JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of a Salt-Independent Pectin Methylesterase Purified from Valencia Orange Peel

BRETT J. SAVARY,*,† ARLAND T. HOTCHKISS,† AND RANDALL G. CAMERON‡

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038-8551, and Citrus and Subtropical Products Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 600 Avenue S, Northwest, Winter Haven, Florida 33883-1909

The pectin methylesterase (PME; EC 3.1.1.11) present in a commercial orange peel enzyme preparation was characterized to establish its identity among the multiple PME isozymes present in Valencia orange (*Citrus sinensis* L.) peel. We show the commercial enzyme corresponds to the major peak 2 PME previously separated by heparin-Sepharose chromatography (Cameron et al., *J. Food Sci.* 1998, *63*, 253). Both PMEs have comparable elution profiles on cation-exchange and hydrophobic-interaction perfusion chromatography columns, molecular weights (ca. 34 kDa) and pI (pH 9.2), and biochemical properties, including a broad pH activity range and activity in the absence of added cations. An identical partial amino terminal peptide sequence was also obtained for the PMEs, which further demonstrated a structural identity with other plant PMEs. The biochemical and structural properties readily distinguish this Valencia orange PME from salt-dependent isozymes and further suggest that it is an ortholog to the salt-independent fruit-specific isozyme of tomato. This work provides a well-defined, enzymatically homogeneous, salt-independent (type 1) plant PME isozyme that is suitable for studying details of the enzyme's mode of action and for use in modifying methylester patterns for studying the structure−functional property relationships in pectin.

KEYWORDS: ***Citrus sinensis*; orange peel; pectin; pectin methylesterase; de-esterification; purification; perfusion chromatography**

INTRODUCTION

Pectin methylesterase (PME) (EC 3.1.1.11) hydrolyzes C_6-methylester groups in homogalacturonan regions of pectin in the plant cell wall. Multiple isozymes of this enzyme are present in fruit and vegetative tissues, and individual isozymes can be distinguished by their expression patterns and by their physical and biochemical properties (*1*). Molecular studies have further demonstrated plant PMEs exist in multigene families (*2*−*4*). Heterogeneity of PME isozymes presumably reflects divergence in tissue-specific and functional specialization. Plant PMEs are generally regarded to de-esterify pectin in a block-wise pattern, but only a few PMEs have been examined in detail (*1*). Recent NMR studies indicate differences in action patterns by individual plant PME isozymes and at different pH values (*5*−*7*).

In tomato plants, two groups of PMEs have been distinguished (*8*): type 1 isozymes that are synthesized specifically in fruit tissues, and type 2 isozymes whose expression is not restricted to fruit tissues but occur generally in vegetative tissues. These two types may be distinguished biochemically in that the type 1 isozymes do not require cations for activity, whereas the type 2 isozymes require cations for activity. Both types of isozymes have been co-isolated from tomato fruit tissue extracts (*9*, *10*). These two PME isozyme types can similarly be distinguished in oranges. In Navel orange, two major PME isozymes were isolated from fruit tissues, and the PE1 was shown to destabilize orange juice cloud, whereas PE2 did not (*11*, *12*). In Valencia orange, four PME activity peaks were resolved chromatographically (*13*). The Valencia peak 2 PME, the most abundant isozyme, destabilized orange juice cloud, but the peak 4 PME, the second major isozyme, did not affect orange juice cloud. Chromatographic behaviors and juice cloud destabilizing properties indicate correspondence of Navel PE1 with Valencia peak 2 and Navel PE2 with Valencia peak 4. In recent molecular studies, a single PME isolated from Valencia orange juice vesicles was used to prepare probes for isolating two groups of cDNA and genomic clones, which were distinguished by sequence homologies, differential hybridization, and tissue expression patterns (*3*). All clones obtained were differentially expressed in nonfruit tissues, indicating the two groups represent two subgroups of vegetative PME isozymes. The PME isolated by Christensen et al. (*14*) from Navel orange peel was shown to be a salt-dependent isozyme. A comparison of nucleotide sequences from the cDNA cloned from this PME reveals it is identical to one of the vegetative PMEs sequenced by Nairn

* To whom correspondence should be addressed (telephone 215-233-6458; fax 215-233-6406; e-mail bsavary@arserrc.gov).
† Eastern Regional Research Center.
‡ Citrus and Subtropical Products Laboratory.

10.1021/jf020060j
Published on Web 04/26/2002

and co-workers (*3*), and it is similarly homologous to the tomato PMEU1 (*14*), thus indicating it represents a type 2 PME isozyme. No genomic or cDNA clones corresponding to type 1 (salt-independent) PMEs have been reported from orange.

