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Identification of Thermolabile Pectin Methylesterases from Sweet Orange Fruit by Peptide Mass Fingerprinting

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The multiple forms of the enzyme pectin methylesterase (PME) present in citrus fruit tissues vary in activity toward juice cloud-associated pectin substrates and, thus, in their impact on juice cloud stability and product quality. Because the proteins responsible for individual PME activities are rarely identified by structural properties or correlated to specific PME genes, matrix-assisted laser desorption−ionization with tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS) was investigated as a direct means to unequivocally identify the thermolabile (TL-) PME isoforms isolated from sweet orange [*Citrus sinensis* (L.) Osbeck] fruit tissue. Affinity-purified TL-PME preparations were separated by SDS-PAGE prior to trypsin digestion and analyzed by MS for peptide mass fingerprinting. The two major PME isoforms accumulated in citrus fruit matched existing accessions in the SwissProt database. Although similar in size by SDS-PAGE, isoform-specific peptide ion signatures easily distinguished the two PMEs.

INTRODUCTION

Pectin methylesterases (PMEs) are cell wall polysaccharide-modifying enzymes (EC 3.1.1.11) that variably act to hydrolyze C6-carboxyl methyl esters decorating homogalacturonan regions of pectin. These enzymes have been extensively reviewed in recent years (*1−3*). Control of PME activities in juice processing from fruit species such as *Citrus* is essential for the quality and stability of fruit juices (*4, 5*) and continues to be a highly active research endeavor (*6−9*). PME action to de-esterify pectin in juice cloud colloidal structures allows calcium-mediated flocculation and subsequent irreversible cloud separation, resulting in degraded juice (*5, 8*). It is well established that *Citrus* species produce multiple forms of PMEs in fruit tissues (*10−14*). PMEs are also now recognized to occur in very large multigene families in plants (*1−3*), and individual isoenzymes may have different action patterns toward pectin methyl esters (*14−16*). Only certain PME forms are responsible for citrus juice cloud separation (*11, 13, 14*). In sweet orange the most abundant PME present in pulp tissue, a thermolabile (TL) form described as salt-independent PME2 (*17*), readily destabilizes juice cloud at room temperature (*14*). Thermal lability is defined for enzymes that are readily inactivated by heating at temperatures below 70 °C. The second major TL-PME form, identified as salt-dependent PME4 (*18*), showed no cloud-destabilizing activity (*14*). These two PMEs correspond, respectively,

to PME-I and PME-II isolated from Navel orange, as designated by Versteeg et al. (*19*). Another minor TL-PME (PME1) detected in orange and grapefruit (*14, 20*) does not bind to a cation-exchange column, but this isoform has not been investigated in detail. A fourth PME, which is also present as a minor activity peak (PME3), is distinguished as a highly thermally tolerant (TT) form that can readily destabilize juice cloud under cold storage, and it is this PME activity that must be strictly controlled in juice processing (*5, 11, 14*). TT-PME is also reported in grapefruit (*12, 20*).

Although considerable research effort has been directed to understanding the biochemical properties of citrus PMEs and for technical means to suppress their activity, rarely are the individual PME forms under investigation structurally identified and correlated to specific gene sequences. Because PME activities measured under standardized assay conditions do not directly correlate to juice cloud destabilizing activity (*14*), ambiguous interpretations may result due to the presence of the multiple forms and their variable affect on juice cloud. We have isolated and characterized individual PMEs present in sweet orange fruit tissues, determining their apparent molecular size and p*I* by electrophoresis, obtaining partial amino terminal sequences, and characterizing general enzymatic properties (*14, 17, 18, 21, 22*). Identification based only on electrophoretic properties (i.e., apparent mass and p*I*) can be confounded by charge variants and posttranslational modifications that may include specific endoproteic cleavage of active enzyme (*17, 23−25*), as well as different buffer systems and

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calibration markers used. It is not known if true size or charge variants differ in activity properties from their structural progenitor(s).

