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Structural Characterization of the Thermally Tolerant Pectin Methylesterase Purified from *Citrus sinensis* Fruit and Its Gene Sequence

Brett J. Savary,*[,†,‡] Prasanna Vasu,[‡,§] Randall G. Cameron,[⊥] T. Gregory McCollum,[⊥] and Alberto Nuñez[†]

[†]Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Wyndmoor, Pennsylvania 19038, United States

[‡]Arkansas Biosciences Institute, Arkansas State University, P.O. Box 639, State University, Arkansas 72467, United States

[§]Food Safety and Analytical Quality Control Laboratory, Central Food Technological Research Institute, CSIR, Mysore 570 020, India

[⊥]U.S. Horticultural Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 2001 South Rock Road, Fort Pierce, Florida 34945, United States

Supporting Information

ABSTRACT: Despite the longstanding importance of the thermally tolerant pectin methylesterase (TT-PME) activity in citrus juice processing and product quality, the unequivocal identification of the protein and its corresponding gene has remained elusive. TT-PME was purified from sweet orange [*Citrus sinensis* (L.) Osbeck] finisher pulp (8.0 mg/1.3 kg tissue) with an improved purification scheme that provided 20-fold increased enzyme yield over previous results. Structural characterization of electrophoretically pure TT-PME by MALDI-TOF MS determined molecular masses of approximately 47900 and 53000 Da for two principal glycoisoforms. De novo sequences generated from tryptic peptides by MALDI-TOF/TOF MS matched multiple anonymous *Citrus* EST cDNA accessions. The complete *tt-pme* cDNA (1710 base pair) was cloned from a fruit mRNA library using RT- and RLM-RACE PCR. *Citrus* TT-PME is a novel isoform that showed higher sequence identity with the multiply glycosylated kiwifruit PME than to previously described *Citrus* thermally labile PME isoforms.

KEYWORDS: *Citrus sinensis, fruit cell wall enzyme, orange juice cloud stability, MALDI-TOF MS, PCR cloning, purification, thermostable pectinesterase*

INTRODUCTION

Processed citrus juice from Florida (predominantly from oranges) is currently valued as a ca. U.S. $3.45 billion industry.[1] Product quality is largely defined by juice cloud properties, such as turbidity, and associated organoleptic properties, such as flavor, aroma, and color.[2] Cloud loss is a defect largely attributable to the enzyme pectin methylesterase (PME).[3] PME acts on pectin associated with juice cloud, leading to aggregation through calcium cross-linking and subsequent cloud separation and loss of flavor attributes.[2,4] Multiple forms of PME are present in citrus fruit tissues, but it is the thermally tolerant (TT-) PME that is most significant in destabilizing juice cloud at 4 °C.[5−8] TT-PME activity must be strictly controlled during processing to stabilize juice products. Pasteurization of juice at approximately 20 °C above the temperature needed to control microbial growth is required to inactivate TT-PME effectively, but high temperatures can contribute taste defects.[5,9] Commercial juices are stabilized at temperature ranges from 90 to 95 °C and holding times of 15−60 s.[10]

There is uncertainty regarding individual citrus PME behavior in juice and in vitro systems due to inadequate or ambiguous identification of isoenzymes and their corresponding genes.[11,12] A 36000 Da protein was associated with thermostable activity,[13] and a new *Citrus* PME gene based on peptide sequences derived from this protein was cloned.[14] We recently determined that this gene sequence actually represents the salt-independent PME by using direct N-terminal peptide sequencing and tryptic peptide mass-fingerprinting using MALDI-TOF MS.[12,15,16] This *Citrus* PME isoform is the major thermally labile (TL-) PME accumulated in citrus fruit.[8,17,18] We describe for the first time in this paper the unequivocal structural identification of the protein responsible for TT-PME activity and its complete nucleotide sequence from mRNA.

Citrus TT-PME molecular mass has been reported to be about 40000−43000 and 51000−53000 Da,[6,11,19,20] whereas TL-PME isoenzymes are commonly estimated in the range of 32000−36000 Da. We previously correlated TT-PME activity to an enriched 41000 Da glycoprotein[7,11,20] in both grapefruit and sweet orange. However, the N-terminal peptide sequences and MALDI-TOF MS spectra obtained from this protein indicated identity to polygalacturonase inhibitor proteins (PGIPs). PGIPs are monomeric plant cell wall-associated proteins that modify activities of fungal pathogen polygalactur-

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onases.[21−23] Activities by certain fungal PGs appear to be stimulated by individual plant PGIPs.[22] This could be explained if plant PGIPs contain a PME catalytic activity: PME action is necessary to demethylesterify pectin for PG binding and hydrolysis of pectin substrates. We were unable to either confirm a novel dual functional activity for orange TT-PME or demonstrate copurification of a second protein (PGIP) having nearly identical chromatographic properties because our efforts were hampered by difficulties in purifying adequate quantities of protein.[7,11] TT-PME in *Citrus* fruit is reported as a small fraction of total extractable activity.[6,8,18,19,24−26] Purification of cationic cell wall proteins such as TT-PME from fruit tissue is further hampered by the presence of soluble pectinates coextracted during isolation procedures.[6,8,26,25,27] This has led to complex purification schemes, insoluble precipitates, and low yields. Although such preparations can be regarded as "monocomponent", that is, having eliminated other PME isoforms and other pectin-active enzyme activities, these are not necessarily regarded as homogeneous proteins.[11]

The purpose of this study was to establish unambiguous identification of the protein responsible for TT-PME activity and to obtain the complete cDNA sequence for the corresponding gene. This required developing improved techniques for efficient enzyme purification from orange fruit tissues and obtaining sequences from isoform-specific peptides. We sought a definitive enzyme preparation for pectin treatment studies to determine TT-PME's action pattern and to modify functional properties.[28] Isolation of the gene sequence will also support molecular studies to determine TT-PME's expression patterns in fruit tissues. We report here a simplified purification method that provided greatly improved yield and purity of TT-PME. We also report detailed structural characterization using MALDI-TOF MS to obtain peptide mass fingerprints and de novo sequences from tryptic peptides. These amino acid sequence tags were then used to clone and sequence the complete cDNA for *Citrus* TT-PME from a fruit mRNA library.

