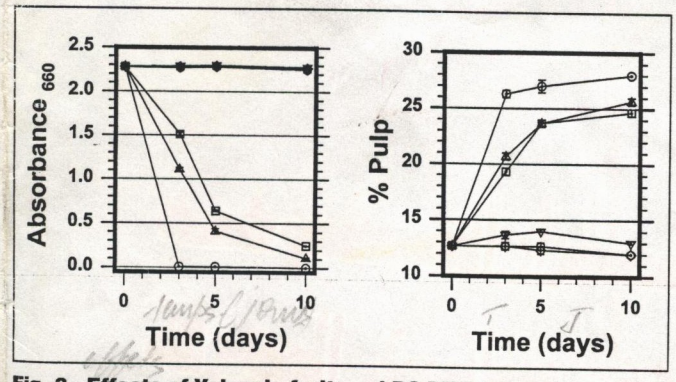

Fig. 3—Effects of Valencia fruit peel DS PME 1-4 on juice cloud stability at 30°C. Absorbance was measured at 660 nm and % pulp = settling pulp. ○ = PME 1; □ = PME 2; △ = PME 3; ▽ = PME 4; ◇ = Control 1 (pasteurized FCOJ only); ⬡ = Control 2 (pasteurized FCOJ plus a volume of TBS equal to the largest volume used for PME sample spiking). Absence of an error bar indicates S.E. was contained within the symbol.

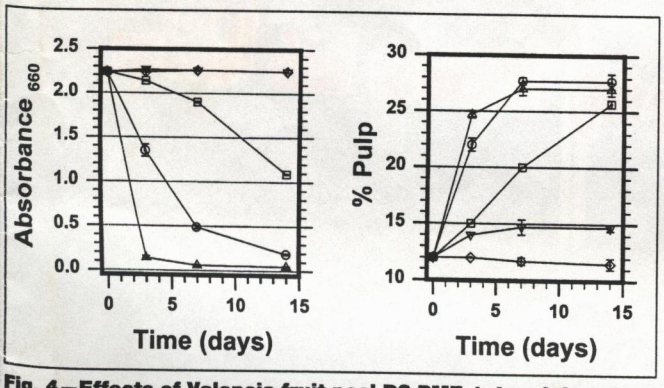

Fig. 4—Effects of Valencia fruit peel DS PME 1-4 on juice cloud stability at 4°C. Measurements, symbols and error bars as in Fig. 3. No buffer control was used.

A 4°C the most rapid cloud loss was measured in the PME 3 (TS-PME) sample (Fig. 4). The cloud was destabilized by PME 3 at 4°C more rapidly than at 30°C. PME 1 (TL-PME) also destabilized the juice cloud at 4°C, although more slowly than at 30°C. PME 2 (TL-PME) reduced the A_{660} of juice from 2.24±0.003 at Day 0 to 1.09±0.006 at Day 14. The increase in settling pulp to 25.7%±0.33% at Day 14 in the PME 2 sample (Fig. 4) suggests that this PME also would precipitate juice cloud. PME 4 had no effect on juice A_{660} at 4°C although settling pulp had increased from 12.0%±0.0% to 14.7%±0.33% at Day 14.

DISCUSSION

A combination of anion exchange and affinity chromatography demonstrated the occurrence of four forms of PME in peel DS. Only one of these was a TS-PME (PME 3). Three of the four (PME 1, 2 and 3) destabilized orange juice cloud within 10 days at 30°C. Juice cloud also was rapidly destabilized (<2 wk) at 4°C by PME 1 and 3. PME 2 destabilized the juice cloud at 4°C but not as rapidly as PME 1 or 3. The increase in settling pulp in samples with PME 2 added suggests that it too may have clarified the juice with longer incubations. PME 4 did not precipitate juice cloud at either 30°C or 4°C.

Results of heparin chromatography of the Valencia peel DS were very similar to those obtained for red grapefruit finisher pulp DS (RG DS; Cameron and Grohmann, 1995) which also contained four chromatographic forms of PME. However, the relative amount of PME 4 activity, compared to PME 3, appeared to be greater in Valencia peel DS than RG DS. In both RG DS and Valencia peel DS only PME 3 possessed TS-PME activity and, like the RG DS PME 3, the Valencia peel DS PME 3 was apparently glycosylated since it bound to concanavalin A.

Comparison of our results to those of Versteeg et al. (1980) is not reliable since Versteeg et al. (1980) inoculated their samples with only 1 U· mL^{-1} while we used 5 U· mL^{-1}. Additionally, our initial A_{660} was 2.24-2.28 while Versteeg et al. (1980) reported an initial A_{660} of only ~ 1.6. Given the differences in amounts of enzyme activity used in our study it is possible that our PME 1 and Pectinesterase 1 (Versteeg et al., 1980) may be the same form of PME. The more rapid juice cloud destabilization due to PME 1 we found (7-14 d) compared to Versteeg et al.'s (1980) Pectinesterase 1 (30-37 d) would likely be due to the greater activity levels we used.

The differential effects of PME 1-4 on juice cloud stability suggest the different forms may have different modes of action on the pectin substrate. Differences in salt effects, kinetics, pI and pH optima have been reported for multiple forms of PME from tomato (Warrilow and Jones, 1995).

The rapid cloud loss associated with PME 1-3 at 30°C and PME 1 and 3 at 4°C indicates that both contribute to the quality defect of cloud loss in citrus juices. Since peel DS extract resulted in the most rapid cloud destabilization (Cameron et al., 1996) and peel DS contains both a TL- and TS-PME capable of rapidly clarifying citrus juice it may be advantageous in citrus juice processing to minimize the amount of peel PME's in products.

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