Partial N-terminal amino acid sequencing from electrophoretically resolved sweet orange TL-PMEs has provided direct correlation of identity with specific gene isoforms (*18*, *21*). However, improved methods for identification are desired due to issues of protein amino termini blockage preventing Edman sequencing chemistry and limited utility for such sequencing to differentiate protein products between highly homologous isoforms. The latter is particularly relevant because there appear to be at least 19 partial PME expressed-sequence tag sequences represented in *Citrus* expressed-sequence tag libraries (*21*). A growing set of *Citrus sinensis* PME genomic and transcript cDNA sequences have been deposited in Genbank (*26−29*), representing at least three distinct translatory products (preproteins) with lengths of 510, 584, and 631 amino acids. The existence of a large PME gene family present in *Citrus* species is further suggested by 66 PME-related gene sequences identified in the *Arabidopsis thaliana* genome and 89 gene copies in the *Populus trichocarpus* genome (*3*). Increasingly larger numbers of PME sequence accessions are available in public sequence databases from various plant genomic programs including *Citrus* (*2*, *3*, *30*), and these sequence resources support application of mass spectrometry such as matrix-assisted laser desorption−ionization with time-of-flight mass spectrometry (MALDI-TOF MS) for direct and unequivocal identification of proteins by peptide mass fingerprinting (PMF) (*31−34*).

The objective of this study was to evaluate MALDI-TOF MS as a tool to routinely and accurately identify the multiple TL-PME forms isolated from sweet orange fruit tissue. The approach was to generate PMFs from tryptic digests for each PME separated by SDS-PAGE and to match them to reference peptide libraries prepared from theoretical digests of known sweet orange PME sequences. High-quality spectra were then evaluated for unique peptide ion signatures for unequivocal identification of individual PME forms. Direct identification of PME isoenzymes based on protein structure will improve our understanding of their function and impact on juice quality as well as their evaluation for technological application to tune the fine-structure of isolated pectins for improved functional performance (*24*, *35*, *36*).

EXPERIMENTAL METHODS

Preparation of Pectin Methylesterases. TL-PME activity peaks were isolated from sweet orange fruit finisher pulp [*C. sinensis* (L.) Osbeck] as described previously (*14*). PME activities and pH-optimum profiles were determined titrimetrically as described previously (*17*) using citrus pectin at pH 7.0 with 1.2% NaCl. Individual PME isoforms were further purified by affinity chromatography using the kiwi fruit PME-inhibitor protein immobilized on activated Sepharose media (*37*) to isolate electrophoretically pure individual TL-PMEs (**Figure 1**).

Evaluation of Heat-Treated Finisher Pulp Protein Extracts. Cell wall-associated proteins were extracted from thoroughly washed finisher pulp using Tris buffer (pH 8.2) with 1.0 M NaCl and exchanged to 20 mM sodium phosphate (pH 7.0). The concentrated extract was placed in a 70 °C water bath, and aliquots were taken at time intervals, cooled on ice, and centrifuged to clarify. PME activity was then measured, and proteins remaining soluble were visualized by SDS-PAGE. Heat treatment of affinity-purified TL-PME2 was performed in the same manner.

Gel Electrophoresis and Trypsin Digestion of Proteins. TL-PME preparations were resolved by SDS-PAGE using the NuPAGE Bis-Tris 12% gel system (Invitrogen, Carlsbad, CA) with MOPS buffer following the manufacturer's instructions. Gels were stained with SimplyBlue (Coomassie Brilliant Blue G250). Individual protein bands (ca. 1 μg) cut from gels were digested overnight with Trypsin-Gold (Promega, Madison, WI) following the manufacturer's instructions. Peptides extracted from gel pieces were subsequently purified with C18 ZipTips (Millipore, Billerica, MA) following the manufacturer's instructions using recrystallized
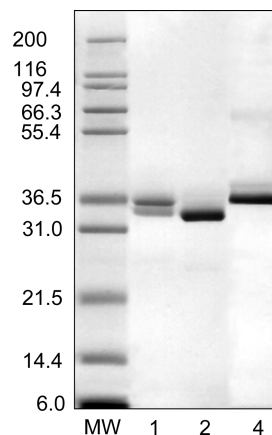

Figure 1. SDS-PME of sweet orange TL-PMEs purified from fruit pulp. Lanes: MW, molecular weight markers (×1000); 1, PME1; 2, PME2; 4, PME4.

α-cyano-4-hydroxycinnamic acid matrix solution (5 mg/mL) in acetonitrile mixed 1:1 with 0.1% trifluoroacetic acid. Approximately 0.7 μL of the peptide−matrix solutions were freshly spotted onto the target plate and dried in a desiccator at room temperature immediately before MS analysis.