We are interested in using pectinases such as the orange peel PME to study the relationship between pectin structure and dependent functional properties. A better understanding of the role of homogalacturonan methylesterification in this relationship may allow the specific manipulation of structure to develop novel pectins with higher performance and greater functionality in food and for new industrial applications. Ultimately, new markets are sought for which pectin can be produced from underutilized peel residues from domestic orange juice production and from sugar beet pulp from sugar production. Because it is evident that multiple isozymes are present in orange peel, and individual isozymes have different biochemical properties and likely different action patterns, it is necessary to isolate individual Valencia PMEs for characterization to establish unequivocal identity. For specific modification of pectin methylester patterns, without other concomitant structural changes in the pectin molecule that can confound interpretations of methylesterification affects on functional properties, the enzyme must be free from contaminating isozymes and depolymerizing enzymes. In this paper we report our efforts to prepare a well-defined and enzymatically homogeneous type 1 (salt-independent) plant PME. In a companion paper (*15*) we demonstrated the equivalency of the salt-independent PME isolated from either the commercial or fresh peel preparations following deesterification of citrus pectins with this enzyme. This PME isozyme was determined to act in a block-wise manner, and deesterification occurred without unintended depolymerization of the pectin.

MATERIALS AND METHODS

PME Activity Assay. PME activity was determined by titration assay in a Radiometer pH-stat following the conditions described by Sigma Technical Services: 1.0% citrus pectin (Sigma, P9135) containing 1.2% (w/v) NaCl, maintaining the solution at pH 7.0 (with 50 mM NaOH), and controlling temperature at 30 °C with a water-jacketed reaction beaker. Enzyme solutions were diluted to give consumption rates of about 10−20 μL NaOH/min. One unit of activity is 1 μmol hydroxide equivalents consumed per min. The affects of salt and pH were determined by assaying enzyme activity (in triplicate) in the absence or presence of 1.2% (w/v) NaCl at intervals from pH 4 to pH 10, otherwise using the standard assay conditions. Activities in the alkaline range were corrected by subtracting hydrolysis rates of pectin solutions in the absence of enzyme. Temperature optimum was determined using the standard assay conditions but with first equilibrating the substrate solution to the indicated temperature prior to addition of the enzyme. For determining the thermal stability, enzyme solution was placed in a capped microfuge tube and placed in a waterbath that was equilibrated at the target temperature. Constant volume aliquots (20 μL) were taken at 1, 5, 10, and 15 min intervals and immediately assayed for activity in the standard assay solution.

Enzyme Preparation. Orange peel PME (P5400) was purchased from Sigma Chemical Co. (St. Louis, MO). This enzyme preparation is prepared from Valencia orange peel and is described by the manufacturer to be essentially free of polygalacturonase and pectin acetylesterase activities. The enzyme was prepared by dissolving the lyophilized power in 50 mM HEPES buffer (pH 7.5) containing 500 mM NaCl, followed by sonication and clarification by centrifugation. The enzyme solution was then diafiltered for three cycles in an Amicon stirred-cell ultrafiltration device (fitted with YM 10,000 NMWL membrane) with 20 mM HEPES buffer (pH 7.5), providing dilution-concentration factors of 5-fold per cycle, and a final protein concentration of about 2 mg/mL. Protein determination was performed with the Biorad (Coomassie blue) dye-binding assay using bovine serum albumin as standard.

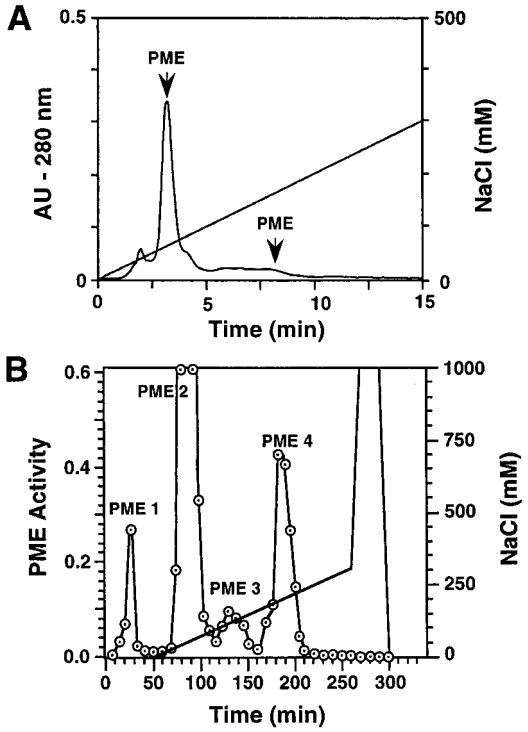

Figure 1. Cation-exchange chromatography of the Valencia orange peel pectin methylesterases. A. Preparative separation of the commercial enzyme preparation on Poros HS/20 perfusion chromatography column (1.7 × 18 cm). Elution gradient performed in 15 min using a flow rate of 55 mL/min. Arrows indicate peaks of PME activity determined in separate analysis of all fractions. B. Separation of Valenica orange peel PME isozyme activities using heparin-Sepharose CL-6B column (1 × 20.5 cm) run at 0.5 mL/min under cation-exchange conditions. PME activity was semiquantitatively measured in all chromatography fractions using a pH-sensitive dye to detect pectin methylester hydrolysis (Materials and Methods).

