pH Affects Marsh Grapefruit Pectinesterase Stability and Conformation

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Thermolabile pectinesterase (TL-PE) and thermostable pectinesterase (TS-PE) of Marsh grapefruit were purified by ion-exchange and affinity chromatography. The effect of pH on stability and solvent accessible hydrophobicity of both isozymes was studied. TL-PE was inactivated irreversibly by 5 min of incubation at pH 2 and pH 12, whereas TS-PE was not inactivated. Extrinsic 8-anilinonaphthalene-1-sulfonate (ANS) fluorescence intensity was an order of magnitude lower for both PE isozymes than for bovine serum albumin (BSA) at neutral pH. At pH values <3.5, ANS fluorescence of both PE isozymes increased and, at pH 2.0, was higher than ANS−BSA fluorescence. Fluorescence increase at low pH was greater in ANS−TL-PE than in ANS−TS-PE. Activity loss and increase in fluorescence after pH 2 treatment was irreversible for TL-PE, but nearly fully reversible for TS-PE. Resistance to conformational change at low pH by TS-PE probably contributes to its enhanced stability.

Keywords: *Thermostable pectinesterase; thermolabile pectinesterase; fluorescence; hydrophobic; 8-anilononaphthalene-1-sulfonate (ANS)*

INTRODUCTION

Pectinesterase (PE; EC 3.1.1.11) deesterifies pectin, which leads to cloud loss in citrus juice. Heat-sensitive PEs are inactivated at temperatures of <70 °C, whereas thermostable PEs (TS-PEs) require heat treatment at 90 °C for nearly 1 min for inactivation (Versteeg et al., 1980). Depending on the citrus variety, TS-PE represents 5−30% of the total PE activity (Rombouts et al., 1982). Adequate microbial pasteurization is achieved in citrus juices at temperatures near 70 °C, but pasteurization times and temperatures are based on inactivation of PE to preserve cloud stability. The high temperature necessary to inactivate TS-PEs has a deleterious effect on the aroma and flavor of the juice (Kew and Veldhuis, 1961).

Previous research on citrus PE has emphasized development of methods to purify the enzyme and to characterize the mechanism of action, kinetics, and stability parameters. Thermostable isozymes of PE (TS-PEs) have been purified and characterized from several cultivars of citrus, including Navel oranges, Marsh grapefruit, and red grapefruit (Versteeg et al., 1980; Seymour et al., 1991b; Cameron and Grohman, 1995). The TS-PEs are more active and more stable to many physical and chemical processing treatments, including low temperature, repeated freeze/thaw cycles, and low pH (Versteeg et al., 1980; Seymour et al., 1991a). Various reports have demonstrated that thermal inactivation rates of PE in citrus juices increased at lower pH values (Rouse and Atkins, 1952; Rouse and Atkins, 1953; Atkins and Rouse, 1954; Kew et al., 1957). To inactivate TS-PE, grapefruit juice must be heated for 0.8 s at 90.5, 93.5, or 96 °C at pH values of 3.0, 3.5, or 3.8, respectively (Rouse and Atkins, 1952). It is possible that the thermal inactivation rate is dependent on the pH effect on PE conformation and/or release from a pectin complex. Development of a less severe thermal process to inactivate TS-PE depends on understanding factors that contribute to the native structure and catalytic activity of PE. Electrostatic complex formation between PE and pectate influences PE activity (Nari et al., 1991; Charnay et al., 1992; Snir et al., 1995). Release from pectin complex adversely affected thermolabile-PE (TL-PE) activity but had no effect on TS-PE activity (Leiting and Wicker, 1996). Because TS-PE has a higher mol% hydrophobic amino acid than TL-PE (Seymour et al., 1991b), we suspected that the hydrophobic nature of TS-PE contributed to greater activity and stability of TS-PE.

