Variable Heat Stability for Multiple Forms of Pectin Methylesterase from Citrus Tissue Culture Cells

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Six forms of pectin methylesterase from *Citrus sinensis* var. Valencia juice sac derived tissue culture cells have been partially purified by anion-exchange and affinity (heparin and concanavalin A) chromatography. Data on thermal stability and thermal denaturation are provided. One of these forms (PME 3b) retained 29% activity after being incubated at 95 °C for 30 s. Two other forms also retained activity at 80–90 °C. The most active form, accounting for 93.6% of total activity at 30 °C, was completely inactivated after 2 min of incubation at 80 °C. The most heat stable form, PME 3b, has a K_m of 0.0309 mg mL^{-1} and a V_{max} of 1.4596 μequiv min^{-1}. PME 3b has a pH optimum of 7.0 and is glycosylated. Denaturing electrophoresis and native gel filtration chromatography suggest molecular weights between 34 000 and 47 000 for the partially purified PMEs. The molecular weight of PME 3b estimated by LDS–PAGE was 37 500 and that estimated by gel filtration chromatography was 40 600.

INTRODUCTION

Pectin methylesterase (PME; EC 3.1.1.11), a cell wall enzyme, catalyzes the demethylation of pectin. Its technological significance is well documented (Pilnik and Voragen, 1991; Rombouts and Pilnik, 1978), and more recently, its biological significance has received increased attention (Nari *et al.*, 1991; Tieman *et al.*, 1992). The enzyme is apparently ubiquitous in angiosperms as it has been found in every plant tissue assayed (Versteeg, 1979; Pilnik and Voragen, 1991). In fact, multiple forms of PME have been reported for several species (Versteeg, 1979; Pressey and Avants, 1972; Giovane *et al.*, 1990). Up to 12 forms of PME have been suggested to occur in citrus (Pilnik and Voragen, 1991).

Variability in heat stability of PME isoforms is important in many food-processing industries (Pilnik and Voragen, 1991) and is especially so for juice cloud maintenance in citrus juice products (Wenzel *et al.*, 1951; Rothschild and Karsenty, 1974; Versteeg *et al.*, 1980). The cloudy material present in citrus juices is composed of several components (Shomer, 1991). In the presence of active PME pectin is demethylated, allowing formation of pectate gels which subsequently precipitate, leaving a clear supernatant liquid (Wenzel *et al.*, 1951; Versteeg *et al.*, 1980; Shomer, 1991). To overcome this quality defect, citrus juices must be pasteurized at temperatures greater than that needed to control microbial growth to inactivate the heat-stable PME. Although Guyer *et al.* (1956) reported PME activity after heating frozen citrus concentrate to 99 °C, the authors dismissed the possibility of a heat-stable PME to explain their observation on juice cloud stability. Versteeg (1979) was the first to report a form of PME, from navel oranges, with unusually high heat stability. Versteeg *et al.* (1980) provided data showing that at 5 °C citrus juice cloud loss was due to the heat-stable PME and not to other heat-sensitive forms. Seymour *et al.* (1991a) and Rillo *et al.* (1992) presented temperature curves for PMEs from marsh grapefruit and mandarin oranges, respectively, that retained activity at 85 °C. Inactivation temperatures for other PMEs are generally much lower (Manabe, 1973; Versteeg *et al.*, 1980; Puri *et al.*, 1982; Seymour *et al.*, 1991a).

The intent of this study was to (1) determine if a heat-stable PME was present in a tissue culture cell line derived from Valencia fruit vesicles and (2) estimate how many PME isoforms were present in the cell line. Our decision to utilize tissue culture cells was motivated by our ultimate goal, which is to develop an understanding of the genetic regulation of heat-stable PMEs in citrus and to provide a defined system for genetic manipulation of PME.