MATERIALS AND METHODS

Sweet orange finisher pulp was obtained from Florida's Natural Growers (Lake Wales, FL, USA) and was stored frozen at −20 °C prior to use. Green kiwifruits (*Actinidia deliciosa*) purchased from a local market were allowed to over-ripen prior to use for isolating and immobilizing the PME inhibitor protein (PME-IP).[29] All chemicals and solvents were of analytical grade from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise specified. Citrus pectin (P9135) was from Sigma-Aldrich. DEAE-Sepharose FF was from Sigma-Aldrich, and CNBr-activated Sepharose 4B was from Amersham Biosciences (Piscataway, NJ, USA).

Protein Determination. Protein concentrations were determined according to the method of Lowry et al.[30] using bovine serum albumin as standard. Other protein assay reagents, Coomassie Brilliant Blue reagent (Bio-Rad Laboratories, Hercules, CA, USA) and the bicinchoninic acid reagent (Pierce Chemical Co., Rockford, IL, USA), were used for purified TT-PME. All determinations were performed according to the standard protocol supplied by the manufacturer. Purified TT-PME was also determined by absorbance at 280 nm using the extinction coefficient calculated from the protein composition of the mature protein using the ProtParam program at the ExPASy proteomics tool Web site (http://au.expasy.org/tools/).

Galacturonic Acid Determination. Soluble pectins in pulp extracts were determined colorimetrically as galacturonic acid (GalA) equivalents using a *m*-hydroxybiphenyl method adapted from Kintner and Van Buren.[31] An aliquot (10 μL) of samples (clarified by centrifugation for 10 min at 17000*g*) was diluted to 200 μL with deionized water and cooled in an ice bath for 10 min, after which 1.2 mL of concentrated H_2SO_4 was added and then vortexed vigorously. Samples were subsequently heated for 5 min at 100 °C and then cooled immediately by placing on ice. Thereafter, 20 μL of 0.15% *m*-hydroxybiphenyl (prepared in 0.5% NaOH) was added, vortexed immediately, and incubated at room temperature for 20 min. Pectin contents were estimated on the basis of a standard curve with GalA (absorbance at 520 nm).

Enzyme Activity Assay. PME activity was determined by pH-stat titration as previously described[15] using a TIM 854 autotitrator (Radiometer Analytical, Loveland, CO, USA) with 1% citrus pectin containing 1.2% sodium chloride maintained at pH 7.0 and 30 °C. Enzyme solutions were diluted to give consumption rates of 20 - 50 μL titrant solution (0.02 M sodium hydroxide) per min. One unit of activity is 1 μmol hydroxide equivalents consumed per minute. Thermally tolerant PME activity was determined as residual enzyme activity following heat treatment at 70 °C for 10 min in a solution of 0.05 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride.[24] Polygalacturonase-inhibitor protein (PGIP) activity was determined according to the method of Kemp et al.[22] using Megazyme *Aspergillus* polygalacturonase M2 (Wicklow, Ireland). All analyses (including protein and GalA determinations) were done in triplicate and repeated at least once.

PME Extraction Trials. Preliminary tissue extractions to solubilize PME activity from finisher pulp (ca. 10 g) compared different buffers (1:3, w/v) and sodium chloride concentrations for determining effective conditions for scaled-up TT-PME isolation. Extractions used either water without adjustment to endogenous pH of pulp slurry (pH 3.6) or two buffered solutions (either 0.02 M sodium acetate, pH 5.0, or 0.1 M Tris-HCl, pH 8.0) containing various sodium chloride contents (0.0, 0.1, 0.5, or 1.0 M). Because no soluble PME activity was detected in salt-free water extracts, finisher pulp was washed with water until a clear-running solution was obtained, and then this treated pulp was used for extraction treatments. Extractions were performed by combining tissue and extraction solution in 50 mL centrifuge tubes and incubating for at least 1 h with intermittent vortexing and sonication. Following centrifugation (Sorvall SS-34 rotor, 20 min at 20000 rpm; DuPont Instruments, Doraville, GA, USA), the supernatants were tested for soluble PME activity. Total and TT-PME activities as well as protein concentrations were determined before and after heat treatments.

