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Isolation, Characterization, and Pectin-Modifying Properties of a Thermally Tolerant Pectin Methylesterase from *Citrus sinensis* Var. Valencia

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The thermally tolerant pectin methylesterase (TT-PME) was isolated as a monocomponent enzyme from sweet orange fruit (*Citrus sinensis* var. Valencia). It was also isolated from flower and vegetative tissue. The apparent molecular weight of fruit TT-PME was 40800 by SDS-PAGE and the isoelectric point estimated as p*I* 9.31 by IEF-PAGE. MALDI-TOF MS identified no tryptic-peptide ions from TT-PME characteristic of previously described citrus PMEs. TT-PME did not absolutely require supplemented salt for activity, but salt activation and pH-dependent activity patterns were intermediate to those of thermolabile PMEs. Treatment of non-calcium-sensitive pectin with TT-PME (reducing the degree of methylesterification by 6%) increased the calcium-sensitive pectin ratio from 0.01 to 0.90, indicating a blockwise mode of action. TT-PME produced a significantly lower end-point degree of methylesterification at pH 7.5 than at pH 4.5. Extensive de-esterification with TT-PME did not reduce the pectin molecular weight or *z*-average radius of gyration, as determined by HPSEC.

KEYWORDS: ***Citrus sinensis*; pectin methylesterase; pectin esterase; homogalacturonan; pectin; polysaccharide; enzyme modification; juice cloud stability**

INTRODUCTION

The presence of a thermally tolerant pectin methylesterase (TT-PME) in citrus fruit tissue was first suggested by Stevens et al. (*1*) and Wenzel et al. (*2*), but it was not conclusively demonstrated until identified by Versteeg et al. (*3*). Versteeg et al. (*3*) reported that only the TT-PME rapidly (<4 weeks) destabilized citrus juice cloud at 5 °C. Of the four chromatographic forms they isolated from Valencia fruit peel, Cameron et al. (*4*) demonstrated that the TT-PME destabilized the juice cloud most rapidly at 4 °C but not at 30 °C, when a thermally labile PME (TL-PME) destabilized the cloud more rapidly. A TT-PME from lemon fruit was the only form that destabilized the cloud of lemon juice (*5*). TT-PME has been isolated from grapefruit pulp (*6*, *7*), citrus tissue culture cells (*8*), and a commercial fresh frozen Valencia orange juice (*9*). It is also present in other sweet orange varieties (*10*).

Citrus juice cloud stability and commercial citrus pectin's functional properties are related to the pectin molecular weight (*11*), degree of methylesterification (DE; *12*), and intramolecular distribution of the methyl ester blocks within the population of pectin molecules (*13*−*16*). Although a minimal block size of nine unesterified galacturonic acid residues has been hypothesized to be necessary for calcium cross-linking, a larger de-esterified block might be necessary for gel formation (*17*, *18*). Approximately 25−30% of cloud-associated pectin has been reported to be calcium pectate (*19*). High-methoxy pectins in which de-esterified blocks allow them to gel in the presence of calcium without the addition of sucrose are termed calcium-sensitive pectins (*12*, *13*). Two TL-PMEs from citrus fruit rag tissue (*20*, *21*) have been demonstrated to be blockwise demethylating enzymes, introducing calcium sensitivity into a non-calcium-sensitive pectin (NCSP) with very limited reduction (6.0−6.5%) in their DE.

In previous papers the TT-PME from citrus has been shown to be *N*-glycosylated (*9*) and have a broad pH optimum in the presence of 100 (*6*) or 200 mM NaCl (*8*). However, its relationship to the salt-dependent (*21*) or salt-independent (*20*) PME has not been determined. Discrepancies regarding molecular mass also have been reported for the citrus TT-PME (*3*, *6*, *8*, *9*, *22*, *23*) with estimates ranging from 36−42 kDa (*4*, *8*, *9*) to 51−53 kDa (*3*, *6*). Our objectives here are to describe previously unreported characteristics of the TT-PME from citrus fruit tissue, demonstrate its presence in vegetative and flower tissue, compare its structural properties to two TL-PMEs, and

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present data on its mode of action, physical properties, and the effects of salt and pH on activity.