The fluorescent probe 8-anilinonaphthalene-1-sulfonate (ANS) can be used to determine the surface hydrophobicity of enzyme/protein. ANS is nonfluorescent in water and other polar environments, but highly fluorescent in nonpolar environments or when bound to hydrophobic domains on proteins (Freifelder, 1982). Changes in protein structure that result in an increase in hydrophobicity can be estimated by the change in fluorescence of ANS. If pH causes a change in the hydrophobic domains of protein, the conformational change may be detected by ANS fluorescence. The relationship of protein structure to activity and stability under various pH conditions can be inferred. The objective of this study was to investigate the pH effects on the conformation and stability of purified TL-PE and TS-PE and to trace conformational changes involving hydrophobic groups. Fluorescence spectroscopy with an external probe (ANS) was used to quantify pH-induced structural changes of TS-PE and TL-PE.

MATERIALS AND METHODS

Materials. PE was extracted from Marsh grapefruit pulp (donated by Citrus World, Lake Wales, FL). Enzyme purification was performed on a fast-performance liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). CM Bio-Gel and Affi-Gel Heparin Gel were purchased from BioRad (Hercules, CA). Pectin was donated by Citrus Colloids (Hereford, U.K.). The hemimagnesium salt of 8-anilino-1-naphthalene-sulfonic acid and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO).

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Enzyme Purification. Frozen pulp was cut by hand and homogenized in a Sorvall Omni Mixer at high speed with four volumes of either 1 M NaCl with no pH adjustment (TS-PE) or 1 M NaCl in 0.25 M Tris-Cl, pH 8.0 (TL-PE); (Wicker, 1992). The homogenate was stirred for 1 h, and the supernatant was collected by centrifugation for 20 min at 7000*g*. All ensuing treatments were carried out at 4 °C unless otherwise mentioned.

The supernatant liquid was brought to 35% saturation with solid ammonium sulfate and stirred for 30 min. This suspension was centrifuged at 7000*g* for 20 min. The pellet was discarded, and the supernatant was brought to 80% ammonium sulfate saturation and stirred for 30 min. Following centrifugation at 7000*g* for 30 min, the supernatant liquid, containing no PE activity, was discarded, and the pellet was solubilized in a minimal volume of deionized water. The solution was dialyzed against three changes of 10 volumes of 10 mM sodium phosphate buffer, pH 7.0. Dialysis tubing was boiled in 10% acetic acid for 10 min and rinsed in water before use.

Enzyme solution (200 mL) was loaded at a flow rate of 1 mL/min onto CM Bio-Gel A (200 mL, preswollen), which was packed in a 2.5 × 40 cm column that was pre-equilibrated in 10 mM sodium phosphate, pH 7.0. The resin was washed at 1 mL/min (total 200 mL) with application buffer. Enzyme activity was eluted at 1 mL/min with 250 mL of a linear NaCl gradient from 0 to 0.6 M in 10 mM sodium phosphate, pH 7.0. Fractions of 3 mL were collected. All fractions were qualitatively tested for PE activity by a modified titrimetric assay. The substrate and conditions are described in the section under pectinesterase assay. Sequential addition of fractions and evaluation of NaOH consumption was used to identify fractions containing PE activity. PE active fractions were then determined quantitatively by titration.

Pooled fractions were dialyzed against 10 mM sodium phosphate, pH 7.4, and then applied at 0.8 mL/min onto Affi-Gel heparin Gel, which was packed in a 2.5 × 10-cm column that was preequilibrated in 10 mM sodium phosphate, pH 7.4. The resin was washed with 50 mL of application buffer at 0.8 mL/min. The bound protein was eluted with a 200-mL linear salt gradient from 0−1.5 M NaCl in the application buffer, and fractions of 2.5 mL were collected.

Buffer Preparation. To minimize ionic strength effects in pH studies, buffer with constant ionic strength (10 mM) was prepared as described by Ellis and Morrison (1982). Buffer for pH studies between pH 2 and 7 were prepared with glycine (3.3 mM), acetic acid (3.3 mM), 2-(*N*-morpholino)ethane sulfonic acid (MES; 3.3 mM), and tris(hydroxymethyl)aminomethane (Tris; 10 mM). Buffer for pH studies between pH 7 and 12 were prepared with Tris (4.4 mM), glycine (3.3 mM), and ethylamine (4.4 mM).

