Purification and Characterization of a Thermally Tolerant Pectin Methylesterase from a Commercial Valencia Fresh Frozen Orange Juice

Randall G. Cameron* and Karel Grohmann

Citrus and Subtropical Products Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 600 Avenue S N.W., P.O. Box 1909, Winter Haven, Florida 33883-1909

A thermally tolerant form of pectin methylesterase (TT-PME) has been purified from a commercially available Valencia fresh frozen orange juice by gel filtration, heparin, and concanavalin A chromatography. TT-PME represented 8.3% of total PME activity in the solubilized dialysis precipitate. This TT-PME retained $83.3 \pm 1.1\%$ relative activity after a 2 min incubation in an 80 °C water bath and $49.2 \pm 4.1\%$ relative activity after a 60 s incubation in a 95 °C water bath. It also retained $3.3 \pm 0.6\%$ and $8.3 \pm 1.5\%$ relative activity at pH 3.5 and 4.5, respectively. It had a native molecular mass of 40.1 kDa (gel filtration chromatography) and a denatured M_r of 42.7 ± 0.1. Binding to concanavalin A and treatment with PNGase F suggests it is an N-linked glycoprotein. After extended deglycosylation with PNGase F, polypeptide bands at M_r 41.7 ± 0.6 ($n = 4$) and 40.1 ± 0.4 ($n = 4$) were observed.

INTRODUCTION

Multiple forms of pectin methylesterase (PME; EC 3.1.1.11) have been found in navel oranges (Versteeg et al., 1980), white grapefruit finisher pulp (Seymour et al., 1991a,b), lemon fruit (Macdonald et al., 1993, 1994), citrus tissue culture cells (Cameron et al., 1994), and red grapefruit finisher pulp (Cameron and Grohmann, 1995). In each of these sources of PME at least one of the forms exhibited tolerance to heat treatment. Thermally tolerant forms of PME (TT-PME) are especially significant for the citrus juice industry. Elevated pasteurization temperature, above that needed to control microbial growth, is required to inactivate PME and prevent juice cloud precipitation and concentrate gelation (Wenzel et al., 1951; Rothschild and Karsenty, 1974).

Precipitation of citrus juice cloud is due to a series of events initiated by the enzymatic cleavage (by PME) of a methyl ester attached to C6 of galacturonic acid moieties of pectin (Rouse, 1949). Baker (1979) determined that a degree of methylation greater than 21% was the critical level of esterification necessary to maintain juice cloud stability. Wenzel et al. (1951) investigated the use of thermal inactivation of PME to maintain juice cloud stability. Eagerman and Rouse (1976) reported that heating juice at 90 °C for 1 min would stabilize the juice for commercial purposes. Chen et al. (1993) stated that temperature ranges from 90 to 95 °C and holding times of 15−60 s are used to stabilize commercial juices.

Versteeg et al. (1980) reported that at 5 °C only a TT-PME was capable of rapidly (less than 4 weeks) precipitating the orange juice cloud. Macdonald et al. (1993, 1994) showed that TT-PME from lemon fruit precipitated lemon juice cloud but thermally sensitive forms

did not. They suggested that the reason the TT-PME precipitated lemon juice cloud is that it retains some activity at the pH of lemon juice. The two major PME forms they found in lemon fruit would precipitate the juice cloud if the pH was first adjusted to 3.3 from 2.7.

Here we report on the purification and characterization of a TT-PME from a commercially available fresh frozen orange juice (FFOJ) that retains activity at the pH of orange juice.

MATERIALS AND METHODS

A commercially available FFOJ, labeled as being made from Valencia oranges, was purchased from a local supermarket. According to the juice processor, prior to juicing, the oranges are washed with water three times to minimize the possibility of fungal contamination; the FFOJ never comes into contact with a heat exchanger, consequently, no pasteurization occurs; and the juice is chilled immediately after squeezing and is stored frozen at −30 °C after packing.

