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Enhanced pectin degradation is associated with the ease of fruit detachment in tabasco pepper

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ENHANCED PECTIN DEGRADATION IS ASSOCIATED WITH THE EASE OF FRUIT DETACHMENT IN TABASCO PEPPER

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ABSTRACT

Pectin metabolism was analyzed in tabasco pepper (*Capsicum frutescens* L.) to determine the metabolic process associated with the ease of fruit detachment from the calyx. Two genotypes that differ in the fruit detachment force (FDF) were used: 'Easy Pick' (EZ)which requires a low force and 'Hard Pick' (HP) which requires higher force. Pectin dissolution in fresh ripe fruit tissue and in extracted fruit cell wall was higher in the EZ genotype than the HP genotype and inversely correlated to the FDF. Size-exclusion chromatography of EDTA-soluble polyuronides indicated that pectin was degraded in ripe tissue from both genotypes, but the degree of depolymerization was more extensive in the EZ genotype. The ease of fruit detachment was, therefore, attributed to pectin ultra-degradation. Polygalacturonase activity, however, was the same in protein extracts from both genotypes. In contrast, pectin methyl-esterase (PME) activity *in vivo* assessed by methanol production was detected in ripe fruit of the EZ genotype only and it was associated with the FDF decline. The decrease in degree of pectin esterification and pH at the fruit junction area detected in EZ ripe fruit was attributed to PME activity *in vivo*. PME activity *in vitro*, however, was detected in protein extracts and disrupted tissue from both genotypes at all ripening stages. This suggests that a PME regulatory mechanism may be blocking PME activity *in vivo*. Two PME isoforms were detected in protein extracts from ripe fruit. The PME-1 form was detected in EZ genotype only and appears to be responsible for methanol production *in vivo.* A predominant 36.7 k protein was associated with localized pH reduction in a pectin-agarose gel. The PME-2 form was detected in both genotypes and appears to be active in disrupted tissue only. A 40.8 k protein was resolved consistently in PME-2 active

fractions. In conclusion, PME-1 appears to be responsible for PME activity *in vivo* and was associated with the ease of fruit detachment in tabasco pepper.

CHAPTER 1. INTRODUCTION

In most cultivated peppers (*Capsicum* spp.) the fruit adheres tightly to the calyx when ripe and the pedicel remains attached to the fruit when harvested (Motsenbocker, 1996). This is a concern in processing for pepper sauce because woody pedicels and green calyxes introduced into the mash impart off-color and lower the quality. Consequently, to comply with the low tolerance for green-woody tissue, more effort and time is required to remove the stem from the fruit affecting the harvest and post-harvest cost. Development of cultivars that facilitate mechanical harvest therefore, will impact modernization and development of cost-efficient harvest technology.

Chile pepper is one of the fastest growing spices in U.S. market due to the changing American diet and the growing influence of Latin-American foods and population (Buzzanell et al., 1995). Chile peppers are used in pickling, relishes, catsup, sauces, and processed meat and fish. The U.S. market for all chile peppers increased from 95 million pounds dry-weight basis in 1980 to 210 million pounds in 1993 (Buzzanell et al., 1995). Domestic pepper production, although expanding, is insufficient to furnish the U.S. demand and imports accounted for 34 % of the market in 1990-93, up from 27 % in 1980-83. In addition, the value of all chile and pepper imports increased from $174 million in 1992 to $568 million in 2001 (FAOSTAT, 2003).

In the past, raw product supply for the Louisiana pepper hot sauce and pickling industry came from local production areas close to the processors. Currently, imports from other countries or states account for almost all the pepper processed in Louisiana. For instance, almost all tabasco pepper (*C. frutescens*) is grown in Central and South America and processed into sauce in Louisiana. One of the several factors that have initiated this shift in production areas is labor

cost and availability. For both processing and fresh market, harvest of pepper fruit is still primarily conducted by hand and the greatest single expense is often harvest costs. Understanding the metabolic process and factors involved in the separation of the fruit from the calyx may assist in the development of cultivars that facilitate and improve pepper harvest for processing, and increase local pepper production near Louisiana processors.