Effect of pH on PE Stability. The pH stability of PE was assessed by incubation of PE at different pH values for the approximate time of an assay followed by activity assay at a single pH (Whitaker, 1972). Aliquots of 0.05 mL of purified PE were added to 0.95 mL of buffer at the appropriate pH, the mixture was incubated at room temperature for 5 min, and the residual PE activity was immediately tested at pH 7.5. The final concentrations of TL-PE and TS-PE were 20 unit/mL.

Fluorescence Spectroscopy. Fluorescence spectra were recorded with a Fluorlog-2 spectrofluorometer (SPEX Instruments, Edison, NJ). The excitation and emission slits were set at 0.25 mm. The binding of ANS was measured by following the fluorescence enhancement of the ligand, ANS, bound to the protein. The final concentration of ANS was 5×10^{-5} M. The excitation wavelength for ANS fluorescence was 380 nm, and the emission spectra were recorded between 400 and 600 nm. An aliquot of 0.05 mL of PE or BSA was added into 0.95 mL of buffer at the indicated pH in a 1-mL cuvette and was incubated for 5 min at room temperature before fluorescence testing. The final concentrations for TL-PE and TS-PE were 16.4 and 20.3 μg/mL, respectively. When the pH was adjusted back to pH 7, 1 N NaOH was used. The final volume changes were <0.015 mL. The pH was measured with a PER-146 Combination Micro pH electrode (Lazar, Los Angeles, CA). The fluorescence of ANS bound to BSA was evaluated as a control. The concentration of BSA was 22 μg/mL. Representative plots of at least triplicate spectra are presented.

Table 1. Purification of Thermolabile Pectinesterase (TL-PE) from Marsh Grapefruit Finisher Pulp[a]

purifn step	PE (units)	sp act. (U/mg of protein)	purifn factor	% yield
crude extract	26 939	22	1	100
ammonium sulfate	12 600	84	3.8	46.77
CM Bio-Gel	8 491	123	14.6	31.52
Affi-Gel heparin	6 886	1 223	55.6	25.56

[a] The crude extract was prepared from finisher pulp using 1 M NaCl in 0.25 M Tris-Cl, pH 8.0. [b] PE activity is defined as microequivalents of ester hydrolyzed per minute under defined conditions.

Table 2. Purification of Thermostable Pectinesterase (TS-PE) from Marsh Grapefruit Finisher Pulp[a]

purifn step	PE (units)	sp act. (U/mg of protein)	purifn factor	% yield
crude extract	12 205	20	1	100
ammonium sulfate	2 430	105	6.2	20.3
CM Bio-Gel	2 140	210	10.5	17.5
Affi-Gel heparin	1 476	987	49.3	12.1

[a] The crude extract was prepared from finisher pulp using 1 M NaCl with no pH adjustment. [b] PE activity is defined as microequivalents of ester hydrolyzed per minute under defined conditions.

PE Assay. The titrimetric method (Kertesz, 1950) was used to determine PE activity in 1% high methoxy pectin, 0.1 M NaCl, pH 7.5, at 30 °C. The amount of standardized NaOH required to neutralize the release of carboxylic groups was recorded with a Brinkman 614 Impulsomat automatic titrator (Switzerland). PE units were defined as microequivalents of ester hydrolyzed per minute.

Protein Assay. The protein concentration was determined according to the method of Bradford (1976), with a calibration curve with IgG as the standard protein.

RESULTS

Purification. Purification of TL-PE and TS-PE are reported in Tables 1 and 2, respectively. The final specific activities of TL-PE and TS-PE were 1223 and 987 U/mg of protein, respectively. For both isozymes, the Affi-Gel heparin was most effective at increasing the specific activity with little loss of yield. The specific activity and yield of TL-PE from Marsh grapefruit pulp in this study was improved compared with that obtained by the method described by Seymour et al. (1991b), who obtained 818 U/mg of protein. The selective extraction of TS-PE at endogenous pH values (Wicker, 1992) and Affi-Gel proved to be an effective method to obtain large quantities of TS-PE of high purity from grapefruit. After Affi-Gel heparin and concanalin A (ConA) chromatography, the specific activities of TS-PE from red grapefruit were 148.8 and 157.9 U/mg of protein, respectively (Cameron and Grohman, 1995). Seymour et al. (1991a) obtained SDS-PAGE pure preparations of TS-PE from 3 kg of Marsh grapefruit pulp, which had a specific activity of 529 U/mg of protein. In this study, TS-PE with a specific activity of 980 U/mg protein was obtained from 1 kg of pulp.

pH Stability. The data in Figure 1 show the effect of pH on the stability of TL-PE and TS-PE. Both isozymes showed stability over a wide pH range. At pH 2 and 12, the extreme pH in our experiment, TL-PE lost all activity, whereas TS-PE maintained almost the same activity as before pH treatment, with slight inactivation at pH 12. Thus, TL-PE was less stable at alkaline and acidic pH conditions than TS-PE.