Protein Extraction. Two liters (final volume) of the thawed juice was adjusted to 100 mM Tris-HCl, pH 8.0 (pH was adjusted with solid NaOH), and 1 M NaCl. The juice was stirred overnight at 6 °C. After centrifugation at 12100*g* for 30 min at 4 °C, the supernatant (containing the PME activity) was brought to 35% ammonium sulfate saturation and stirred for 2 h at 6 °C. This solution was centrifuged as described above. The supernatant, containing PME, was then brought to 70% ammonium sulfate saturation and stirred overnight at 6 °C. The next morning this solution was centrifuged at 12100*g* at 4 °C for 60 min. The supernatant was discarded (no PME activity) and the pellet solubilized in a minimal volume of 10 mM Tris-HCl, pH 7.5, 20 mM NaCl, and 0.02% (w/v) sodium azide (TBS). This solution was dialyzed (6000 molecular weight cutoff dialysis tubing) against 2 L of TBS for approximately 18 h. Four changes of TBS were made during this period. During dialysis a precipitate formed within the dialysis tubing. Upon completion of dialysis the suspension within the dialysis tubing was centrifuged at 27200*g* for 15 min at 4 °C. The pelleted precipitant contained TT-PME activity and was solubilized in 10 mM Tris, pH 7.5, 1 M NaCl, and 0.02% (w/v) sodium azide. This solution was centrifuged at 27200*g* for 15 min at 4 °C to pellet any remaining insoluble

* Author to whom correspondence should be addressed [e-mail RCAMERON@ASRR.ARSUSDA.GOV; fax (941) 299-8678].

TT-PME from Commercial Orange Juice

J. Agric. Food Chem., Vol. 44, No. 2, 1996 **459**

Table 1. Purification of a TT-PME from Commercially Available Valencia FFOJ

sample	units loaded	activity recovered (units)	volume (mL)	% recovery	specific activity (μequiv min^{-1} μg^{-1} pro)	% TT-PME[f]	purification factor[g]
solubilized dialysis precipitate		0.0433 ± 0.0029[a] (0.0037 ± 0.0001)[b]	5.0		0.0181[a] (0.0015)	8.3	
gel filtration	216.5[a]	0.0123 ± 0.0003[a] (0.0012 ± 0)[b]	16.0	90.9	0.0201[a] (0.0020)	9.9	1.3
heparin	196.8[a] (19.2)[d]	0.0021 ± 0.0001[a,c] (0.0019 ± 0.0001)[b]	9.8	97.0[e]	0.0102[a] (0.0092)[b]	90.2	6.1
concanavalin A	18.6[d]	0.0013 ± 0.00001[a] (0.0015 ± 0.00006)[b]	6.0	48.4[e]	0.0187[a] (0.0216)[b]	115.5	14.4

[a] Activity before a 2 min incubation in an 80 °C water bath. [b] Activity remaining after a 2 min incubation in an 80 °C water bath. [c] A thermally sensitive peak also was eluted but not pursued. [d] Units loaded based on activity remaining after a 2 min incubation in an 80 °C water bath. [e] % of 80 °C stable activity recovered. [f] Based on specific activity. [g] Based on specific activity remaining after a 2 min incubation in an 80 °C water bath.

material. The supernatant (containing TT-PME activity) was frozen at −75 °C in 10 mL aliquots.

Chromatography. A combination of gel filtration and affinity chromatography was used to purify the TT-PME. The columns were attached to an FPLC (Pharmacia LKB) system and the eluent monitored at 280 nm. All chromatography was performed at room temperature, and individual fractions were refrigerated at 4 °C immediately after collection.

Gel Filtration Chromatography. Five milliliters of the solubilized dialysis precipitant was loaded onto a Superdex 75 HiLoad 16/60 (Pharmacia LKB) gel filtration column. The column previously had been equilibrated with 10 mM Tris, pH 7.5, 1 M NaCl, and 0.02% (w/v) NaN$_3$. The flow rate was 1 mL·min^{-1}, and 1 mL fractions were collected during protein elution. Individual fractions were qualitatively assayed for PME activity. Fractions containing TT-PME (determined by a qualitative assay, see Enzyme Assays) activity were pooled and concentrated using Centricon microconcentrators (Amicon, 30 kDa cutoff membrane). The buffer was changed to TBS using Centricon microconcentrators. The TT-PME remained in solution in TBS after gel filtration chromatography.