The peak 2 PME was prepared from fresh Valencia orange peel as previously described (*13*). Briefly, this involved homogenizing tissue in 100 mM Tris buffer (pH 8.0) with 1.0 M NaCl, precipitation with 75% saturated ammonium sulfate, dialysis in Tris-buffered saline, and finally concentration with a 10,000 NMWL ultrafiltration cartridge. Following chromatography on DEAE Sephacel (Sigma), PME peaks were separated by chromatography on heparin-Sepharose CL-6B. A 1 cm × 20.5 cm length column was used with a 0.5 mL/min flow rate, and elution was achieved with a linear gradient of 20 mM to 320 mM NaCl in Tris buffer. PME activity was assayed in all fractions using a kinetic microplate reader as described previously (*13*). Rechromatography was performed on peak 2, then this sample was worked up similarly to the commercial orange peel preparation.

Perfusion Chromatography. Chromatographic separations were performed with a Biocad 700E workstation (Applied Biosystems, Foster City, CA), monitoring UV 280 nm and conductivity of the flow stream. All separations were conducted at room temperature. Preliminary preparative separation of the commercial orange peel PME preparation was performed with Poros HS/20 cation-exchange media packed in a 1.7 cm × 18 cm column. The sample was injected into the 20 mM HEPES buffer (pH 7.5) mobile phase running at 55 mL/min. After the absorbance (280 nm) returned to baseline, the column was eluted with a 15-min linear gradient run to 300 mM NaCl. Fractions of 10 mL were collected and immediately assayed for PME activity. Relative PME activity was determined spectrophotometrically with a pH-sensitive dye as previously described (*10*). Active fractions were pooled and concentrated by ultrafiltration. The presence of endopolygalacturonase activity in this preparation was tested by assaying for any increase in reducing sugar end-groups as previously described (*10*) and by any decrease in viscosity (Gary Luzio, Hercules Food Gums) − no activities were detected by either method.

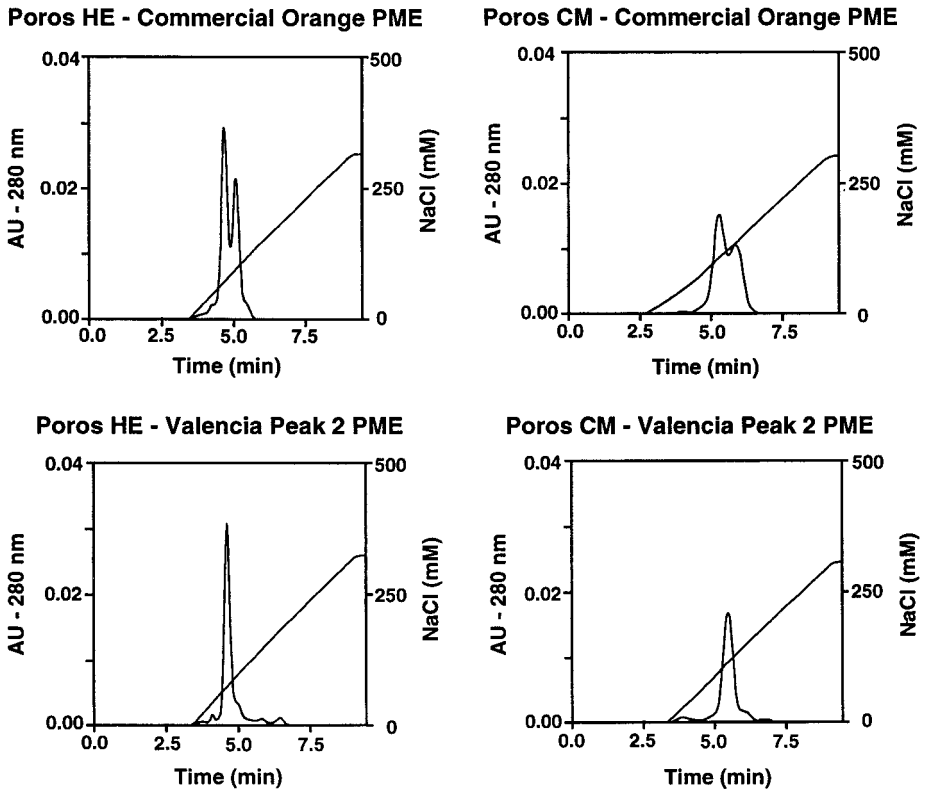

Figure 2. Analytical cation-exchange chromatography separations of the commercial orange peel PME and Valencia peak 2 PME. Left panel shows the elution of the PME peaks from both preparations (commercial orange PME above and Valencia peak 2 PME below) on a Poros HE (heparin) column. Right panel shows the PME peak elution in both preparations (commercial orange PME above and Valencia peak 2 below) on Poros CM (carboxymethyl) columns.