MATERIALS AND METHODS

Tissue Extracts. Fresh Valencia oranges were used to prepare extracts from peel flavedo and albedo tissue as previously described (*21*). Additionally, a mixture of young flush to mature leaves (38.9 g) was homogenized in a blender with 2 volumes of deionized water for 2 min and then stirred for 3 h at 4 °C. After centrifugation at 15700*g* for 30 min at 4 °C, the supernatant was discarded (no PME activity observed), and the pellets were resuspended in 500 mL of 0.1 M Tris (pH 8.5, 28 °C), 1 M NaCl, and 0.02% NaN_3 (w/v). After 2 h of stirring at 4 °C, the material was centrifuged as described above. The supernatant was brought to 75% ammonium sulfate and stirred overnight. Following centrifugation as previously described, the pellets were solubilized in a minimal volume of 10 mM Tris (pH 7.5, 28 °C), 20 mM NaCl, and 0.02% NaN_3 and then dialyzed against the same buffer (1 L × three changes at 1 h, 2 h, and overnight). Extracts were also prepared from anther and pollen tissue, as well as mature leaves or young flush leaves, for determination of TT-PME activity. Anther and pollen tissue was collected from Robinson tangerines, red grapefruit, Pineapple orange, Temple orange, and Hamlin orange flowers. Young flush and mature leaf extracts were prepared from Hamlin leaves. The tissue was ground in 3 volumes of 100 mM Tris (pH 8.0 at 31 °C), 150 mM NaCl, 20 mM MgCl, 5 mM DTT, 2% PVP-40 (w/v), 15% glycerol (v/v), 50 μM leupeptin, 0.005% TLCK (w/v), and 10 μM PMSF.

Chromatography and Enzyme Activity Assay. DEAE-Sephacel, heparin CL-6B, and concanavalin A−Sepharose (mannose-type *N*-glycan affinity) chromatographies were run as previously described (*4*). Column fractions were screened for relative activity using a colorimetric assay as previously described (*4*). Standard PME activity in crude extracts and pooled column fractions were determined titrimetrically on 0.5% Sigma citrus pectin (59% DE) with a Radiometer PHM290 pH-stat controller (assayed at pH 7.5, 200 mM NaCl, 30 °C, using 10 mM NaOH as the titrant). Enzyme activity was determined as a function of pH and supplemented NaCl (0, 25, and 200 mM NaCl). Heat-stable activity was estimated after the sample had been heated for 2 min (for chromatography peaks; *8*) or 10 min (leaf, anther plus pollen tissue; *26*) in an 80 °C water bath (*7*). All activity estimates are the means of two or more replicates. Protein concentrations of fruit tissue extracts and chromatography peaks were estimated with the BCA protein assay (Pierce Endogen) according to the manufacturer's directions using IgG as the standard. Protein concentrations in anther, pollen, mature leaf, and young flush leaf tissue extracts were estimated with the Bio-Rad protein assay reagent, also using IgG as the standard. All estimates are based on the means ± SD of three replicates.

Electrophoresis. Monocomponent enzyme preparations were resolved by SDS-PAGE using the 12% NuPAGE Bis-Tris gel system (Invitrogen) with MOPS buffer following the manufacturer's instructions. Gels were calibrated with SeeBlue Plus2 standards (188, 98, 62, 49, 38, 28, 17, 14, 6, and 3 kDa) and stained with SimplyBlue (Coomassie G250). Isoelectric focusing (IEF)-PAGE, followed by zymogram activity staining, was run on a Bio-Rad mini-IEF gel apparatus as previously described (*20*, *24*), but with narrow-range ampholytes (pH 8−10). Edman N-terminal amino acid sequencing of proteins electroblotted to PVDF membrane was performed as previously described (*20*).

MALDI-TOF MS. Stained protein bands were cut from PAGE gels and digested with Trypsin Gold (Promega). Peptides were recovered with Millipore C18 ZipTips with 60% acetonitrile−0.1% TFA containing α-cyano-4-hydroxycinammic acid matrix. Peptide-matrix samples were analyzed with the ABI 4700 MALDI-TOF to obtain peptide mass spectra (range from *m*/*z* 800 to 4000) for the TT-PME. Theoretical tryptic peptide libraries were created using deduced protein sequences from citrus PMEs in the public gene and protein databases at the NCBI (www.ncbi.nih.gov/Entrez/) and compared with spectra generated with previously purified salt-dependent (peak 2) and salt-independent (peak 3) orange PME isoenzymes (*20*, *21*).

