Secondary Structure and Stability of Marsh Grapefruit Thermolabile Pectinesterase

D. Sun,[†] T. Grant,[‡] L. Dure,[‡] and L. Wicker*,[§]

Department of Biochemistry and Department of Food Science and Technology, University of Georgia, Athens, Georgia 30602

Thermolabile pectinesterase (TL-PE) was inactivated completely after 8 h of dialysis at pH 3.3 but retained activity at pH 4.1 and pH 7.0. Circular dichroism (CD) spectra and secondary structural analysis showed similar secondary structure between pH 7.0, pH 4.1, and pH 3.3. The CD spectra of TL-PE were compared at pH 7.0 and 4.1 and between 25 and 70 °C. Heating TL-PE at pH 7.0 and 4.1 resulted in irreversible protein precipitation. The thermal transition temperature for destabilization of TL-PE structure occurs at a lower temperature at pH 4.1 than at pH 7.0, suggesting some change in the secondary structure of TL-PE.

Keywords: *Pectinesterase; stability; activity; thermal transition; denaturation*

INTRODUCTION

Pectinesterase (PE; EC 3.1.1.11) has a major impact on citrus processing because of its effect on the quality of the finished products (Pilnik and Rombouts, 1978). Multiple forms of citrus PE have been identified (Versteeg et al., 1980; Rombouts et al., 1982; Wicker et al., 1988; Seymour et al., 1991b; Cameron et al., 1994; Cameron and Grohmann, 1995, 1996). Most recently, Macdonald et al. (1993, 1994) postulated that some PE isozymes, which are separated by ion exchange or isoelectric focusing, may actually be pectin−pectinesterase complexes. Chen et al. (1998) reported that pectin−pectinesterase complexes prevented separation of PE from pectin on preparative ion exchange disks. Attempts to inactivate PE have usually focused on heat treatment and/or a combination of heat and pH (Bisset et al., 1953; Eagerman and Rouse, 1976; Versteeg et al., 1980; Owusu-Yaw et al., 1988; Arreola et al., 1991). Thermostable PE (TS-PE) is more stable than thermolabile PE (TL-PE) at low pH, and pH stability coincides with heat stability in PE isozymes (Seymour et al., 1991a; Sun and Wicker, 1996). In juices, less severe heating was required to inactivate PE at lower pH values in pH-adjusted juices extracted from Duncan and Marsh grapefruit and from Valencia, Pineapple, Parson Brown, and Hamlin oranges (Rouse and Atkins, 1952, 1953; Atkins and Rouse, 1953, 1954).

PE genes have been cloned and sequenced from *Erwinia* (Plastow et al., 1988), *Pseudomonas* (Spok et al., 1991), *Arabidopsis* (Richard et al., 1994), tomato (Gaffe et al., 1997), and Valencia orange (Nairn et al., 1998). There are several conserved regions in the deduced PE amino acid sequence, and two domains exist in plant PE (Nairn et al., 1998; Markovic and Jornvall, 1986). The N terminus shows only about ∼12−19% homology, and the C terminus shows ∼30−69% homology (Gaffe et al., 1997). A variable 200−275 amino acid N-terminal domain is absent in fungal and prokaryotic PE (Nairn et al., 1998). Other information on the higher structure of plant PE is limited to conjecture of quaternary structure based on estimation of molecular weight (MW). Marsh grapefruit TS-PE and TL-PE have approximate MWs of 53000 and 30000 Da, respectively, and are thought to be single polypeptides (Seymour et al., 1991b). The MW of isolated mung bean PE is close to 75000 Da by equilibrium sedimentation, but the MW is near 32000 Da by SDS−PAGE electrophoresis, suggesting dimeric structure (Goldberg et al., 1992). Fayyaz et al. (1994) showed that papaya PE is composed of two subunits with estimated MWs by SDS−PAGE of 15010 and 16976 Da, respectively.