Analytical separations were performed with Poros 20 CM (carboxymethyl), HE (heparin), and PE (phenyl ether) media packed in 4.6 mm × 100 mm columns (1 column volume, CV, is 1.66 mL). Cation exchange chromatography used 20 mM HEPES buffer (pH 7.5) at 5 CV/min with elution using a 30 CV gradient of 0 to 300 mM NaCl. Gradients are indicated by the conductivity response of the salt gradients. Hydrophobic-interaction chromatography with Poros PE used 2.0 M ammonium sulfate in HEPES buffer at 5 CV/min with elution using a 15 CV linear gradient to 0 M ammonium sulfate in HEPES buffer.

Electrophoresis. Chromatographically purified PME preparations were resolved by SDS−PAGE using 12% Ready Gels in a Mini-Protean electrophoresis cell (Biorad) following the manufacturer's instructions. Molecular sizes were calculated with Biorad broad range molecular weight marker proteins. Isoelectric-focusing PAGE was performed with a Biorad Mini-IEF gel apparatus using pH 3 to 10 Biolytes. Samples were spotted at the anode and focused for 1 h at 450 V. The focusing gel was then overlayed with a pectin-agar gel for about 4 min, followed by staining with Coomassie blue R250 stain (Biorad IEF stain protocol). The overlay gel (1.5 mm) contained 1% agarose and 1% pectin in 50 mM HEPES (pH 7.0). Strong activity bands were readily detected within 2 min of contact with resolving gel. Activity bands were visualized by 1-min immersion in 0.05% ruthenium red followed by de-staining with water. Biorad pI markers were used to estimate PME pI.

PME preparations resolved in a 12% SDS−PAGE gel were further treated for sequencing analysis by subsequent electroblotting to Biorad Trans-Blot PVDF membrane during 1 h at 150 V (constant) in 10 mM 3-(cyclohexylamino) propanesulfonic acid (pH 11.0 with NaOH) buffer containing 10% MeOH (*16*). The membrane was stained with Coomassie brilliant blue R-250, and individual bands were cut out and submitted for N-terminal amino acid sequencing. Sequencing was performed with an Applied Biosystems 477A protein sequencer at the Macromolecular Core Facility, Milton S. Hershey Medical Center (Hershey, PA). Sequences obtained were subsequently examined by BLAST database search (www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

In a preparative separation of the commercial orange peel PME preparation on the Poros HS column, a large protein peak eluted early in the gradient at about 100 mM NaCl (**Figure 1A**). This peak represented PME by enzyme assay of the fractions (data not shown). The PME peak was resolved from residual protein contaminants, which included a minor amount of a second PME activity detected in fractions eluting with higher salt concentration at about 8 min. After the fractions containing the major PME peak were pooled, this preparation was assayed for polygalacturonase activity by extended treatment of polygalacturonic acid under conditions used to measure tomato polygalacturonase (*10*). No activity was detected by reducing sugar or viscometry assays. The commercial orange peel PME showed chromatographic behavior that was similar to that of the Valencia peak 2 PME resolved from other isozyme activities using a heparin-Sepharose CL6B column with about 100 mM NaCl (**Figure 1B**). The ion-exchange separation using perfusion media provided an approximate 100-fold higher flow rate over that using the conventional Sepharose-based media. This speed advantage with the Poros HS column was achieved even though it had a column geometry and load capacity similar to that of the heparin-Sepharose CL6B column. The commercial orange peel PME peak and the Valencia peak 2 PME were collected and used for direct comparison of chromatographic, electrophoretic, and biochemical properties.

Common retention profiles of the two PME preparations obtained with analytical-scale Poros HE (heparin) and Poros CM (carboxymethyl) columns are shown in **Figure 2**. The commercial orange peel PME preparation was partially resolved into two peaks with both media, whereas the Valencia peak 2 PME eluted as a single peak at a time identical to that of the

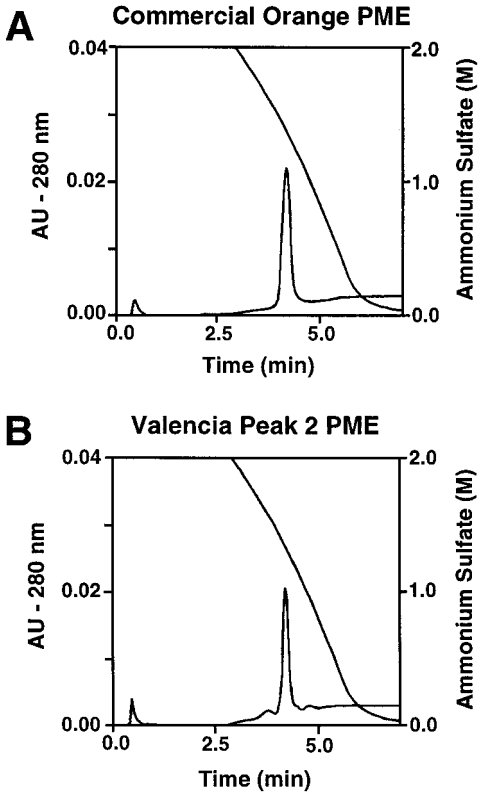

Figure 3. Analytical hydrophobic-interaction chromatography separations of the commercial orange PME (A) and Valencia peak 2 PME (B) using a Poros PE column. Gradient line represents conductivity measured for decreasing gradient of ammonium sulfate concentration.