Purification of TT-PME. Preparative extraction of washed finisher pulp (1.3 kg) for TT-PME purification was performed with 0.02 M acetate buffer containing 0.5 M sodium chloride. Thawed finisher pulp was washed five times (2 h each treatment) with 4.5 L of water containing 0.05 M sodium chloride (resulting in a clear-running final filtrate). This washed pulp was extracted overnight at 4 °C with continuous stirring using 4.5 L of 0.02 M sodium acetate containing 0.5 M sodium chloride and adjusting to pH 5 with sodium hydroxide. The extract was filtered through two layers of Miracloth (Calbiochem) and clarified by centrifugation at 8000 rpm (Sorvall SLC-6000 rotor) for 40 min (4 °C). Suspended particulates were removed by filtration with Gelman GF prefilters under low vacuum. The extract was then concentrated by tangential-flow ultrafiltration using a 30000 NMWL regenerated cellulose membrane (Millipore, Bedford, MA, USA). The retentate was diluted with deionized water to reduce the sodium chloride concentration to 0.2 M and then reconcentrated. Sodium phosphate monobasic was added to bring the solution to 0.02 M and then adjusted to pH 7.0 with NaOH. This solution was heated to 70 °C in a water bath for 30 min, then cooled on ice for 1 h, and finally centrifuged at 15000 rpm (Sorvall SLA-1500 rotor) for 40 min to clarify the solution.

The clarified heat-treated extract was subsequently treated with DEAE-Sepharose to remove coextracted soluble pectins having high charge density (i.e., pectinates). DEAE-Sepharose was preconditioned to pH 7.0 batchwise in a beaker following the manufacturer's recommendations. The extract solution was treated in portions (150 mL) by mixing with the DEAE-Sepharose (ca. 150 mL settled bed volume) in a 2 L beaker, maintaining pH 7.0, then recovered with a fine glass-frit Buchner funnel. The adsorbant was washed once with 1 volume of buffer solution. This wash and the extract were combined

and concentrated by ultrafiltration (30000 NMWL YM membrane in an Amicon stirred cell) and finally clarified by centrifugation. Sodium chloride was added to return the solution to 0.5 M. PME activity was separated using a PME-IP affinity chromatography column (7.5 mL bed volume with binding capacity of ca. 14000 PME units) prepared following methods described by Denés et al.[29] The affinity column was pre-equilibrated with 0.02 M phosphate buffer (pH 7.0) containing 0.5 M sodium chloride, and the bound TT-PME was eluted with 0.02 M carbonate buffer (pH 10.0) containing 1.0 M sodium chloride. The eluted TT-PME solution was immediately neutralized and concentrated to at least 1 mg/mL with an Amicon Centricon-10. This sample was exchanged into sodium phosphate buffer (pH 7.0, 0.1 M) using a Bio-Rad Econopak 10DG desalting column and reheated at 70 °C for at least 20 min, followed by centrifugation. The final preparation was stored with 0.02% sodium azide (w/v) at 4 °C.

Electrophoresis Methods. SDS-PAGE was performed using a NuPAGE Novex 12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) with MOPS buffer as previously described.[12] Gels were calibrated with Mark-12 wide-range unstained protein standards or SeeBlue Plus2 prestained protein standards and stained with Simply Blue Coomassie G-250 staining according to the instruction manual. For N-terminal protein sequencing, TT-PME bands were resolved on a 10% SDS-PAGE gel and electroblotted to Bio-Rad Trans-Blot PVDF membrane in CAPS buffer as previously described for orange PMEs.[15] Direct N-terminal amino acid sequencing of blotted proteins was performed with an Applied Biosystems Procise 491 protein microsequencing system. Initial yields averaged 5 pmol per cycle. The sequence obtained was subsequently used for BLASTp searching of the nonredundant plant database (www.ncbi.nlm.nih.gov/) and the Plant Genome Central Large Scale *Citrus* EST Sequencing Project database (www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html) at the National Center for Biotechnology Information Web site.

MALDI-TOF Mass Spectrometry and Peptide Sequence Analysis. Samples were prepared for MALDI-TOF MS as described recently for orange TL-PMEs.[12] Coomassie Brilliant Blue-stained protein bands were excised from the SDS-PAGE gels, digested with Trypsin Gold (Promega, Madison, WI, USA), and the extracted peptides were recovered from Millipore C18 Zip Tips using 60% acetonitrile/0.1% TFA containing α-cyano-4-hydroxycinammic acid matrix prior to spotting a mixture with a saturated matrix solution [α-cyano-4-hydroxycinnamic acid in 0.1% TFA/acetonitrile (70:30, v/v)] on the target plate. MS and MS/MS spectra were acquired with a 4700 Proteomics analyzer (Applied Biosystems, Framingham, MA, USA) and evaluated with the MASCOT search engine associated with the GPS Explorer program. De novo peptide sequences for eight of the tryptic peptides were accomplished by MS/MS spectrum interpretation using PEAKS (Bioinformatics Solutions Inc., Waterloo, ON, Canada) automatic de novo algorithm software,[32] with the following parameters: precursor ion mass tolerance, 0.05 Da; fragmented ion mass tolerance, 0.1 Da; and trypsin as digestion enzyme. Oxidized methionine was selected as a variable modification, and de novo sequences carrying methionine were verified versus the detection of the oxidized/not-oxidized ion in the MS spectrum for additional confirmation. Guanidination reagent (Sigma-Aldrich, MS0100) was used to confirm assignments between Q and K for internal positions. Each peptide was subsequently used for BLAST search of the nonredundant protein and *Citrus* EST databases as performed for the N-terminal peptide sequence.

