Stability of Pectinesterases of Marsh White Grapefruit Pulp†

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Differences in thermal and storage stability, as well as susceptibility to proteolysis, were demonstrated in two forms of pectinesterase (PE) isolated from Marsh white grapefruit. It was shown previously that the two forms contain different amounts of attached carbohydrate. Partial removal of the carbohydrate moiety decreased thermostability of thermostable (TS) PE. The presence of an O-glycosidic linkage was demonstrated in TS PE. The O-linked oligosaccharides have an average degree of polymerization of 2.8 and were composed of four different monosaccharides.

INTRODUCTION

Pectinesterase (PE) is of significance to the citrus industry since it has been definitively established as the causative agent for juice clarification and gelation of frozen concentrates (Joslyn and Pilnik, 1961; Krop, 1974). Thermostable forms of PE have been demonstrated previously and are thought to be primarily responsible for clarification of underpasteurized citrus juice (Versteeg et al., 1980). The high temperature necessary to inactivate these forms has a deleterious effect on the flavor and aroma of the juice (Kew and Veldhuis, 1961).

Two forms of PE were purified from Marsh white grapefruit pulp (Seymour et al., 1991). It was shown that the two forms contain different amounts of attached carbohydrate. Several workers have reported that attached carbohydrate contributes to pH and thermal stability of glycoproteins (Chu et al., 1978; Hayashida and Yoshioka, 1980; Tashiro and Trevithick, 1977). This paper is concerned with stability and the molecular basis for the differences in stability of Marsh white grapefruit PE.

MATERIALS AND METHODS

Storage Stability. Thermostable (TS) and thermolabile (TL) PE were prepared according to the method of Seymour et al. (1991). The stability of PEs was tested under typical industry storage conditions. To either reconstituted single-strength grapefruit juice (10.8 °Brix) (Citrus World, Inc., Lake Wales), both at nonadjusted pH (3.3) and at pH adjusted to 2.0 with hydrochloric acid, or 20 parts of grapefruit juice concentrate (40 °Brix) was added 1 part of purified TL or TS PE. Sufficient enzyme was added to each juice sample to obtain an initial activity of 2.5 units/mL. Single-strength juice samples were stored at 4 °C, and 1-mL aliquots of concentrate samples were frozen and stored at −20 °C. At time intervals, samples were taken and the residual activity was measured by titrimetric assay as described by Seymour et al. (1991).

The stability of TS PE to freeze–thaw treatments was also determined. An aliquot of 0.5 mL of TS PE, initially about 80 units/mL, in 10 mM sodium phosphate, pH 7.0, was quickly frozen in a dry ice–ethanol bath and then thawed in a 40 °C waterbath. Aliquots were assayed titrimetrically for total activity and thermostability (activity after 70 °C for 5 min) after every fifth freeze–thaw cycle. This process was repeated up to 40 freeze–thaw cycles.

Thermostability Studies. Residual activities of PEs after incubation for 5 min at various temperatures were determined. To tubes of identical dimensions (13-mm diameter) containing 1 mL of single-strength grapefruit juice (10.8 °Brix), pH 3.3, was added 0.05 mL of purified enzyme containing approximately 1 PE unit. After mixing, a thermocouple probe was placed in the tube and it was placed in a waterbath adjusted to a temperature at least 10 °C higher than the incubation temperature to bring the temperature up rapidly. The tubes were then incubated for 5 min in a waterbath at the desired various temperatures. Residual activity was measured immediately after the tubes were cooled on ice.

The rate of heat inactivation of TS PE in single-strength grapefruit juice (10.8 °Brix), pH 3.3, was determined at 75, 80, and 85 °C. Pectinesterase, 5 units in 0.05 mL, was added to test tubes containing 1 mL of reconstituted grapefruit juice of pH 3.3 and heat treated as described above for various times at the different temperatures. The log of percent residual activity was plotted against heating time.

