Separation of Thermostable Pectinmethylesterase from Marsh Grapefruit Pulp

Milena Corredig, William Kerr, and Louise Wicker*

Department of Food Science and Technology, University of Georgia, Athens, Georgia 30602

A rapid, ion-exchange method that isolates thermostable pectinmethylesterase (TS-PME) from Marsh grapefruit pulp is presented. TS-PME was selectively extracted with 1 M NaCl and equilibrated at low pH (3.1 ± 0.04). After dilution to a final concentration of 50 mM acetate buffer, pH 5.5, and 0.1, 0.25, or 0.5 M NaCl, the extract was applied to an ion-exchange column without ammonium sulfate precipitation and dialysis. The percent yield varied from an average of 70% (0.1 M NaCl) to 14% (0.5 M NaCl), with a specific activity of >400 U/mg protein in the latter sample. TS-PME activity of some column fractions varied compared with crude extracts before column separation. Some fractions lost thermostability after separation if loaded at 0.1 M or 0.25 M NaCl. In extracts that were loaded at 0.5 M NaCl, the TS-PME activity was significantly higher than that observed in the heated crude extracts. Ion-exchange chromatography may have separated an unidentified protective factor in PME fractions.

Keywords: *Pectin complexes, cation activation, thermostable, ion exchange*

INTRODUCTION

In citrus, pectinmethylesterase (PME), is tightly associated with the cell wall, and amounts of detectable PME vary after extraction (Wicker et al., 1988; Snir et al., 1996; Cameron et al., 1996). After release from the cell wall during juice extraction, PME initiates a complex series of reactions, that result in cloud destabilization and undesirable appearance of the juice (Baker and Cameron, 1999).

Thermostable (TS-) and thermolabile (TL-) PME were first reported by Versteeg et al. (1978, 1980). Since then, various isozymes of PME have been isolated from citrus (Seymour et al., 1991a; Macdonald et al., 1993; Cameron and Grohmann, 1996; Cameron et al., 1996, 1998). These isozymes differ in isoelectric points, molecular weights and catalytic properties (kinetics, affinity for substrate, pH/temperature sensitivity) (Bordenave, 1996). Although TS-PME represents a small fraction of the total PME present in citrus juice, it seems to be the most active clarifying enzyme at the pH of juice and the cold temperatures used during storage (Versteeg et al., 1980; Snir et al., 1996). One non-thermostable form of PME caused cloud instability at refrigeration temperatures (Cameron et al., 1998).

Evidence is accumulating that some isozymes may be PME−pectin complexes (Macdonald et al., 1993; Chen et al., 1998) and some complexes are not completely separated (Macdonald et al., 1994). Further, the nature of thermostability of the enzyme is not completely understood, and multiple forms of TS-PME have been reported (Macdonald et al., 1993; Cameron et al., 1998). Compared with TL-PME, TS-PME has higher surface hydrophobicity and greater glycosylation (Seymour et al., 1991b), and is stabilized by interaction with pectin (Macdonald et al., 1994). The complex of PME in the cell wall has a stabilizing effect (Wicker et al., 1988; Macdonald et al., 1993; Bordenave, 1996). Cations compete for the carboxylic groups of pectin and displace the enzyme from the PME−pectin complex (MacDonnell et al., 1945; Nari et al., 1991). Cation type and concentration and pH influence PME activity (Leiting and Wicker, 1997; Sun and Wicker, 1999).

The enzyme is most completely solubilized by high salt and high pH buffers (Wicker et al., 1988; Fayyaz et al., 1994; Macdonald et al., 1994). After PME extraction, the conventional method of isolation requires various steps, including one- or two-step ammonium sulfate precipitation, dialysis, ion-exchange chromatography, and affinity and/or gel permeation chromatography (Seymour et al., 1991a,b; Rillo et al., 1992; Macdonald et al., 1993; Cameron et al., 1996, 1998).