CSPR Assay. Methylated lime pectin (82% DE), a non-calcium-sensitive pectin (NCSP) kindly provided by Danisco Coultor (Brabrand, Denmark), was used for determining calcium-sensitive pectin ratios (CSPRs). Pectin was treated with TT-PME and with the salt-independent TL-PME, to reduce DE by 5.6−6%, and CSPR was determined as described previously (*11*). Following treatment, warm EtOH (40 °C; no residual activity was observed for either PME isoform) was added to pectin−enzyme solutions to inactivate enzyme and precipitate pectin, then following overnight chilling at 4 °C, the pectin was recovered and dialyzed (1000 MWCO, Spectrum) in deionized water at 4 °C and finally lyophilized prior to further analysis. Untreated NCSP was prepared as a control using the same conditions but without any enzyme. Pectin solutions were adjusted to pH 4.0. The CSPR was determined by dividing the amount of pectin that gels in 30 mM calcium and 8% 2-propanol (IPA) by the total amount of pectin which precipitates in 60% IPA. Therefore, CSPR values close to 1.0 indicate a CSP, whereas a NCSP has low CSPR values. Reported values are the means from three to eight replicates.

Molecular Properties of De-esterified Pectin. Enzymatic end-point demethylation was performed titrimetrically at pH 4.5 and 7.5 (at 32 °C) on 0.5% Sigma citrus pectin (59% DE) with 200 mM NaCl and 1 unit of enzyme (1 μequiv min^{-1}) per milligram of pectin for all four peaks of PME activity. The titrations were run for 25 h at pH 4.5 and for 60 min at pH 7.5. The reported values are the means of two to seven replicates (only one measurement was available for the salt-independent TL-PME at pH 4.5). A statistical comparison of means for each PME at pH 4.5 versus 7.5 was made with Student's *t* test assuming unequal variance.

High-performance size exclusion chromatography (HPSEC) of enzyme-treated pectin samples was performed as described previously (*11*). HPSEC separation utilized two PL-Aquagel OH-60 and one OH-40 column (Polymer Labs) in series and a 0.05 M $NaNO_3$ mobile phase, at 45 °C and 0.7 mL/min. Detection was with a Dawn DSP multiangle laser light scattering (MALLS) photometer (Wyatt Technologies), a model 100 differential pressure viscometer (Viscotek Corp.), and an Optilab DSP interferometric refractometer (Wyatt). A value of 0.132 mL/g in 0.05 M $NaNO_3$ was used for the concentration dependence of the differential refractive index (d*n*/d*c*) of pectin. The d*n*/d*c* was determined off-line using the HPLC pump, the autoinjector, and the Optilab refractometer. The weight-average molecular weight (M_w), intrinsic viscosity (η_w, weight-average intrinsic viscosity for a polydisperse collection of macromolecules), and *z*-average radius of gyration (Rg_z, the effective radius of a polydisperse collection of macromolecules rotating in solution under Brownian motion) were determined for triplicate HPSEC runs for each pectin sample.

RESULTS

Isolation of TT-PME. Four peaks of PME activity (see **Figure 1** and ref *4*) were resolved by heparin chromatography of the DEAE flow-through material from fruit rag tissue extracts, as previously described (*4*, *7*, *8*, *20*, *21*). The third PME activity peak contained the thermally tolerant activity (based on residual activity after heating at 80 °C for 2 min). Following heparin rechromatography and lectin affinity chromatography on concanavalin A−Sepharose (*4*, *9*), the fruit tissue TT-PME was purified by a factor of 34.9 (**Table 1**). Specific elution of TT-PME from the concanavalin A affinity column by methyl α-D-glucopyranoside indicated TT-PME is an *N*-glycosylated protein, which is consistent with previous demonstrations of a shift in molecular weight following PNGase F treatment (*9*) and glycan detection analysis (*7*). Separation of Valencia orange leaf tissue extracts similarly by heparin chromatography (**Figure 1A**) demonstrated four peaks of PME activity, corresponding to citrus fruit tissues (*4*, *7*, *20*, *21*), except that the salt-dependent TL-PME (peak 4) is the dominant form. Concanavalin A chromatography separated TT-PME from leaf tissue extracts (**Figure 1B**). In young flush leaves from Hamlin oranges, a higher percentage of TT-PME (65.1% of total PME activity) was observed to be present in protein extracts (**Table 2**) than found in Valencia fruit (0.2%). This value dropped to 33.9% in mature

