Purification and Properties of Pectinesterases of Marsh White Grapefruit Pulp†

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Thermolabile (TL) and thermostable (TS) pectinesterases (PE) were purified 124- and 309-fold, respectively, from Marsh white grapefruit pulp by ion-exchange and gel filtration chromatography. Thermolabile PE accounted for the majority of PE activity (90%) in crude extracts. Native M_r values of TL PE and TS PE were estimated to be 36 000 and 51 000, respectively, and both enzymes were composed of a single polypeptide chain. Carbohydrate content was estimated at 2% for TL PE and 14.2% for TS PE. Amino acid content, antigenic properties, and UV spectra served to distinguish the enzymes. The K_m values (Sunkist pectin substrate) of TL and TS PEs were 0.274 and 1.02 mg/mL, respectively. Turnover numbers were 26 354 and 30 621 mol/(mol·min) for TL and TS PEs, respectively.

INTRODUCTION

Pectinesterase (PE) (pectin pectylhydrolase, EC 3.1.1.11) is present in abundance (Rexova-Benkova and Markovic, 1976; Macmillan and Sheiman, 1974) and exists in multiple forms (Evans and McHale, 1978; Korner et al., 1980; Rombouts et al., 1982; Versteeg et al., 1978) in all citrus fruits tested. The enzyme is firmly associated with the cell wall fraction and is located mainly in peel, rag, and juice sac tissues (Joslyn and Pilnik, 1961). PE is of great concern to the citrus industry since it has been definitively established as the causative agent for clarification of citrus juices and gelation of concentrates (Joslyn and Pilnik, 1961; Krop, 1974). To prevent these quality defects, the enzyme must be inactivated by pasteurization at 90 °C for 1 min (Rouse and Atkins, 1952). However, the high processing temperature produces a cooked off-flavor in the juice (Kew and Veldhuis, 1961).

Thermostable forms of citrus PE have been isolated (Versteeg, 1979). Although representing a minor fraction of the total activity in citrus fruits (Rombouts et al., 1982; Versteeg, 1979), these forms are the most active in chilled juice and account for the severe time/temperature profile needed to inactivate PE (Versteeg et al., 1980). It has been suggested that thermostable PE is largely responsible for cloud loss in underpasteurized citrus juices (Versteeg et al., 1980). The objectives of this study were to purify, characterize, and compare the structural properties of the thermostable and thermolabile forms to gain some insight into the observed differences in thermostability.

MATERIALS AND METHODS

Purification of Thermolabile Pectinesterase (TL PE). Crude extract was prepared according to the method of Wicker et al. (1987). Marsh white grapefruit pulp (300 g) was homogenized in a 1:5 ratio (w/v) of pulp to 0.25 M Tris-HCl, 0.3 M NaCl, and 1 mM sodium azide, pH 8.0 (precooled to 4 °C), for 1 min in a blender. All ensuing treatments were carried out at 4 °C. The mixture was stirred for 1 h and then centrifuged at 16000*g* for 25 min. To the supernatant was added solid ammonium sulfate to 75% saturation. After standing overnight, the precipitate was collected by centrifugation at 10000*g* for 20 min. The solution was dialyzed against four changes of 10 volumes of 10 mM sodium phosphate buffer containing 1 mM sodium azide, pH 7.5.

The enzyme solution (200 mL) was adsorbed onto CM-Sephadex C50 (200 mL; 5 g), preequilibrated in 10 mM sodium phosphate, pH 7.5, in a beaker. After 1 h, the supernatant was decanted and the resin washed with 1000 mL of the above phosphate buffer. After adsorption, the resin was packed in a 2.6 × 40 cm column and washed at 25 mL/h (total 500 mL) until absorbance at 280 nm (A_{280}) was less than 0.1. Enzyme activity was eluted with 0.2 M NaCl (pH 7.5) at a flow rate of 25 mL/h. Fractions (6 mL) were collected and assayed for PE activity and A_{280}. Selected fractions were pooled (18 mL) and applied to a Sephacryl 200 (475 mL; preswollen) gel filtration column (2.6 × 90 cm) and eluted at 3 mL/h. Fractions of 1.5–2 mL were collected and assayed for PE activity and A_{280}. Fractions selected for PE activity were pooled and concentrated in an Amicon stirred-cell equipped with a PM 10 (10 000 MW cutoff) membrane. Sample purity was evaluated by SDS-PAGE and HPLC gel filtration as described under characterization.