The Superdex 75 HiLoad 16/60 column also was used to estimate native molecular weight of the TT-PME after heparin and concanavalin A chromatography. The column was calibrated with cytochrome *c*, carbonic anhydrase, bovine serum albumin (BSA), and β-amylase ($r^2 = 0.99$).

Heparin Chromatography. TT-PME material from gel filtration chromatography (pooled, concentrated, and buffer changed to TBS) was loaded onto the heparin Sepharose CL-6B column (1 × 20.5 cm) at 0.5 mL·min^{-1}. After loading, the column was washed with 15 mL of TBS at 0.5 mL·min^{-1} to remove unbound protein. Elution of bound proteins was accomplished with a linear gradient of 0.02−0.3 M NaCl in TBS. One milliliter fractions were collected; those containing TT-PME activity were pooled and concentrated, and the buffer was changed as described above. The buffer was changed to concanavalin A loading buffer containing 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 0.02% (w/v) NaN$_3$ (buffer A).

Concanavalin A Chromatography. The pooled, concentrated TT-PME (now in buffer A) was loaded onto a concanavalin A−agarose (Sigma) column (1 × 19 cm) at 0.5 mL·min^{-1}. The column was washed with 20 mL of buffer A, and bound proteins were eluted with a linear gradient of 0−50 mM α-D-glucopyranoside (Sigma) in buffer A over 30 mL. The flow rate was held at 0.5 mL·min^{-1}, and 1 mL fractions were collected and assayed for TT-PME activity. Fractions containing TT-PME activity were pooled and concentrated and the buffer was changed to TBS. All quantitative enzyme assays and electrophoretic analyses were performed on the TT-PME (in TBS) after concanavalin A chromatography.

Enzyme and Protein Assays. Column fractions were initially screened for PME activity at two temperatures, i.e. at room temperature and after being incubated for 2 min in an 80 °C water bath (to determine which fractions contained TT-PME activity). To 20 μL of a column fraction were added 180 μL of a reaction mixture, containing approximately 0.5% (w/v) citrus pectin (63.6% DM, Sigma), 0.2 M NaCl, and 0.05% (w/v) bromothymol blue (as a pH change indicator). The time required for a color change to occur (from blue to yellow) was recorded.

Quantitative assays were conducted according to the procedure of Cameron et al. (1992) utilizing either an automatic titrator (pH profile) or a kinetic microplate reader (all other assays). Microplates were read at 15 s intervals for either 15 or 30 min (depending on activity levels) with a 10 s plate shake immediately before each reading. The reaction mixture contained 0.3% (w/v) citrus pectin (63.6% DM, Sigma), 200 mM NaCl, and 0.026 unit of TT-PME. Each reported value is the mean of a minimum of three replicates except the pH 3.5 assay, which had only two replicates. One unit of activity equals 1 microequivalent of acid released per minute per microliter when assayed with 0.3% (w/v) citrus pectin (63.6% DM, Sigma) and 200 mM NaCl at 30 °C.

The TT-PME pH profile was constructed by assaying 0.033 (pH 6.0 and 7.0), 0.066 (pH 4.5 and 5.5), or 0.099 unit (pH 3.5) of the concanavalin A bound enzyme by continuous titration. Assays were run for 10 min (pH 5.5, 6.0, and 7.0) to 16 h (pH 3.5 and 4.5). The extended runs at pH 3.5 and 4.5 were required due to the relatively low levels of activity present at these pH values. Control runs at pH 3.5 and 4.5 demonstrated no base utilization over a 2 h period before enzyme was added.

Thermal stability and thermal inactivation curves were performed according to the method of Cameron and Grohmann (1995).

Protein amounts were estimated with a Micro BCA protein assay reagent kit (Pierce) using BSA as the standard.