Stability of Pectinesterases to Proteolysis. Pectinesterases (10 μg) were incubated with 40 units of trypsin (Sigma) or pepsin (Sigma) at the respective pH optima (as provided by Sigma) of the proteases. Incubation with pepsin at room temperature was carried out in 100 mM acetic acid, pH 3, for up to 48 h. Incubation with trypsin was carried out at room temperature in 100 mM sodium phosphate buffer, pH 7, for 48 h. Aliquots were assayed for activity at time intervals along with control samples (incubated without protease under identical conditions).

Carbohydrate Assays. Hydrolysates of purified TS PE or of O-linked oligosaccharides of TS PE were assayed for reducing sugar content according to the method of Waffenschmidt and Jaenicke (1987). A working reagent of equal volumes of reagent A and reagent B was made fresh daily. Reagent A was a 5 mM solution of disodium 2,2′-bicinchoninate (Sigma) dissolved in 0.80 M sodium carbonate buffer, pH 10.1. Reagent B was 5 mM cupric sulfate pentahydrate (Fisher Scientific) and 12 mM serine (Sigma, S-4500). To approximately 1 μg of carbohydrate in 100 μL of distilled water in a screw-cap vial was added 0.5 mL of working reagent. The vials were tightly capped and kept for 15 min at 100 °C in an aluminum heating block. After the vials were cooled to room temperature for 20 min, the absorbance at 560 nm was read in a Beckman DU 40 spectrophotometer. Glucose (1–10 mmol) was used as a standard.

Preparation of Alditol Acetates. Monosaccharides in both a total hydrolysate of purified TS PE and a hydrolysate of O-linked oligosaccharide were converted to their respective alditol acetates and analyzed by gas chromatography/mass spectroscopy. Purified TS PE (100–200 μg) or O-linked oligosaccharide (5 μg) was hydrolyzed for 4 h in 2 N HCl at 100 °C in

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a stoppered vial and then cooled to room temperature and neutralized with equimolar NaOH. The total hydrolysate of TS PE was then deionized by passage through an Amberlite MB3 column (1.4 × 5 cm). Fractions of 0.5 mL were collected and pooled on the basis of reducing sugar content. Sugars were acetylated according to the method of Blakeney et al. (1983). Sodium borohydride was dissolved in anhydrous dimethyl sulfoxide (20 mg/mL). Sugars were reduced at 40 °C for 90 min by adding 1 mL of sodium borohydride solution to 0.1 mL of sample in 1 M ammonia. After reduction, excess sodium borohydride was degraded by the addition of glacial acetic acid (0.1 mL). To the solution was added 1-methylimidazole (0.2 mL) (Fluka) followed by acetic anhydride (2 mL) with mixing. After 10 min at room temperature, 5 mL of water was added to degrade excess acetic anhydride. After cooling, 1 mL of dichloromethane was added and the solution was vortexed. After the phases had separated, the lower phase was removed with a Pasteur pipet and dried in a stream of N_2. After the residue was dissolved in 50 μL of dichloromethane, 5–10 μL was subjected to analysis by gas chromatography/mass spectroscopy.

Gas Chromatography/Mass Spectroscopy. Gas chromatography was performed on a Finnegan 4500 GC/MS instrument. Derivatized sugars were separated on a DB5 GC column (30 m × 0.25 mm). A temperature program of 100–250 °C at 5 °C/min was chosen initially to ensure elution of all alditol acetates, including amino sugar derivatives. The temperature program was later modified to 150–220 °C at 2 °C/min to optimize separation of the types of alditol acetates known to be present on the basis of the first analysis. Peaks were fragmented by electron impact at an electron energy of 70 eV. Chromatograms were constructed by monitoring the intensity of the fragment ion of m/z 115, a fragment ion which is diagnostic for alditol acetates. Alditol acetates were tentatively identified by comparison to the mass spectra of the following derivatized sugars: D-glucose (Sigma), D-galactose (Fisher Scientific), D-mannose (Sigma), L-arabinose (Sigma), D-xylose (Sigma), D-ribose (Sigma), D-fucose (Sigma), and L-rhamnose (Sigma).