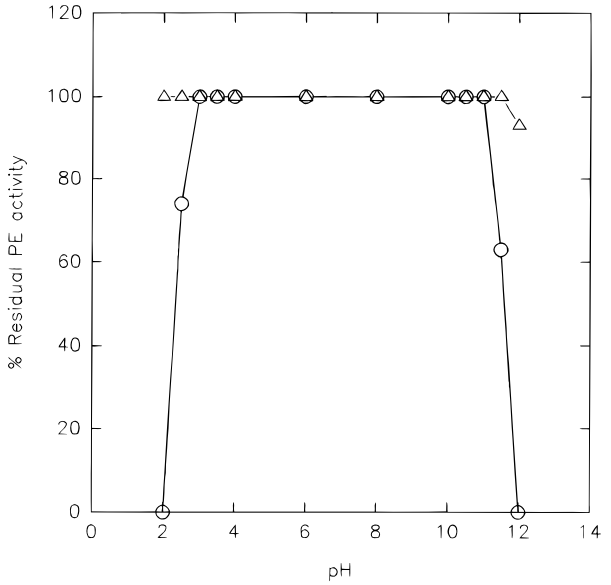

Figure 1. Effect of pH on enzyme stability of TL-PE (○) and TS-PE (△). Aliquots of TS-PE or TS-PE were incubated at the indicated pH at room temperature for 5 min prior to assay at pH 7.5 and 30 °C.

Extrinsic Fluorescence. The fluorescence spectra of TL-PE and TS-PE in glycine-acetic-MES-Tris buffer are shown in Figures 2a and 2b, respectively. For both PE isozymes, the ANS fluorescence intensity was very low at pH 7. As pH was decreased between pH 7 and pH 4, there was no fluorescence change in either TL-PE or TS-PE (data not shown). When the pH was adjusted to 3.5, an increase in fluorescence of ANS−TL-PE was observed (Figure 2a). At pH 3.5, ANS−TS-PE fluorescence showed a slight increase in intensity (Figure 2b). At pH 2.0, the average increase in fluorescence of ANS−TL-PE was ∼47-fold higher than at neutral pH (Figure 2a). The average increase in fluorescence of ANS−TS-PE was 28-fold higher at pH 2 than at pH 7. The fluorescence of ANS−TL-PE was about twice that of ANS−TS-PE at pH 2. Alkaline pH (as high as pH 12) had no effect on ANS fluorescence of either TL-PE or TS-PE. Tryptophan fluorescence (excitation at 280 nm and emission at 300−400 nm) of TL-PE or TS-PE was not affected by alkaline or acidic pH (data not shown). Controls of fluorescence in the absence of PE or in the absence of ANS were conducted. The fluorescence intensity was low (∼600−700 counts/s) and did not change significantly over the emission spectra range. The spectra depicted in the figures have been corrected for background counts per second (cps).

ANS fluorescence spectra of PE isozymes were compared with BSA at neutral and acidic pH (Table 3). BSA is a hydrophobic protein that gives a large fluorescence signal at 475 nm with ANS (Freifelder, 1982). The fluorescence of ANS−BSA was higher than that of ANS−TL-PE or ANS−TS-PE at neutral pH. As the pH was decreased, the fluorescence of ANS−PE increased and surpassed that of ANS−BSA at pH 2.

After pH 2 treatment, the TL-PE and TS-PE solutions were titrated back to pH 7 and ANS fluorescence was tested. The original fluorescence intensity was not obtained following pH adjustment back to pH 7 (Table 3). Rather, the fluorescence of ANS−TL-PE was almost 30 times the original fluorescence at pH 7. There was complete loss of TL-PE activity after titration back to pH 7.0 after incubation at pH 2.0. For TS-PE, there was also a fluorescence increase compared with original (pH 7) after the same treatment as for TL-PE, but it was much lower than that of TL-PE. There was full recovery of TS-PE activity after titration back to pH 7.