The main objective of this study was to determine the metabolic processes occuring during ripening and the factor(s) leading to the ease of fruit detachment from the calyx in tabasco pepper. Two tabasco genotypes that differ in the ease of ripe fruit detachment from the calyx were used: Easy Pick (EZ) which requires low force (0.6 N) and 'Hard Pick' (HP) which requires higher force (8.9 N) (Motsenbocker, 1996). Ripening in tabasco pepper is represented by changes in the fruit external color as determined by colorimetric analysis, so the ripening stage when the fruit detachment force (FDF) decreased was established. In order to accomplish the main objective, studies were conducted to analyze pectin characteristics in association with FDF throughout ripening. Activity of cell wall degrading enzymes in protein extracts were evaluated also throughout ripening. The results of these experiments suggested that pectin methyl-esterase (PME) plays a role in the ease of fruit separation. Therefore, studies to investigate PME activity *in vivo* and *in vitro* assessed by methanol production were conducted. The ripening specific PME associated with the ease of fruit detachment was then partially characterized. In addition, the role of PME activity on pectin metabolism in ripening tabasco pepper fruit is discussed.

CHAPTER 2. REVIEW OF LITERATURE

The ease of fruit detachment in tabasco pepper is characterized by a dramatic decrease in the force required to separate ripe fruit from the calyx, which facilitates pepper harvest while the pedicel remains on the plant (Motsenbocker, 1996). A study conducted to determine differences in mechanically harvest tabasco pepper resulted in higher harvest yield of easy-detachable fruits than hard-detachable fruits (Davis, 1980). The ease of fruit detachment in peppers was reported to be inherited as a single dominant gene (Smith, 1951). This characteristic was later designated "soft-flesh" since it is the consequence of fruit tissue disintegration. A similar phenotypic characteristic has been described in other crops. Fruit of the tomato cultivar 'San Marzano' separates very easily at both the pedicel joint and the fruit-calyx junction (Rick and Sawant, 1955). However, the jointless character was usually accompanied by a very tight attachment of the pedicel to the fruit so that fruit tissue was torn away at harvest. In peach, the melting flesh / non-melting flesh (M/m) gene was associated with pectin degradation (Scorza and Sherman, 1996; Pressey and Avant, 1978).

Several studies have been conducted to investigate the role of fruit characteristics on the ease of fruit detachment. In an inheritance study of pepper (*C. annuum*), Werner and Honma (1980) reported a positive and significant correlation between FDF and fruit length, width, and weight. Histological studies of fruit detachment zones in pepper attributed the ease of fruit detachment to structural differences between genotypes. Studies in cayenne pepper and tabasco pepper reported higher sclereids content in the junction of the calyx and fruit of a non-detachable in comparison to a readily detachable genotype (Gersch et al., 1998; Sundberg et al., 2003). However, the peripheral parenchyma cells in the easy detachable tabasco genotype stretched and

the intercellular space enlarged as less and less middle lamella was bound between adjacent cells (Sundberg et al., 2003). Also, residual fruit tissue remained in the calyx after ripe tabasco pepper fruit was detached (Motsenbocker, 1996). These results are similar to those reported in peach fruits where fruit abscission from the receptacle occurred by disintegration of the middle lamella and adjacent primary cell wall which was attributed to the activity of polygalacturonase (PG) and endo-$-1,4-glucanase (EGase) or commonly called cellulase (Bonghi et al., 1993). In addition, antisense suppression of a tomato EGase increased the force required to break the fruit abscission zone (Brummell et al., 1999). These results suggest that the ease of fruit detachment is associated with the level of cell wall degradation in ripe fruit.

Cell wall architecture and metabolism play a role in the integrity of plant tissue. (Brummell and Harpster, 2001). Cell wall is composed mainly of crystalline micro-fibrils of cellulose (~30%) embedded in a noncrystalline amorphous matrix of pectin (~35%) and hemicellulose (~30%) with small quantities of intercalated structural proteins (Fischer and Bennett, 1991; Fry, 1995). Pectin is composed of alternate branched blocks of methyl-esterified polyuronides with unbranched blocks of varying degrees of esterification (Jarvis 1984). The unbranched blocks aggregate through Ca^{2+} bridges between de-esterified carboxylic groups of adjacent polymers keeping the cell wall matrix coherent and maintaining cell to cell adhesion. As the calcium concentration increases, the number of polyuronides that aggregate increases which is directly related to gel stiffness (Tibbits et al. 1998). In contrast, as free calcium ions decrease the eventual dissolution of the gel occurs as a result of dissociation of calcium bridges. Therefore, the degree of pectin esterification and apoplastic conditions play an important role in the integrity of the cell wall structure and in the texture of fresh and processed fruit products.