Effect of Alkali on Serine and Threonine Residues. To test for the presence of O-glycosidic bonds, 50 μg of TS PE in 0.25 mL of distilled water was mixed with 0.25 mL of 0.2 N NaOH at room temperature. The absorbance at 241 nm against a blank of 0.1 N NaOH was measured as a function of time by a Beckman DU8 spectrophotometer to determine the production of α-aminoacrylic acid and α-aminocrotonic acid by the β-elimination reaction (Neuberger et al., 1966). The number of O-glycosidic linkages per molecule of TS PE was estimated from the molar extinction coefficient at 241 nm [6050, given by Greenstein and Winitz (1961)] of the olefinic amino acids produced by the β-elimination reaction.

Isolation and Analysis of O-Linked Oligosaccharides. TS PE was subjected to alkaline borohydride reduction according to the method of Tanaka et al. (1964). Approximately 50 μg of protein or 7 μg of carbohydrate (on the basis of an estimate of 14%) in 0.25 mL of distilled water was exhaustively dialyzed against 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM sodium azide. The dialysate was added to 0.5 mL of a solution containing 0.45 M sodium borohydride and 0.15 N NaOH. After 48 h at 4 °C in the dark, the solution was acidified to pH 5.3 with 1 N acetic acid to degrade excess borohydride. The solution was placed in a centricon ultrafilter (10-kDa exclusion) and centrifuged until 90% of the solution had filtered through the membrane. The oligosaccharide (in filtrate) was then hydrolyzed with 2 N HCl for 4 h at 100 °C in a screw-cap vial and assayed for reducing equivalents. The remaining portion of the acid-hydrolyzed oligosaccharide, about 0.5 μg, was acetylated and analyzed by GC/MS as described under Gas Chromatography/Mass Spectroscopy.

Deglycosylation of TS PE. Samples of pure TS PE [125 μg as carbohydrate according to the method of Dubois et al. (1956)] were subjected to periodate oxidation in 0.02 M sodium periodate in 0.05 M sodium acetate buffer, pH 5.0, for 2 h in the dark according to the method of Pazur et al. (1963). After dialysis against deionized water at 4 °C overnight, aliquots were assayed for protein, carbohydrate, and enzyme activities. In a separate experiment, further degradation of the periodate-oxidized enzyme was carried out as follows: the pH was adjusted to 9.5 with sodium borohydride after periodate oxidation and then dialyzed again against water and adjusted to pH 1.0 with HCl (Unrau and Smith, 1957).

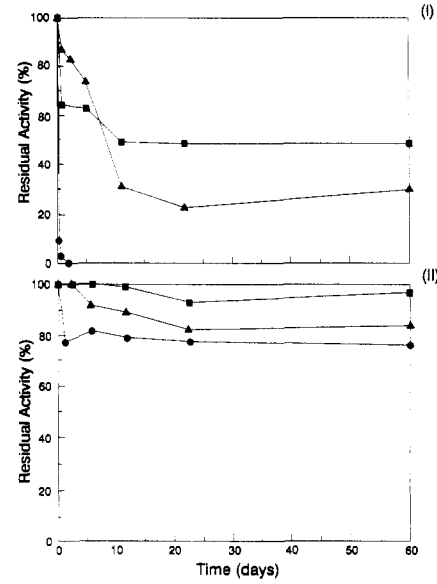

Figure 1. Storage stability of TL PE (I) and TSPE (II) in grapefruit juice. Activity was measured titrimetrically after storage for various times in single-strength grapefruit juice at 4 °C at pH 2 (●) or pH 3.3 (▲) and in frozen concentrate at −20 °C (■) for both PE forms.

TS PE was also deglycosylated according to the method of Sojar and Bahl (1987). This method consisted of incubating freeze-dried samples with 150 μL of trifluoromethanesulfonic acid/mg of protein, at 0 °C for 1 h followed by neutralization of the acid with aqueous pyridine at −20 °C. The neutralized reaction mixture was dialyzed at 4 °C against 10 mM sodium phosphate, 0.1 g/L azide, pH 7.0, and assayed for activity.

Enzymatic deglycosylation of TS PE was also attempted by using *N*-glycosidase (Genzyme Corp., Boston, MA) and α-mannosidase (Sigma, M7257). Enzyme concentrations and conditions used were as recommended by the manufacturer.