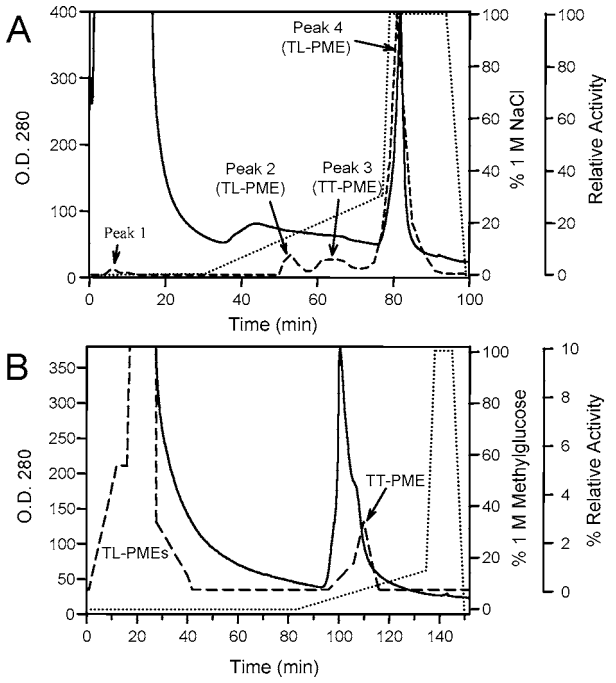

Figure 1. Chromatography of Valencia leaf extracts: (—) OD 280; (- - -) relative activity; (•••) NaCl gradient. (**A**) heparin–Sepharose cation exchange separation of four peaks of PME activity; (**B**) concanavalin A–Sepharose affinity separation of mannose-type glycoproteins, including TT-PME activity.

Table 1. Purification of TT-PME from Valencia Fruit Rag Tissue

purifn step	enzyme activity (μequiv·min^{-1})	heated activity[a] (μequiv·min^{-1}·mg^{-1})	purifn factor[b]
DEAE	1679.44	2.50	1
heparin	32.30	22.14	8.6
heparin rechromatography	38.98	25.86	10.3
concanavalin A	108.56	87.28	34.9

[a] Activity remaining after heating for 2 min at 80 °C. [b] Purification factor based on heated activity.

Table 2. Total and TT-PME Activity Present in Leaf and Anther plus Pollen Tissue Extracts from Citrus

tissue	variety/type	total PME activity (mequiv·min^{-1}·mg^{-1} of protein ± SD)	TT-PME activity (mequiv·min^{-1}·mg^{-1} of protein ± SD)
pollen + anther	Robinson tangerine	0.223 ± 0.022	0.212 ± 0.011
	red grapefruit	0.775 ± 0.081	0.238 ± 0.049
	Pineapple orange	0.313 ± 0.041	0.210 ± 0.021
	Temple orange	0.386	0.229 ± 0.014
	Hamlin orange	0.117 ± 0.026	0.045 ± 0.030
Hamlin leaf tissue	young flush	1.297 ± 0.102	0.845 ± 0.072
	mature flush	0.227 ± 0.024	0.077 ± 0.015

Hamlin leaves (**Table 2**). The TT-PME was also observed in extracts from anther and pollen tissue of tangerine, grapefruit, and three varieties of sweet oranges (Pineapple, Temple, and Hamlin; **Table 2**).