Purification of Thermostable Pectinesterase (TS PE). Crude extract was prepared by a modification of the method of MacDonnel et al. (1945) and Krop (1974). Marsh white pulp (3 kg) was homogenized in 2 parts of buffer (precooled to 4 °C) containing 0.25 M Tris-HCl, 0.3 M NaCl, and 1 mM sodium azide, at pH 8.0, for 1 min in a blender. All subsequent treatments were carried out at 4 °C. The mixture was stirred for 1 h and then centrifuged at 16000*g* for 20 min. Solid ammonium sulfate to 30% saturation was added to the supernatant. After standing overnight at 4 °C, the solution was centrifuged at 10000*g* for 20 min. A floating oil layer was decanted off and discarded, along with the pellet. The supernatant was filtered through two layers of cheesecloth, and solid ammonium sulfate to 75% saturation was added. After standing overnight, the precipitate was collected by centrifugation at 10000*g* for 20 min. The precipitate was redissolved in 10 mM sodium phosphate buffer, pH 7.0, with the aid of an ultrasonic bath, and dialyzed against three changes of 20 volumes of 10 mM sodium phosphate, pH 7.0, with 1 mM sodium azide. After dialysis, 1 mM sodium azide was added to the enzyme solution as a preservative. Heat treatment of crude PE at 70 °C for 5 min was carried out to inactivate the thermolabile PE fraction. Enzyme solution was heated in aliquots of 30 mL in large test tubes (25 × 200 mm). Solutions were rapidly brought up to 70 °C by placing them in a water bath

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adjusted to 85 °C and were then shifted to a 72 °C water bath for 5 min. The solution was then centrifuged at 10000g for 20 min.

The heat-treated enzyme (570 mL) was adsorbed onto CM-Sephadex C50 (175 mL; 4.4 g) preequilibrated in 10 mM phosphate buffer, pH 7.0. After 1 h, the supernatant was decanted and the resin washed with 1000 mL of the above phosphate buffer. After adsorption, the resin was packed into a 5 × 9 cm column and washed at a flow rate of 25 mL/h (total 500 mL) until A_{280} was less than 0.1. Enzyme was eluted by increasing the NaCl concentration of the eluant buffer linearly up to 300 mM over 1000 mL. Fractions of 7–8 mL were collected and assayed for PE activity as well as A_{280}. Fractions were pooled (200 mL) for further purification, dialyzed to remove NaCl, and adsorbed in a beaker with CM-Bio-Gel A (35 mL; preswollen). The resin was packed in a 2.6 × 7 cm column and washed at a flow rate of 50 mL/h (total 800 mL) until A_{280} of the eluant was less than 0.1. Enzyme was eluted by increasing NaCl concentration linearly to 100 mM over 1000 mL, at a flow rate of 50 mL/h. Four-milliliter fractions were collected and assayed for PE activity and A_{280}. Fractions were pooled and concentrated in an Amicon stirred-cell (PM 10). The concentrated activity (11 mL) was applied to a Sephadex G-75 (500 mL; 33 g) column (2.6 × 95 cm) and eluted at 2 mL/h. Fractions of 1–1.5 mL were collected and assayed for PE activity and A_{280}. Samples were analyzed for purity as described under characterization.

Enzyme Assays. A spectrophotometric assay for plant pectinesterase [modified method of Hagerman and Austin (1986)] was used to assay column chromatography fractions. This assay gave accurate measurement of relative but not absolute activities due to lack of reproducibility of standard curves. A 0.5% (w/v) solution of Sunkist pectin was prepared with the addition of 0.1 M NaCl. A 4-fold increase in bromthymol blue (0.04%) to that of Hagerman and Austin (1986) was used in the assay (Sigma Chemical Co., St. Louis, MO). Just before assays were carried out, 7.5 mL of the bromthymol blue solution was mixed together with 100 mL of the pectin solution and the mixture adjusted to pH 7.8 with sodium hydroxide. Distilled water was then added to make a total volume of 150 mL. To a cuvette was added 1.5 mL of the mixture; the initial A_{620} was 1.1–1.2 vs a water blank. The reaction was started by addition of up to 20 μL of PE solution to the cuvette, and the rate of decrease in A_{620} over time was recorded in a Beckman DU7 spectrophotometer (Beckman Instruments Inc., Palo Alto, CA). The assay was calibrated with galacturonic acid, and the decrease in A_{620} is linear when up to 0.2 μmol of acid is added per 1.5 mL of pectin solution. Solution pH was closely monitored to ensure reproducible color changes.