Mass Spectrometry. MALDI-TOF/TOF mass spectra of trypsin-digested proteins (i.e., PMFs) were acquired with a 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Framingham, MA) in the positive reflectron mode with a 200 Hz Nd:YAG (355 nm) laser. At least three sample replicates were examined for each protein digest. Spectra were obtained by averaging 1000 acquired spectra in the MS mode or 2500 in the MS/MS mode. Collision-induced dissociation (air as the collision gas) was used at approximately 1×10^{-6} Torr and a 1 keV acceleration voltage to generate the MS/MS spectra of selected peptides. MS spectra and MS/MS spectra of selected peptides were combined and queried against primary sequence databases using the Mascot search engine (*32*) (www.matrixscience.com/search_form_select.html) and GPS Explorer program (Applied Biosystems). MS/MS interpretations and fragmentation assignments of selected peptides were performed with GPS Explorer (Applied Biosystems) and PEAKS (Bioinformatics Solutions Inc., Waterloo, ON, Canada).

Average molecular masses of intact proteins were acquired with a Waters Maldi Micro MX mass spectrometer (Milford, MA) in the positive linear mode with a 20 Hz nitrogen laser (337 nm). Spectra were obtained from 50 scans and calibrated with trypsinogen (23981.9 Da) and aldolase (39213.3 Da) (Sigma-Aldrich, St. Louis, MO), which were also used for internal lock mass. Protein solutions were mixed 1:1 (v/v) with 10 mg/mL sinapinic acid in 0.1% aqueous trifluoroacetic acid containing 40% acetonitrile, and 1 μL of each sample was spotted onto the stainless steel target plate and air-dried. All data were processed with Waters MassLynx software, and the average molecular weights were determined using at least three independent experiments for each protein.

PME Peptide Reference Libraries. Protein sequences translated from PME genes [Q8GS16 (*28*) and P83948 (*26*)] were downloaded from the ENTREZ database at the NCBI site (www.ncbi.nlm.nih.gov). Protonated peptide masses $(M + H)^+$ from theoretical trypsin digestion were obtained with the PeptideMass tool (www.expasy.ch/tools/peptide-mass.html), and peptides with $m/z > 500$ were tabulated for reference libraries. Signal peptide and N-terminal pro-protein sequence domains were omitted from libraries, using N-terminal amino acid sequences determined directly from mature TL-PME proteins (*17*, *18*, *21*). Peptide coverage was examined by manual screening of MS spectra for each protein and compared with corresponding PME libraries. Observed PME-derived peptides confirmed by MS/MS de novo sequencing were used for additional BLAST searches (www.ncbi.nlm.nih.gov/BLAST) to assess their frequency in protein sequence databases.

RESULTS AND DISCUSSION

Identification of Major TL-PMEs by MS Analysis. The two predominant TL-PMEs activity peaks (PME2 and PME4), as well as

a secondary minor TL-PME (PME1), were separated by cation-exchange chromatography as described previously (*14*). Affinity purification with immobilized PME-inhibitor protein provided highly purified individual sweet orange TL-PME proteins, as observed by SDS-PAGE (**Figure 1**). **Figure 2** shows representative MALDI-TOF MS spectra for trypsin digests from individual TL-PMEs. The peptide ion profiles in spectra for PME2 and PME4 readily distinguish a unique PMF for each protein. Intensities of individual peptide ions in spectra for sample replicates varied somewhat, and occasionally weak peaks were not observed between replicate samples, but all major peptide ions were consistently observed between replicate samples. Combined with MS/MS sequence data for major peptide ions (four for each protein), Mascot searching of the Swiss-Prot database routinely
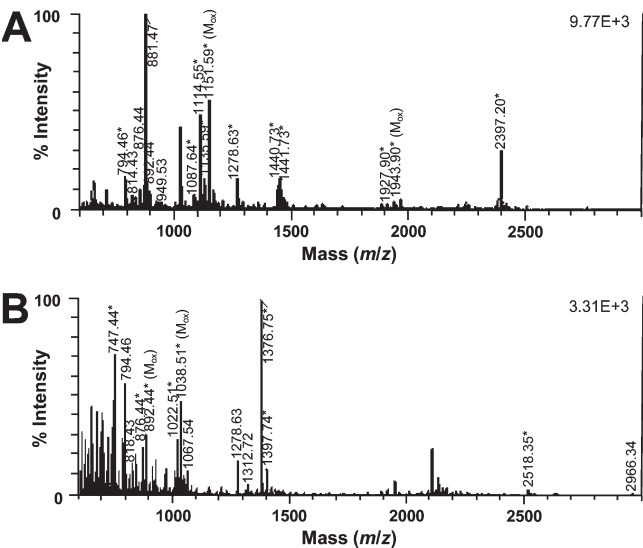
provided 100% confidence (typically, protein scores of ca. 400, with at least twelve peptides detected) in identification for PME2 and PME4 with existing gene accession for sweet orange PMEs. PME4 closely matched the major PME protein isolated from Navel orange peel by Christensen et al. (*26*), UniProtKB P83948, whereas ==PME2 matched the sequence designated CsPME4 by Arias and Burns (*28*),== UniProtKB Q8GS16, a protein that they associated with a heat-stable PME activity in finisher pulp extract.