Electrophoresis and Deglycosylation. Samples for electrophoresis were precipitated at 20 °C with a minimum of 10 volumes (v/v) of cold (−20 °C) acetone. Protein was pelleted by centrifugation at 13000*g* for 15 min at 4 °C. The acetone was decanted and the pellet solubilized in 10% (v/v) glycerol, 2.3% (w/v) sodium dodecyl sulfate (SDS), 62.5 mM Tris-HCl, pH 6.8 at 27 °C, and 5% (v/v) 2-mercaptoethanol for SDS−polyacrylamide gel electrophoresis (SDS−PAGE). SDS−PAGE was run on a 12.5% vertical slab gel (Mini Protean II, Bio-Rad) according to the method of Laemmli (1970). Gels were silver stained (Blum et al., 1987).

Enzymatic deglycosylation was performed with a GlycoShift Protein De-N-Glysosylation Kit (Oxford GlycoSystems) according to the provided instructions. Total deglycosylation was attempted by doubling the initial concentration of PNGase F (20 vs 10 μL), extending the reaction time (48 vs 18 h), and adding a second aliquot of 20 μL of PNGase F after the first 24 h incubation period.

RESULTS

TT-PME represented 8.3% of the total PME specific activity present in the solubilized dialysis precipitate (Table 1).
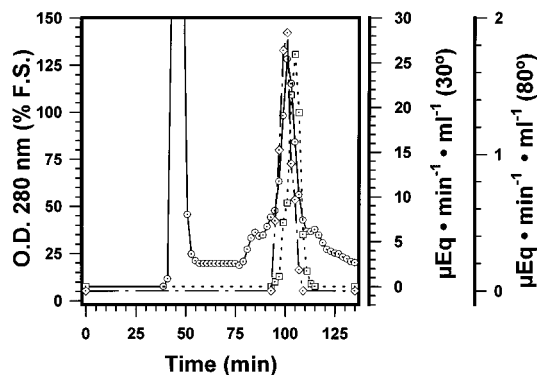
460 *J. Agric. Food Chem.,* Vol. 44, No. 2, 1996

Cameron and Grohmann

Figure 1. Gel filtration chromatography of crude FFOJ dialysis precipitate: (F.S.) full scale = 0.5 absorbance units; (○) OD 280 nm; (□) PME activity assayed at 30 °C with no prior heat treatment; (◇) PME activity assayed at 30 °C after a 2 min incubation in an 80 °C water bath.
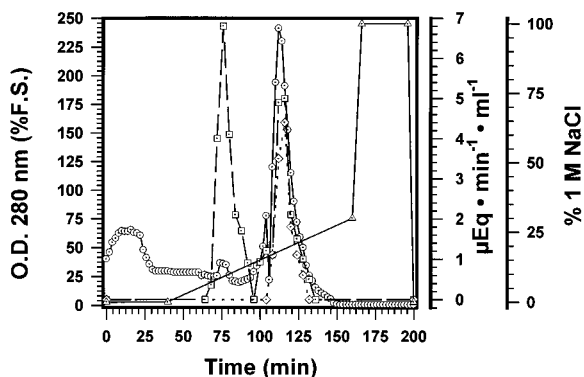

Figure 2. Heparin chromatography of TT-PME from gel filtration chromatography: (F.S.) full scale = 0.1 absorbance unit; symbols same as in Figure 1; (△) % 1 M NaCl in elution buffer.

Chromatography. *Gel Filtration.* A total of 90.9% of PME activity was recovered after gel filtration chromatography (Table 1). The TT-PME was 9.9% of the total PME specific activity.

Heparin. Two peaks of PME activity were eluted from the heparin column by a linear NaCl gradient (Figure 2). The first eluted at approximately 80 mM NaCl. This peak did not contain any TT-PME activity but did exhibit thermally sensitive PME activity. The TT-PME activity eluted at approximately 200 mM NaCl (Figure 2). The recovery percentage (97.0) and percent of activity due to TT-PME (90.2, Table 1) suggest this peak consisted almost totally of TT-PME.

Concanavalin A. TT-PME that was bound to concanavalin A eluted with 27 mM methyl α-D-glucopyranoside (Figure 3). This peak consisted entirely of TT-PME (Table 1; Figure 7).