larger peak in the commercial preparation. PME peaks eluted early (<100 mM) in the elution gradient on both columns, with the Poros HE column providing sharper peaks than those eluted from the Poros CM column. Peaks also eluted about a minute earlier on Poros HE than on Poros CM.

Retention profiles of the two PME preparations were also examined in a second adsorption mode using Poros PE hydrophobic-interaction chromatography media (**Figure 3**). The commercial orange peel PME eluted as a single, wide peak with about 1 M ammonium sulfate. The Valencia peak 2 PME eluted at the same time as the commercial orange peel PME peak. Note that this PME isozyme approaches its precipitation point in 2 M ammonium sulfate at 4 °C, however there were no solubility problems while performing this separation at room temperature. PME binding to Poros HP (a more hydrophobic matrix) occurred at lower ammonium sulfate molarity with PME elution at a correspondingly lower molarity (data not shown).

Electrophoretic analyses of the commercial orange peel and Valencia peak 2 PME preparations following chromatographic purification are shown in **Figure 4**. SDS−PAGE (**Figure 4A**) showed the commercial preparation contained a dominant band estimated at 34 000 Da, and secondary bands at 27 000 Da and about 8000 Da (lane 2). A single band of 34 000 Da was also observed for the purified Valencia peak 2 PME (lane 3). Further resolution of the two PME preparations by IEF−PAGE followed by Coomassie staining (**Figure 4B**) showed both contained a common protein band focusing at pI 9.2. The enzyme activity overlay of the IEF gel (**Figure 4C**) showed intense PME activity for this common band. The purified commercial orange peel PME also contained a second major band focusing at pI 8.7. This band was also strongly active. The second protein and corresponding enzyme activity observed in the commercial orange PME presumably represents the second PME charge variant that was partially resolved during analytical ion-exchange chromatography separations (**Figure 2**). Type 2 PME was reported previously with a much higher pI than type 1 PME (*11*). No traces of PME activity were detected at pH >9.2.

Amino acid sequencing experiments provided structural identification of the purified PMEs and also showed the PME present in the commercial enzyme preparation is partially cleaved into two smaller polypeptides, which are observed by SDS−PAGE (**Figure 4A**). The partial sequences obtained from the 34 000 Da protein in the commercial orange PME and peak 2 PME preparations were identical (**Table 1**). The partial sequence obtained for the 27 000 Da protein present in the commercial PME was further determined to be identical to that of the 34 000 Da proteins. Alignment of this sequence was located at the N-terminus determined for other orange and tomato PMEs. A partial sequence was also obtained for the 8000 Da polypeptide, aligning with an internal sequence located toward the C-terminus of the other PMEs (data not shown). The 27 000 and 8000 Da polypeptides remained associated under nondenaturing conditions, since the smaller fragment was not separated by gel filtration chromatography, ultrafiltration, or by either adsorption chromatography separation. Furthermore, the partial cleavage of PME does not appear to inactivate the enzyme as no differences in specific activities were observed

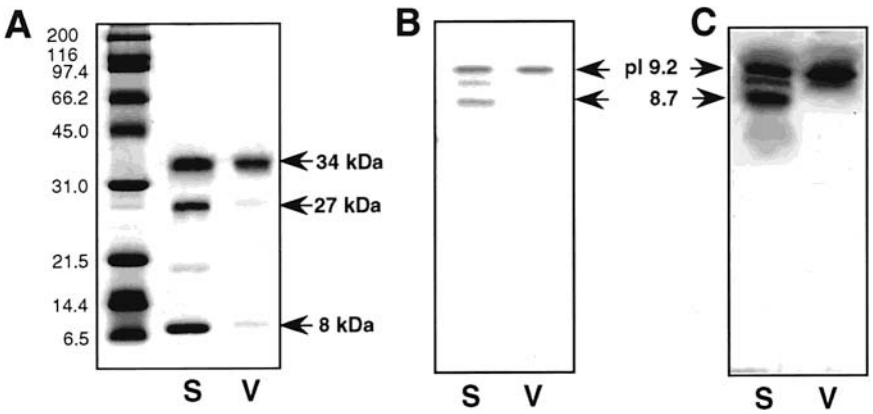

Figure 4. Electrophoretic analysis comparing the purified commercial orange PME (S) and the Valencia peak 2 PME (V). A. Separation by SDS–PAGE on a 12% gel with Coomassie brilliant blue staining. Molecular mass calculated from Biorad broad range standards loaded in the first lane (size in kDa indicated to left of gel). B. Separation by IEF–PAGE using pH 3–10 ampholines and Coomassie brilliant blue staining. The pIs were calibrated from Pharmacia standard proteins (not shown). C. PME activity overlay gel (of IEF gel), stained with ruthenium red.