Theoretical tryptic peptides determined from the two TL-PME isoforms were tabulated and compared with those observed by MALDI-TOF MS. Active PMEs are processed from prepro-proteins (*1*−*3*), so only polypeptides representing the mature enzyme domain were included. The N-terminal sequences of purified TL-PMEs were determined previously by direct amino acid sequencing (*17, 21, 26*). There are twenty peptides that can potentially be observed in the mass range of m/z 500−5000, but only as many as fifteen peptide ions were observed in highest quality spectra from both PMEs. Whereas the two PME isoforms share 81% sequence identity for the mature protein domains (315 total amino acids), there are only four common tryptic peptides. **Figure 3** summarizes the tryptic peptide ion masses, $(M + H)^+$, and amino acid sequences for the theoretical peptides and those observed in TL-PME spectra. Differences in peptide profiles between PME2 and PME4 are due primarily to single substitutions of amino acids within sequences (four peptides − e.g., peptides 15 and 16, respectively) and for arginine and lysine substitutions that alter trypsin cleavage sites (eight peptides, for example, peptide 7 and peptides 8 and 9, respectively). Additional peptide ions were observed in spectra that represent modified peptides containing oxidized methionine (+16 Da) (e.g., peptides 5 and 6, respectively) and for several single miss-cleaved peptides (e.g., AGEYR + ENVEVPK, m/z 1390.72, in PME2).

De Novo Peptide Sequences by MS/MS. Detailed MS/MS analysis of major peptide ions was used to assess both common and isoenzyme-specific peptide signatures in PME2 and PME4. Computer-assisted interpretation of MS/MS spectral data provided the de novo amino acid sequence assignments. Representative MS/MS spectra and amino acid sequence results are presented in **Figure 4**. The fragmentation pattern from the ion series generated for peptide ion m/z 1278.63 provided the sequence DITFQNTAGPSK (**Figure 4A**), which is one of the four

Figure 2. Peptide mass fingerprints obtained by MALDI-TOF MS from trypsin digests of sweet orange TL-PMEs: (**A**) PME2; (**B**) PME4. Mass range covered m/z 600−3000. Ion masses are indicated for those ions matched to PME peptides (see **Figure 3**). Peptide ions providing MS/MS sequencing are indicated by asterisk. Peptides with oxidized methionines (+16 mass) are indicated by M_{ox}.

A

#	Sequence Position	Mass (M + 1)	PME2 Peptide Sequence
2	33-36	564.35	YIIR
4	44-50	814.43	ENVEVPK
5	53-62	1135.59	*INLMFIGDGR (+ M$_{ox}$)*
6	63-71	949.53	TTTIITGSR
7	72-95	2397.20	*NVVDGSTTFNSATVAVVGDGFLAR*
8	96-107	1278.63	**DITFQNTAGPSK**
9	108-114	794.46	**HQAVALR**
10	115-124	1114.55	*VGSDLSAFYR*
11	125-140	1927.90	**CDMLAYQDTLYVHSLR (+ M$_{ox}$)**
13	172-178	881.47	RPNPNQR
14	179-186	876.44	**NMVTAQGR (+ M$_{ox}$)**
15	187-199	1441.73	DDPNQNTGIVIQK
16	202-212	1087.64	IGATSDLLAVK
17	213-224	1440.73	GSFETYLGRPWK

B

#	Sequence Position	Mass (M + 1)	PME4 Peptide Sequence
2	19-33	1312.72	TVAA$\underline{A}$VAAAPQGGTK
3	35-38	564.35	**YIIR**
5	46-52	818.43	ENVEVTK
6	56-64	1022.51	*NIMFIGDGR (+ M$_{ox}$)*
7	67-73	747.44	**TIITGSR**
8	74-83	1067.54	NVVDGSTTFK
9	84-97	1376.75	*SAT$\underline{V}$AVVGEGFLAR*
10	98-109	1278.63	DITFQNTAGPSK
11	110-116	794.46	HQAVALR
12	117-142	2966.34	VGADLSAFYNCDMLAYQDTLYVHSNR (+ M$_{ox}$)
15	181-188	876.44	**NMVTAQGR (M$_{ox}$)**
16	189-201	1397.74	*ADPNQNTGIVIQK*
17	204-226	2518.35	**IGATSDLKPVQGSFPTYLGRPWK**