Effect of Deglycosylation on Stability of TS PE. Thermostability of the periodate-oxidized enzyme was tested by incubation at 70 °C for various periods of time in 10 mM phosphate buffer, pH 7.0. The reaction was stopped by cooling on ice and assayed for PE activity. To evaluate storage stability, periodate-oxidized enzyme was spiked into single-strength grapefruit juice, pH 3.3, and stored at 4 °C for 1 week. Enzyme activity was tested at various time intervals.

RESULTS AND DISCUSSION

Storage Stability. Enzyme activity of TL and TS PE was monitored under various storage conditions for up to 60 or 120 days (Figure 1). Storage conditions were chosen for this study to simulate those normally encountered after processing. Storage stability in single-strength grapefruit juice adjusted to pH 2 was included, because low pH inactivation of PE has been suggested as an alternative to pasteurization in citrus juice (Owusu-Yaw et al., 1988). TS PE was more stable than TL PE under all conditions tested. Particularly striking is the difference in stability in single-strength juice at pH 2; TL PE was immediately inactivated, while TS PE lost only 5% of activity after 60 days. Thermostable PE was also found to be extremely stable to repeated freezing and thawing. After 40 freeze–thaw cycles, no loss of activity or thermostability was observed in TS PE (data not shown).

Thermal Stability. Thermostability of both enzymes was tested by heating for 5 min at various temperatures from 40 to 85 °C at pH 7.0 in 10 mM phosphate buffer and measuring residual activity. A large difference in ther-

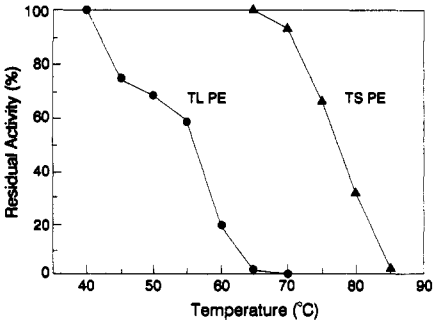

Figure 2. Heat stability of pectinesterases in grapefruit juice. Pectinesterases were heated at various temperatures in single-strength grapefruit juice of pH 3.3 for 5 min. Residual activity was measured titrimetrically.

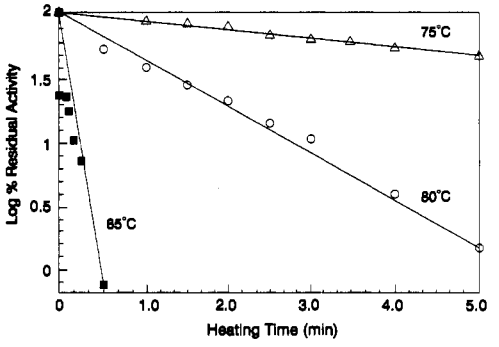

Figure 3. Thermal inactivation rates of TS PE in grapefruit juice at 75, 80, and 85 °C. The activity of TS PE was measured after heating in single-strength grapefruit juice of pH 3.3 for various times.

mostability was observed between TL and TS PE (Figure 2); TL PE and TS PE were inactivated completely at 65 and 85 °C, respectively. Thermal inactivation rates of TS PE at 75, 80, and 85 °C in single-strength grapefruit juice, pH 3.3, were 13.66, 2.77, and 0.3 min, respectively (Figure 3). From these data, the change in temperature required to increase the inactivation rate 10-fold (z value) was calculated to be 6 °C. These data agree with other heat inactivation studies of grapefruit PE (Eagerman and Rouse, 1976). TS PE appears to be less thermostable than the counterpart in navel orange (Versteeg et al., 1980); however, this could be due to the pH of grapefruit juice (pH 3.3) being lower than that of orange juice (pH 4.0).

Inactivation of Pectinesterases by Proteases. The susceptibility of both enzymes to inactivation by trypsin and pepsin was investigated. These two proteases have different bond specificity; pepsin cleaves at the carboxyl side of a wide variety of hydrophobic and acidic amino acids, while trypsin cleaves at the carboxyl side of Lys and Arg. Both PEs were fairly resistant to the action of trypsin; however, pepsin almost completely inactivated TL PE within 5 h but had little effect on TS PE (Figure 4). This result suggests that, unlike TL PE, the cleavage sites recognized by pepsin in TS PE are either absent or inaccessible. The inability of trypsin to significantly inactivate the enzymes suggests that the Lys and Arg residues are inaccessible at pH 7.0.