Circular dichroism (CD) is a technique for the rapid determination of the secondary structure content of proteins (Chen et al., 1972; Provencher, 1982; Compton and Johnson, 1986). This method is very sensitive to changes in structure and is used to study effects of environmental changes or ligand binding on the secondary structure. Quantitative information about the distributions of different elements of secondary structure can be obtained by fitting the far-UV CD spectrum to those of proteins and peptides with known secondary structure or to spectra of synthetic peptides containing only one kind of secondary structure.

Heat- or pH-induced loss of activity is likely due to a change in the native structure of TL-PE. CD can be used to follow secondary structural changes during heat and pH inactivation of TL-PE. Information on the structure−function and structure−stability relationship of TL-PE would aid in the understanding of denaturation. The objectives of this research were to evaluate the secondary structure of TL-PE at pH values near the pH optimum for activity (pH 7.0) and at pH values near citrus juice pH (pH 4.1 and 3.3) and to determine the change in secondary structure with heating.

* Author to whom correspondence should be addressed [telephone (706) 542-1055; fax (706) 542-1050; e-mail lwicker@arches.uga.edu].

[†] Present address: Department of Citrus, Citrus Research and Education Center, Lake Alfred, FL.

[‡] Department of Biochemistry.

[§] Department of Food Science and Technology.

S0021-8561(98)00209-X CCC: $15.00
Published on Web 08/19/1998

MATERIALS AND METHODS

Enzyme Purification and Chemicals. TL-PE was purified from Marsh grapefruit pulp as described from previous work (Sun and Wicker, 1996). The enzyme activity after CM BioGel and AffiGel chromatography was 196 U/mL. The concentration of PE was determined with the BioRad dye binding protein assay kit using IgG as standard (Bradford, 1976). All other chemicals were of analytical grade.

PE Assay. The titrimetric method (Kertesz, 1950) was used to determine PE activity in 1% high-methoxyl pectin and 0.1 M NaCl, pH 7.5, at 30 °C. The pectin had a degree of esterification of 72% and was donated by Citrus Colloids (Type 104, Hereford, U.K.). The amount of standardized NaOH required to neutralize the release of carboxylic groups was recorded with a Brinkman 614 Impulsomat automatic titrator (Westbury, NY). PE units were defined as micromoles of ester hydrolyzed per minute.

Sample Preparation. Sample buffer was changed to 10 mM sodium phosphate, pH 7.0, 10 mM sodium citrate, pH 4.1, or 10 mM sodium citrate, pH 3.3, by using Slide-A-Lyzer 10 K dialysis cassettes (Pierce, Rockford, IL). Thermal denaturation studies were performed on a solution of TL-PE (0.1 mg/mL) in 10 mM sodium phosphate buffer, pH 7.0, or in 10 mM sodium citrate buffer, pH 4.1.

CD. CD spectra were performed on a Jasco J-710 spectropolarimeter interfaced to a 386 computer and controlled by Jasco software. The temperature of a thermostated cell holder was controlled with a water bath to within 0.1 °C. The sample solution was equilibrated at 25 °C for 30 min, and then the temperature was incrementally raised to 70 °C. At each temperature, the solution was allowed to equilibrate for 30 min, and the CD spectrum was recorded five times. The reported results are the smoothed average over the five measurements. The voltage to the photomutiplier tube was kept below 600 V to prevent distortion of the CD spectrum. The quartz cuvette was washed sequentially with acetone, ethanol, and water and dried between each sample. Each spectrum was collected at intervals of 0.2 nm and a dwell time of 5 s. A solvent spectrum was subtracted from each protein spectrum at each temperature. The data are reported as mean residue ellipticity [θ].

Secondary Structure Estimation. Secondary structure content was estimated by using the convex constraint algorithm (CCA) program and K2d (Kohone neural network with a two-dimensional output layer) program. The CCA program is a general deconvolution method for a CD spectra set for any variety of conformational types (Perczel et al., 1992). The algorithm, based on a set of three constraints, is able to deconvolute a set of CD curves to its common "pure" component curves and conformational weights (Perczel et al., 1992). To analyze a single CD spectrum with this method, the spectrum is appended to the data set used as a reference data set (Perczel et al., 1992). The K2d program is based on a self-organizing neural network to extract from a set of CD spectra the secondary structure features present in the data (Andrade et al., 1993). This information is kept in a matrix result of 100 different trainings of the neural network (Andrade et al., 1993). K2d uses this precalculated data to estimate the secondary structure in the test sample.