MATERIALS AND METHODS

Tissue Culture. A nonembryogenic cell line (Val88-1) was developed from immature fruit vesicles of *Citrus sinensis* (L.) Osbeck cv. Valencia. An immature fruit was collected 6-weeks postpollination in the spring of 1988 in Orlando, FL. The vesicles were removed and cultured on Murashige and Tucker's (1969) basal medium supplemented with 1 μM 6-benzylaminopurine, 2.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), and 100 mg L^{-1} casein hydrolysate to induce callus. The vesicle callus cultures were subcultured every 21 days and grown in a growth cabinet under low light (15–20 microeinsteins m^{-2} s^{-1}), provided by cool-white fluorescent lamps, constant 27 °C, and 4-h photoperiod. After 8 months of selection, a rapidly growing, slightly friable callus (Val88-1) was obtained. For maintenance, Val88-1 was transferred to the same culture medium but with the 2,4-D reduced to 1 μM and the subculture period increased to 28 days.

Tissue Preparation. Three-week-old tissue culture cells were harvested, weighed, and frozen in liquid nitrogen. The cells were stored at −75 °C until needed. Tissue cell homogenization was accomplished by adding 2 volumes (w/v) of cold (4 °C) deionized water to frozen cells in a commercial blender. After homogenization, the resulting slurry was centrifuged at approximately 20000*g* for 30 min at 4 °C. The supernatant liquid (total soluble protein, TSP) was collected and qualitatively assayed for PME activity. The supernatant liquid (positive for PME activity) was then brought to 35% saturation with solid ammonium sulfate and stirred for 2 h at 6 °C. This suspension was centrifuged (20000*g*, 30 min, 4 °C), the pellet was discarded (no PME activity), and the supernatant liquid was brought to 75% ammonium sulfate saturation and stirred overnight at 6 °C. Following centrifugation (20000*g*, 30 min, 4 °C) the supernatant liquid, containing no PME activity, was discarded and the pellets were solubilized in a minimal volume of a buffer containing 10 mM TrisCl, pH 7.5

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at 27 °C, and 20 mM NaCl (TBS). The solubilized ammonium sulfate pellets were dialyzed against two changes of 1.5 L of TBS at 6 °C overnight and then frozen in 10-mL aliquots at −75 °C until needed. Since no protease inhibitors were included during this extraction, the dialyzed preparation was tested for protease activity with a protease test kit (Bio-Rad) and by incubating a commercial citrus peel PME (Sigma) with the dialyzed TSP for 1, 3, 6, 24, and 48 h at 4 °C. The commercial PME also was incubated with alkaline protease (Promega) for 1 h as a positive control. These samples were precipitated at −20 °C with 90% acetone and then subjected to LDS–PAGE and stained with Coomassie Brilliant Blue R to visually determine if the commercial PME underwent protease degradation during the incubation.

The pellets (cell wall material) from the initial homogenization were resuspended in 1 L of extraction buffer [100 mM Tris, pH 8.0 at 28 °C, 1 M NaCl, pH 8.0 at 28 °C, 1 M NaCl, 10% (v/v) glycerol, 2% (w/v) polyvinylpolypyrrolidone, 5 mM EDTA, 5 mM DTT, 5 mM leupeptin, and 50 μg mL^{-1} (w/v) N^{α}-p-tosyl-L-lysine chloromethyl ketone]. After stirring for 1 h at 6 °C, the suspension was centrifuged as described above. The supernatant liquid (salt-extracted protein, SEP) was pooled and brought to 35% saturation with solid ammonium sulfate and stirred for 2 h at 6 °C. After centrifugation (20000*g*, 30 min, 4 °C), the supernatant was pooled and brought to 75% ammonium sulfate saturation and stirred overnight at 6 °C. This suspension was centrifuged as described above and the supernatant liquid discarded. The pellets were solubilized in a minimal volume of TBS and dialyzed as described above. During dialysis of the SEP a precipitate formed, so the dialyzed suspension was centrifuged (25000*g*, 15 min, 4 °C). The supernatant liquid (SEP DS) was collected and frozen in 10-mL aliqnots at −75 °C. The pellets (SEP DP), which contained PME activity, were not soluble in TBS but could be solubilized in 5 mL of 0.1 M sodium phosphate buffer containing 1 M NaCl. The SEP DP was frozen at −75 °C.