Carbohydrate Content. Isolation of an O-linked oligosaccharide, followed by hydrolysis and assay of reducing equivalents, gave a value of 20 ± 1.4% carbohydrate content for TS PE. An average M_r of the reducing equivalents of 167, based on the average M_w of the component sugars, was used in the calculations. The fact that the total neutral sugar content is approximately equal to the amount of reducing sugar removed by alkaline borohydride reduction suggests that all the neutral sugars are

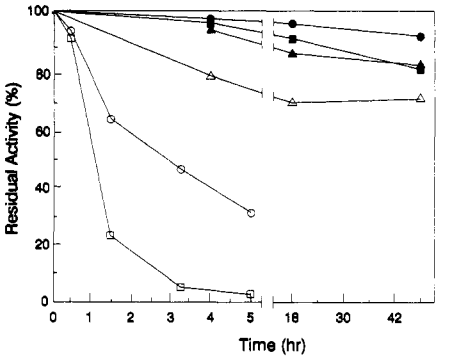

Figure 4. Effect of proteases on activity of pectinesterases. TL PE was incubated with 40 units of pepsin at pH 3.0 (□) or trypsin at pH 7.0 (△). TS PE was incubated with 40 units of pepsin at pH 3.0 (■) or trypsin at pH 7.0 (▲). Control samples of TL PE (○) and TS PE (●) were incubated at pH 3.0 to correct for the low pH inactivation.

alkali labile and, therefore, O-linked. This rules out the possibility of large N-linked structures as they are stable under the mild alkaline conditions used.

Composition and Partial Structural Analysis of the Carbohydrate Moiety of TS PE. The carbohydrate of TS PE was removed either by acid hydrolysis to release all sugars as monosaccharides or by reductive β-elimination to release O-linked oligosaccharides. Gas chromatography and electron impact spectra identified an alditol acetate of a hexose, two pentoses, and a deoxy sugar. Accurate retention times were not obtained due to problems with the data collection system, but the identities of the sugar acetates were tentatively made by comparison of the mass spectra to those of standard sugar acetates, as galactose, arabinose, xylose, and fucose at peak height ratios of 5:3:1:1, respectively. This result further substantiates the presence of O-linked carbohydrate attached to TS PE. Additional support for the O-linkage comes from the fact that aminohexoses, which are present in N-glycosidic linkages, were not detected in TS PE.

Effect of Alkali on Serine and Threonine Residues. The cleavage of the O-glycosidic linkage between protein and carbohydrate moieties by a β-elimination reaction increases the absorbance at 241 nm as time proceeds due to formation of α-aminoacrylic acid and α-aminocrotonic acid from serine and threonine, respectively (Neuberger et al., 1966). The increase in absorbance of TS PE, when placed in 0.1 N NaOH, indicated that alkali-labile O-glycosidic bonds were involved in the linkage of protein to oligosaccharide. N-Linked carbohydrates are stable to mild alkali treatment. From the molar extinction coefficient of α-aminoacrylic acid at 240 nm (6050; Greenstein and Winitz, 1961), it was estimated that there are 23 O-glycosidic bonds per molecule of TS PE. On the basis of a carbohydrate content of 14.2% determined from the phenol–sulfuric acid assay, the average degree of polymerization was calculated as 2. On the basis of a carbohydrate content of 20%, determined as reducing equivalents in the hydrolyzed O-linked oligosaccharide, the average degree of polymerization was calculated to be 2.8.