Activity was determined titrimetrically in pooled chromatography fractions and in all kinetic studies. PE solution is added to 25 mL of 1% Sunkist pectin in 0.1 M NaCl at 25 °C, which had been adjusted to pH 7.0 or 7.5 (with NaOH), depending on the pH optimum of the enzyme being assayed. While the solution was stirred with a magnetic stirrer, the initial reaction velocity is measured by automatic titration of the liberated carboxyl groups with standardized 0.05 N NaOH in a Combi recording pH stat (Metrohm Ltd., Herisau, Switzerland). One unit of PE activity is defined as the amount of enzyme that liberates 1 μmol of carboxyl groups/min at standard assay conditions. The effect of pH on PE activity on Sunkist pectin was determined by carrying out titrimetric assays as described above at different pH values (from 5 to 8.5). Kinetic parameters were determined on Sunkist pectin by measuring activity at different substrate concentrations. K_m and V_{max} were calculated from Lineweaver–Burk plots. The effect of degree of esterification (DE) of substrate on activity was examined by using the following substrates: HP Bulmer pectin (samples A and C–E), Kodak pectin, and Sunkist pectin.

Protein Assays. Protein concentrations were estimated according to the method of Smith et al. (1985) by using bovine serum albumin as a standard. Protein was estimated in chromatography eluant fractions by UV absorbance at 280 nm.

Pectin Analyses. Pectins used in activity measurements and kinetic analysis were obtained from Sunkist Growers, Inc. (Ontario, CA; lot A7216), Eastman Kodak (Rochester NY; lot P2569), and HP Bulmer Ltd. (Hereford, England; samples A and C–E). The content of anhydrogalacturonic acid (AGA) of Sunkist and Kodak pectins was determined according to the method of Blumenkrantz and Asboe-Hansen (1973). Degrees of esterification (DE) of Sunkist and Kodak pectins were estimated according to the method of Wood and Siddiqui (1971). The DE of HP Bulmer pectin was provided by the manufacturer.

The following characteristics were found: for Sunkist pectin, DE, 70%, and AGA content, 69%; for Kodak pectin, DE, 62%; and AGA content, 64%.

Analysis of Purity and Relative Molecular Mass (M_r). Estimation of M_r and analysis of purity were carried out by two methods: HPLC gel filtration and SDS–PAGE. Aliquots were subjected to HPLC gel filtration on a Superose 6 HR 10/30 column run at room temperature with a buffer of 10 mM sodium phosphate, pH 7.0, containing 200 mM NaCl at a flow rate of 0.3 mL/min. The column was fitted to a Dionex system; protein was detected by a variable-wavelength detector at 254 nm. SDS–PAGE was carried out according to the method of Laemmli (1970). Separations were performed by using a Bio-Rad Protean II vertical slab unit for electroblotting or a Bio-Rad minigel unit for analysis of purity and M_r. Running gels (10 mL for minigel and 20 mL for large slab) were composed of 12% acrylamide, 0.3% bis(acrylamide) (both Bio-Rad). For analysis of cyanogen bromide cleavage products, SDS–PAGE by the method of Giulian et al. (1985) [running gel 17.5% acrylamide, 0.08% bis(acrylamide) stacking gel 10% acrylamide, 0.5% bis(acrylamide)] was carried out under the same running conditions. Proteins were stained with a solution containing 0.25% Coomassie brilliant blue R-250 (Eastman Kodak) and 0.05% amido black (ICN Biomedicals, Costa Mesa, CA).

Estimation of Isoelectric Point. Isoelectric points were estimated by isoelectric focusing on premade polyacrylamide isoelectric focusing gels, pH 3–10, 0.6 mm thick (FMC Bioproducts, Rockland, MN). Purified enzymes and standards (broad p*I* kit, pH 3–10, Pharmacia) were run at constant power for 2 h at 8 W (500-V limiting) on a Hoefer Isobox flatbed system (HE 950) at 10 °C. Protein bands were visualized as described under SDS–PAGE.

Amino Acid Analysis. Purified PE and standards were first separated by SDS–PAGE by the method of Laemmli (1970) on a Protean II vertical slab unit. SDS–PAGE separated proteins were transferred by electroblotting onto PVDF membranes according to the method of Matsudaira (1987). Blotted protein bands corresponding to the R_f of the enzymes were cut out of the membranes with a clean razor and hydrolyzed in 6 N HCl under N_2 in sealed tubes for 24 h at 110 °C. Cystine plus cysteine content was determined as cysteic acid by carrying out the hydrolysis in the presence of dimethyl sulfoxide. Tryptophan was determined after hydrolysis in 3 N mercaptoethanesulfonic acid. Typically, 0.3–0.4 nmol of proteins was recovered from 3–4 nmol of purified protein after the electroblotting and hydrolysis procedures for amino acid analysis. The amino acids were resolved and quantified by using a Beckman 6300 amino acid analyzer with a Nelson analytical data acquisition system. The amino acid analyses were carried out at the Protein Chemistry Core facility, University of Florida.