Chromatography. A combination of anion-exchange (DEAE-Sephacel, Sigma), affinity (heparin–Sepharose CL-6B, Pharmacia LKB; Econo-Pac heparin cartridge, Bio-Rad; and concanavalin A agarose, Sigma; Giovane *et al.*, 1990), and gel filtration (Superdex 75 HiLoad 16/60, Pharmacia LKB) chromatography was used to partially purify different forms of PME. The columns were attached to an FPLC (Pharmacia LKB) system, and the eluent was monitored at 280 nm. All chromatography was performed at room temperature, and individual fractions were refrigerated (4 °C) immediately after collection.

Anion-Exchange Chromatography. A 10-mL volume of either SEP or TSP was loaded onto the DEAE-Sephacel column (2.5 × 9 cm). After loading, the column was washed with 40 mL of TBS to remove unbound protein. Bound proteins were eluted with a 75-mL linear salt gradient from 0 to 1 M NaCl in TBS. The flow rate was 1 mL min^{-1}, and fractions of 1 mL were collected throughout the run. Fractions corresponding to UV-absorbing peaks were qualitatively assayed for PME activity. Fractions from individual peaks containing PME activity were pooled and concentrated (typically to less than 1 mL) at 10 °C with a Centricon microconcentrator (Amicon, 30-kDa cutoff membrane). The pooled and concentrated material was frozen at −75 °C.

Heparin Chromatography. Pooled and concentrated material from anion-exchange chromatography was diluted to 10 mL with TBS and loaded onto the heparin–Sepharose CL-6B column (1 × 19 cm) at 0.5 mL min^{-1}. After loading, the column was washed with 15 mL of TBS at 0.5 mL min^{-1} to remove unbound protein. Bound proteins were eluted with a 60-mL linear gradient from 0 to 0.3 M NaCl in TBS. Fractions of 1 mL were collected throughout the run. Fractions containing PME activity were treated as described above.

Pooled and concentrated samples from heparin–Sepharose CL-6B chromatography were diluted to 10 mL with TBS and then rechromatographed on a Econo-Pac heparin cartridge. Two different elution profiles were used. In the first, used on PME 1–4, the column was washed with 10 mL of TBS after loading to remove unbound material, followed by a 80-mL linear gradient of 0–0.2 M NaCl in TBS to elute bound proteins. Fraction size was 1 mL, and the flow rate was 1 mL min^{-1}. The second elution profile differed by utilizing a 90-mL linear gradient from 0 to 1.0 M NaCl in TBS following a 10-mL wash with TBS. Fraction size was 1 mL, and the flow rate was 1 mL min^{-1}.

Concanavalin A Chromatography. Pooled and concentrated material from heparin chromatography was loaded onto a concanavalin A–agarose column (Sigma, 1 × 22 cm) in a buffer containing 10 mM Tris, pH 7.5 at 27 °C, 0.5 M NaCl, 0.02% (w/v) NaN_3, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (buffer A). After washing with 25 mL of buffer A, bound proteins were eluted with a 25-mL linear gradient of 0–0.5 M glucose in buffer A. Fraction size was 1 mL, and the flow rate was 0.5 mL min^{-1}. Fractions were assayed qualitatively for PME activity, and active fractions were pooled, concentrated, and frozen at 75 °C.

Gel Filtration Chromatography. Two milliliters of SEP DP was loaded onto a Superdex 75 HiLoad 16/60 (Pharmacia LKB) gel filtration column. The column had been equilibrated in 0.1 M sodium phosphate buffer, pH 7.5, and 1 M NaCl. The flow rate was 1 mL min^{-1} and 1-mL fractions were collected during protein elution. Fractions were qualitatively assayed for PME activity. Active fractions were pooled and concentrated as described above and frozen at −75 °C.

For estimation of native molecular weight, samples from Econo-Pac heparin or concanavalin A chromatography were loaded onto the Superdex 75 HiLoad 16/60 column. The flow rate was 1 mL min^{-1}, and 1-mL fractions were collected. Column fractions were qualitatively assayed for PME activity. The column was calibrated with cytochrome *c*, carbonic anhydrase, BSA, and β-amylase (Sigma). The buffer contained 0.1 M sodium phosphate, pH 7.5, and 1 M NaCl.