The CCA program estimates four structures: α-helix, β-sheet, β-turn, and random coil. The K2d program estimates three structures: α-helix, β-sheet, and random coil. In this work, we reported the estimations by both programs.

RESULTS AND DISCUSSION

pH Effect on PE Secondary Structure. After 8 h of dialysis at 4 °C against pH 4.1 and 7.0 buffer, TL-PE retained 83 and 89%, activity, respectively, whereas all activity was lost after dialysis against pH 3.3 buffer. The far-UV CD spectra of TL-PE, recorded at pH values of 3.3, 4.1, 7.0, are shown in Figure 1. Differences in the CD spectra of TL-PE at pH 7.0 and 4.1 were not distinguishable. The CD spectrum of TL-PE at pH 3.3 is slightly different from spectra at pH 7.0 or 4.1, suggesting some structural differences might exist. The predicted distribution of secondary structures at these pH values is listed in Table 1. For the CCA result, the sigma (σ) (error) values are between 0.1 and 1. Results by both programs suggest that α-helix and β-sheet components are present substantially at all pH values, but the confidence level is not high enough to assign absolute values for percentage of secondary structure. Changes in predicted secondary structure of TL-PE with pH could not be estimated because the CD spectra (Figure 1) and secondary structural component results (Table 1) were similar. Loss of activity at pH 3.3 could not be associated with changes in the secondary structure component results (Table 1).

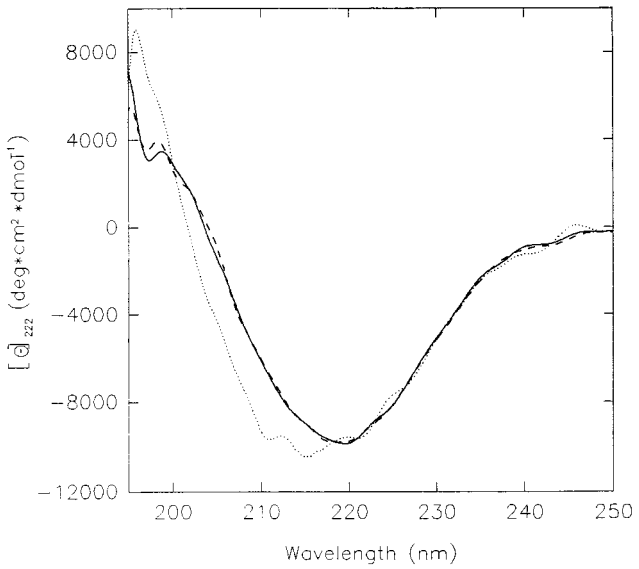

Figure 1. CD spectra of TL-PE at 25 °C measured at pH 7.0 (—), pH 4.1 (- - -), and pH 3.3 (• • •). The protein concentration was 0.1 mg/mL. The spectra are representative of two trials.

Table 1. Predicted Secondary Structure Components of TL-PE at Different pH Values at 25 °C[a]

	α-helix		β-sheet		β-turn[b]		coil		CCA
	CCA	K2d	CCA	K2d	CCA	K2d	CCA	K2d	σ value
pH 3.3	21	32	19	16	13		47	52	0.484
	27	31	18	19	17		38	49	0.330
pH 4.1	27	31	21	16	20		32	53	1.020
	32	32	11	18	17		40	51	0.321
pH 7.0	23	32	33	17	14		30	51	0.696
	22	30	26	18	0		52	51	0.379

[a] Predicted distribution of secondary structure is given for each replication. [b] K2d program does not give information on β-turn.