Because clarification is a major defect affecting the appearance of juices and concentrates, it is important to identify the role and properties of PME that are relevant to the citrus industry. Consistently obtaining sufficient quantities of TS-PME has limited the ability to conduct clarification, structural, and interaction studies. Extraction, recovery, and purification of TS-PME in high yields are likely influenced by the formation of PME−pectin complexes. The objective of this research was to develop a rapid method to isolate TS-PME from Marsh grapefruit pulp in high yield.

EXPERIMENTAL PROCEDURES

Preparation of Crude TS-PME from Grapefruit. TS-PME was selectively extracted from unstabilized, frozen Marsh grapefruit pulp (donated by Citrus World, Lake Wales, FL) with 4 volumes (w/v) of 1 M NaCl and homogenized (Pro300A, Proscientific Inc., Monroe, CT) for 25 s at 4 °C (Wicker, 1992). After stirring for 1 h at 4 °C, the suspension was centrifuged at 5000g for 20 min with a Sorvall RC-5B centrifuge (Dupont Instruments, Doraville, GA). The extract was filtered through two layers of cheesecloth and kept at 4 °C. Thimerosol was added to the extracts as a preservative, to a final concentration of 0.05 g L^{-1}.

* Corresponding author [telephone (706) 542-1055; fax (706) 542-1050; e-mail lwicker@arches.uga.edu].

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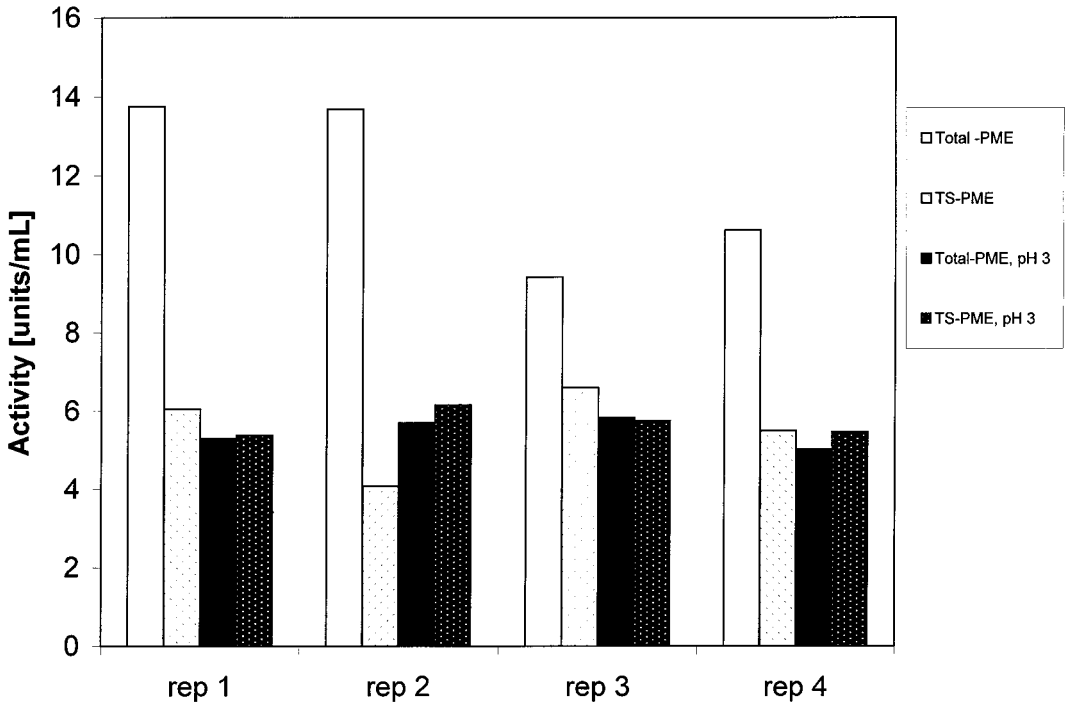

Figure 1. PME activity extracted from Marsh grapefruit pulp in Units/ml measured the same day of extraction, before (white), and after (black dots) heating at 70 °C for 5 min; PME activity measured after 16 h storage at 4 °C, pH 3.1 before (black) and after (white dots) heating at 70 °C for 5 min. Results are shown for four independent extractions.