Enzyme and Protein Assays. Column fractions were screened for PME activity at three temperatures, i.e., room temperature and after being incubated for 2 min at 70 and 80 °C, respectively. Enzyme activity was qualitatively determined by adding 25 μL from each fraction of interest to 175 μL of a reaction mixture containing approximately 0.5% pectin (68–73% degree of methylation and high polymerization, HP Bulmer Pectin Ltd., Hereford, England), 0.2 M NaCl, and 0.05% bromothymol blue. The pH of the reaction mixture was adjusted to give a blue to blue-green color (visual determination) upon addition of the column eluent. The time required for a color change to occur (blue to green to yellow) was recorded.

Quantitative assays were carried out according to the method of Cameron *et al.* (1992) using a kinetic microplate reader set at 30 °C [adapted from Hagerman and Austin (1986)]. Microplates were read every 30 s for 10 min with a 5-s plate shake immediately before each reading.

Temperature stability curves were determined by incubating samples in a constant-temperature water bath at 40, 50, 60, 70, 80, 85, 90, and 95 °C for 2 min. The heated samples were then assayed for activity as described above. The temperature at which the enzyme was inactivated was used for the determination of thermal denaturation curves. For determination of thermal denaturation curves centrifuge tubes (1.5 mL) were floated in a water bath set at the desired temperature for 5 min prior to addition of sample. Samples were heated for the appropriate time and then immediately placed on wet ice.

The effect of pH on the activity of the heat-stable PME (after Econo-Pac heparin) was estimated by continuous titration against 10 mM NaOH. The reaction mixture contained 0.35% pectin and 0.2 M NaCl. pH values used were 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, and 10.0. Activity estimates at pH 8.0, 8.5, 9.0, and 10.0 were corrected for chemical alkaline deesterification by subtracting the amount of base required to maintain the set pH with no enzyme added from the value obtained when enzyme was included. Protein quantities were estimated with a Micro BCA protein assay reagent kit (Pierce) using BSA as the standard.

Estimates of K_m and V_{max} for the heat-stable PME (after concanavalin A chromatography) were made using five different concentrations of pectin (0.01–0.3%). The reported values refer to initial activity.

Electrophoresis. Samples for electrophoresis were precipitated with a minimum of 10 volumes (v/v) of cold (−20 °C) 70% (v/v) acetone and placed at −20 °C for a minimum of 2 h. After centrifugation (13000*g*, 15 min, 4 °C), the acetone was decanted and the pellet was solubilized in a sample buffer containing 10% glycerol (v/v), 2.3% (w/v) lithium dodecyl sulfate (LDS), 62.5 mM Tris-HCl, pH 6.8 at 27 °C, and 5% (v/v) 2-mercaptoethanol

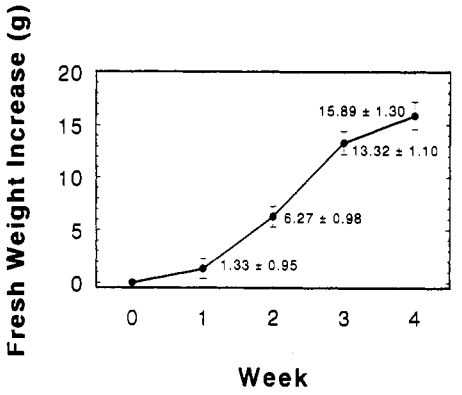

Figure 1. Growth curve for Val88-1 tissue culture cells.

for lithium dodecyl sulfate–polyacrylamide gel electrophoresis (LDS–PAGE). LDS–PAGE was run on 12.5% and 10% verticle slab gels (Mini-Protein II, Bio-Rad) according to the procedure of Laemmli (1970) and Mini-Protein II instructions. Gels were stained with Coomassie Brilliant Blue R (Sigma).

RESULTS

Tissue Preparation. The occurrence of PME activity in the TSP from 3-week-old tissue culture cells (Figure 1) was unexpected. Water extraction is used to separate water-soluble cytoplasmic proteins from ionically bound cell wall proteins (Fry, 1988). In preliminary trials with smaller amounts of tissue, no PME activity was observed in the water extract, but during scale-up PME activity was observed in the TSP. Therefore, we decided to include this material in our study. No protease activity was observed in the TSP by commercial protease test kits or incubation of a commercial PME with the TSP (data not shown). Incubating the commercial PME with a commercially obtained alkaline protease did result in degradation of the commercial PME.