Table 1. Alignment of the Partial Amino-Terminal Sequences Obtained from the Valencia Orange Proteins with PME Sequences from the ENTREZ Database

source	type[a]	aligned sequence	accession (or ref.)[b]
Valencia peel PME[c]	I	1–vvpdvtvaadgsg	
Navelina orange PME	II	267–sssvtpnvvvaadgs[d]	(*14*)
Valencia orange, PECS-C1	II	267–sssvtpnavvaadgsg[d]	AAB57670, (*3*)
Valencia orange, PECS-C2	II	196–qttpranivvaqdgsg	AAB57671, (*3*)
tomato, fruit-specific PME	I	1–iianavvaqdgtg	A25010, (*9*, *10*)
tomato, fruit-specific PME	I	227–gkdiianavvaqdgtg	S46527
tomato, PMEU1	II	266–sstvtpdvvvaadgsg[d]	AAD09283

[a] Type: I, salt-independent PME isozymes; and II, salt-dependent PME isozymes. [b] Accession number for ENTREZ protein sequence database search at NCBI (http://www.ncbi.nlm.nih.gov/Database/index.html). [c] Sequence obtained directly from 34 kDa and 27 kDa proteins in the commercial PME and the 34 kDa Valencia peak 2 protein. [d] Isolated proteins reported to be blocked. The blocked residue in the Navelina PME was chemically removed to allow partial sequencing.

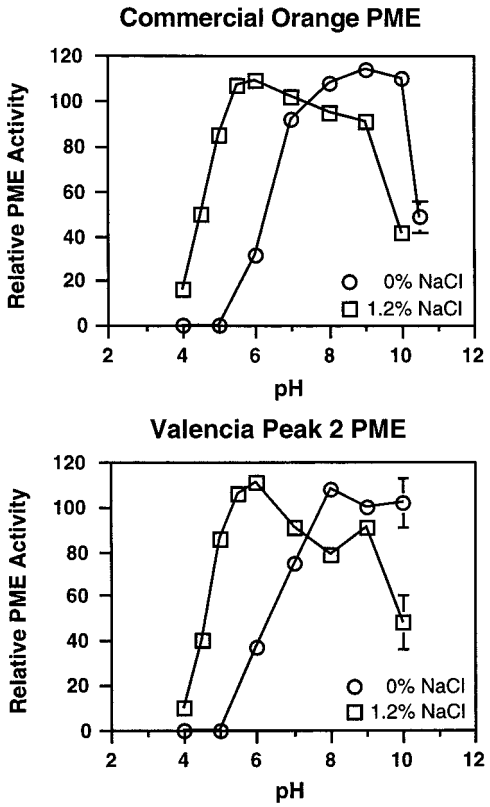

Figure 5. Enzyme activity patterns for the commercial orange peel PME and Valencia peak 2 PME. PME activities for both enzyme preparations were determined from pH 4 to pH 10 in the absence or with addition of 1.2% (w/v) NaCl. Activities calculated as PME activity relative to commercial orange PME activity determined at pH 7.0 and 1.2% NaCl. Activities were corrected for background alkali hydrolysis.

between the two PME preparations (about 1000 units PME activity/mg protein).

The commercial orange peel PME and the Valencia peak 2 PME showed common enzyme activity patterns over the pH range 4 to 11, both in the presence or absence of 1.2% (w/v) NaCl (**Figure 5**). In the presence of NaCl, the enzymes displayed an activity optimum at about pH 6. The activity range was broad and suggestive of two activity optima. In the absence of NaCl, the two PMEs remained active, indicating they are not dependent on supplemented NaCl for activation. The pH optimum in the absence of NaCl shifted to the alkaline range with an optimum at about pH 9.

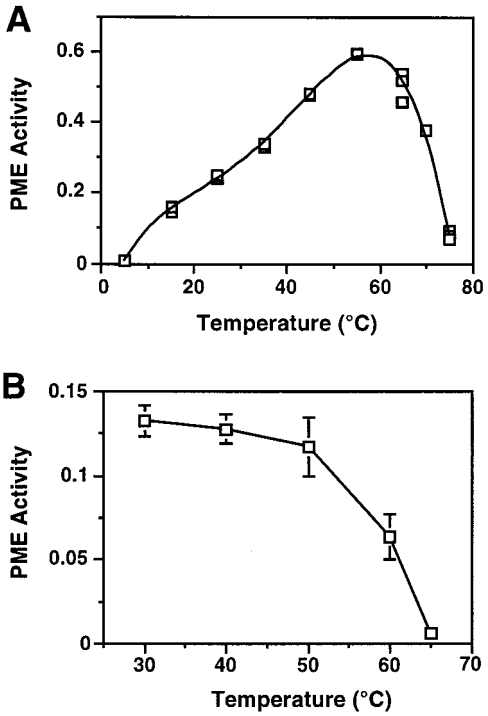

Figure 6. A. Temperature activity curve for the commercial orange peel PME (temperature of assay mixture). B. Thermal stability curve for commercial orange peel PME (5 min incubation at indicated temperature).