PME Isolation. In all experiments, crude PME extracts were diluted to various concentrations of NaCl just before loading onto a cation-exchange column (HI-Trap SP; Pharmacia Biotech, Piscataway, NJ). The 5-mL column was preequilibrated in start buffer (50 mM acetate, pH 5.5). The same start buffer was used for each of the diluted PME extracts. After loading, the column was washed with start buffer and eluted with a 0−1 M NaCl linear gradient in start buffer. PME extracts were diluted with pH 5.5, acetate buffer to obtain a final concentration of 50 mM acetate and 0.1, 0.25, or 0.5 M NaCl. The diluted extract was filtered through a No. 1 Whatman filter (Whatman Int., Maidstone, UK). The pH of the extract was adjusted to 5.5 with 1 M NaOH before column loading if necessary. Ion-exchange chromatography was conducted at 4 °C using an FPLC system and column (Pharmacia Biotech, Piscataway, NJ) equipped with P-500 pumps and GP-250 Plus gradient programmer. The extract was loaded at 5 mL/min, washed with 5 volumes of filtered (0.45 μm filters, Gelman Scientific, Ann Arbor, MI) start buffer, and eluted with a linear gradient (15 column volumes) from 0 to 1 M NaCl in start buffer. The column was regenerated with 5 volumes of 50 mM sodium acetate, 1 M NaCl, pH 5.5 followed by 5 volumes of 50 mM sodium acetate, pH 5.5. Absorption at 280 nm was measured for each 5-mL fraction eluted during chromatography. Fractions containing PME were qualitatively identified by adding 20 μL of each fraction to 1 mL of 10 g L^{-1} pectin, 0.1 M NaCl, and 0.2 g L^{-1} bromthymol blue, pH 7.5. Fractions, which changed color, were quantified for PME activity by the titrimetric assay.

Total and TS-PME Activity Determination. Enzymatic activity was determined by a titrimetric assay (Rouse and Atkins, 1955) in 10 g L^{-1} high methoxyl pectin (Citrus Colloids, Hereford, U.K.), 0.1 M NaCl, 30 °C and pH 7.5 using a pH stat system (Brinkmann 614 Impulsomat, Brinkmann Instruments, Westbury, NY). An enzyme unit was defined as the amount of PME that hydrolyzed 1 μmol of ester per minute. Total and thermostable PME activity was defined as the residual activity before and after heating at 70 °C for 5 min, respectively. A 2-mL aliquot of 50 mM phosphate, 0.1 M NaCl, pH 7.0, was preheated in a water bath at 70 °C, and 500 μL of the eluted fraction was added to the glass tubes. The change in pH of the heating buffer was determined to be <0.1 pH unit. Following heating, samples were rapidly cooled in an ice bath. Enzymatic activity was determined for control and heated samples within 30 min after the heating treatments.

Protein Analysis. Protein was determined by the Bradford (1976) microprotein assay, using bovine immunoglobulin (IgG) as the standard (Bio-Rad, Hercules, CA), and quantified with a BioRad microplate reader and software (model 550).

Replications and Statistical Analysis. The extractions and chromatographic isolations were repeated at least three times. Representative chromatograms are reported. Significant differences between sample means were tested by ANOVA (SAS, 6.2).

RESULTS AND DISCUSSION

Selective Extraction of TS-PME. If PME were assayed within a few hours, greater amounts of activity were detected, but high variability in total and TS-PME activity between extractions was observed. Total PME activity measured within a few hours after extraction averaged 11.8 ± 2.2 U/mL. The amount that was heat stable was 5.5 ± 1.1 U/mL. Variability in the amount of citrus PME recovered after extraction was previously reported (Wicker et al., 1988; Snir et al., 1996). Among the sources of variability, juice particle size, time of storage, and mode of extraction have been considered (Wicker et al., 1988; Snir et al., 1996).