Chromatography. *Gel Filtration Chromatography.* Two 280-nm peaks were present on the gel filtration chromatogram of the SEP DP (not shown). The first eluted with the void volume and contained no PME activity. The second peak, eluting between 90 and 120 min, contained PME activity. Prior to gel filtration, the PME in this material was not soluble in low-salt buffers; consequently, it could not be run on ion-exchange or heparin columns. However, after gel filtration, it could be concentrated and the buffer changed to TBS without precipitation of PME activity.

Anion-Exchange Chromatography. Similar chromatograms were obtained with DEAE-Sephacel chromatography of TSP and both SEP DS and SEP DP. PME activity was located in both flow-through and bound factions (Table 1).

Heparin Chromatography. Concentrated and pooled DEAE bound or unbound PMEs containing fractions from anion-exchange chromatography were next loaded onto the heparin–Sepharose CL-6B column. Several differences were evident between the chromatograms (Figure 2). PME activity from the unbound DEAE fractions had major peaks with activity in the flow-through material (PME 1) and that eluted between 75 and 95 min (PME 2, Table 1). Additionally, two minor peaks of activity were eluted at 110–130 min (PME 3, Table 1) and at 130–150 min (PME 4, Table 1). The peak that contained PME stable at 80 °C eluted at 110–130 min (PME 3, Table 1), while the peak at 130–150 min contained a PME stable at 70 °C but not at 80 °C. Heparin–Sepharose chromatography of the DEAE bound material resulted in a chromatogram having five major peaks (Figure 1B), all of which contained PME activity. The peak containing the 80 °C stable PME (PME 3, Table 1) eluted between 95 and 120 min, while the peak containing PME stable at 70 °C (PME 4, Table 1) eluted between 125 and 145 min. Peaks common to heparin–Sepharose chromatography of both DEAE bound and unbound material were pooled and rechromatographed on the Econo-Pac heparin cartridge.

PME 1, which did not bind to heparin–Sepharose, did not bind to the Econo-Pac heparin either. PME 2, a single peak on heparin–Sepharose, was separated into four major peaks by the Econo-Pac heparin cartridge. The majority of PME activity eluted between 10 and 20 min with less active material eluting between 20 and 30 min. The second peak containing PME activity has not been pursued further. PME 3 was separated into two peaks of activity on the Econo-Pac heparin cartridge, one of which did not bind to this matrix (PME 3a, Table 1) and the second of which eluted at 45–60 min (PME 3b, Table 1). PME 4 (Table 1) produced a single peak when rechromatographed on Econo-Pac heparin. Inspection of Table 1 indicates that the majority of PME activity stable at 70 °C is associated with PME 3b and that the dominant form at 30 °C was PME 4.

Concanavalin A Chromatography. PME 3b (from Econo-Pac heparin chromatography) activity binds to concanavalin A and could be eluted with a glucose gradient.

Thermal Characterization. *Temperature Curves.* The six different peaks of PME activity from Econo-Pac heparin chromatography produced six different temperature curves (Figures 3and 4). Three of these retained measurable levels of activity at 80 °C after 2-min incubations (PME 1, 3a, and 3b). PME 3b was not inactivated until 95 °C, and PME 1 had trace levels of activity up to 95 °C.

Thermal Denaturation Curves. PME 3b, the most stable form, retained measurable levels of activity after being incubated at 95 °C for 30 s. A 60-s incubation was sufficient to reduce activity to below measurable levels. However, by allowing the assay microplate to sit at room temperature after the 10-min activity assay (data not shown), lower, residual levels of activity could be observed. By 1.5 h after the assay, wells loaded with PME 3b incubated for 2.5 min at 95 °C had turned bright yellow, while standard curve wells (no PME added) were still a green-blue color, indicating the presence of active enzyme. PME 1 also had trace levels of activity after being incubated at 95 °C for 2 min. PME 3a was inactivated after a 30-s incubation at 85 °C. PME 5 was inactivated by a 60-s incubation at 90 °C.