Enzyme Activity. *Temperature Curve.* The concanavalin A purified TT-PME retained 83.3 ± 1.1% relative activity after a 2 min incubation in a 80 °C water bath (Cameron and Grohmann, 1995; Figure 4). After 2 min in a 90 °C water bath, it retained 49.4 ± 1.2% relative activity.

Thermal Inactivation. Incubation in a 95 °C water bath was used to approximate processing pasteurization temperatures. After a 60 s incubation in a 95 °C water bath (Cameron and Grohmann, 1995; Figure 5), the purified TT-PME retained 49.2 ± 4.1% relative activity. A 30 s incubation in the 95 °C water bath had no effect on enzyme activity [see Cameron and Grohmann (1995) for thermal response curves for incubation at 95 °C].
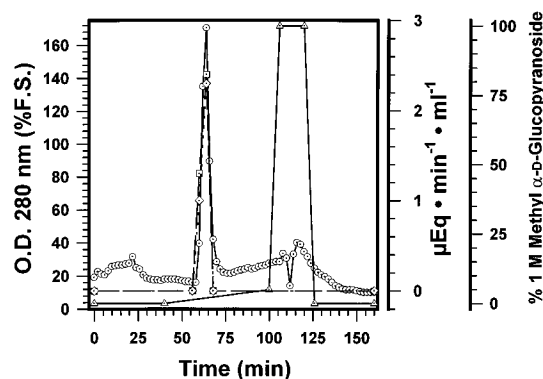

Figure 3. Concanavalin A chromatography of TT-PME from heparin chromatography: (F.S.) full scale = 0.1 absorbance unit; symbols same as in Figure 1; (△) % 1 M methyl α-D-glucopyranoside in elution buffer.
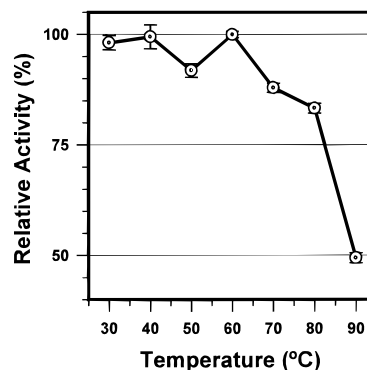

Figure 4. Temperature curve for TT-PME from concanavalin A chromatography. Each data point is the mean ± SE for a minimum of three replicates with 0.026 unit of enzyme used per replicate.
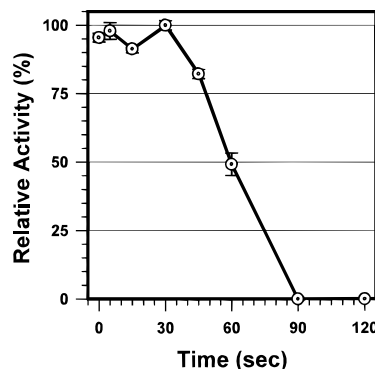

Figure 5. Thermal inactivation curve for TT-PME (incubated in a 95 °C water bath) from concanavalin A chromatography. Each data point is the mean ± SE for a minimum of three replicates with 0.026 unit of enzyme used per replicate.

After a 90 s incubation, the TT-PME retained residual activity, i.e., the activity was too low to assign a numerical value, but the pH indicator used (bromothymol blue) did change color from blue to yellow over a 4.5 h period. Control wells, in which no enzyme was added, and wells containing enzyme incubated for 2 min in a 95 °C water bath exhibited no residual activity (the color remained blue).

pH Profile. Relatively low levels of activity were measured at pH 3.5 and 4.5 (Figure 6). Higher enzyme loadings (0.099 unit at pH 3.5 and 4.5 vs 0.033 unit at pH 6.0 and 7.0) and longer assay times (up to 16 h at pH 3.5 and 4.5 vs 10 min at pH 5.5, 6.0, and 7.0) were required to observe the levels of activity reported in Figure 6. At pH 3.5 and 4.5 the TT-PME retained 3.3
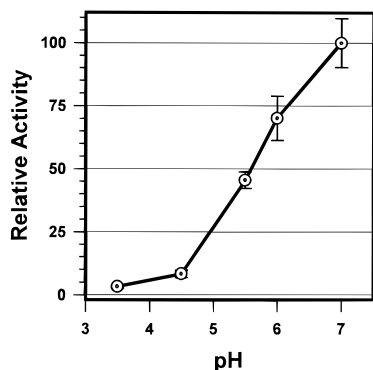
TT-PME from Commercial Orange Juice