Antibody Preparation. Immunological cross reactivity between the enzymes was tested by using purified TL PE as an antigen to induce antibody formation in chickens. The animals were injected with 100 μg of TL PE and boosted in 2 weeks with an additional 100 μg. The antibodies were purified from egg yolk according to the method of Jensenius et al. (1981). Titers were determined by ELISA.

ELISA Procedure. Antigens were diluted to the appropriate concentration (1–100 ng) into coating buffer, 0.1 M sodium carbonate, pH 9.6, and 100 μL was added to each well of an Immulon plate (Fisher Scientific Co., Fair Lawn, NJ). After 2 h at room temperature (or overnight at 4 °C), the Immulon plate was washed four times with phosphate-buffered saline, pH 7.2, containing 0.5% Tween (PBS–Tween). Antibody solution (100 μL), diluted with PBS–Tween, was then added. After 1 h at room temperature (or overnight at 4 °C), the plate was washed with PBS–Tween four times. Anti-chicken alkaline phosphatase conjugate (Sigma) was diluted 1/2000 with PBS–Tween, and 100 μL/well was added. After 1 h at room temperature, the plate was washed four times with substrate buffer (0.1 M sodium carbonate and 0.5 mM magnesium chloride, pH 9.4). Alkaline phosphatase substrate, *p*-nitrophenyl acetate (Sigma), was

dissolved in substrate buffer (1 mg/mL), and 100 μL/well was added. The absorbance at 405 nm was read with an ELISA reader (Bio-Rad) when it reached 0.5–1.0 (within 0.5–2 h).

In competitive ELISA experiments, either TL PE or TS PE (0–100 ng) was added as competitor to the anti-TL PE antibody. Various amounts of competitor are mixed with the primary antibody solution prior to addition to the wells containing bound antigen. Anti-TL PE antibody was used at 1/1000 and 1/2000 dilutions, and 10 ng of TL PE was plated as antigen.

Ultraviolet Spectra. The ultraviolet spectra of the purified enzymes was obtained by scanning from 220 to 400 nm in a Beckman DU7 spectrophotometer. The scans were carried out in quartz cuvettes of 10 mm path length in 10 mM sodium phosphate buffer, pH 7.0.

Carbohydrate Content of Pectinesterases. To test for the possibility that the enzymes were glycosylated, staining for carbohydrate on SDS–PAGE gels was carried out with dansylhydrazine both before and after periodate oxidation (Eckhardt et al., 1976). Glycoprotein and non-glycoprotein controls were stained under the same conditions.

Total neutral sugar content was assayed according to the phenol–sulfuric acid method (Dubois et al., 1956) using glucose as a standard. In the assays, 0.1–0.2 mg of TS PE and 0.5–1 mg of TL PE was analyzed.

RESULTS

Purification of TL PE. Ammonium sulfate fractionated crude grapefruit PE was further purified by ion-exchange chromatography on CM-Sephadex. One activity peak was observed upon elution with 0.2 M NaCl. Fractions were pooled on the basis of specific activity and then applied to a column of Sephacryl 200. Most of the PE activity eluted in a single large peak; however, a minor activity peak was also observed. The fractions of the major peak were pooled, and the activity was found to be completely inactivated by heating at 70 °C for 5 min. The activity of the minor peak, however, was stable to this treatment. On the basis of Versteeg's (1979) results that PE forms in orange can be differentiated by heat stability, the major activity peak was designated thermolabile pectinesterase (TL PE), while the minor activity peak was designated thermostable pectinesterase (TS PE). The specific activity of TL PE could not be increased by rechromatography. TL PE appeared to be pure when analyzed by SDS–PAGE and HPLC gel filtration (Figure 1). The total yield of TL PE was 19%. The purification of TL PE is summarized in Table I. The yield (0.3%) of TS PE activity by this procedure was too low for further analysis.

Purification of TS PE. Purification of thermostable PE was achieved by thermal inactivation followed by chromatographic methods. When crude PE was heated at 70 °C for 5 min, the residual PE activity was generally found to be 5–10% of the original activity; therefore, it was necessary to start with a 10-fold greater amount of pulp in the purification of TS PE. The best yields were obtained if the heating was carried out at pH 7.0 rather than at pH 7.5 or 8.0. The heat-treated crude PE solution was then applied to CM-Sephadex. One activity peak was observed on the CM-Sephadex elution profile. From this step, a 50-fold purification of heat-treated crude PE was achieved. This material was pooled and applied to a CM-Bio-Gel A column. The elution condition for the CM-Bio-Gel A column increased the resolution over that of the CM-Sephadex column such that a significant amount of bound contaminating protein was separated. A single broad activity peak was observed in the elution profile. The fractions were pooled on the basis of specific activity, concentrated, and applied to a G-75 gel filtration column. A major activity peak of nearly uniform specific activity was observed which appeared to be pure when analyzed by SDS–PAGE and HPLC gel filtration (Figure 1). Rechromatography did not increase the specific activity. The total yield of TS PE was 25%. The purification procedure of TS PE is summarized in Table II.