Cloning of *Citrus* TT-PME. TT-PME peptide sequences (two tryptic peptides TMLMFVGDGIGK and SATVAVVGTGFIAK and the N-terminal peptide LQKSVXLTKFDLIVAK) were used to query the *Citrus* EST cDNA database by translated BLAST (tblastn). EST accession hits were first aligned manually using peptides to anchor overlapping amino acid sequences, and then the contiguous nucleotide sequence alignment was deduced. Specific PCR primers (Table 1) were used in combination with *Citrus reticulata* "Clementine" DNA to conduct initial RT-PCR experiments to first verify *Citrus* EST nucleotide sequences. DNA was extracted from leaf tissue following the protocol of Dellaporta et al.[33] Three primer combinations were used: PME3A-100F/963R, PME3A-100F/689R, and PME3B-298F/723R. Amplicons generated from these primer combinations were sequenced (method of Sanger et al.[34]) using an ABI PRISM Big Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an automated fluorescent DNA sequencer (model 3730XL, Applied Biosystems). DNA sequences were analyzed using Sequencher software (GeneCodes, Ann Arbor, MI, USA) and BLASTn at the NCBI. Amplicon sequences were found to be identical to sequences contained within NCBI accession DY285162 (and contained an 81 bp intron).

Table 1. PCR Primers

primer	5′−3′ sequence
RT-PCR	
PME3A:100F	CCAGCATTGGACAGAGTTAC
PME3A:963R	AATCAACGGTGCCATATACG
PME3A:689R	TCCCATCTCCTACGAACATC
PME3B:298F	TTGATGGGTTCGCTTACAGT
PME3B:723R	ACGCTTCTATTGGCCTTCAC
RACE-PCR	
3′ GS outer primer 1	GGCTTATTTTGAGAACGTGGA
3′ GS outer primer 2	GGATCGGCAAAACAGTAGTGA
5′ GS inner primer	TCCCTCACGTTTCCGTCACTGT
5′ GS outer primer	TCACCGGGGATTTTCTTGAGCA

RACE-PCR was then used to generate the cDNA for *tt-pme*. Primer pairs PME3A−100F/698R and PME3B−298F/732R were used in combination with sweet orange (*Citrus sinensis*) total RNA (prepared from whole immature fruit, ca. 90 days after anthesis) for RT-PCR using the Titan One-Step RT-PCR kit (Roche Diagnostics, Indianapolis, IN, USA). Total RNA was isolated using the method of Stromer et al.[35] Two RT-PCR amplicons were sequenced as described above and found to be identical to sequences contained within NCBI accession DY285162. On the basis of this sequence, gene-specific primers were designed (Table 1) for subsequent RNA ligase-mediated rapid PCR amplification of cDNA ends (RLM-RACE PCR, First-Choice kit, Ambion, Austin, TX, USA) to generate a full-length cDNA clone of the TT-PME gene. To obtain the complete cDNA encoding the pre- and pro-peptide sequences, the gene-specific primers (5′ GS outer primer and 5′ GS inner primer for 5′ RACE and 3′ GS outer primer and 3′ GS inner primer; Table 1) were used for RLM-RACE PCR. The resulting amplicons (ca. 700 bp for the 5′ amplicon and ca. 1000 for the 3′ amplicon) were purified from agarose gels following electrophoresis using the QIAquick gel extraction kit (Qiagen, Germantown, MD, USA) and cloned into *Escherichia coli* (pCR2.1-TOPO cloning vector and TOPO Top10F′ cells, Invitrogen) following the manufacturer's protocol. The RACE amplicons were sequenced and analyzed as described above. By combining the sequences obtained from the 5′ and 3′ RACE, a putative full-length coding sequence for the TT-PME was assembled.

RESULTS AND DISCUSSION

PME Extraction from Pulp Tissue. Plant PMEs isolated from fruit tissues are typically cationic cell wall-associated proteins.[6,16] We addressed improving purification efficiency first by manipulating solution pH and salt concentration during extraction. Figure 1 compares recoveries of total and TT-PME activities from orange finisher pulp with acetate (pH 5.0) and Tris (pH 8.0) buffers and with addition of 0.5 or 1.0 M sodium chloride. No PME activity (assayed at pH 7.0) was detected in salt-free water washes of pulp (measured at pH 3.8). Although some activity was solubilized by increasing the pH with acetate buffer, highest activities were detected at alkaline pH (Tris buffer at pH 8.0) with 1 M NaCl. These results are comparable with previous results for PME extraction from citrus finisher pulp showing differential solubility between thermolabile and thermostable PME activities.[24] We selected extraction at pH 5.0

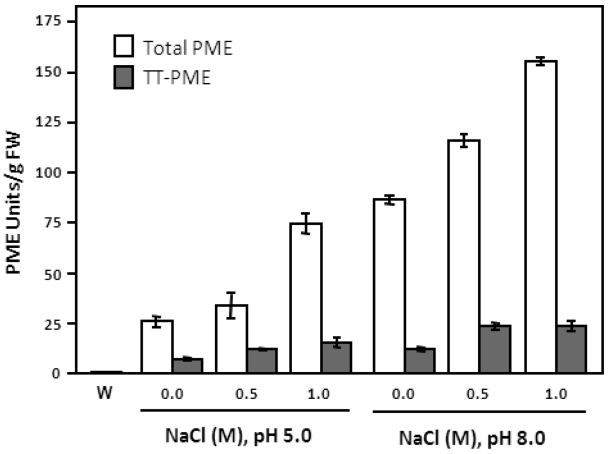

Figure 1. Evaluation of pH and salt content for PME extraction from orange finisher pulp. Finisher pulp was washed with salt-free water (W, wash solutions measured pH 3.8) prior to extraction with acetate (100 mM, pH 5.0) or Tris (100 mM, pH 8.0) buffers containing either 0, 0.5, or 1.0 M sodium chloride. Clarified extracts were assayed for total PME activity and then heated for 10 min at 70 °C to determine the fraction of TT-PME activity.

with 0.5 M sodium chloride, because this provided the lowest ratio of total PME to TT-PME activity while reducing contents of total coextracted total protein (data not shown). This pH and salt content would also be compatible for subsequent direct application to affinity chromatography media, eliminating manipulations such as dialysis and ammonium sulfate precipitation, which generally lead to excessive losses of enzyme activity and are not amenable to scaled-up preparative enzyme purifications.