Thermal Denaturation. Figure 2 shows the CD signal at 222 nm recorded at different temperatures for TL-PE at pH 7.0 and 4.1. The negative CD signal at 222 nm primarily corresponds to the backbone peptide bond nπ* transition, and changes reflect alterations in the secondary structure, especially the α-helix content of the protein. Because a visible precipitate was observed after heating, the decrease of $[\theta]_{222}$ negativity may be due to loss of secondary structure, loss of protein from solution, or a combination. Protein destabilization may be due to loss of secondary structure and/or precipitation, either of which results in a decrease in $[\theta]_{222}$ negativity.

At pH 7.0, $-[\theta]_{222}$ does not change from 25 to 55 °C (Figure 2). However, $-[\theta]_{222}$ decreases at 59 °C. At 65

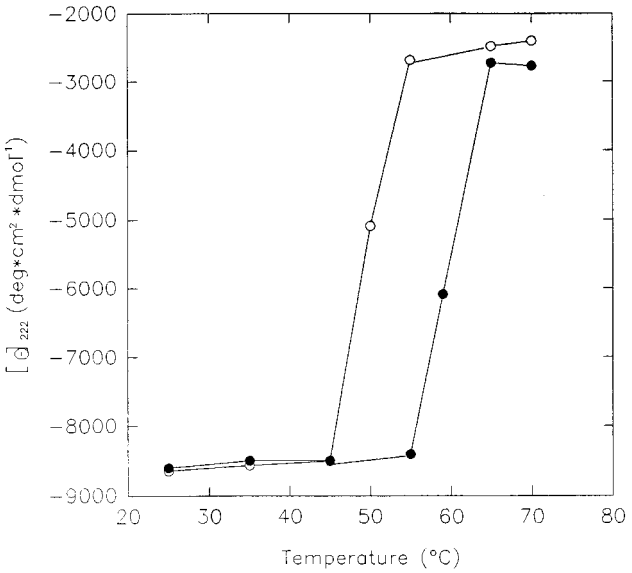

Figure 2. Plot of mean residue ellipticity at 222 nm versus temperature recorded at pH 7.0 (●) and pH 4.1 (○). The protein concentration was 0.1 mg/mL. The values are representative of two trials.

°C, there is no signal change with wavelength. Most likely, precipitation of TL-PE by heat is irreversible because, after cooling to 25 °C, there is no recovery of CD spectra. At pH 4.1, the changes in the spectra were qualitatively similar to spectra at pH 7.0, but the temperatures were different. There is no decrease of $-[\theta]_{222}$ from 25 to 45 °C (Figure 2). A decrease in $-[\theta]_{222}$ occurs in the temperature range of 45−55 °C, with no further signal decrease with increasing temperatures to 65 °C. It is evident that heat destabilization of TL-PE structure occurs at lower temperatures at pH 4.1 than at pH 7.0. There is no CD spectrum recovery after the heated solution cools to 25 °C, suggesting the structural change and/or precipitation is also irreversible at pH 4.1. Although there is no discernible secondary structural difference in TL-PE between pH 7.0 and 4.1 as shown by CD analysis (Figure 1; Table 1), the thermal transition temperature for TL-PE is higher at pH 7.0 than at pH 4.1 (Figure 2). The lower thermal stability at pH 4.1 than at pH 7.0 may be due to differences in side change ionization of amino acid residues.

Surface hydrophobicity is also related to protein stability and conformation (Kato and Nakai, 1980). Sun and Wicker (1996) have shown that TL-PE has higher surface hydrophobicity at pH values of 3.5 and 2.0 than at neutral pH of pH 7.0. At all pH values tested, TL-PE shows higher surface hydrophobicity than TS-PE (Sun and Wicker, 1996). They postulated that low pH causes unfolding of TL-PE, increasing hydrophobic surface area, decreasing internal hydrophobic residues, and decreasing heat stability. In a pH stability study, Sun and Wicker (1997) reported no significant effect on stability when TL-PE was incubated at pH values from 7.0 to 3.0 for 5 min, the time of an assay, and assayed at pH 7.5. However, an increase in surface hydrophobicity was observed at pH 3.5. The loss of activity at pH 3.3 without heating in this study compared to the earlier study (Sun and Wicker, 1997) is likely due to the extended time at low pH. In this study, no discernible effect on secondary structure was observed at pH 7.0 or 4.1, but TL-PE was clearly less heat stable at pH 4.1 than at pH 7.0.