Kinetics and Effects of pH on Heat-Stable PME. The K_m for PME 3b was 0.0309 mg mL^{-1} pectin and its V_{max} of 1.4596 μequiv min^{-1}. The pH optimum for PME 3b was 7.0 (Figure 5), and levels of activity were higher between pH 8.0 and 9.0 compared to that between 5.0 and 6.0.

Estimation of Molecular Weights. Denaturing electrophoresis (LDS–PAGE, Figure 6) and native gel filtration chromatography (Table 2) of the six partially purified PMEs suggest molecular weight differences (Table 2) among the various PME forms. With PME 1, 2, and 3b there is close agreement between gel filtration estimates of molecular weight and a densely stained band visualized by LDS–PAGE (Table 2; Figure 6). However, there is a wide disparity in the molecular weight estimates for PME 3a, 4, and 5. A lightly stained polypeptide band is present in these samples (Figure 6) but is also present in PME 1 and 2. Additionally, the gel filtration samples used for LDS–PAGE had been concentrated with a 30-kDa cutoff

Table 1. Purification Steps for PME from Valencia Juice Sac Derived Tissue Culture Cells[a]

sample	μg of protein μL^{-1}	μequiv min^{-1} (μg of protein)$^{-1}$ × 10^{-2} (30 °C)	μequiv min^{-1} (μg of protein)$^{-1}$ × 10^{-2} (70 °C)	% activity remaining at 70 °C
DEAE FT[b]	10.66 ± 0.51	42.33 ± 2.68	4.84 ± 0.27	11.4
DEAE BD[c]	3.58 ± 0.47	51.94 ± 0.30	1.27 ± 0.05	2.4
heparin CL-6B				
PME 1	6.17 ± 0.17	3.20 ± 0.004	0.01 ± 0.001	0.3
PME 2	3.04 ± 0.01	0.16 ± 0.001	0.02 ± 0.001	12.5
PME 3	1.08 ± 0.05	8.72 ± 0.07	1.97 ± 0.07	22.6
PME 4	0.80 ± 0.01	117.89 ± 2.11	12.37 ± 0.54	10.5
PME 5	0.07 ± 0.01	1.29 ± 0.07	0.14 ± 0.01	10.9
Econo-Pac heparin of pooled CL-6B				
PME 1	3.97 ± 0.52	0.24 ± 0.001	0.01 ± 0.00003(n = 2)	4.2
PME 2	0.17 ± 0.005	15.59 ± 1.00	0.021(n = 1)	0.1
PME 3a	0.06 ± 0.001	7.14 ± 0.71	1.90 ± 0.04	26.6
PME 3b	0.18 ± 0.03	1.17 ± 0.11	0.83 ± 0.05	70.9
PME 4	0.15 ± 0.01	251.30 ± 7.93	21.13 ± 0.27	8.4
PME 5	0.03 ± 0.0004	67.67 ± 2.00	5.33 ± 0.33	7.9

[a] All values for protein concentration and PME activity are means of three replicates unless otherwise noted. [b] FT, flow through. [c] BD, bound.

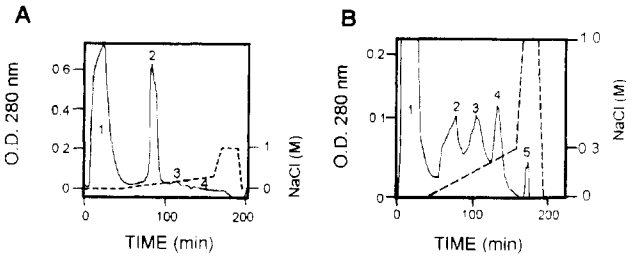

Figure 2. Heparin–Sepharose CL-6B chromatograms of DEAE FT (A) and DEAE BD (B) fractions. The DEAE FT samples were pooled material from TSP, SEP DS, and SEP DP DEAE chromatography as were the DEAE BD samples.