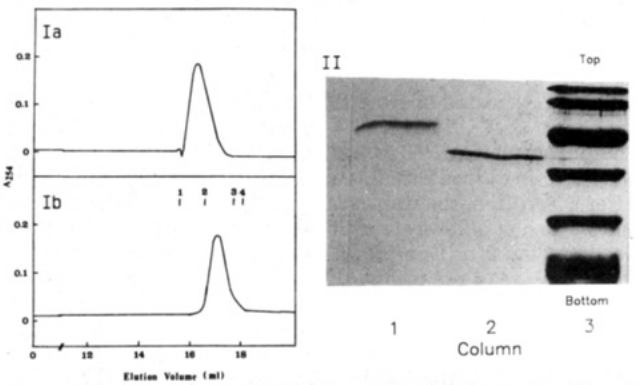

Figure 1. Gel filtration HPLC and SDS–polyacrylamide gel electrophoresis of grapefruit PEs. The native MW of TS PE (panel Ia) and TL PE (panel Ib) was determined by gel filtration on HPLC Superose 6. Protein (100 μg) was eluted in 0.2 M sodium chloride in 10 mM sodium phosphate buffer at pH 7.0. Standard proteins shown for comparison were (1) bovine serum albumin (67 000), (2) ovalbumin (43 000), (3) carbonic anhydrase (29 000), and (4) chymotrypsinogen (25 000). Purified TS PE and TL PE were denatured in the presence of SDS and 2-mercaptoethanol, analyzed on 12% SDS–PAGE minigel, and stained with Coomassie blue/amido black (panel II). Lane 1 contains 5 μg of TS PE. Lane 2 contains 5 μg of TL PE. Lane 3 contains the following standard proteins from top to bottom: phosphorylase *b*, 94 000; bovine serum albumin, 67 000; egg albumin, 43 000; carbonic anhydrase, 29 000; soybean trypsin inhibitor, 21 000; α-lactalbumin, 14 000.

Table I. Extraction and Purification of Thermolabile Pectinesterase[a]

purif step	act.,[b] units	protein,[b] mg	units/mg of protein	purif factor	yield, %
crude extract	6848[c]	978[d]	7		100
ammonium sulfate precipitation	4413	83	53	7.6	64
CM-Sephadex	3100	7.7	403	58	45
Sephacryl-200	1309[e]	1.6[f]	818	117	19

[a] The results of two purification procedures were averaged. [b] Protein and activity values were calculated per 100 g of grapefruit pulp. [c] Replicates: 6163, 7533 units. [d] Replicates: 879, 1076 mg. [e] Replicates: 996, 1622 units. [f] Replicates: 1.03, 2.12 mg.

Table II. Extraction and Purification of Thermostable Pectinesterase[a]

purif step	act.,[b] units	protein,[b] mg	units/mg of protein	purif factor	yield, %
crude extract	3690[c]	1032[d]	3.6		
ammonium sulfate precipitation	2664	134	20		
heat treatment	217[e]	124[f]	1.7	1	100
CM-Sephadex	160	2	80	46	74
CM-Bio-Gel A	139	0.65	214	122	64
Amicon conc	134	0.38	353	202	62
G-75	74[g]	0.14[h]	529	302	34

[a] The results of two purification runs were averaged. [b] Protein and activity values were calculated per 100 g of grapefruit pulp. [c] Replicates: 3330, 4053 units. [d] Replicates: 927, 1136 mg. [e] Replicates: 191, 242 units. [f] Replicates: 115, 134 mg. [g] Replicates: 70, 79 units. [h] Replicates: 0.130, 0.146 mg.