Figure 3. Peptides observed in spectra from sweet orange PME2 (**A**) and PME4 (**B**) proteins by MALDI-TOF MS. Peptide libraries were created from UniProtKB accessions Q8GS16 (*27*) for PME2 and P83948 (*25*) for PME4. (Peptide 2 in PME4 contains a variant amino acid reported in PME Sequence I.D. 1 in PCT patent WO 97/03574 (*36*). Protonated ion mass $(M + H)^+$ for tryptic peptides was calculated using the PeptideMass tool (peptide sequences < m/z 500 are omitted). The amino-terminal sequence was determined directly from purified mature protein (*17,18*). All peptides analyzed by MS/MS are indicated in bold. Peptides in italics represent abundant signature peptides. Conserved peptides common to both PME isoforms are indicated by shading of masses.
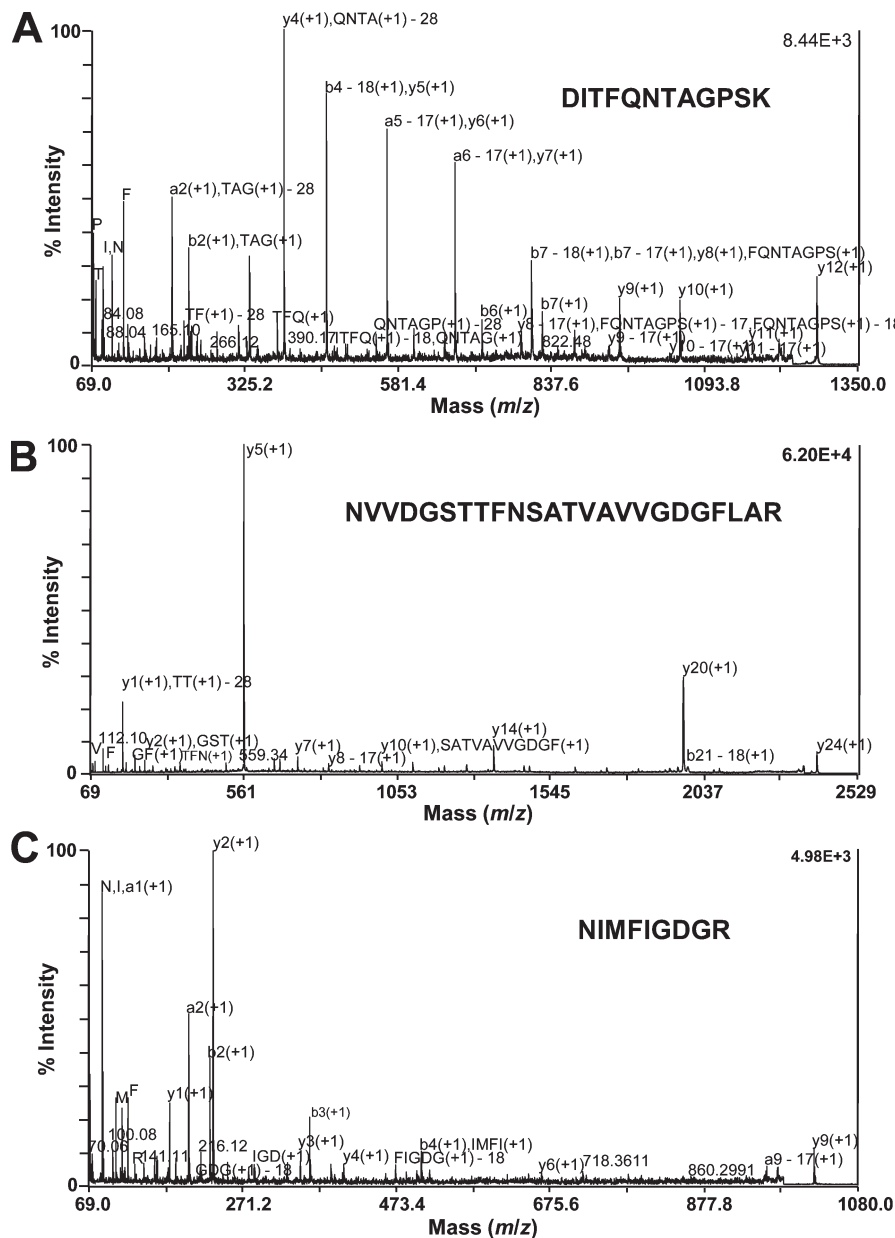
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Figure 4. Representative MS/MS spectra and de novo sequences of peptides from sweet orange PME2 and PME4: (**A**) Peptide ion *m/z* 1278.63 (M + H)$^+$ is one of four tryptic peptides common to both PME2 and PME4; (**B**) Peptide ion *m/z* 2397.20 is a signature peptide specific to PME2. (**C**) Peptide ion *m/z* 1022.51 is specific to PME4.