TT-PME Structural Properties. Electrophoretic analysis of the TT-PME isolated from Valencia orange fruit tissue indicated an apparent molecular mass of 40.8 kDa for the dominant peptide band by SDS-PAGE (**Figure 2A**). This is clearly greater than the 34 kDa observed for peak 2 TL-PME (**Figure 2A**; *20*) and the peak 4 TL-PME (33.5 kDa; *21*). IEF-PAGE provided an estimate of isoelectric point at p*I* 9.31 (**Figure 2B**). This is

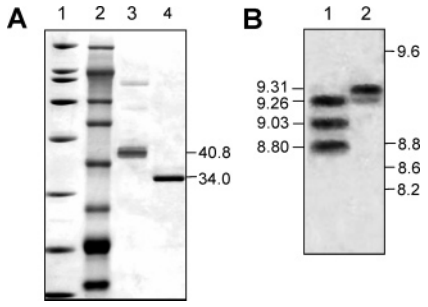

Figure 2. Electrophoretic analysis of TT-PME isolated from Valencia orange fruit tissue. (**A**) SDS-PAGE determination of apparent molecular mass. TT-PME, estimated at 40.8 kDa, is compared to peak 2 TL-PME (34.0 kDa). Separation used 12% NuPAGE gel with MOPS buffer system (Invitrogen): (lane 1) Bio-Rad broad range molecular mass standards (200, 116.3, 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa); (lane 2) Invitrogen SeeBlue Plus2 molecular mass standards (188, 98, 62, 49, 38, 28, 17, 14 kDa); (lane 3) TT- PME; (lane 4) peak 2 TL- PME. (**B**) PME activity zymogram from IEF-PAGE. IEF gel was focused using pH 8–10 ampholytes (Bio-Rad model 111 system) and pectin–agarose overlay gel stained with ruthenium red: (lane 1) Valencia peel salt-independent TL-PME (from Sigma) with three charge variants [upper activity band represents the primary PME isoenzyme corresponding to the peak 2 TL-PME (*20*)]; (lane 2) Valencia orange TT-PME, with estimated p*I* 9.31 [position of p*I* calibration marker proteins indicated at right edge of gel (Sigma, cytochrome *c*, p*I* 9.6, and lentil lectins, p*I* 8.2, 8.6, 8.8)].

only slightly higher than that for the peak 2 TL-PME (p*I* 9.26 on this gel; previously estimated as 9.20 using a broad pH 3−10 IEF gel; *20*), but significantly lower than the p*I* 10.1 reported (*21*) for the peak 4 TL-PME. The peak 2 TL-PME in **Figure 2B** is represented by the homologous enzyme previously isolated from a commercial orange peel preparation, which was demonstrated to contain two charge variants of lower p*I* (*20*).

A gene recently isolated from Valencia orange was putatively identified to represent the thermostable form of pectin methylesterase (*28*). We obtained a partial N-terminal amino acid sequence from the peak 3 TT-PME protein (**Figure 2A**): D L (−) N P N D. This amino acid sequence showed no corresponding sequence with the predicted translated protein from the new PME gene sequence (*28*). In contrast, the extensive partial sequences we obtained previously for peak 2 TL-PME (*20*) are identical with corresponding sequences in the predicted translated thermostable PME gene product (*28*). No peptide ions observed for TT-PME (**Figure 3**) corresponded with any theoretical trypsin-digest peptides generated from the translated "thermostable PME-associated" gene product or for other Valencia orange PME genes previously reported (*29*), including one corresponding to peak 4 TL-PME (*21*, *30*).

TT-PME Activity Profiles. Previous characterization of peak 2 and peak 4 TL-PMEs for salt-dependent enzyme activities demonstrated a general pattern for salt independence (*20*) and salt dependence (*21*), respectively. The effect of supplemented salt on enzyme activity was similarly determined for TT-PME prepared from fruit tissue following concanavalin A chromatography (**Figure 4A**). The results show it is a salt-independent PME; however, there are distinct differences in the relative activity profiles compared to the peak 2 salt-independent TL-PME (*20*). There was no significant enzyme activity by the latter at pH 7.5 between 0 and 1.2% NaCl (176 mM), whereas TT-PME was only ∼35% as active in the absence of corresponding supplemented NaCl at this pH. Maximal activity was achieved with at least 50 mM NaCl (**Figure 4A**). The pH activity profiles with varying salt concentrations are shown for