The Valencia peak 2 PME was previously shown to be a thermally labile enzyme (*13*). The commercial orange peel PME was examined in further detail here. The temperature optimum was determined to be about 55−60 °C (**Figure 6A**). It lost nearly half of its activity after heating for 5 min at 55 °C, and it was nearly completely inactivated at 65 °C (**Figure 6B**), demonstrating it is a thermally labile isozyme, like the Valencia peak 2 PME.

DISCUSSION

Individual PME isozymes have been previously isolated from orange fruit tissues and shown to vary in their ability to destabilize juice cloud (*12*, *13*). This variation presumably reflects differences in biochemical properties of individual isozymes and may further reflect differences in their pectin de-esterification action patterns. Except for the salt-dependent (type 2 PME) isozyme recently reported by Christensen et al. (*14*), no other individual orange PME isozyme has been characterized in detail beyond kinetic, thermostability, and juice cloud destabilizing properties (*11*−*13*). Solms and Deuel (*17*) presented evidence that an orange PME acts on methylesters adjacent to free carboxyl groups, and Markovic and Kohn (*18*) showed calcium ion binding to pectin partially de-esterified by an orange PME was consistent with a block-wise action pattern. However, it is not clear which isozyme was used in these previous studies, and no direct studies on action pattern by NMR spectroscopic analysis have been reported for any orange PME isozyme. As it is likely that individual PME types may vary in their action patterns (*6*), we sought to determine to which of the PME multiple isozymes present in Valencia orange peel the PME in the commercial orange peel preparation corresponds. We also wanted to ensure catalytic purity of the PME so there would be no contaminating isozyme or depolymerizing activities that would confound our efforts for discrete pectin methylesterification pattern modification with the subject PME.

By preliminary chromatographic treatment of the commercial PME preparation, we separated low levels of a second PME isozyme activity (**Figure 1A**). The commercial orange PME was found to be a type 1 isozyme, as demonstrated by its activity at neutral pH in the absence of salt (**Figure 5**). With an array of comparative analyses, we demonstrated unequivocally that it is equivalent to the dominant peak 2 PME previously isolated from fresh Valencia peel (*13*). This conclusion is based on common chromatographic profiles (**Figures 2** and **3**), identical molecular size and pI (**Figure 4**), partial N-terminal sequences (**Table 1**), and common biochemical properties (**Figures 5** and **6**).

The properties determined for this salt-independent PME isozyme clearly distinguish it from the type 2 orange PMEs (*3*, *14*), and the properties are consistent with those of the well-characterized fruit-specific PME from tomato (*9*, *10*). From sequencing studies, we observed a common structural feature between parallel PME isozymes (**Table 1**). The type 1 tomato and orange PMEs were both sequenceable and align commonly at their N-terminus. In contrast, the type 2 isozyme paralogs from orange and tomato were both found to be N-terminally blocked, which was attributed to a terminal acetylated serine occurring at several residues preceding the terminus of the type 1 PMEs (*14*). It is not clear at this time whether this structural feature is a general property for distinguishing PME types. We are now seeking to isolate a cDNA clone corresponding to the type 1 orange PME from fruit tissue, which will be useful for full sequence analysis and establishing tissue-specific expression patterns.

Following limited deesterification of citrus pectin with the purified salt-independent orange PME isozyme (*15*), we demonstrated a block-wise mode of action by ^{1}H NMR analysis and a rapid shift to calcium sensitivity. Both the salt-dependent PME purified from the commercial and fresh orange peel commonly introduced blocks of free galacturonic acids without subsequent degradation of the pectin's molecular weight. This provides separate evidence of the functional equivalence and enzymatic purity of the PME prepared from the two sources. The availability of the type 1 PME for preparation from either the commercial source or fresh orange peel should facilitate its further use as a convenient and standardized enzyme between different laboratories interested in its use for pectin structure modification studies. Further specific characterization of this type 1 plant PME's action mechanism is now possible. This PME provides a means to prepare calcium-sensitive high-molecular-weight pectins (*15*), in the absence of cations or under moderately acidic conditions (**Figure 5**), and will be useful for developing an enzymatic process for preparing pectins with improved performance for new applications.