Separation of Soluble Pectinates and PGIP Activity. Concanavalin A lectin affinity chromatography was used to separate TT-PME from nonglycosylated TL-PME activities by Cameron and Grohmann[7,20] and to purify the grapefruit PGIP.[21] In preliminary trials during this study, we obtained only partial binding of TT-PME activity to Con-A Sepharose upon direct application of clarified extracts. TT-PME similarly failed to bind to PME inhibitor protein immobilized on Sepharose[29] (Supporting Information). We associated this behavior with strong interaction in solution with coextracted pectinates. Consistent with this was poor permeation of PME activity through a 100,000 NMWL ultrafiltration membrane at pH 5 with 0.5 M as well as at pH 8.0 with 1.0 M sodium chloride. Similar ultrafiltration results were described by Snir et al.[26] Coextracted pectinates appear to be pervasive in citrus fruit extracts and have confounded efforts to dialyze or separate proteins by chromatography.[20,25−27,36] Ammonium sulfate precipitation treatments were used in previous PME purifications, but they generally resulted in large activity losses and did not completely remove the soluble pectinates.

To address issues with coextracted pectinates, we attempted selective removal of soluble pectinates using anion-exchange chromatography in the presence of moderate salt contents. We hypothesized that the pectinates with high charge density (such as block demethylesterifed pectin) were primarily responsible for the strong protein-binding interactions in solution, particularly for cationic protein such as PMEs, and would be selectively separated. This supposition was drawn from Cheng and Kindel's report[37] that citrus polygalacturonic acid was largely retained on anion-exchange resin following washing with 0.5 M sodium chloride. We found that GalA contents in the extract (1.93 mg GalA equiv/mL) were reduced 25-fold (to <0.08 mg/mL residual GalA) by simple batchwise treatment with DEAE-Sepharose. The sodium chloride molarity in the extract solution was reduced to 0.2 M by simple dilution prior to addition of the chromatography medium to the solution. Viscosity of the extract solution was also reduced following the DEAE-Sepharose treatment, which is also consistent with separation of coextracted pectin.

Once treated with the anion-exchange medium, the TT-PME activity readily bound to the PME-IP-Sepharose affinity column (Supporting Information). PME-IP affinity chromatography has been used routinely to purify fruit PMEs,[29,38] including those from citrus.[12] No PGIP activity was detected in the bound-protein fraction eluted from the PME-IP affinity column, but it was recovered in the flow-through volume. The specificity of PME and PME-IP interaction provided the selectivity necessary to separate the abundant PGIP (a moderately thermostable protein, data not shown), which has strikingly similar physical and chromatographic properties to the TT-PME.[11] These treatments provided a similar approach to obtain a highly purified PGIP from sweet orange fruit tissue, for which a complete description will be reported separately.[39]

Optimized Purification of TT-PME. A 1.3 kg batch of finisher pulp was processed to demonstrate TT-PME purification in high yield by combining ultrafiltration, heat treatment, separation of pectinates with DEAE-Sepharose, and selective affinity binding to immobilized PME-IP. The results using this optimized purification scheme (Figure 2) are

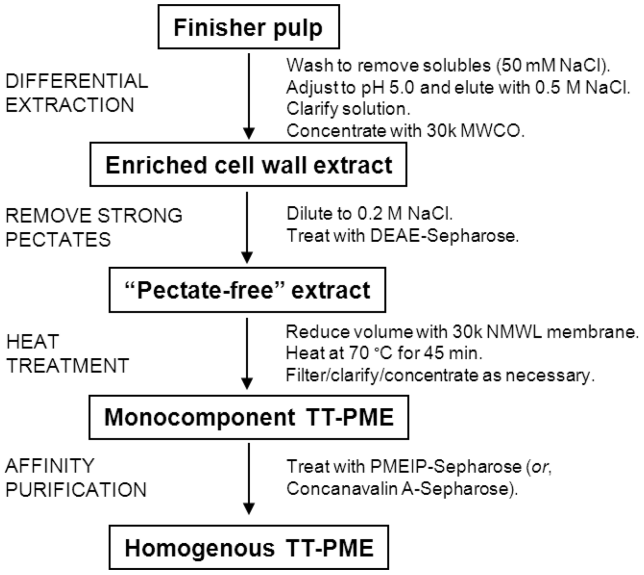

Figure 2. Optimized method for purifying TT-PME with high yield.

summarized in Table 2. TT-PME initially represented 43% of total PME activity extracted from finisher pulp (at pH 5). It was purified 163-fold with a yield of 72% to provide 8 mg of purified protein with specific activity of 963 units/mg protein (protein determined by using the Lowry method[30]). This yield is better than a 20-fold improvement over prior reports for any citrus PME purification. Total protein determined for TT-PME with the Coomassie Brilliant Blue reagent provided a higher specific activity of 1600 units/mg, which is the same as TL-PME assayed under identical conditions. (TT-PME specific activity determined using different protein assay methods is