By contrast, when PME activity was measured ~16 h after extraction and stored at 4 °C at low pH (3.1 ± 0.04), the activity averaged 5.5 ± 0.4 U/mL. The data in Figure 1 also illustrated the residual activity after heating at 70 °C for 5 min for four extractions. If the heating treatments were carried out within a few hours after extraction, a higher and more variable amount of total PE activity was observed. In the extracts, which were stored overnight, lower total PME was observed, but it was primarily thermostable. The low pH of the extracts probably mainly inactivated TL-PME. Other authors (Owusu-Yaw et al., 1988) reported a loss of PME

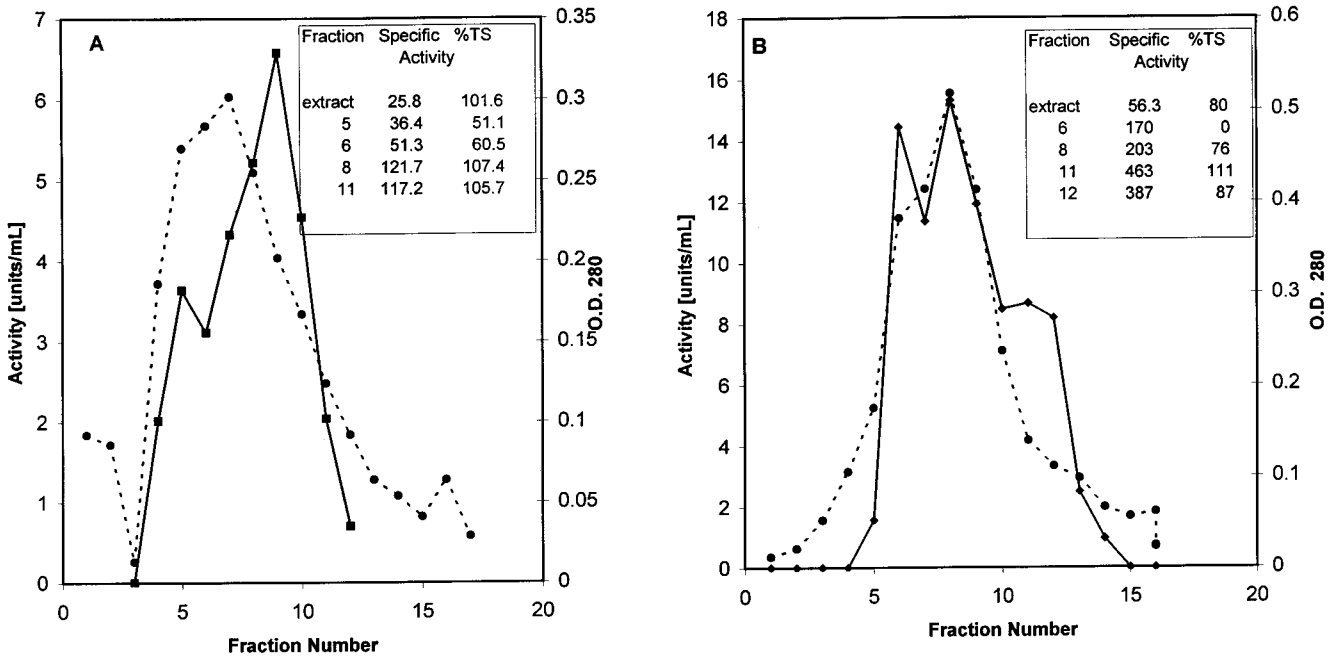

Figure 2. Fractionation of crude TS-PME extract from Marsh grapefruit pulp on Hi-Trap SP (Pharmacia). PME activity [units/mL] (left-hand axis, solid line) and absorbance at 280 nm (right-hand axis, dotted line). Figure 2a. Extract containing 0.1 M NaCl was loaded after overnight storage at 4 °C. Figure 2b. Extract containing 0.1 M NaCl was loaded the same day as extracted. Inset: specific activity [units/mg protein] and %TS-PME (as activity recovered after heating at 70 °C for 5 min versus unheated controls) for selected fractions. Representative results of at least three chromatograms are presented.

activity at low pH. Sun et al. (1998) demonstrated by circular dichroism that TL-PME from Marsh grapefruit pulp undergoes structural changes at low pH, which led to complete inactivation after 8 h at pH 3.3. Another possibility is the formation of inactive PME−pectin complexes during extraction and overnight equilibration.