J. Agric. Food Chem., Vol. 44, No. 2, 1996 **461**

Figure 6. pH profile for TT-PME from concanavalin A chromatography. Each data point is the mean ± SE for a minimum of three replicates (except pH 3.5, *n* = 2). Enzyme loadings were 0.033 (pH 5.5, 6.0 and 7.0), 0.066 (pH 4.5), and 0.099 unit (pH 3.5).
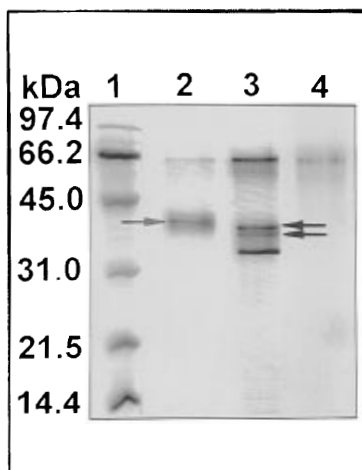

Figure 7. SDS−PAGE of concanavalin A purified TT-PME: (lane 1) molecular weight standards; (lane 2) concanavalin A purified TT-PME (0.4 μg); (lane 3) concanavalin A purified TT-PME (0.4 μg) after treatment with PNGase F; (lane 4) sample buffer only which was run on a separate gel.

± 0.6% and 8.3 ± 1.5%, respectively, of the activity observed at pH 7.0.

Molecular Weight Estimates and Deglycosylation. Gel filtration of the concanavalin A purified TT-PME was used to estimate the native molecular weight of the TT-PME. A value of M_r 40.1 was obtained. After SDS−PAGE (Figure 7), a single polypeptide band could be visualized. It had a M_r of 42.7 ± 0.1 (mean ± SE, *n* = 3). After treatment with PNGase F, its mobility was increased (Figure 7). Two polypeptide bands were observed at M_r 40.1 ± 0.4 (*n* = 4) and 41.7 ± 0.6 (*n* = 4). The heavily stained band at M_r 36.2 ± 0.6 (*n* = 4) is the enzyme PNGase F (Tretter et al., 1991; data not shown). Extended incubations with increased loading of PNGase F failed to completely deglycosylate the enzyme (data not shown). The stained band at M_r 61.5 is artifactual as it is present in lanes in which only the SDS−PAGE sample buffer was added (Figure 7).

DISCUSSION

Extraction of a commercially available FFOJ with a high-salt (1 M NaCl) buffer demonstrates the presence of at least one TT-PME. This TT-PME precipitates from the high-salt buffer (1 M) during dialysis against a low-salt buffer (20 mM NaCl). The PME activity present in this precipitate can be resolubilized in a high-salt

buffer, and after gel filtration, it will remain in solution in a low-salt buffer. A TT-PME also was present in the dialysis supernatant (low salt). Preliminary studies suggest this second TT-PME may be of a different molecular form from the TT-PME described here (data not shown). Further purification and characterization of this TT-PME are required to determine if it is different from the TT-PME reported here.