Physical Characterization. SDS–PAGE of TL PE in the presence of 2-mercaptoethanol showed a single band estimated at 37 300 when stained with Coomassie blue/amido black (Figure 1). A M_r of 35 500 was estimated for native TL PE by HPLC gel filtration on Superose 6 (Figure 1). The agreement of these two methods indicates that the active enzyme is composed of a single polypeptide

Table III. Amino Acid Composition of Purified Grapefruit Pectinesterases

amino acid	mol %	TS PE, mol/mol[a]	mol %	TL PE mol/mol[a]
Asp	10.8	46	12.3	35
Thr	7.2	31	8.5	24
Ser	6.3	27	7.2	21
Glu	8.8	38	7.9	23
Pro	4.0	17	2.8	8
Gly	10.4	45	10.5	30
Ala	9.7	42	12.5	36
Val	8.1	35	6.6	19
Met	0.7	3	0.7	2
Ile	4.7	20	4.4	13
Leu	7.2	31	5.7	16
Tyr	5.2	22	3.9	11
Phe	5.7	24	4.4	13
His	1.4	6	1.3	4
Lys	5.9	25	3.1	9
Arg	3.9	17	5.9	17
Cys	1.6	7	1.7	5
Trp	ND		ND	
sum		436		286
mol wt		46 623		31 460
+carbohydrate		54 340		32 102

[a] The calculations were based on Met as the lowest common denominator. Ratios of the other amino acids to Met were multiplied by 2 (TL PE) or 3 (TS PE).

chain. SDS-PAGE of TS PE in the presence of 2-mercaptoethanol showed a single band estimated at 53 500 when stained with Coomassie blue/amido black (Figure 1); an estimate for the native M_r of 50 300 was obtained from HPLC gel filtration on Superose 6 (Figure 1), thus indicating that the TS PE also is composed of a single polypeptide chain.

Both enzymes appear to be strongly basic as both ran to the cathode on an IEF polyacrylamide gel of pH range 3–10 (data not shown). This result is not unexpected, since Versteeg (1979) found that all pectinesterases found in orange had isoelectric points greater than 10.

UV Spectra. The spectra of TL PE showed a maximum plateau between 274 and 281 nm, TS PE at 276–278 nm, with both spectra having minima at 250 nm. The ratio of absorbance at 280 to 250 nm was 1.55 for TL PE and 2.10 for TS PE. The extinction coefficient (1%, 1 cm) at 280 nm was 13.9 for TS PE and 15.1 for TL PE. These differences in spectra suggest that these enzymes possess unique structures.

Amino Acid Composition. The amino acid compositions of the pectinesterases are listed in Table III. TS PE had a notably higher concentration of hydrophobic amino acids (Phe, Tyr, Leu, Ile, Pro, and Val) than did TL PE; on a mole percent basis, TS PE contained approximately 35% hydrophobic residues to 28% for TL PE. The average hydrophobicities, calculated according to the method of Bigelow (1967), are 1152 cal/residue for TS PE and 934 cal/residue for TL PE.

The grapefruit PEs were similar in content of certain amino acids. Both enzymes showed a high content of potential sites of glycosylation: Asx (Asp + Asn), 33 residues (12.3%) for TL PE and 54 (10.8%) for TS PE; Ser, 19 residues (7.2%) for TL PE and 32 (6.3%) for TS PE; Thr, 23 residues (8.5%) for TL PE and 36 (7.2%) for TS PE. The content of potentially acidic residues (Asx and Glx) was high: 20.2% and 19.6% for TL and TS PE, respectively. Since the pI of both enzymes is greater than 10, these amino acids would most probably be amidated. The content of basic residues, Lys and Arg together, is surprising low in both enzymes given their basic properties. From the amino acid analyses, it was estimated that TL PE and TS PE contain 286 and 436 amino acids, respectively. The M_r of TS PE was estimated on the basis of amino acid composition and was similar to the SDS-PAGE and gel filtration estimates. The estimate for TL PE was off by greater than 10% from the other methods, however.

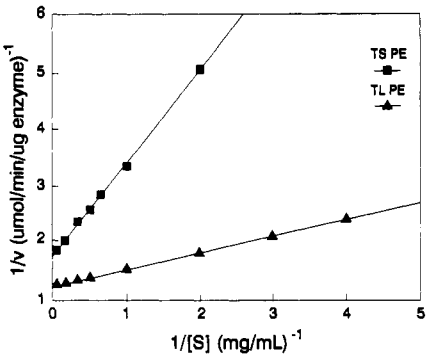

Figure 2. Lineweaver–Burk plot of the activity of TS and TL PE at various concentrations of Sunkist pectin. Activity was measured by titrimetric assay. After adjusting the pH of 1% Sunkist pectin (25 mL) to pH 7.0 or 7.8 for TL or TS PE, respectively, enzyme solution (2 units/assay) was added. One PE unit was defined as 1 μmol of protons released/min.