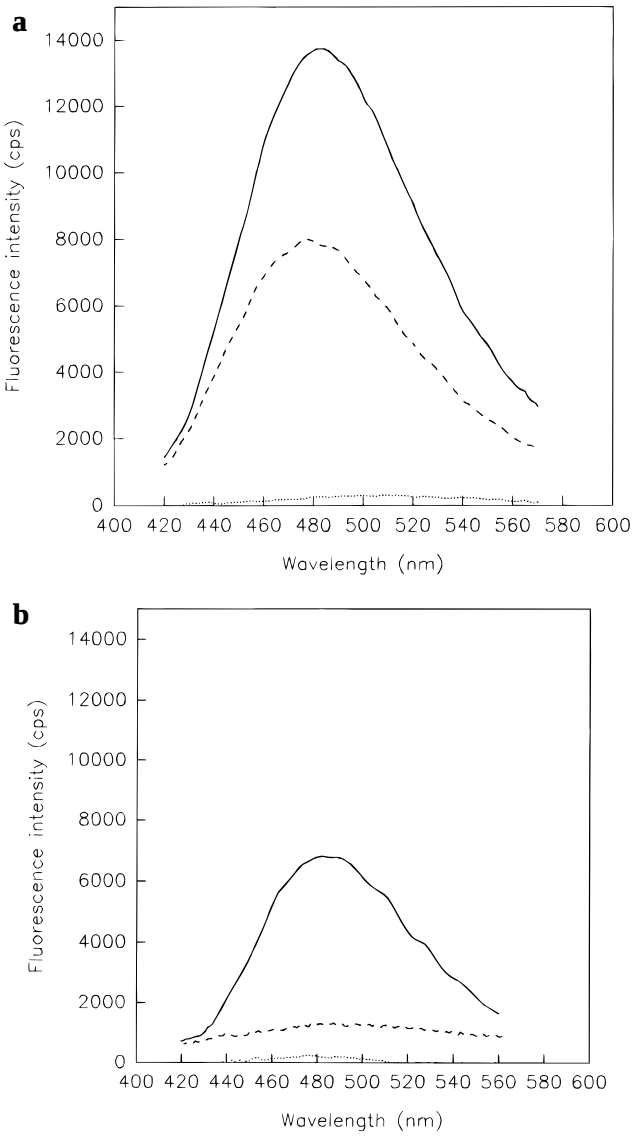

Figure 2. Fluorescence emission spectra of (a) ANS−TL-PE and (b) ANS−TS-PE at pH 7.0 (...), pH 3.5 (---), and pH 2.0 (—) at enzyme concentrations of (a) 16.4 mg/mL and (b) 20.3 mg/mL. The excitation wavelength was 380 nm, and spectra are representative of three trials.

Table 3. Extrinsic ANS Fluorescence of Thermolabile (TL) and Thermostable (TS) Pectinesterase at Different pH Values[a]

pH	ANS−TL-PE	ANS−TS-PE	ANS−BSA
7.0	271 ± 66	243 ± 57	2781 ± 216
3.5	8742 ± 753	1196 ± 48	6926 ± 301
2.0	12732 ± 1487	6886 ± 946	6975 ± 158
7.0[b]	7945 ± 1055	1535 ± 168	

[a] The fluorescence at the maximum wavelength of 475 nm is reported as the mean of triplicate assays; the concentrations of TL-PE, TS-PE, and BSA were 16.4, 20.3, and 22 μg/mL, respectively; the excitation and emission wavelengths were 380 and 475 nm, respectively. [b] The fluorescence ANS−PE was measured after incubation at pH 2.0, titration back to pH 7.0, and incubation at pH 7.0 for 5 min.