Table 2. Purification of TT-PME3 from Sweet Orange Finisher Pulp[a]

			PME activity				
treatment	volume (mL)	protein[b] (mg)	total (units)	TT (units)	specific activity (units/mg)	purity factor	recovery (%)
cell wall extract	4330	1808	24300	10700	5.9	1.0	100
DEAE, 30k NMWL concentrate	206	883	21500	10000	11.3	1.9	94
heat treatment, 30k NMWL	134	141	9170	9170	65.0	11	86
PME-IP affinity (homogeneous)	5.60	8.00	7710	7710	963[c]	163	72

[a]Extraction from 1.3 kg of washed pulp. [b]Protein determined by using the Lowry reagent. [c]Final specific activity was 1600 units/mg with protein determined by Coomassie Brilliant Blue reagent.

compared in the Supporting Information.) Thermal stability, sodium chloride activation, and pH-dependent activity profiles determined with this pure TT-PME preparation were identical to the monocomponent preparation previously reported.[11]

Electrophoretic purity of the TT-PME preparation was assessed by SDS-PAGE (Figure 3). Two doublet bands are

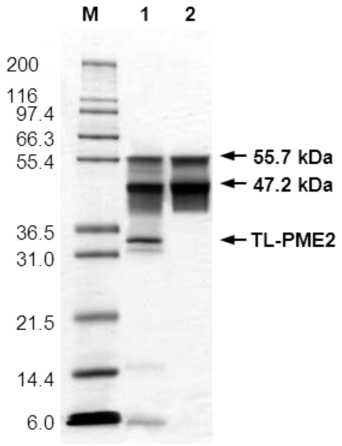

Figure 3. TT-PME protein preparation separated by SDS-PAGE (12% gel), before (lane 1) and after (lane 2) reheating to 70 °C. Replicate gels were run to recover protein bands (indicated by arrows at right margin of gels) for direct N-terminal sequencing and MALDI-TOF MS peptide mass fingerprinting. Molecular weight markers (in kDa) are indicated at left margin. Salt-independent TL-PME is indicated by arrow.[12]

observed with molecular masses of approximately 56000 and 47000 Da. These two bands were partially resolved as two glycovariants of approximately 55300 and 56500 Da and 47000 and 48200 Da in separation trials using Con-A Sepharose chromatography and a 10% PAGE gel (results not shown). Analysis of the purified TT-PME preparation by MALDI-TOF MS in linear mode indicated protein masses of 53000 and 48000 Da (Figure 4A). No traces of the 48000 Da band corresponding to co-isolated PGIP[11] were observed by SDS-PAGE or MALDI-TOF MS. A minor amount of protein at 34000 Da was readily removed by an additional heat treatment. We identified it as the salt-independent TL-PME by MALDI-TOF MS peptide mass fingerprinting.[12] We recently demonstrated this TL-PME isoform corresponds to the protein band that was used to isolate a *Citrus* PME gene associated with thermostable PME activity.[12,14]

Structure Characterization. N-Terminal peptide sequences were generated directly from the four variant TT-PME glycoisoforms. Edman sequencing chemistry indicated an identical 17 amino acid sequence for the N-terminal peptide: LQKSVXLTKFDLIVAKD. BLASTp search[40] (all protein databases at the NCBI Web site queried, blast.ncbi.nlm.nih.gov/) and tBLASTn provided no match to any previously described *Citrus* PME.