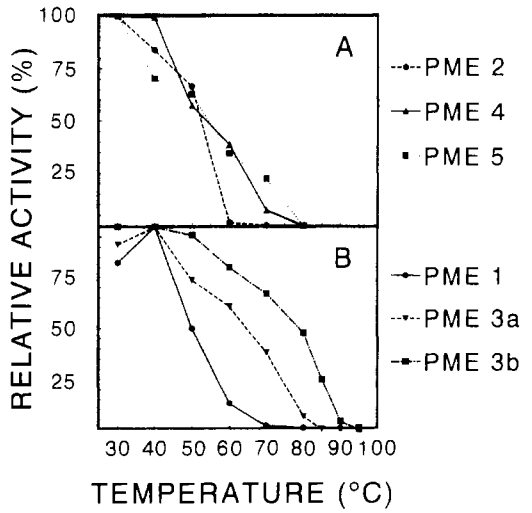

Figure 3. Temperature curve for PME 1, 3A, and 3b (A) and for PME 2, 4, and 5 (B). Fractions used for activity estimates were from rechromatography over Econo-Pac heparin.

membrane. It may be that even in the presence of 1 M NaCl these PMEs interacted nonspecifically with the gel filtration column matrix, retarding their elution and resulting in an underestimate of their molecular weight (MacDonald *et al.*, 1993).

DISCUSSION

PME has been purified previously from several citrus varieties or species (Manabe, 1973; Versteeg, 1979; Seymour *et al.*, 1991; Rillo *et al.*, 1992). Multiple forms of PME have been reported from tomato (Pressey and Avants, 1972), apple (Castaldo *et al.*, 1989), and citrus

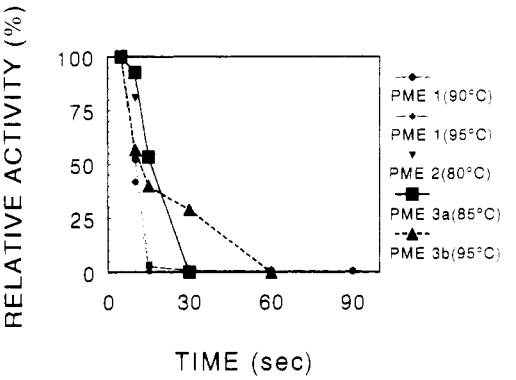

Figure 4. Thermal denaturation curve for PME 1 (90 and 95 °C), PME 2 (80 °C), PME 3a (85 °C), and PME 3b (95 °C). Fractions used for activity estimates were from rechromatography over Econo-Pac heparin.

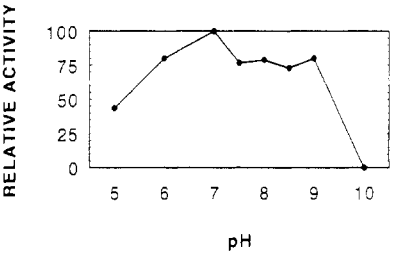

Figure 5. Effect of pH on the activity of PME 3b. Fractions used for activity estimates were rechromatography over Econo-Pac heparin.

(Versteeg *et al.*, 1980; Seymour *et al.*, 1991a,b). In fact, Pilnik and Voragen (1991) suggest a total of 12 PMEs may be present in citrus. Results presented here indicate a minimum of six PMEs occur in Valencia juice sac derived tissue culture cells. These six forms of PME can be distinguished by their chromatographic behavior on DEAE-Sephacel and heparin. A seventh PME was observed but not pursued.

As might be expected, the thermal properties of the previously reported multiple forms of PME are variable. The most heat-stable form was reported by Versteeg (1979). This PME retained activity to 95 °C and was the only form studied by Versteeg to cause precipitation of the juice cloud in orange juice at 5 °C (Versteeg *et al.*, 1980). The heat stablity of PMEs from marsh grapefruit (Seymour *et al.*, 1991a) and mandarin orange (Rillo *et al.*, 1992) was slightly lower, the enzyme being inactivated at