Pectin metabolism affects the texture of fruit and vegetable as well as the texture of processed fruit products. In tomato fruit homogenate processed to paste, pectin degradation is enhanced in comparison to fresh fruit affecting the textural characteristics of the product (Brummell and Labavitch, 1997; Hurtado et al., 2002). The capacity of pectin to form gels and influence the viscosity of solutions depends on the integrity of pectin polymers. In fact, one of the assays to study PG activity is by viscosity reduction of a pectin solution (Bonghi et al., 1993). Therefore, pectin integrity in the harvested fruit destined for processing is an important factor that influences the quality of the processed product and every effort is made to preserve the desired pectin characteristics.

Plant cell wall degrading enzymes associated with abscission and/or fruit softening have been reviewed previously (Fisher and Bennett, 1991; Fry, 1995; Brummell and Harpster, 2001). The main cell wall hydrolytic activities that have been subject of intense study in relation to fruit softening and abscission are PG (EC 3.2.1.15), EGase (EC 3.2.1.4), PME (EC 3.1.1.11), exo-$-D-galactosidase (EGal) (EC 3.2.1.23), and xyloglucan endotransglycosylase (XET) (EC 2.4.1.207). Other hydrolytic activities such as $-mannanase and glycosidases have secondary roles. All these enzymes have been identified and studied in tomato, a relative of pepper in the Solanaceae family. In addition, EGase, PG, and PME have been identified also in pepper (Ferrarese et al., 1995; Harpster et al., 1997; Sethu et al., 1996). EGase hydrolyzes internal linkages of (1-4)$-D-linked glucans present in cellulose and xyloglucan. This is difficult to demonstrate in cellulose though, because of its semi-crystalline nature and water insoluble characteristic (Brummell and Harpster, 2001). In contrast, pectin depolymerization and dissolution is attributed to PG activity (Gross and Wallner, 1979; Huber, 1983; DellaPenna, et

al., 1990; Brummell and Harpster, 2001). According to activity studies in vitro and with isolated cell wall however, PG-mediated pectin degradation is enhanced by the action of PME (Pressey and Avants, 1982; Koch and Nevins, 1989). PME catalyzes the cleavage of the ester bond between the methyl and the C6 carboxyl group of galacturonosyl units in of the polyuronide chain. As a result, methanol is released and the de-esterified polygalacturonic acid is exposed to PG action. PG catalyzes the hydrolysis of the "-(1–4) linkages between adjacent galacturonic acid units, leaving smaller size polyuronides associated with fruit softening (Brummell and Harpster, 2001).

Hemicellulose, one of the main components of the cell wall matrix, undergoes depolymerization during fruit ripening which is attributed to several enzymes including EGase (Huber, 1983). However, ripening specific EGases isolated from pepper and tomato have been associated only with fruit abscission (Ferrarese et al., 1995; Harpster et al., 1997; Brummell et al., 1999). In fact, 80% EGase mRNA reduction in fruit abscission zone by antisense suppression increased the force required to break the abscission zone but did not affect fruit softening (Brummell et al., 1999).

Another mayor cell wall component, pectin, is degraded by PG during fruit ripening. Pectin degradation in tomato is characterized by an increase in soluble uronide and a decrease in polyuronide molecular size (Brummell and Harpster, 2001). Although there is hydrolysis of polyuronides covalently attached to the wall, only a portion of polyuronides become water soluble because most remain associated to the wall by ionic bonds to other insoluble molecules (Brummell and Harpster, 2001). Chelating agents such as CDTA and EDTA can remove calcium from the wall increasing the solubility of pectin held to the wall by ionic bonds, and pectin held