Fractionation of TS-PME by Ion-Exchange Chromatography. The volume of the Hi-Trap SP column was low compared with the volume of crude extract loaded onto the column. During loading of crude extracts of PME, more PME was bound when the crude extract was loaded at 0.1 M NaCl than when loaded at 0.5 M NaCl. Elution of bound PME began after at least 5−7 column volumes of elution buffer. When Marsh grapefruit extract containing 0.1 M NaCl was stored overnight before separation using Hi-Trap SP columns, activity was detected between fractions 5 and 12 (Figure 2A). A distinct shoulder in the elution peak was observed between fractions 5 and 6, which had lower specific activity and thermal stability. The percent TS-PME in fractions 5 and 7 was estimated at <50%. Fractionation using Hi-Trap SP of the crude extract, which was thermostable initially, resulted in loss of TS-PME activity of some fractions after separation. The presence of TL-PME in these fractions was supported by the chromatograms of extracts that were not stored overnight (Figure 2B). There is evidence of three, partially resolved peaks of PE activity. Comparing with Figure 2A, no residual activity in fraction 6 was observed in after heating. Only fraction 11 showed no loss of activity after heating. The percent TS-PME was lower in fractions depicted in Figure 2B than percent TS-PME depicted in Figure 2A.

When the extract was diluted to contain 0.25 M NaCl and stored overnight before loading onto the SP column, most of the PME activity eluted as a single peak between fractions 7 and 12, and high absorbance (280 nm) readings were observed between fraction 5 and 10 (Figure 3A). Fraction 8 contained <100% TS-PME, and later eluting fractions (fractions 10 and 12) contained TS-PME, showing >100% residual activity after heating (see inset in Figure 3A). When Marsh grapefruit extract was diluted to contain 0.25 M NaCl and applied to the Hi-Trap SP column within hours of extraction, the PME eluted in two partially resolved peaks (Figure 3B). The crude extract contained 80% TS-PME and only fraction 12 had <80% TS-PME.

When PME extracts containing 0.5 M NaCl were applied after overnight storage, PME eluted between fractions 7 and 13 (Figure 4A). No detectable TL-PME bound the column matrix. Unlike the loadings at 0.1 or 0.25 M NaCl when the extract contained 0.5 M NaCl, the time of loading did not change the enzyme elution pattern (Figure 4A,B). Only one peak was observed, which eluted between fractions 7 and 15. Fraction 8 had close to 100% TS-PME, and fractions 9 and 11 had >100% TS-PME (Figure 4A). A similar trend was observed when PME was loaded the same day as extraction.

The specific activity was increased by fractionation in all cases. When crude PME was loaded at 0.25 M, the specific activity increased from 50 U/mg protein to >400 U/mg if loaded after overnight storage (fraction 11, inset, Figure 3A). When loaded the same day, the specific activity increased to 240 U/mg (fraction 11, inset, Figure 3B). Similarly, when crude extract was loaded at 0.5 M NaCl, the specific activity increased to >400 U/mg protein (fraction 11, inset, Figure 4A) and over 500 U/mg protein (fraction 11, inset, Figure 4B). All these fractions contained 100% or greater residual activity to heat at 70 °C. Concentration of PME from Marsh grapefruit by ammonium sulfate addition and subsequent dialysis is a time-consuming task, and periodically results in a large loss of yield (Sun and Wicker, unpublished observations). During dialysis against low ionic strength buffer, some TS-PME becomes insoluble that was soluble at high ionic strength (Cameron and Grohmann, 1996; Cameron et al., 1998).