ACKNOWLEDGMENT

We thank Pamela L. Rockwell-Warner for technical assistance, Anne Stanley for amino acid sequencing, and Gary Luzio for the PG-viscometry assay. We also thank Kevin Hicks for his critical reading of this manuscript.

LITERATURE CITED

(1) Bordenave, M. Analysis of Pectin Methyl Esterases. In *Plant Cell Wall Analysis*; Linskens, H. F., Jackson, J. F., Eds.; Springer-Verlag: Berlin, 1996; Modern Methods of Plant analysis, Vol. 17, pp 165−180.

(2) Gaffé, J.; Tiznado, M. E.; Handa, A. K. Characterization and functional expression of a ubiquitously expressed tomato pectin methylesterase. *Plant Physiol.* **1997**, *114*, 1547−1556.

(3) Nairn, C. J.; Lewandowski, D. J.; Burns, J. K. Genetics and expression of two pectinesterase genes in Valencia orange. *Physiol. Plant.* **1998**, *102*, 226−235.

(4) Micheli, F.; Hollinger, C.; Goldberg, R.; Richard, L. Characterization of pectin methylesterase-like gene AtPME3: a new member of a gene family comprising at least 12 genes in *Arabidopsis thaliana*. *Gene* **1998**, *220*, 13−20.

(5) Grasdalen, H.; Andersen, A. K.; Larsen, B. NMR spectroscopy studies of the action pattern of tomato pectinesterase: generation of block structure in pectin by a multiple-attack mechanism. *Carbohydr. Res.* **1996**, *289*, 105−114.

(6) Catoire, L.; Pierron, M.; Morvan, C.; Hervé du Penhoat, C.; Goldberg, R. Investigation of the action patterns of pectin-methylesterase isoenzymes through kinetic analyses and NMR spectroscopy. *J. Biol. Chem.* **1998**, *273*, 33150−33156.

(7) Denès, J. M.; Baron, A.; Renard, C. M. G. C.; Péan, C.; Drilleau, J. F. Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. *Carbohydr. Res.* **2000**, *327*, 385−393.

(8) Gaffé, J.; Tieman, D. M.; Handa, A. K. Pectin methylesterase isoforms in tomato (*Lycopersicon esculentum*) tissues: effects of expression of a pectin methylesterase antisense gene. *Plant Physiol.* **1994**, *105*, 199−203.

(9) Warrilow, A. G. S.; Jones, M. G. Different forms of tomato pectinesterase have different kinetic properties. *Phytochemistry* **1995**, *39*, 277−282.

(10) Savary, B. J. Perfusion chromatography separation of the tomato fruit-specific pectin methylesterase from a semipurified commercial enzyme preparation. *Prep. Biochem. Biotechnol.* **2001**, *32*, 241−258.

(11) Versteeg, C.; Rombouts, F. M.; Pilnik, W. Purification and some characteristics of two pectinesterase isoenzymes from orange. *Lebensm.-Wiss. Technol.* **1978**, *11*, 267−274.

(12) Versteeg, C.; Rombouts, F. M.; Spaansen, C. H.; Pilnik, W. Thermostability and orange juice cloud destabilizing properties of multiple pectinesterases from orange. *J. Food Sci.* **1980**, *45*, 969−971

(13) Cameron, R. G.; Baker, R. A.; Grohmann, K. Multiple forms of pectinmethylesterase from citrus peel and their effects on juice cloud stability. *J. Food Sci.* **1998**, *63*, 253−256.

(14) Christensen, T. M. I. E.; Nielson, J. E.; Kreiberg, J. D.; Rasmussen, P.; Mikkelsen, J. D. Pectin methyl esterase from orange fruit: characterization and localization by *in-situ* hybridization and immunohistochemistry. *Planta* **1998**, *206*, 493−503.

(15) Hotchkiss, A. T.; Savary, B. J.; Cameron, R. G.; Luzio, G. A.; Chau, H. K.; Fishman, M. L. Enzymatic modification of pectin to increase its calcium sensitivity while preserving its molecular weight. *J. Agric. Food Chem.* **2002**, *50*, xxxx−xxxx.

(16) LeGendre, N.; Matsudaira, P. T. Purification of proteins and peptides by SDS−PAGE. In *A practical guide to protein and peptide purification for microsequencing*; Matsudaira, P. T., ed.; Academic Press: New York, 1989. pp 49−69.

(17) Solms, J.; Deuel, H. Über den Mechanismus der enzymatischen Verseifung von Pektinstoffen. *Helv. Chim. Acta* **1955**, *38*, 321−329.

(18) Markovic, O.; Kohn, R. Mode of pectin deesterification by *Trichoderma reesei* pectinesterase. *Experientia* **1984**, *40*, 842−843.

Received for review January 18, 2002. Revised manuscript received March 27, 2002. Accepted March 27, 2002. This research was partly supported through a USDA-ARS Cooperative Research and Development Agreement with Hercules Inc., Wilmington, DE. Mention of brand or firm name in this publication does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

JF020060J