common peptides. A second common peptide ion, *m/z* 794.46, provided fragmentation patterns consistent with the sequence HQAVALR, but ion fragments provided weak intensities for MS/MS spectra. This peptide is particularly highly conserved in plant PMEs (*2*), consistent with BLAST search (results not shown). Using automated screening features of instrument software, combination of these four common peptide ions (*m/z* 1278.63, 876.44, 794.46, and 564.35) may be useful for initial MALDI-TOF MS screening of proteins for putative identification as a PME.

Representative MS/MS spectra for abundant peptide ions that are isoform-specific peptides are also presented in **Figure 4**. Whereas as many as seven peptides ions unique for PME2 could be selected and MS/MS spectra obtained, three peptides ions (*m/z* 1114.55, 1135.59, and 2397.20) routinely provided high-quality spectra. Peptide ion *m/z* 2397.20 provided a reliably intensive peptide ion, and its presence confirms the identity of this PME isoenzyme (**Figure 4B**). Similarly, there were six peptides unique for PME4, and three peptide ions (*m/z* 1022.51, 1376.75, and

1397.74) were of consistent intensity to serve as signature peptides for screening samples to confirm the presence of this PME isoenzyme (**Figure 4C**). The latter two peptide ions (for peptides SAT**V**AVVGEGFLAR and **A**DPNQNTGIVIQK) were critical in matching PME4 to protein accession P83948 (*26*) from that translated from a highly homologous gene isolated by Nairn et al. (*27*). Single amino acid differences in both (V for A and A for T, respectively, as underlined in the sequence) provided distinguishable detectable mass shifts, as well as unambiguous assignments for the amino acid substitutions. These are 2 of 6 variant amino acids in the 318 amino acids present in the mature protein sequences (98% identity between the two forms). Peptide mass matching further distinguished the PME isozyme purified from Valencia orange from a second homologous PME sequence reported in Navel orange (*38*). In this case, the single amino acid difference in peptide 2 (**Figure 3B**) was sufficient to distinguish protein products from a nearly identical homologous gene. These results indicate that isoenzyme-specific peptides can be used as

molecular signatures to confirm PME presence and isoform identity in protein preparations by MALDI-TOF/TOF MS.

Characterization of TL-PME1. PME1 is observed as a minor *Citrus* TL-PME that does not bind to a cation-exchange column at neutral pH (*14, 20*), but no description has been made of this enzyme. Two bands were resolved by SDS-PAGE in the affinity-purified PME1 preparation (**Figure 1**). Both bands provided spectra common to PME2 (**Figure 2A**) with confident identification by Mascot with the PME accession reported by Arias and Burns (*28*). However, protein scores were consistently lower with fewer peptides obtained with MS/MS, compared to PME2. The peptide ion *m/z* 2397.20 is greatly reduced (no MS/MS confirmation), and no other variant peptides with distinguishable mass shifts were evident in the spectra. Manual inspection of spectra demonstrated no PME4-specific peptide ions unique to PME4 were present, particularly in the upper band. In contrast to PME2, which provided extensive N-terminal peptide sequence coverage by Edman sequencing chemistry (*21*), the N-termini were blocked for both PME1 protein bands. The pH−activity profiles for PME1 in the presence and absence of 1.2% NaCl were similar to those observed for PME2 (*17*), indicating PME1 is also a salt-independent form (**Figure 5**). Results from Southern blotting analysis by Arias and Burns (*28*) indicated a second homologous gene copy for PME2. The structural and biochemical similarities with the PME2 protein therefore suggest that PME1 may be the translational product of this second homologous gene. However, we have no certain explanation for the larger size variant

Figure 5. pH−activity profiles for purified PME1 activity assayed in the presence (circles) and absence (triangles) of 1.2% NaCl. 100% PME activity is determined at pH 7 with 1.2% NaCl.

observed in PME1. Confirming this tentative identification will require isolation and sequencing of individual variant peptides from the protein, which would also support subsequent RT-PCR cloning of the second homologous gene copy.