DISCUSSION

Electrostatic interactions between PE and substrate are important in cell wall extension and growth (Moustacas et al., 1991), activity (McDonnell et al., 1945;

Charnay et al., 1992), permeation through ultrafiltration membranes (Snir et al., 1995), and stability (Leiting and Wicker, 1996). Competitive displacement of PE from an inactive complex by metal and organic cations has been well documented (Moustacas et al., 1991; Charnay et al., 1992). The concentration and type of cation affected the magnitude of PE activation and peak shape (Leiting and Wicker, 1996). If only electrostatic interactions were involved, then cations of similar size and charge should show similar activation effects. Furthermore, solubilization of PE from a pectin complex by cation displacement had no effect on TS-PE but adversely affected TL-PE (Leiting and Wicker, 1996). The specific effect of cations on the magnitude of PE activation and differential solubilization of PE isozymes suggested that nonelectrostatic interactions contribute to PE interaction with pectin, activity, and stability.

Based on ANS fluorescence, both TL-PE and TS-PE molecules are more hydrophillic at neutral and alkaline pH than at acidic pH values. The ANS fluorescence at different pH suggests that most hydrophobic amino acid groups in TL-PE and TS-PE are folded away from solvent accessibility at neutral or alkaline pH. The change in hydrophobicity of TL-PE was irreversible and resulted in irrecoverable loss of activity after incubation at low pH. Less change and more complete recovery in the hydrophobic nature of TS-PE was observed. Furthermore, the stability of TS-PE was not affected by low pH incubation. Seymour et al. (1991a) observed less residual activity during a 60 day incubation of TS-PE in grapefruit juice at pH 2.0 than at pH 3.3, but greater residual activity in TS-PE than TL-PE at pH 3.3. The greater fluorescence increase and exposure of hydrophobic groups to solvent of TL-PE compared with TS-PE at low pH may contribute to the greater thermal sensitivity of TL-PE at low pH. Support for this suggestion is found in the results of Seymour et al. (1991a) who showed that at pH 3.0, pepsin inactivated most of the TL-PE within 5 h, but had little effect on TS-PE. Pepsin cleaves hydrophobic and acidic amino acid linkages. The authors speculated that at pH 3.0, TL-PE has more hydrophobic amino acids exposed than TS-PE, thus TL-PE is more susceptible to pepsin degradation at this pH.

ANS is an excellent tool to estimate hydrophobicity and often has been used to estimate conformational changes in proteins and enzymes (Stryer, 1965). At the acidic pH values where a change in hydrophobicity was observed, PE has a net positive charge and is 6 pH units from the lowest estimated, isoelectric point (p*I*) for citrus PE (Rombouts et al., 1982). Based on apparent pK_a ranges for pectin, pectin has a net negative or zero charge and ANS is negatively charged over the pH range of this study. Thus, it is not likely that anionic interactions of ANS with PE or pectin were responsible for changes in fluorescence intensity. The results are consistent with those of previous studies in which ANS was used as a probe of hydrophobic sites and intermediate conformational states in proteins and enzymes (Ptitsyn, 1995).

ANS has been used to estimate the folding patterns and intermediate conformational states of proteins and enzymes (Goto and Fink, 1989; Semisotnov et al., 1991; Couthon et al., 1995). ANS has highest affinity to the "molten globule" conformation of several enzymes, in which the secondary structure was largely unaffected by perturbants but the tertiary structure was disrupted. It was speculated that ANS affinity was low when either hydrophobic groups were folded away from solvent accessibility in native protein or when the hydrophobic groups were fully exposed in an unfolded protein (Semisotnov et al., 1991). Although the amino acid sequence of tomato PE is known, no one has yet reported the secondary or tertiary structure of PE. To obtain a clearer picture of the structure of TL-PE and TS-PE at different pH values, we are continuing with binding studies with ANS and analysis by Scatchard plots to estimate the number of binding sites and magnitude of binding. In conjunction with other methods to measure secondary structure, an estimate of intermediate conformational states of TL-PE and TS-PE at low pH can be made.

SUMMARY

Clearly, pH affected the stability and accessibility of hydrophobic domains to solvent for TL-PE. TS-PE stability and conformation was less likely to be changed by low pH treatment and the conformational change was nearly reversible. The resistance to irreversible conformational change by TS-PE may explain why TL-PE is more sensitive to low pH thermal inactivation than TS-PE.

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