Tolerance to elevated temperatures by the TT-PME purified from FFOJ suggests it is in part responsible for the elevated pasteurization temperatures required to stabilize orange juice products (Chen et al., 1993). The temperature curve (Figure 4) and thermal inactivation curve (Figure 5) for this TT-PME are very similar to those reported for TT-PME from the dialysis supernatant of extracts from red grapefruit finisher pulp (Cameron and Grohmann, 1995). The temperature curve for the FFOJ TT-PME also is similar to one from citrus tissue culture cells (Cameron et al., 1994), but the thermal inactivation curve for FFOJ TT-PME demonstrates that it is more stable than the tissue culture form which was essentially inactivated by a 60 s incubation in a 95 °C water bath. It is difficult to compare the TT-PME reported here to that reported by Seymour et al. (1991a) since their TT-PME was assayed at pH 3.3 with grapefruit juice as the pectin-containing reaction cocktail. Versteeg (1979) reported complete inactivation of a TT-PME from navel oranges after a 5 min incubation at 90 °C (assayed at pH 7.5). The 5 min incubation, compared to 2 min in this study, may account for their lower inactivation temperature than we have observed with this Valencia orange TT-PME, which retained 49.4 ± 1.2% relative activity after 2 min at 90 °C. Also, this Valencia TT-PME apparently is more stable than one from mandarin oranges (Rillo et al., 1992). The mandarin orange PME was totally inactivated after a 1 min incubation at 90 °C.

The retention of low levels of activity at pH values comparable to those found in orange juice (pH 3.5−4.5; Chen et al., 1993) also suggests that this TT-PME from FFOJ may contribute to cloud loss and concentrate gelation. Low levels of activity at the pH of citrus juices also have been reported by Versteeg (1979) and Seymour et al. (1991a,b). Macdonald et al. (1993) showed that the two major lemon fruit PMEs (thermally sensitive) would not precipitate lemon juice cloud unless the pH was raised from 2.7 to 3.3. However, the TT-PME from lemon fruit (Macdonald et al., 1994) would precipitate lemon juice cloud at pH 2.7. Both Seymour et al. (1991a) and Macdonald et al. (1994) demonstrated that their respective TT-PMEs were more stable at low pH values (pH 2.0−2.5) than the thermally sensitive PMEs.

Glycosylation has been suggested as an important factor for maintaining enzyme stability (Faye, 1988; Varki, 1993). Seymour et al. (1991a) suggested that O-linked glycosylation of their TT-PME was in part responsible for both thermal and pH tolerance. Macdonald et al. (1994) reported that their TT-PME stained positively for glycosylation, but the authors could not rule out the possibility that their positive staining for glycosylation was not due to associated pectin. We have demonstrated here that TT-PME from FFOJ is glycosylated and contains at least one N-linked glycan (binding to concanavalin A and increased mobility after treatment with PNGase F). The presence of two polypeptide bands after extended treatment with PNGase F indicates total deglycosylation was not achieved. Possible reasons for incomplete deglycosyla-

tion include glycan microheterogeneity (Faye et al., 1986; Michaud et al., 1993) and incomplete denaturation prior to deglycosylation (Alexander and Edler, 1989). Fucose linked α 1→3 to Asn-GlcNAc also prevents deglycosylation by PNGase F (Tretter et al., 1991). Fucose has been identified from orange peel hydrolysates (Grohmann et al., 1996), but its source in the peel is unknown. Further studies are planned to probe the relationship between glycosylation and stability of this enzyme.

In summary, Valencia FFOJ contains at least one TT-PME. After a 2 min incubation in an 80 °C water bath, it retained 83.3% relative activity. The enzyme maintained approximately a 50% level of relative activity after a 60 s incubation in a 95 °C water bath. This TT-PME is a N-linked glycoprotein with a native M_r of 40.1. It also retained low levels of activity at pH 3.5.

ACKNOWLEDGMENT

LITERATURE CITED

Alexander, S.; Edler, J. H. Endoglycosidases from *Flavobacterium meningosepticum.* Application to biological problems. *Methods Enzymol.* **1989**, *179*, 505–518.

Baker, R. A. Clarifying properties of pectin fractions separated by ester content. *J. Agric. Food Chem.* **1979**, *27*, 1387–1389.

Blum, H.; Beier, H.; Gross, H. J. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **1987**, *81*, 93–99.

Cameron, R. G.; Grohmann, K. Partial purification and thermal characterization of pectin methylesterase from red grapefruit finisher pulp. *J. Food Sci.* **1995**, *60*, 821–825.

Cameron, R. G.; Buslig, B.; Shaw, P. Adaptation of a spectrophotometric assay for pectin methylesterase to a kinetic microplate reader. *J. Food Sci.* **1992**, *57*, 1006–1008.