PME2 Protein Solubility in Heat-Treated Enzyme Preparations. Our preparation of highly pure PME2 in this study and a previous one (*17*) and mass spectrometry with previous partial direct N-terminal sequencing (*21*) demonstrate unequivocally that this protein represents the product from the gene sequence reported by Arias and Burns (*28*) for a PME protein they isolated from a heat-treated extract. With improved separation techniques and these new structural analysis tools, we have also purified and characterized the protein responsible for TT-PME activity (PME3). The structural properties and cloning of the TT-PME isoform will be reported in a separate paper (Savary et al., unpublished data). To understand how the PME2 protein was originally associated with a thermally stable PME activity (*28, 39*) and demonstrate the power of using MS to readily identify enzymes important in food chemistry, we investigated PME2 solubility properties in pure and crude extract forms following heat treatment (**Figure 6**).

PME2 is the major TL-PME isoenzyme accumulated in citrus pulp tissue (*10, 13, 14, 19*). **Figure 6A** shows that following heating of a crude pulp tissue cell wall extract at 70 °C, the total PME activity was rapidly reduced to a 20% level, which represents the TT-PME that is stable with continued heating. These results are similar to those reported by others (*39, 40*). Consistent with the inactivation of TL-PME activity with heating at 70 °C, the activity for the purified PME2 was rapidly lost during heat treatment at this temperature. In purified form, this 35 kDa protein becomes completely insoluble during heat inactivation (**Figure 6B**). However, in the SDS-PAGE profile for the time course for crude protein extract treated at 70 °C (**Figure 6C**), there is no pronounced change in the solubility of a common 35 kDa protein; it is observed though the entire heat treatment time course. This major 35 kDa protein is identified as TL-PME2 by its MALDI-TOF MS spectrum (same as **Figure 2**). These results show that although inactivated by heat, it has not lost solubility and is maintained in the total protein profile observed by SDS-PAGE, which explains its observation in previous studies (*27, 39*). Pulp tissue extracts contain measurable amounts of soluble pectinates, which we suggest here are responsible for maintaining heat-denatured TL-PME2 in solution. Soluble pectinates can interact with active PMEs in solution and confound
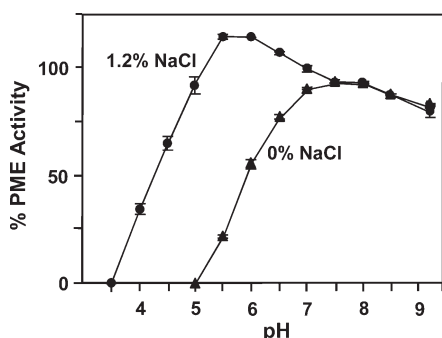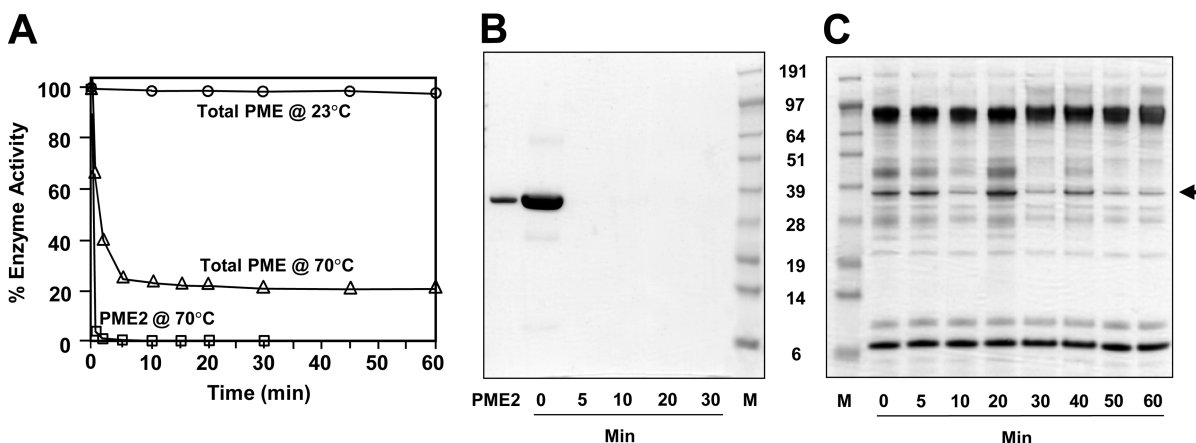