in the wall by covalent bonds can be released by sodium carbonate. Size exclusion chromatography profiles of chelator-soluble uronide showed a molecular size downshift from almost exclusively large polymers in mature-green tomato into a wide range of large to medium size molecules in fully ripe fruit (Huber, 1983; DellaPenna et al., 1990; Tieman et al., 1992; Huber and O'Donoghue, 1993; Brummell and Labavitch, 1997). In contrast, an almost complete switch into very small oligouronides that elute at the end of the separation range was detected in ripe fruit of 'Hass' and 'Lula' avocado, and in disrupted tomato tissue (paste and active cell wall extract) after incubation (Huber and O'Donoghue, 1993; Brummell and Labavitch, 1997). Studies of pectin degradation in situ using non-softening *rin* (ripening inhibitor) mutant tomato which was transformed to express a chimeric PG resulted in slight increase in pectin depolymerization and pectin dissolution reached levels similar to the wild-type, but fruit softening was not affected (Giovannoni et al. 1989; DellaPenna et al. 1990). In addition, suppression of PG activity by antisense RNA to less than 1% in protein extract delayed slightly pectin depolymerization *in vivo* and did not prevent polyuronide dissolution (Smith et al., 1990; Brummell and Labavitch, 1997). The suppression of pectin depolymerization by PG antisense RNA, however, was more distinguishable in tomato paste than in intact fruit (Brummell and Labavitch, 1997). Based on these results, it was suggested that PG-mediated pectin degradation is normally restricted *in vivo* but in homogenized tissue the restriction is removed and pectin disassembly results. PG affects directly polyuronide molecular size but pectin dissolution and fruit softening appear to be influenced by additional factors.

During growth and development, pectin is synthesized fully esterified so it can be transported in solution and the process of de-esterification occurs at the destination site by PME

as required (Jarvis 1984). This is supported by the detection of PME activity in protein extracts from various tissues (Gaffe et al, 1994). Multiple PME isoforms have been identified in tomato depending on fruit ripening stage and variety. At least three isoforms have been identified by ion-exchange chromatography in immature-green tomato fruit, one additional isoform appeared in ripe fruit only, and another isoform was present in one variety out of three studied (Pressey and Avants, 1972, Tucker et al.,1982, Warrilow et al., 1994). In another study, three immunologically related PME isoforms were found to be specific to tomato fruit in addition to several other isoforms found in all tissues including fruit (Gaffe el al. 1994). In tomato, PME activity in protein extract increased two to three fold in early ripening stages, but reports on the pectin esterification decline in cell wall extracts are inconsistent. The degree of pectin esterification (DPE) was reported as being reduced extensively (Koch and Nevins, 1989), reduced slightly (Tieman et al. 1992), or not at all (Koch and Nevins 1990), though different varieties were used. In addition, antisense transformed tomato with PME activity *in vitro* reduced to less than 10% had only a slight effect on the DPE and on the reduction of polyuronide molecular size in comparison to wild type Rutgers (Tieman et al., 1992). In contrast to tomato, PME activity in bell pepper protein extract increased from immature-green to mature-green stage and then decreased to even lower levels in turning and red-ripe fruit, but there was no indication of the degree of pectin esterification (Sethu et al., 1996). Therefore, the role of PME in pectin degradation *in vivo* remains unclear.

Apoplastic conditions influence pectin metabolism during fruit ripening. Protein binding and enzyme kinetics are influenced by the ion composition and strength due to shielding effects on molecular charges. In tomato, optimal pH for PG-mediated pectin dissolution and

depolymerization *in vitro* is between pH 4 and pH 5 and is influenced by the ion type and concentration used in the reaction solution (Pressey and Avants, 1982; Chun and Huber, 1998). Optimal pH for PME activity *in vitro* however, is pH 7 and higher (Pressey and Avants, 1982). Apoplastic pH and ionic conditions in tomato pericarp change dynamically. The pH of apoplastic fluids from pericarp decreased from pH 6.7 in mature-green to pH 4.4 in fully-ripe fruit (Almeida and Huber 1999). In contrast, K^+, PO_4^{3-}, and Cl^- increased, and Na and divalent cations remained relatively constant. Therefore, Almeida and Huber (1999) suggested that the cell wall dissolution and depolymerization by PG during fruit ripening is under the control of local pH and ionic conditions.

Recently, an expansin-type protein was found also to be expressed specifically during tomato fruit ripening, and it was proposed to have a role in cell wall softening (Cosgrove 1997, Rose et al. 1997). Expansins are a class of cell wall proteins that have been proposed to disrupt hydrogen bonds at the cellulose/hemicellulose interface and to allow "cell wall creep" in expanding cells (Cosgrove, 1997). Recently, suppression and over-expression of a ripening-regulated tomato expansin in transgenic plants was investigated (Brummell et al., 1999). Fruits in which expansin accumulation was suppressed to 3% were firmer and fruits over-expressing high levels of recombinant expansin were much softer throughout ripening than the control wild type. Therefore, there are still aspects of cell wall degradation and fruit softening during ripening that need to be elucidated.