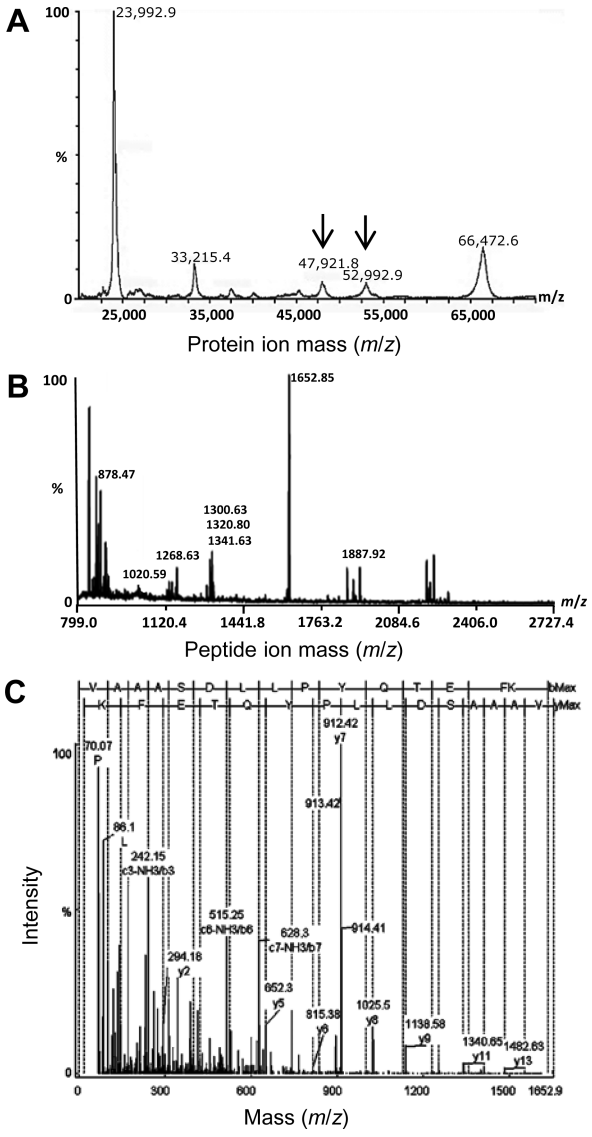

Figure 4. MALDI-TOF MS analysis of TT-PME: (A) MALDI-TOF MS spectrum (linear mode) from TT-PME native protein (two glycoforms indicated by arrows) [internal calibration with bovine pancreas trypsinogen (23982.05) and bovine serum albumin (66430.1; M + 2 at 33, 215)]; (B) mass spectrum (*m*/*z* 799 to 2727) of trypsin digest from the affinity-purified TT-PME separated by SDS-PAGE (masses indicated for eight peptide ions further analyzed by MALDI-TOT/TOF MS); (C) MS/MS mass spectrum for base peptide ion (*m*/*z* 1652.85) with amino acid assignments indicated for daughter fragment ions.

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TT-PME3
Ovary       1   GAMTNQYTCLDGFAYSDGNVRDVIKSSLYNISRHVSNSLVMLKKIPGDNM
Flavedo    42   GAMTNQYTCLDGFAYSDGNVRDVIKSSLYNISRHVSNSLVMLKKIPGDNM
Mixed      89   GAMTNQYTCLDGFAYSDGNVRDVIKSSLYNISRHVSNSLVMLKKIPGDNM

TT-PME3                                        LQKSVXLTKFDLIVAKD
Ovary      51   SSKYEVFPEYGRIKRGFPTWLSLNDRKLLQKSVNLTKFDLIVAKDGSGNF
Flavedo    92   SSKYEVFPEYGRIKRGFPTWLSLNDRKLLQKSVNLTKFDLIVAKDGSGNF
Mixed     139   SSKYEVFPEYGRIKRGFPTWLSLNDRKLLQKSVNLTKFDLIVAKDGSGNF

TT-PME3                                                TMLMFVGDGIGK
Ovary     101   TTITEAVEAAPNKSNTRFVIYIKAGAYFENVEVDKKKTMLMFVGDGIGKT
Flavedo   142   TTITEAVEAAPNKSNTRFVIYIKAGAYFENVEVDKKKTMLMFVGDGIGKT
Mixed     189   TTITEAVEAAPNKSNTRFVIYIKAGAYFENVEVDKKKTMLMFVGDGIGKT

TT-PME3                              SATVAVVGTGFIAK             HQAVALR
Ovary     151   VVKANRSVVDGWTTFRSATVAVVGTGFIAKGITVENSAGPSKHQAVALR
Flavedo   192   VVKANRSVVDGWTTFRSATVAVVGTGFIAKGITVENSAGPSKHQAVA
Mixed     239   VVKANRSVVDGWTTFRSATVAVVGTGFIAKGITVENSAGPSKHQAVALR
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Figure 5. Alignment of 199 common amino acid sequence from three translated *Citrus* EST cDNAs matched from TT-PME peptides. TT-PME, peptides determined directly from purified protein (N-terminal peptide is underlined, three de novo peptides used in BLAST also in box); Ovary, from *C. sinensis* ruby orange ovary at anthesis cDNA clone (CF833608) (5′3′ frame 1 translation); Flavedo, from *C. sinensis* developing fruit flavedo at 80 DAFB cDNA clone (CK936641) (5′3′ frame 2 translation); Mixed, from *C. clementina* adult, mixed tissue cDNA (DY285162) (5′3′ frame 3 translation).

Figure 6. Nucleic acid for *Citrus* TT-PME3 cDNA (1710 bp) (GenBank accession number pending) with translated protein sequence separated into signal peptide (33 amino acids; A), propeptide (212 amino acids; B), and catalytic domain (324 amino acids; C). Cleavage site between signal peptide and propeptide was predicted by SignalP tool.[44] The 17 amino acid peptide N-terminal sequence determined directly from the mature protein is indicated in brackets. Eight peptides determined from the TT-PME by MS/MS de novo sequencing tryptic digest are underlined. Thirteen N-X-S/T N-glycosylation sequons are indicated in bold. Five cysteine residues and peptide sequence conserved in propeptide of Clade 1 of plant PMEs[41] are marked in boxes. Five additional sequence elements conserved in plant PMEs are also indicated in boxes in the catalytic domain.[41] Residues identified in the active site of enzyme are indicated by an asterisk (*).[41]

MALDI-TOF MS for trypsin digests from the four partially resolved TT-PME glycovariants indicated a common peptide mass fingerprint (Supporting Information), consistent with the identical N-terminal peptide sequence. Twelve significant peptide ions were consistently observed. A representative peptide mass fingerprint for the 47000 Da band is shown in Figure 4B. MASCOT search using the peptide ion set provided protein scores indicating relationship to plant PME sequences, but TT-PME did not match with any previously reported *Citrus* PME sequence. Further investigation by MALDI-TOF/TOF MS to generate de novo peptide sequences provided high-confidence amino acid assignments for eight peptides. The MS/MS spectrum representing the base ion, mass 1652.85 Da, is shown in Figure 4C. One of the sequenced peptides, 794.46 Da, gave the best fit to HQAVALR, which is a common peptide observed in the *Citrus* TL-PMEs,[12] and it is present in a highly conserved sequence region in plant PMEs.[41,42]