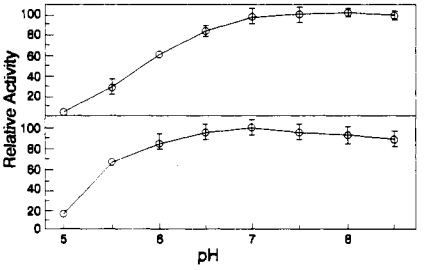

Figure 3. Effect of pH on reaction rate of TS and TL PEs on Sunkist pectin. Enzyme activity measured titrimetrically at all pHs as described for Figure 2. Three units of enzyme activity (measured at optimal pH) was used per assay. Relative activity is expressed in relation to the activity of PE at pH 7 or 7.5.

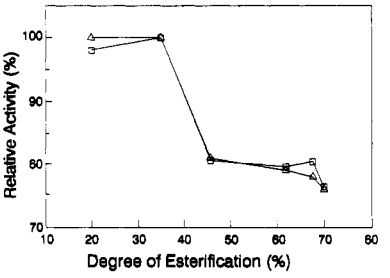

Figure 4. Effect of degree of esterification on the reaction rate of TL and TS PEs. Activity was measured titrimetrically on substrates with varied DEs (see Materials and Methods for list of substrates).

Effect of pH, DE, and Substrate Concentration on Initial Rates. Initial reaction rates were measured at various concentrations of Sunkist pectin (Figure 2). The K_m values of TL and TS PE were 0.274 ± 0.04 and 1.02 ± 0.083 mg/mL, respectively, and V_{max} values were 0.724 ± 0.056 and 0.59 ± 0.046 μmol/(min·μg), respectively. Turnover numbers on Sunkist pectin were 26 354 ± 2038 and 30 621 ± 2,387 mol/(mol·min) for TL PE and TS PE, respectively. The optimal pH values of pectinesterases on Sunkist pectin were around 7.0 for TL PE and between 7.5 and 8.0 for TS PE (Figure 3). The effect of DE on reaction rates is shown in Figure 4. The measured activity of both enzymes increased similarly with decreasing DE.

Carbohydrate Content. Both enzymes, after periodate oxidation, were able to bind dansylhydrazine, as

Table IV. Characteristics of Pectinesterases

source	mol wt	isoelectric point	sp act., units/mg	K_m, mg/mL	optimal pH	turnover no., mol/(mol·min)
grapefruit						
TL PE	37 300[d]	≥10	776	0.274	7.0	26 354
TS PE	53 500[d]	≥10	540	1.02	7.8	30 621
orange[a]						
PE I	36 200[d]	10	694	0.083	7.6	20 400
PE II	36 200[d]	≥11	762	0.0046	8.0	20 280
HMWPE	54 000[e]	10.2		0.041	8–8.5	
orange[b]						
PE I			222	0.25		
PE II			444	0.21		
orange[c]			2200	2.3	8.0	
tomato	23 700	7–9.3[g]	704	2.4[h]	8.0[h]	39 134[i]
	35 500[e,f]		1150[h-j]	0.74[i]	8.5[i]	

[a] Versteeg et al. (1978) Versteeg (1979). [b] Evans and McHale (1978). [c] Manabe (1973a). [d] Estimated by SDS-PAGE. [e] Estimated by gel filtration chromatography. [f] Pressey and Avants (1972). [g] Delincee (1976). [h] Nakagawa et al. (1970). [i] Lee and Macmillan (1968). [j] Markovic (1974).

visualized by fluorescence of bands with the expected R_f values on SDS-PAGE (data not shown). The bands of both a non-glycoprotein control (chymotrypsinogen) and unoxidized PEs did not fluoresce, indicating specific binding of dansylhydrazine to the carbohydrate moiety of the PEs had occurred.

To quantify the carbohydrate, the enzymes were assayed for total neutral carbohydrate. Total neutral carbohydrate content as estimated by the phenol–sulfuric acid assay using glucose as a standard gave the following results: TL PE, 2 ± 0.9%; TS PE, 14.2 ± 2.8%. If monosaccharides other than glucose are present, differences in color yields among the various sugars could introduce a source of error in this measurement.

Antigenic Properties. In the competitive ELISA, both TL PE and TS PE were used as competitors for anti-TL PE antibodies against plated antigen, TL PE. As can be seen from Figure 5, color intensity was reduced greatly (75–80% at both antibody dilutions) when 25 ng of TL PE was used as the competitor. When up to 100 ng of TL PE was added, little additional effect on intensity was seen. When TS PE was added as the competitor at 0–100 ng, the reduction in color intensity was nearly linear over the range. When 100 ng of TS PE was added, intensity dropped by 59 and 66% at antibody dilutions of 1/1000 and 1/2000, respectively. These results indicate that the pectinesterases are partially cross reactive and, therefore, have shared structural components.