Figure 6. Evaluation of PME2 solubility following heat treatment at 70 °C. (**A**) PME activity during time course of heat treatment: total PME activity in finisher pulp extract at 23 °C (control treatment, circles) and at 70 °C (triangles). Purified PME2 in neutral buffer was heated at 70 °C (squares). (**B**) Time course of soluble protein following heat treatment of purified PME2 (lane 1): aliquots (initially containing 5 µg of protein) taken from solution heated for up to 30 min at 70 °C, chilled and centrifuged, and separated by SDS-PAGE. (**C**) Time course of soluble proteins in finisher pulp extract heated at 70 °C: samples treated in same manner as purified PME2. M, molecular weight markers are ×1000. Arrow to right indicates PME2 band.

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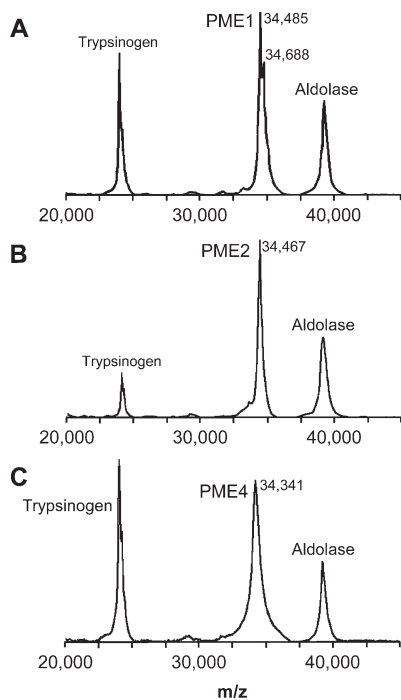*J. Agric. Food Chem.,* Vol. 58, No. 23, 2010   **12467**

Figure 7. Molecular weight (average) determined for purified TL-PMEs by MALDI-TOF MS operating in linear mode. Internal two-point calibration used trypsinogen (23982) and aldolase (39213) standards.

efforts to purify them (*41−43*). We find these charged polysaccharides are effectively separated from basic proteins such as PMEs by bulk pretreatment with anion-exchange resin in the presence of moderate NaCl concentrations (Savary et al., unpublished data).

Mass Determination for TL-PMEs. We determined the average molecular weight for the three purified TL-PME preparations using MALDI-TOF MS in linear mode (**Figure 7**). Consistent with the two bands observed by SDS-PAGE, PME1 provided a split ion peak with average molecular weights of 34485.2 (±17.3) and 34687.7 (±9.2). The average molecular weight observed for PME2 was 34467.0 (±73.6) and for PME4 was 34340.9 (±27.4). The polypeptide sequences representing mature PME2 and PME4 lack glycosylation sequons, and the mass values determined by MS are in close agreement with the molecular weights calculated from their known amino acid sequences. In **Figure 1**, PME4 appears to migrate as a slightly higher molecular weight protein compared to PME2. We previously observed PME4 to run slightly lower than PME2 (*17, 18*). This difference is associated with the use of Bis-Tris/MOPS buffer (Invitrogen NuPAGE gel system) in place of the traditional Laemmli buffer (*44*) we used previously. We suspect the migration of PME4 is slightly retarded in the Bis-Tris buffer system due to its very alkaline p*I* [calculated at pH 9.68, experimentally observed at pH 10.24 (*18*)]. Similar anomalous results have occurred with other high p*I* PMEs and some polyhistidine-tagged proteins (unpublished observations). These results show why relative masses determined by SDS-PAGE are an unreliable means to identify PMEs.

In conclusion, we have demonstrated how biochemical and molecular foundations developed previously for *Citrus* PMEs can be combined with current proteomics tools (mass spectrometry, bioinformatics, and sequence databases) to define diagnostic peptide ions in PMFs and use them to obtain highly specific structure-based identification of PME isoforms. These tools are now accessible to laboratories with common biochemical capabilities (chromatography and electrophoresis) to generate samples from

SDS-PAGE gels that can be submitted to now widely available service and core facilities for MALDI-TOF MS analysis (*45*). Unequivocal identification of individual PMEs present in enzyme preparations will enable a better understanding of their functional properties and determination of appropriate targets for biotechnological manipulation of plant PME expression in crop production and food processing systems.

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