The N-terminal peptide from direct sequencing and de novo peptide sequences generated by MALDI-TOF/TOF MS were used to further BLAST search of the translated PlantEST database for homologous *Citrus* sequences deposited from the Large-Scale EST Sequencing Project at NCBI. This resulted in matching two accessions from *C. sinensis*, one obtained from developing fruit flavedo cDNA and another from ovary tissue at anthesis, and three accessions from *Citrus clementina* (*C. reticulata*) EST cDNAs generated from reproductive and vegetative tissues. Sequences from EST cDNA accession hits were aligned, and a common nucleotide sequence of 597 base pairs was deduced from three overlapping sequences. The translated 199 amino acid polypeptide determined from this is shown in Figure 5 with positions of the peptides generated from MALDI-TOF/TOF MS and the direct N-terminal peptide sequences. The results indicate a sequence region overlapping the junction of the pro-peptide and catalytic domain. The undesignated residue (X) in the TT-PME N-terminal peptide is indicated to corresponded to asparagine. This residue falls within an N-X-T sequon, and it is likely glycosylated because no amino acid was recovered during that sequencing cycle in TT-PME protein and the tryptic peptide for SVNLTK (m/z 661.39) was not observed in MALDI-TOF MS spectra.

Cloning and Sequence Analysis. Initial PCR experiments with primers selected from the common EST nucleotide sequence and subsequent RLM-RACE PCR experiments enabled the generation of a full-length *Citrus* TT-PME cDNA sequence from sweet orange. The *tt-pme* nucleotide sequence (GenBank accession KF696665) of 1710 base pairs and the translated amino acid sequence are presented in Figure 6. The TT-PME protein sequence structure indicates it represents a type I PME[41] having a 33 amino acid signal peptide, a 212 amino acid propeptide region, and a 324 amino acid catalytic domain. The calculated molecular weight (av) for the TT-PME catalytic domain (aglycan) is 35629.47 with a p*I* of 9.18. There are 13 N-glycosylation sequons in the propeptide and catalytic domains, with 9 potential sites in the catalytic domain. Sequence elements observed in TT-PME indicate it falls within clade 1 of plant PMEs,[41] based on the 5 conserved cysteine residues and the 14 amino acid sequence ending with the RKLL motif. The latter feature is the putative target for subtilisin-like serine protease processing of the propeptide from the catalytic domain.[42] BLAST search using both nucleotide and protein sequences showed no matching homologue in the databases. BLASTp search with the TT-PME 324 amino acid catalytic domain sequence showed the closest described PME is that sequenced directly from the kiwifruit (*Actinidia delisiosa*) PME[43] (UniProtKB/Swiss-Prot accession P85076) with a 78% sequence identity for 96% sequence coverage and *E* value of 1e-180 (Supporting Information). Kiwifruit PME is a multiply (five sites) glycosylated isoform with a mass of 50000 Da by SDS-PAGE, and most of its glycosylation sites align with those present in the TT-PME sequence (Jose Tovar, personal communication). The role of glycosylation in *Citrus* TT-PME thermal stability properties can now be investigated through in vitro expression studies of the gene.

There are 23 theoretical tryptic peptides with masses >600 Da determined from the translated TT-PME amino acid sequence (Supporting Information). Direct peptide sequencing by MALDI-TOF/TOF MS (Figure 4C) resulted in about 28% coverage of the mature protein. All peptide sequences determined directly by MS/MS were confirmed from the translated nucleotide sequence from *Citrus tt-pme*. MALDI-TOF/TOF MS sequencing of tryptic peptides generated multiple peptides that were sufficient to correlate identity to other plant PMEs in sequence databases and to identify translated nucleotide sequences for homologous EST fragments. These sequences from anonymous accessions promoted expeditious cloning of the full cDNA sequence by PCR techniques.

In summary, we demonstrated TT-PME can be purified in high yield from finisher pulp under conditions that are directly amenable to preparative scaleup. Cumbersome ammonium sulfate precipitation and dialysis steps were eliminated by separating problematic pectinates using a high-capacity anion exchanger in the presence of at least 200 mM sodium chloride. Although a practical-grade monocomponent TT-PME can be obtained with extended heat treatment at 70 °C, an additional affinity chromatography treatment provided TT-PME of highly pure composition. Sequence analysis of TT-PME by MALDI-TOF/TOF MS enabled PCR cloning to obtain the *tt-pme* nucleotide sequence demonstrated that it is highly distinct from previously described *Citrus* PMEs. Multimilligram quantities of purified enzyme can now be readily produced for determining substrate specificity and action pattern[28] and to evaluate performance for modifying functional properties of commercially viable pectins and pectin-rich plant fiber. Availability of the TT-PME gene sequence will support development of molecular tools for controlling gene expression in citrus fruit for the purpose of improving juice quality. We have generated antisera specific to the TT-PME and cross-reactive to plant PMEs, based on unique and conserved peptide sequences (B. J. Savary and J. C. Tovar, unpublished results), that will support new molecular studies.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental figures include chromatography profiles for protein and PME activity binding to a PMEIP-Sepharose affinity column, protein determination for affinity-purified TT-PME comparing different assay methods, MALDI-TOF MS spectra from the four partially resolved TT-PME glycoforms, and the theoretical tryptic peptide ions generated from the translated TT-PME cDNA sequence. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*(B.J.S.) Phone: +(870) 680-4792. Fax: +(870) 972-2026. E-mail: bsavary@astate.edu.

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Notes

The authors declare no competing financial interest.

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