Objective color measurements of the fruit external color is an important consideration in research analysis. The International Commission on Illumination [Commission Internationale de l'Eclairage (CIE)] established a tristimulus color system commonly used for surface color

measurements (McGuire, 1992). This system is based on a three dimensional color space with three coordinates (L* a* b* or CIELAB). The three elements of perceived color are value (lightness from black to white), chroma (degree of departure from gray to pure chromatic color), and hue (the chromatic colors) and they are determined from the CIELAB coordinates. The L* coordinate measures directly the value or lightness of the color. The coordinates a* and b* locate the color on a rectangular-coordinate grid perpendicular to L*, so they reflect indirectly on chroma and hue. Chroma is represented by the hypotenuse of a right triangle created by joining points (0,0), (a*, b*), and (a*, 0), and the hue angle is the angle (0° to 360°) between the hypotenuse and 0° on the a* (blue-green / red-purple) axis. Quantitative representation of the ripening process by colorimetric analysis of the fruit external color may help to identify the precise stage when an event such as FDF reduction or cell wall degradation occurs.

CHAPTER 3. FRUIT DETACHMENT FORCE AND CELL WALL DEGRADING ENZYMES IN TABASCO PEPPER

INTRODUCTION

Easy detachment of ripe pepper fruit is characterized by a dramatic decrease in the force required to separate the fruit from the calyx (Motsenbocker, 1996). This characteristic facilitates pepper harvest while the pedicel remains on the plant (Smith,1951; Davis, 1980). Studies with two tabasco pepper genotypes that differ in the ease of fruit separation showed differences in FDF and in visual aspects of the separation zone only in ripe fruit (Motsenbocker, 1996). However, the precise ripening stage when the decrease in FDF occurred was not defined because only four predetermined ripening stages were used.

Anatomical studies of detachment zones in these tabasco genotypes reported reduced cell to cell adhesion in easily detached fruit (Sundberg et al., 2003). These observations were comparable to those found in peach fruits where cell separation by cell wall degradation at the fruit-pedicel abscission zone was attributed to PG and EGase activity (Bonghi et al., 1993). These findings suggest that fruit separation in tabasco pepper depends on cell wall degradation associated with fruit ripening. Cell wall degradation in tomato has been studied extensively and it has been attributed to the action of PG and EGase (Brummell and Harpster, 2001). In tomato, PG was expressed at the onset of fruit ripening as induced by ethylene action. EGase activity was however, present at all developmental stages although it increased during ripening and several isoforms were reported (Huber, 1983; Brummell and Harpster, 2001). These enzymes were present also in pepper (Ferrarese et al., 1995; Harpster et al., 1997; Sethu et al., 1996).

The first objective of this study was conducted to determine the association of the ease of fruit detachment from the calyx with changes in the fruit external color during ripening. The

second objective was to analyze the activity of cell wall degrading enzymes (PG and EGase) in relation to the ease of fruit separation in tabasco pepper during ripening.

MATERIALS AND METHODS

Plant Material. Two tabasco pepper genotypes that differ in the ease of fruit detachment were used: 'Easy Pick' (EZ) which required a low force to separate the fruit from the calyx, and 'Hard Pick' (HP) which requires a higher force (Motsenbocker 1996). Seed was sown in 98 cell trays filled with soilless medium (Metro Mix 200; Scott Sierra, Marysville, OH) and kept under greenhouse conditions. Four weeks old seedlings were transplanted into 12 L pots filled with soilless medium (Metro Mix 700, Scott Sierra, Marysville, OH) supplemented with slow-release fertilizer (3.8 g"L^{-1} Osmocote 14-14-14, Scott Sierra, Marysville, OH), dolomitic limestone (5.1 g"L^{-1} Easy Lime, Sylacauga, AL), and micro-nutrients (0.64 g"L^{-1} Micromax, Scott Sierra, Marysville, OH). Plants were grown under greenhouse conditions with temperatures set to 30 ^{o}C / 15 ^{o}C day / night. Plants were micro-irrigated automatically twice a day in winter and three times a day during the summer based on water requirement. Sidedress fertilization was supplemented with slow-release fertilizer as needed. Plants were randomly distributed in the greenhouse with each plant considered an experimental unit. Assays were performed with fruit tissue from each genotype simultaneously and repeated at least three times.