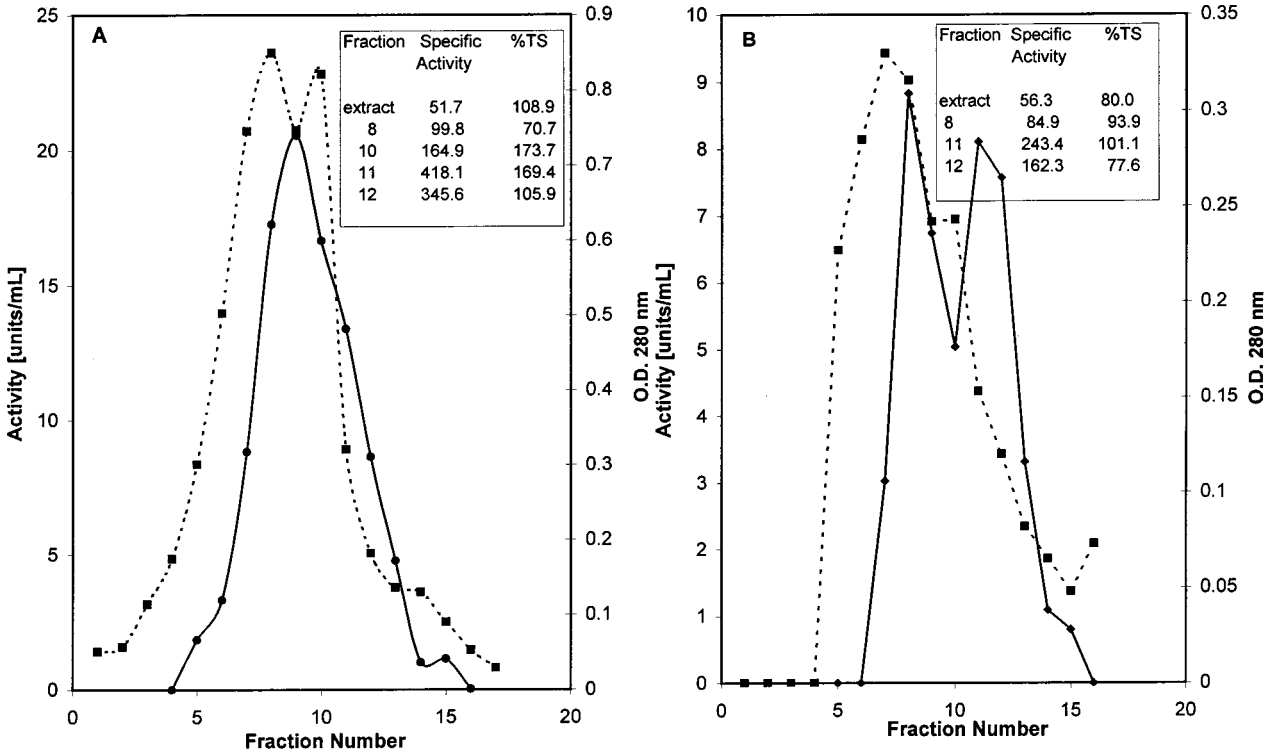

Figure 3. Fractionation of TS-PME as described in Figure 2 except that extracts were diluted to 0.25 M NaCl. Figure 3a. Extracts were stored overnight. Figure 3b. Extracts were fractionated the same day as extracted.