Summary. The CD spectra of TL-PE indicate the presence of α-helix and β-sheet at all pH values. There are no discernible differences in the CD signal of TL-PE at pH 7.0 or 4.1 and only a slight difference at pH 3.3. Heating TL-PE at pH 7.0 or 4.1 to 70 °C results in irreversible protein precipitation. The $-[\theta]_{222}$ signal is lost at a lower thermal transition temperature at pH 4.1 than at pH 7.0. The loss of signal at $-[\theta]_{222}$ is due to either loss of structure or precipitation of protein.

LITERATURE CITED

Andrade, M. A.; Chacon, P.; Merelo, J. J.; Moran, F. Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network. *Protein Eng.* **1993**, *6*, 383−390.

Arreola, A. G.; Balaban, M. O.; Marshall, M. R.; Peplow, A. J.; Wei, C. I.; Cornell, J. A. Supercritical carbon dioxide effects on some quality attributes of single strength orange juice. *J. Food Sci.* **1991**, *56*, 1030−1033.

Atkins, C. D.; Rouse, A. H. Time−temperature relationships for heat inactivation of pectinesterase in citrus juices. *Food Technol.* **1953**, *7*, 489−491.

Atkins, C. D.; Rouse, A. H. Time−temperature concentration relationships for heat inactivation of pectinesterase in citrus juices. *Food Technol.* **1954**, *8*, 498−500.

Bisset, O. W.; Veldhuis, M. K.; Rushing, N. B. Effect of heat treatment temperatures on the storage life of Valencia orange concentrates. *Food Technol.* **1953**, *7*, 258−260.

Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

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

Cameron, R. G.; Grohmann, K. Purification and characterization of a thermally tolerant pectinmethylesterase from a commercial Valencia Fresh Frozen Orange Juice. *J. Agric. Food Chem.* **1996**, *44*, 458−462.

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, R.; Sims, K. A.; Wicker, L. Ion exchange membrane separation of pectinesterase. *J. Agric. Food Chem.* **1998**, *46*, 1777−1782.

Chen, Y.; Yang, J. T.; Martinez, H. M. Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry* **1972**, *11*, 4120−4131.

Compton, L. A.; Johnson, Jr., W. C. Analysis of protein circular dichroism spectra for secondary structure using a simple matrix multiplication. *Anal. Biochem.* **1986**, *155*, 155−167.

Eagerman, R. A.; Rouse, A. H. Heat inactivation temperature−time relationships for pectinesterase inactivation in citrus juices. *J. Food Sci.* **1976**, *41*, 1396−1397.

Fayyaz, A.; Asbi, B. A.; Ghazali, H. M.; Man, Y. B. C.; Jinap, S. Purification and molecular-properties of papaya pectinesterase. *Food Chem.* **1994**, *49*, 373−378.

Gaffe, J.; Tiznado, M. E.; Handa, A. K. Characterization and functional expression of a ubiquitously expressed tomato pectin methylesterase. *Plant Physiol.* **1997**, *114*, 1547−1556.

Goldberg, R.; Pierron, M.; Durand, L.; Mutaftschiev, S. *In Vitro* and *In Situ* properties of cell wall pectinmethylesterases from mung bean hypocotyls. *J. Exp. Bot.* **1992**, *246*, 41−46.

Kato, A.; Nakai, S. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochim. Biophys. Acta* **1980**, *624*, 13−20.

Kertesz, Z. I. *The Pectic Substances*; Interscience-Wiley: New York, 1950.

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 pectinesterases in lemon fruits (*Citrus limon*). *J. Sci. Food Agric.* **1994**, *64*, 129−134.