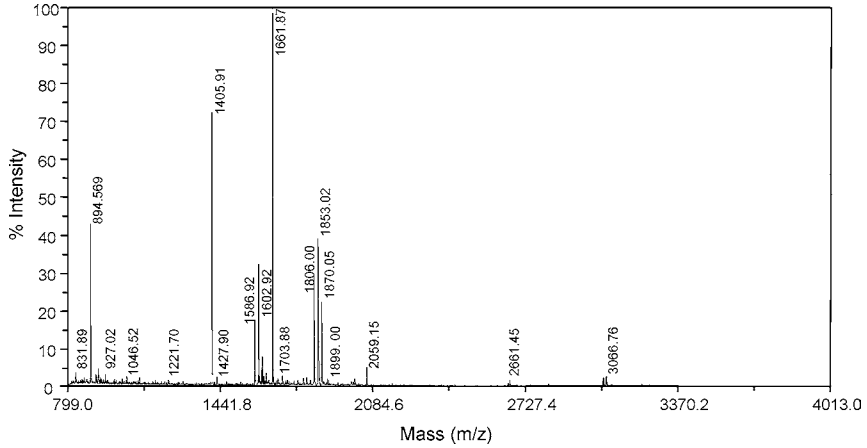

Figure 3. MALDI-TOF mass spectrogram of peptide ions generated from trypsin-treated TT-PME isolated from Valencia orange fruit tissue.

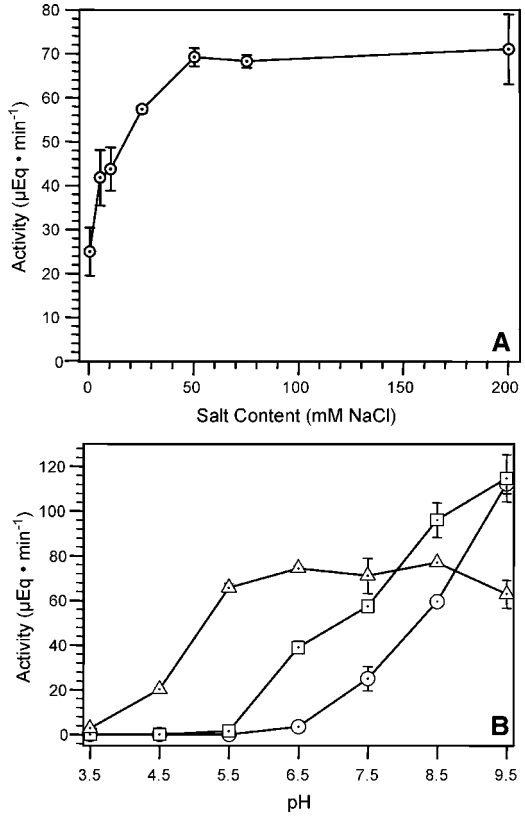

Figure 4. (**A**) Effect of NaCl concentration on peak 3 TT-PME activity at pH 7.5 and (**B**) effect of pH on peak 3 TT-PME activity at 0 (○), 25 (□), and 200 (△) mM NaCl.

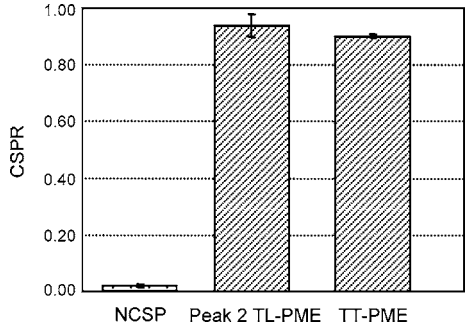

Figure 5. Calcium-sensitive pectin ratio of non-calcium-sensitive pectin before and after treatment with peak 2 TL-PME and peak 3 TT-PME.