Deglycosylation of Thermostable Pectinesterase. To evaluate the contribution of the carbohydrate moiety to the exceptional thermal stability of TS PE, deglycosylation of the enzyme was carried out by various treatments. The use of periodate oxidation for this purpose was first suggested by Kleppe (Pazur et al., 1963) and was later used by others (Pazur et al., 1970; Tashiro and Trevithick, 1977). Treatment of TS PE with periodate appeared to oxidize a portion of the carbohydrate residues while partially maintaining PE activity. During period-

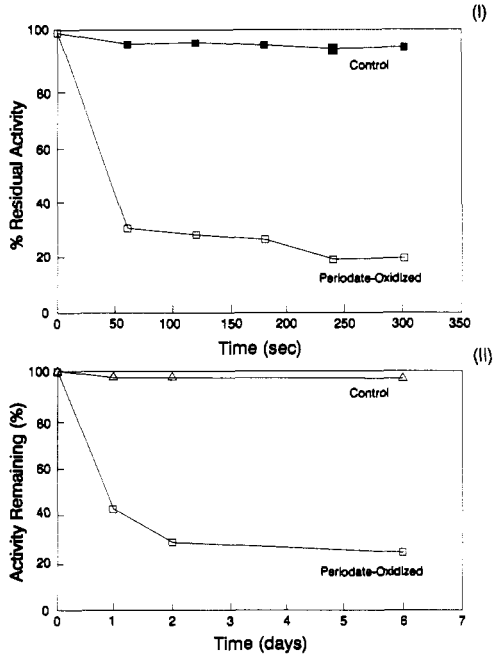

Figure 5. Heat stability of native and periodate-treated TS PE. TS PE was treated with periodate for 2 h as described under Materials and Methods. Enzyme activity was determined after various times at 70 °C in single-strength grapefruit juice (pH 3.3) (I) and at 4 °C in 10 mM sodium phosphate, pH 7.0 (II).

ate oxidation of TS PE for 2 h, no change in amount of protein occurred; approximately 40% of the carbohydrate residues were oxidized as determined by the phenol–sulfuric acid assay. During this period, PE activity dropped to about 53% of the original activity. The periodate-oxidized enzyme was significantly less thermostable than native enzyme, when tested at 70 °C in single-strength grapefruit juice of pH 3.3 (Figure 5, part I). At 4 °C and pH 7.0, periodate-oxidized enzyme lost 75% activity after 6 days (Figure 5, part II). It is possible that partial oxidation of Cys, Met, and Trp may be contributing to the decrease in stability of periodate-oxidized TS PE. This has been shown to occur but at higher concentrations (0.08 N) of periodate (Lee and Montgomery, 1961) than those used in this experiment (0.02 N).

Further degradation of periodate-oxidized TS PE was carried out by reduction with sodium borohydride and mild acid hydrolysis (Unrau and Smith, 1957). This treatment, which should remove nearly all of the attached carbohydrate, resulted in total loss of activity and precipitation. Trifluoromethanesulfonic acid treatment of TS PE, a process that removes all carbohydrate from glycoproteins except N-linked hexoseamines, also resulted in total loss of activity and precipitation of the enzyme. These results appear to suggest a certain amount of carbohydrate may be necessary to maintain solubility and/or stability of TS PE. However, one can also not rule out that these treatments could result in protein denaturation, thereby causing a loss of TS PE activity. The use of specific glycosidases could be a useful technique in the evaluation of the contribution of carbohydrates to protein stability.

Enzymatic treatment with *N*-glycosidase and α-mannosidase had no effect on activity, stability, or migration on SDS gels of TS PE. These results suggest that TS PE does not contain a significant amount of either N-glycosidic or α-mannosyl linkages.

SUMMARY

TS PE was shown to be more thermostable, to be more resistant to acid pH and proteolysis, to have a longer storage life in grapefruit juice, and to be more stable to freeze–thawing than TL PE. TS PE was found to contain 7-fold more carbohydrate than TL PE. Thermostability analysis of periodate-oxidized TS PE suggests that the carbohydrate moiety may aid the stability of TS PE. The increased carbohydrate content could also account for the greater resistance to proteolysis shown by TS PE. It has been shown that carbohydrate moieties provide protection against proteolysis (Coffey and de Duve, 1968; Tashiro and Trevithick, 1977). These results support previous research that TS PE is the technologically significant form of PE in regard to citrus processing (Versteeg, 1979; Versteeg et al., 1980).

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