Markovic, O.: Jornvall, H. Pectinesterase—the primary structure of the tomato enzyme. *Eur. J. Biochem.* **1986**, *158*, 455−462.

Nairn, J. C.; Lewandowski, D. J.; Burns, J. Two groups of pectinesterase genes in Valencia orange. *Plant Physiol.* **1998**, in press.

Owusu-Yaw, J.; Marshall, M. R.; Koburger, J. A.; Wei, C.-I. Low pH inactivation of pectinesterase in single strength orange juice. *J. Food Sci.* **1988**, *53*, 504−507.

Perczel, A.; Park, K.; Fasman, G. D. Analysis of the circular dichroism spectrum of proteins using the convex constraint algorithm: a practical guide. *Anal. Biochem.* **1992**, *203*, 83−93.

Pilnik, W.; Rombouts, F. M. Pectic enzymes. In *Polysaccharides in Food*; Blanchard, J. M. V., Mitchell, J. R., Ed.; Butterworth: London, 1978; pp 109−126.

Plastow, G. S. Molecular cloning and molecular sequence of the pectin methyl esterase gene of *Erwinia chrysanthemi* B374. *Mol. Microbiol.* **1988**, *2*, 247−254.

Provencher, S. W. CONTIN—a general-purpose constrained regularization program for converting noisy linear algebraic and integral-equations. *Comput. Phys.* **1982**, *27*, 229−241.

Richard, L.; Qin, L. X.; Gadal, P.; Goldberg, R. Molecular cloning and characterization of a putative pectinmethylesterase cDNA in *Arabidopsis thaliana* L. *FEBS Lett.* **1994**, *352*, 135−139.

Rombouts, F. M.; Versteeg, C.; Karman, A. H.; Pilnik, W. Pectinesterases in component parts of citrus fruits related to problems of cloud loss and gelation in citrus products. In *Use of Enzymes in Food Technology*; Dupuy, P., Ed.; Technique et Documentation: Paris, 1982; pp 483−487.

Rouse, A. H.; Atkins, C. D. Heat inactivation of pectinesterase in citrus juices. *Food Technol.* **1952**, *6*, 291−294.

Rouse, A. H.; Atkins, C. D. Further results from a study on heat inactivation of pectinesterase in citrus juices. *Food Technol.* **1953**, *6*, 221−224.

Seymour, T. A.; Preston, J. F.; Wicker, L.; Lindsay, J. A.; Wei, C.-I.; Marshall, M. R. Stability of pectinesterase of Marsh white grapefruit pulp. *J. Agric. Food Chem.* **1991a**, *39*, 1075−1079.

Seymour, T. A.; Preston, J. F.; Wicker, L.; Lindsay, J. A.; Marshall, M. R. Purification properties of pectinesterases of Marsh white grapefruit pulp. *J. Agric. Food Chem.* **1991b**, *39*, 1080−1085.

Spok, A.; Stubenrauch, G.; Schörgendorfer, K.; Schwab, H. Molecular cloning and sequencing of a pectinesterase gene from *Pseudomonas solanacearum*. *J. Gen. Microbiol.* **1991**, *137*, 131−140.

Sun, D.; Wicker, L. pH affects Marsh grapefruit pectinesterase stability and conformation. *J. Agric. Food Chem.* **1996**, *44*, 3741−3745.

Versteeg, C.; Rombouts, F. M.; Spansen, C. H.; Pilnik, W. Thermostability and orange juice cloud destabilizing properties of multiple pectinesterases from orange. *J. Food Sci.* **1980**, *45*, 969−971, 998.

Wicker, L.; Vassallo, M.; Echeverria, E. Solubilization of cell wall bound, thermostable pectinesterase from Valencia orange. *J. Food Sci.* **1988**, *53*, 1171−1174, 1180.

Received for review March 2, 1998. Revised manuscript received July 6, 1998. Accepted July 7, 1998. This research was partially supported by BARD Project US-2222-92R.

JF980209K