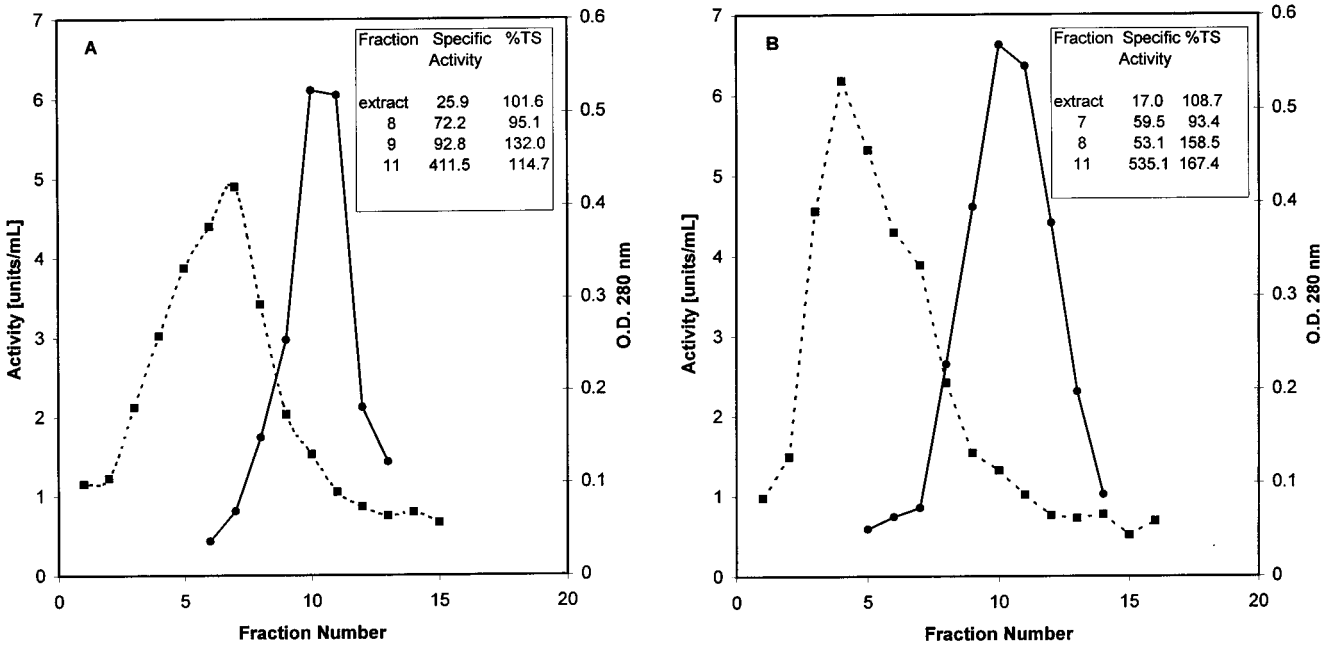

Figure 4. Fractionation of TS-PME as described in Figure 2 except that extracts were diluted to 0.5 M NaCl. Figure 4a. Extracts were stored overnight. Figure 4b. Extracts were fractionated the same day as extracted.

In the present work a simple, rapid purification procedure of TS-PME from Marsh grapefruit pulp was developed. Total activity recovered from gradient elution after loading onto the Hi-Trap SP column varied from an average of 70% (0.1 M NaCl), to 14% (0.5 M NaCl), with a specific activity of over 400 U/mg protein in the latter example. Most likely, use of a high ionic strength start buffer prevented the formation of a TS-PME−pectin complex and allowed binding to the SP column. In all separations, fraction 11 had the highest specific activity (Figures 2−4). When 2 units/mL of fractions 6, 8, or 11 where added to orange juice, fraction 11 from 0.5 M NaCl extracts produced the most rapid clarification, showing a clear serum within 5 days of storage at 5 °C (data not shown).