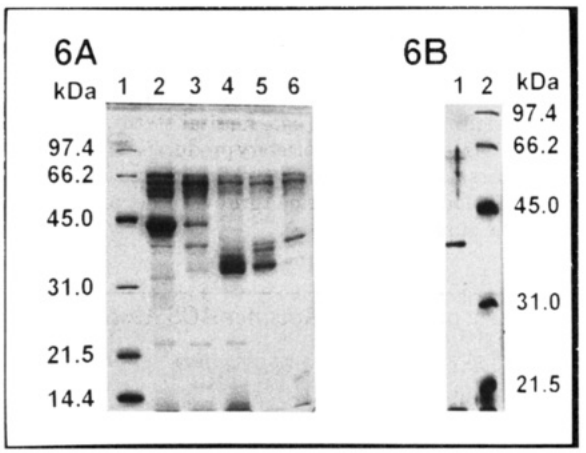

Figure 6. LDS–PAGE of pooled and concentrated active peaks from gel filtration (PME 1, 2, 3a, 4, and 5) or concanavalin A (PME 3b) chromatography. (A) (12.5% acrylamide): lane 1, molecular weight standards (Bio-Rad); lane 2, PME 1 (15 μL); lane 3, PME 2 (100 μL); lane 4, PME 3a (100 μL); lane 5, PME 4 (100 μL); lane 6, PME 5 (100 μL). (B) (10% acrylamide): lane 1, molecular weight standards (Bio-Rad); lane 2, PME 3b (100 μL).

Table 2. Estimated Molecular Weights ($\times 10^{-3}$) of Partially Purified PMEs[a]

	GF[b]	LDS–PAGE[c,d]
PME 1	44.0	44.8, 39.9, 32.7
PME 2	49.4	46.2, 39.9, 35.0
PME 3a	21.2	35.6, 34.6
PME 3b	37.5	40.6
PME 4	19.4	42.3, 39.9, 36.6
PME 5	19.8	43.1

[a] PMEs 1, 2, 3a, 4, and 5 were from Econo-Pac heparin chromatography, and PME 3b was from concanavalin A chromatography. [b] Gel filtration chromatography. [c] All densely stained polypeptide bands are listed. [d] Stained bands below 30 kDa in Figure 6 are most likely due to degradation since the sample had been concentrated with a 30-kDa cutoff membrane.

85 °C. Two of the six partially purified PMEs we have investigated retained activity at 90 °C and one (PME 3b) was still active after a 30-s incubation at 95 °C. The relationship of this extremely stable PME to the one purified by Versteeg *et al.* (1980) is unclear.

The kinetic values determined for the most heat-stable form (PME 3b) were in close agreement with values reported by Versteeg (1979) for navel orange heat-stable PME and by MacDonald *et al.* (1993) for two forms of PME from lemon tissue. The pH optimum reported here for PME 3b differs from that reported by Versteeg (1979) for navel orange heat-stable PME, by Rillo *et al.* (1992) for mandarin orange PME, and by Seymour *et al.* (1991) for marsh white grapefruit heat-stable PME. The major differences were a narrower optimum range for the tissue culture cell PME 3b and a different pH optimum compared to that for the mandarin orange PME reported by Rillo *et al.* (1992), *i.e.*, pH 7.0 for PME 3b vs pH 9.0 for mandarin orange PME.

Versteeg (1979) reported a native molecular weight (gel filtration) of 54 000 for navel heat-stable enzyme, and Seymour *et al.* (1991b) reported that heat-stable PME from marsh grapefruit was a 51 000 protein. Results from native gel filtration chromatography and denaturing electrophoresis reported here suggest a lower molecular weight (40 500, and 37 500, respectively) for the most stable form, PME 3b. None of the six PMEs we have partially purified appear to have a molecular weight greater than 50 000. If the most stable PME reported here (PME 3b) is also present in citrus juice, it could very well play a detrimental role in product quality, leading to cloud loss or concentrate gelation in underpasteurized product. The other form described here that may be of concern to the citrus (and other) industry is PME 3a, which also retains relatively high activity levels after a 70 °C incubation (35–40%). The temperature curve for this PME appears similar to ones described by Seymour *et al.* (1991a) and Rillo *et al.* (1992). However, its relationship to citrus juice cloud loss has not been studied.

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