Cameron, R. G.; Niedz, R. P.; Grohmann, K. Variable heat stability for multiple forms of pectin methylesterase from citrus tissue culture cells. *J. Agric. Food Chem.* **1994**, *42*, 903–908.

Chen, C. S.; Shaw, P. E.; Nagy, S. Orange and tangerine juices. In *Fruit Juice Processing Technology*; Nagy, S., Chen, C. S., Shaw, P. E., Eds.; Agscience: Auburndale, FL, 1993.

Eagerman, B. A.; Rouse, A. H. Heat activation temperature-time relationships for pecinesterase inactivation in citrus juices. *J. Food Sci.* **1976**, *41*, 1396–1397.

Faye, L. Structure, biosynthesis, and function of plant glycoprotein glycans. In *Current Topics in Plant Biochemistry and Physiology*; Proceedings, Plant Biochemistry and Physiology Symposium; University of Missouri: Columbia, MO, 1988; pp 62–82.

Faye, L.; Mouatassim, B.; Ghorbel, A. Cell wall and cytoplasmic isozymes of raddish β-fructosidase have different N-linked oilgosaccharides. *Plant Physiol.* **1986**, *80*, 27–33.

Grohmann, K.; Cameron, R. G.; Buslig, B. S. Fractionation and pretreatment of orange peel by dilute acid hydrolysis. *Bioresour. Technol.* **1996**, in press.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

Macdonald, H. M.; Evans, R.; Spencer, W. J. Purification and properties of the major pectinesterases in lemon fruits (*Citrus limon*). *J. Sci. Food Agric.* **1993**, *62*, 163–168.

Macdonald, H. M.; Evans, R.; Spencer, W. J. The use of continuous-flow electrophoresis to remove pectin in the purification of the minor pectinesterase in lemon fruits (*Citrus limon*). *J. Sci. Food Agric.* **1994**, *64*, 129–134.

Michaud, D.; Seye, A.; Driouich, A.; Yelle, S.; Faye, L. Purification and partial characterization of an acid β-fructosidase from sweet-pepper (*Capsicum annum* L.) fruit. *Planta* **1993**, *191*, 308–315.

Rillo, L.; Castaldo, D.; Giovane, A.; Servillo, L.; Balestrieri, C.; Quagliuolo, L. Purification and properties of pectin methylesterase from Mandarin orange fruit. *J. Agric. Food Chem.* **1992**, *40*, 591–593.

Rothschild, G.; Karsenty, A. Cloud loss during storage of pasteurized citrus juices and concentrates. *J. Food Sci.* **1974**, *39*, 1037–1041.

Rouse, A. H. Gel formation in frozen citrus concentrates thawed and stored at 40 °C. *Proc. Fla. State Hortic. Soc.* **1949**, *62*, 170–173.

Seymour, T. A.; Preston, J. F.; Wicker, L.; Lindsay, J. A.; Wei, Cheng-i; Marshall, M. R. Stability of pectinesterases of Marsh White grapefruit. *J. Agric. Food Chem.* **1991a**, *39*, 1075–1079.

Seymour, T. A.; Preston, J. F.; Wicker, L.; Lindsay, J. A.; Marshall, M. R. Purification and properties of pectinesterases of Marsh White grapefruit pulp. *J. Agric. Food Chem.* **1991b**, *39*, 1075–1079.

Tretter, V.; Altmann, F.; März, L. Peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidase F cannot release glycans with fucose attached α1→3 to the asparagine-linked N-acetylglucosamine residue. *Eur. J. Biochem.* **1991**, *199*, 647–652.

Varki, A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **1993**, *3*, 97–130.

Versteeg, C. Pectinesterases from the orange: their purification, general characteristics and juice cloud destabilizing properties. Ph.D. Thesis, Department of Food Science, Agricultural University, Wageningen, The Netherlands, 1979.

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, 998.

Wenzel, F. W.; Moore, E. L.; Rouse, A. H.; Atkins, C. D. Gelation and clarification in concentrated citrus juices I. Introduction and present status. *Food Technol.* **1951**, *5*, 454–457.