Combining the selective extraction procedure with ion-exchange chromatography after dilution of crude extract and without an ammonium sulfate precipitation consistently resulted in higher yields as well as higher specific activity than previously reported. The recovery and specific activity of citrus TS-PME ranged from 48% yield and 18.7 U/mg from Valencia juice (Cameron and

Grohman, 1996) to 2% yield and 217 U/mg from Marsh grapefruit pulp (Seymour et al., 1991). Sun and Wicker (1996) reported a 12% yield of TS-PME from Marsh grapefruit with a specific activity 987 U/mg. Macdonald et al. (1993) reported a 9−12% yield from lemon, with a specific activity of 11.4−14.5 U/mg. High ionic strength buffers during loading onto the SP column allowed selective binding of TS-PME. Even at the lowest yields obtained with extracts containing 0.5 M NaCl, the separation resulted in ∼1000 units of TS-PME per kilogram of Marsh grapefruit pulp. Macdonald et al. (1993) reported the recovery of ∼6000 units (of different isozymes of PME) per kilogram from lemon fruit after two ammonium precipitation steps. Seymour et al. (1991a) reported the recovery of 72 units of TS-PME per kg of pulp. Cameron and Grohman (1996) reported the recovery of TS-PME with a specific activity of 9.35 U/mg per L of Valencia juice. More than 12 000 units of TS-PME with a specific activity of 987 U/mg was recovered from 1 kg of Marsh grapefruit pulp (Sun and Wicker, 1996.)

During separation with 0.25 or 0.5 M NaCl, a peak of absorbance at 280 nm was observed at the beginning of the gradient elution. Interestingly, these OD_{280} absorbing fractions were eluted at a lower ionic strength than that used during loading of the extract. By selective use of column loading ionic strength, non-PME protein was separated from PME-containing fractions. Binding and elution from ion-exchange occur due to charge primarily, but size and hydrodynamic volume differences affect ion exchange chromatography (Scopes, 1987). The OD_{280} absorbing material, which eluted between fractions 3 and 5, might be a complex of non-PME protein and pectin. If a large molecular weight complex was formed as a result of salt-induced aggregation, it may dissociate and be released at the beginning of the salt gradient (0.1−0.3 M NaCl). Previously, protein−pectin interactions were implicated as a possible cause of cloud loss (Shomer et al., 1999), but it is unknown if the OD_{280} absorbing material in this study is related to the cloud proteins described by Shomer.

Effect of Heating on PME Activity. In extracts loaded at 0.25 or 0.5 M NaCl, the activity after heating was markedly higher in some fractions. TS-PME activity detected in fractions that eluted between fractions 10 and 12 was significantly higher ($p < 0.05$) than activity detected before heating. No significant effect of heating was observed for fractions eluting before fraction 9. The nature of the increase in TS-PME activity after heating is unknown, but Cameron and Grohman (1996) also reported an increase in thermostable PME after 2 min at 80 °C. The increase in activity may be related to limitations of the PME assay as suggested by Snir et al. (1995). We estimated that the maximum change in pH was 0.1 by addition of acetate buffer to the phosphate heating buffer, which should not affect the stability of the enzyme (Sun and Wicker, 1996). It is possible that heat solubilizes an inactive form of TS-PME. Other possibilities may be that TS-PME may be activated by heat, either by changes in conformation or release from an insoluble, inactive complex. Perhaps ion-exchange chromatography removed a protein or other compound, which imparted thermolability or inhibited TS-PME. Work is underway to clarify the causes of these heat-induced effects on the activity of PME.

Conclusions. A rapid, consistent method to selectively extract and isolate TS-PME from Marsh grapefruit pulp has been presented. Some of the previous disparity of TS-PME isolation is probably related to formation of PME−pectin complexes, which influence binding to ion-exchange columns. The time-consuming ammonium sulfate precipitation step and the coprecipitation of non-PME proteins were avoided. The specific activity and yield of TS-PME was comparable to or higher than previous methods. A single-column separation using Hi-Trap SP selectively fractionated TS-PME with a specific activity between 350 and 400 U/mg and at least 14% yield. TS-PME without TL-PME can be obtained after overnight storage, dilution to 0.25 or 0.5 M NaCl, and separation by ion-exchange. An unexpected finding of this research is that an increase in percentage of TS-PME was observed in some fractions. Future study will focus on the role of ionic interactions between TS-PME, pectin, and non-PME protein on detection of PME activity and clarification of juices.

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