Table 3. End-Point Demethylation of a 59% DE Citrus Pectin by Multiple Forms of PME Isolated from Valencia Orange Fruit Tissue

	pH	
PME	4.5	7.5
heparin peak 1[a]	18.9 ± 1.8 (n = 2)	8.5 ± 0.03 (n = 2)
salt-independent thermally labile	15.1 (n = 1)	10.4 ± 0.1 (n = 2)
thermally tolerant[b]	28.1 ± 2.2 (n = 3)	11.7 ± 2.0 (n = 7)
salt-dependent thermally labile[b]	22.2 ± 0.7 (n = 2)	11.8 ± 0.6 (n = 4)

[a] Probability that means are significantly different = 0.05. [b] Means are significantly different at P < 0.001.

TT-PME in **Figure 4B**. These appear intermediate to those observed for the two TL-PMEs (*20*, *21*). Maximal activity was observed at high pH and low NaCl content, and this was better than 55% greater than maximum activity with 200 mM NaCl. In the absence of salt, no activity was detectable for TT-PME below pH 6.5, whereas no activity was detectable for salt-independent TL-PME below pH as low as 5.5. Enzyme activity was detectable at pH as low as pH 3.5 with high NaCl content for both salt-independent PMEs, but not for salt-dependent TL-PME.

Pectin Demethylation with TT-PME. Treatment of methylated lime pectin (NCSP) with the TT-PME, decreasing the DE by up to 6%, resulted in a dramatic shift in calcium sensitivity (**Figure 5**). The CSPR was measured at 0.902 ± 0.008, from an initial value of 0.015 ± 0.005. This showed no significant difference from that measured for peak 2 TL-PME (0.938 ± 0.041) or peak 4 TL-PME that was previously shown not to be significantly differently from peak 2 PME following limited de-esterification of a naturally NCSP from lemon peel (*21*). These results indicate the TT-PME similarly acts in a blockwise mode of action at pH 7.0 in the presence of 150 mM NaCl.

Enzyme end-point demethylation of a 59% DE pectin produced values that were pH-dependent (**Table 3**). At pH 7.5 the TT-PME reduced the DE to 11.7 ± 2.0 and 28.1 ± 2.2% at pH 4.5. Data reported here suggest that all four PME forms present in citrus fruit tissue are able to reduce the pectin DE to lower values at pH 7.5 than at pH 4.5. The means for both the TT-PME and the salt-dependent TL-PME end-point DE at pH 4.5 versus 7.5 were significantly different. The small sample size was likely responsible for the lack of a statistical difference in the end-point DEs for the peak 1 PME and the salt-independent TL-PME.

Similar to our previous results examining molecular properties of pectin treated with peak 2 and peak 4 TL-PMEs (*11*, *21*), extensive de-esterification of a NCSP with the monocomponent

Table 4. Molecular Properties of Enzyme End-Point De-esterified NCSP Pectin (Treated at pH 7.0)

pectin treatment	M_w[a] ($\times 10^5$)	Rg_z[b] (nm)	η_w[c] (dL/g)
TT-PME	2.3 ± 0.02	48.7 ± 1.0	5.0 ± 0.1
untreated NCSP	2.4 ± 0.1	49.5 ± 2.0	5.9 ± 0.03

[a] Weight-average molecular weight, determined by multiangle laser light scattering. [b] *z*-average radius of gyration, determined by multiangle laser light scattering. [c] Weight-average intrinsic viscosity for a polydisperse collection of macromolecules, determined by a combination of light scattering at 90° and viscometry.

TT-PME (to enzyme limit) had little, if any, effect on weight-average molecular weight (M_w) or the *z*-average radius of gyration (Rg_z; **Table 4**). Also like the TL-PMEs (*21*, *31*), the intrinsic viscosity ($[\eta]_w$) of NCSP extensively de-esterified was significantly reduced by 14.5%.