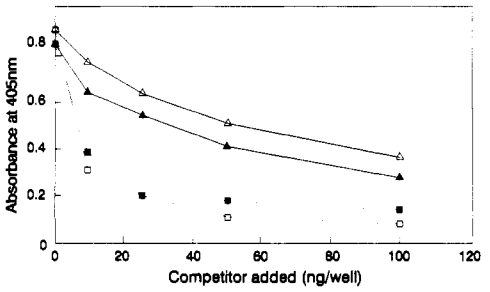

Figure 5. Analysis of antigenic properties of purified TS and TL PEs by competitive ELISA. TL PE (10 ng) was plated and reacted with anti-TL PE antibody in the presence of 0–100 ng/well of TL PE or TS PE as competitor. The antibody was diluted 1/1000 (□) or 1/2000 (■) for TL PE and 1/1000 (△) or 1/2000 (▲) for TS PE.

DISCUSSION

The presence of two forms of pectinesterase in grapefruit pulp was established by this work. The forms differed in molecular weight, amino acid composition, carbohydrate content, pH–activity profile, turnover number, K_m, and specific activity. These enzymes appear to have much in common with other PEs (Table IV). In particular, the M_r, isoelectric point, amino acid composition, and thermostability of TL PE are very similar to PE I purified from navel orange by Versteeg (1978), while TS PE shows similarities in M_r, isoelectric point, and thermostability to the HMW pectinesterase isolated by Versteeg (1979).

This work represents the first time that a high M_r, thermostable pectinesterase (TS PE) has been purified to homogeneity from citrus and also marks the first time that citrus pectinesterase enzymes have been shown to be glycosylated. Versteeg (1979) reported the presence of multiple forms of high M_r pectinesterase in orange. However, in this study, no evidence for the existence of multiple forms of high M_r, thermostable PE in grapefruit was found.

While the turnover numbers of the grapefruit PEs are similar to those of navel orange (Versteeg, 1979), the K_m values are much higher. Differences in DEs of substrates can produce marked effects in K_m values of PEs, however; the K_m of navel orange increased 50-fold as substrate DE was increased from 32 to 96% (Versteeg, 1979). Substrate DE was also shown to affect V_{max} of both grapefruit PEs (this study) and navel orange PE (Versteeg, 1979). Therefore, it is imperative to include the DE of the substrate when the kinetic parameters of PEs are discussed.

Versteeg (1979) showed that affinity for substrate was reduced greatly when the pH was decreased from 7.0 to 4.0. Since free carboxyl groups are necessary for enzyme–substrate complex formation (Solms and Deuel, 1955), the reduced affinity at low pH values can be explained by the decreased dissociation of the free carboxyl groups (the pK_a for pectin is about 4). On substrates with higher DEs, i.e., fewer carboxyl groups, the pH effect should be even more pronounced. The very low activity of the enzymes at pH 5.0 on Sunkist pectin is undoubtedly due to the high DE of the substrate (70%). The pH–activity relation at lower pH values shown here probably does not reflect the activities of these enzymes in citrus juice, since citrus juice would undoubtedly contain some pectin with lower DEs than that of the substrate used here.

Amino acid composition served to distinguish TS and TL PEs (Table III). In particular, TS PE has a 20% greater average hydrophobicity than TL PE and a 2-fold lower volume ratio of polar/nonpolar residues. TL PE is very similar to navel orange PEs described by Versteeg (1979) in amino acid composition, number of amino acids, and

average hydrophobicity, as well as thermostability properties. Average hydrophobicities calculated from the amino acid composition from orange PE I and PE II (Versteeg, 1979) and tomato (Markovic and Jornvall, 1986) were 1030, 1033, and 1022 cal/residue, respectively. TS PE is considerably more hydrophobic (1152 cal/residue) than orange and tomato PEs, while TL PE has a slightly lower hydrophobicity (934 cal/residue) than orange and tomato PEs. Orange PE I and PE II had inactivation temperatures of 60 and 70 °C, respectively; therefore, the reported correlation of increased protein hydrophobicity with greater thermostability (Mozhaev and Martinek, 1984) seems to hold up in the case of orange and grapefruit PEs.

Comparison of the electrophoretic patterns of the cyanogen bromide cleaved enzymes (data not shown) suggests differences in primary structure. However, it is also possible that the differences in mobility of the cleavage products are due solely to variation in carbohydrate content. Differences in amino acid content, UV spectra, and kinetic parameters, however, argue against the hypothesis that both enzymes are derived from a single gene product solely by differential glycosylation. A limited amount of immunochemical cross reactivity of the enzymes with anti-TL PE antibody was shown by competitive ELISA. Since immunochemical cross reactivity indicates the presence of homologous structures, it is a possibility that limited proteolytic processing could account for the observed differences in these enzymes.

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