DISCUSSION

The TT-PME present in Valencia orange fruit rag tissue (*4*, *7*) was prepared as a monocomponent enzyme by a combination of ion-exchange and lectin affinity chromatography (**Table 1**). It was isolated similarly in leaf tissue and appears to be generally distributed in other citrus tissues besides fruit (**Figure 1** and **Table 2**). Because of discrepancies in reports on characteristics of citrus TT-PME, we investigated the purified enzyme in more detail to establish means for unequivocal identity. The apparent molecular mass determined by SDS-PAGE was 40.8 kDa (**Figure 2A**). This is close to the previous molecular mass estimates from size exclusion chromatography or SDS-PAGE reported by Braddock et al. (*22*) and Cameron et al. (*7*−*9*) that ranged from 37.5 to 42.7 kDa. In this study, we used the bis-TRIS gel system with MES buffer for SDS-PAGE as it provided a much improved resolution and reduction in sample artifacts observed with the traditional alkaline pH buffer system (*20*, *21*). The 34.0 kDa estimated for the peak 2 TL-PME is close to the 34665 Da determined by MALDI-TOF MS (data not shown). The isoelectric point for TT-PME was determined as p*I* 9.31. This is very close to that of the peak 2 TL-PME (p*I* 9.26), but nearly a complete pH unit lower than that estimated for peak 4 TL-PME.

The effects of NaCl on TT-PME activity and the action of TT-PME pattern on pectin were examined and compared to the two major TL-PMEs previously characterized (*11*, *21*, *30*). TT-PME was found to be a "salt-independent" PME, like the peak 2 TL-PME, but its salt-pH activity patterns (**Figure 3**) were generally intermediate of those observed for the TL-PMEs (*11*, *21*). TT-PME showed a pattern of lower end-point DE by enzyme treatment at pH 7.5 than at pH 4.5, which was similar to the other PME forms isolated from Valencia orange fruit. TT-PME was determined to act in a blockwise pattern on pectin, as demonstrated by the CSPR functional assay (**Figure 5**), and this introduction of calcium sensitivity into high-methoxyl pectin is not distinguishable from the two TL-PMEs (*11*, *21*). Similarly to the two TL-PMEs, end-point enzyme de-esterification did not lead to dramatic changes in pectin's weight-average molecular weight or radius of gyration, but did significantly reduce the intrinsic viscosity (**Table 4**; *11*, *21*). The extensive carboxyl groups introduced by enzyme action were hypothesized to increase the cross-link density of the pectin network by intramolecular hydrogen bonding, causing the network to be less free-draining and thus decreasing intrinsic viscosity (*30*).

The overall properties of TT-PME suggested a closer relationship to the peak 2 TL-PME than to the peak 4 TL-PME. Both are salt-independent and have similar p*I* values, and the K_m estimated for TT-PME (0.0309 mg·mL^{-1}; *8*) was very similar to the K_m reported for the salt-dependent TL-PME (0.0487 mg·mL^{-1}; *21*). Both have a strong effect on destabilizing juice cloud. Peak 4 TL-PME has no effect on juice cloud, but this property may simply be due to pH-related activity rather than action pattern, because all three forms appear to de-esterify pectin in a blockwise manner. K_m values have not been determined by comparing the various PMEs as a function of assay pH or temperature. TT-PME and peak 2 PME were examined in more detail structurally to better understand their relationship with each other. For this we have applied MALDI-TOF MS, taking advantage of now having the multiple forms isolated and advances in citrus genomics (*28*−*30*). We have now generated tryptic-petide mass fingerprints for each PME form and have compared the experimentally observed peptide ions with theoretical peptides generated from translated peptide sequences for the various PME-related genes reported, which now allow unequivocal structural identification of citrus PMEs and polypeptide fragments derived from them (Savary et al., manuscript in preparation). The tryptic-peptide ion mass spectrum obtained for TT-PME (**Figure 3**) and subsequent de novo sequencing of individual peptides by second-dimension TOF-MS with post-source decay (data not shown) revealed no sequence relationship with peak 2 TL-PME or any other translated PME gene product of EST cDNA (*28*, *29*, *31*). TT-PME clearly does not represent the product from the "thermostable PME-associated gene" (*29*). The translated gene product contains no *N*-glycosylation sites, whereas TT-PME is now well established to be an *N*-linked glycoprotein. The partial N-terminal amino acid sequence from the 40.8 kDa band resolved by SDS-PAGE further showed no alignment relationship with any translated PME gene products or the N termini of TL-PMEs we have isolated (*30*). We will report separately our evidence from mass spectroscopy analysis that the "thermostable PME-associated gene" actually represents the peak 2 TL-PME and the identification and expression patterns for TT-PME in EST cDNA libraries.

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