Ripening and Fruit Detachment Force. Fruits of different ripening stages from immature-green to overripe were harvested for analysis and the fruit external color (hue) was determined by colorimetric analysis in a spectrophotometer (Minolta CM-3500d, Ramsey, NJ). In addition, the FDF in Newton (N) required to separate the fruit from the calyx was determined using a push-pull force gauge (Chatillon CE Digital Force Gauge DFIS 10, Greensboro, N.C.).

The fruit pedicel was firmly held by the gauge and the fruit was pulled by hand parallel to the fruit axis until separation (Motsenbocker 1996). The peak force for separation was recorded for analysis.

Enzyme Extraction. After removing the pedicel from the fruit, a 3mm fruit section was excised from the fruit-calyx junction end [detachment zone (DZ)] and used for assays. The tissue was ground in liquid nitrogen and homogenized in 0.5 mL extraction buffer (20 mM NaOAc buffer and 1.25 M NaCl, pH 6.0). The suspension was stirred for 2 h (RotoMix type 50800, Thermolyne, Dubuque, IA) and centrifuged at 16000 g for 10 min in a micro-centrifuge (Eppendorf 5415C, Hamburg, Germany). The supernatant was used to test for EGase and PG activity. Protein content was determined by the method of Bradford (1976) using BSA as standard. All steps during protein extraction were conducted at 5 °C.

Enzyme Activity Assays. Activity of EGase and PG was determined by viscosity reduction (Bonghi et al., 1993). EGase activity was tested by incubating 200 ：L of the extract in 8 ml EGase-substrate [0.6 % carboxymethylcellulose (CMC; Sigma Chemical Co., St. Louis, MO.) in 20 mM NaOAc, pH 6.0] at room temperature in an Oswald 100 viscometer. The flowing time of the reaction mixture was determined every hour up to 5 h. PG activity was tested similarly in 8 mL PG-substrate [0.5 % pectin from apple (Sigma Co.) in 20 mM NaOAc, pH 5.5] for 12 hours. An activity unit was defined to reduce the flowing time 1% in 1 h.

RESULTS

Ripening. Under greenhouse conditions, the ripening process of tabasco pepper from mature-green fruit until purple-red lasted between 13 and 16 days. The fruit color changed progressively from green to yellow, orange, red, and culminated in purple-red. The fruit external

color was used as ripening indicator and it was reported quantitatively by the hue (angle in the CIE a*b* chromaticity diagram; Fig. 3.1). The a* value represents green (-) to red (+) color in the CIELAB color space. This scale was not suitable as a ripening indicator because it was unable to separate red fruit from purple-red fruit. A comparative description of fruit ripening stage based on color and the hue angle is as follows: immature- and mature-green fruit (hue 102 to 95), yellow fruit (hue 90 to 80), breaker stage (hue 75 to 65), orange (hue 60 to 50), red-mature (hue 45 to 35), and purple-red fruit (overripe; hue <35). In comparison, the red painted stick used as a commercial harvest index for tabasco peppers has hue 34.

Ease of Fruit Detachment. The dramatic decrease in the force required to separate ripe fruit from the calyx is characteristic of the EZ tabasco pepper genotype. Based on the data, The FDF during ripening in the EZ tabasco genotype followed a reverse sigmoidal pattern with three phases (Fig. 3.1). Fruit color in phase I was between hue 102 and hue 52 and FDF ranged between 17 and 26 N. In phase II, fruit color ranged between hue 52 and hue 48 where FDF dropped to 5.4 N or lower as will be shown in following studies. This phase lasted approximately 12 h. In phase III, the mean FDF was 3 N and fruit color changed from hue 48 to hue 30 at the end of the ripening process. In contrast, the pattern of FDF in the HP genotype was comprised of two phases (Fig. 3.1). In Phase I the FDF stayed in the range between 18 and 27 N until hue 45, and then in phase II the FDF decreased to approximately 10 N when fruit color reached hue 30. Fruits of both genotypes softened when ripe, but the EZ fruit became much softer after the decline in FDF. In addition, the placenta and pericarp of EZ fruit disintegrated almost completely. Measuring the shear force of tabasco pepper fruit was not reliable in determining fruit softening because of the seed content (data not presented).

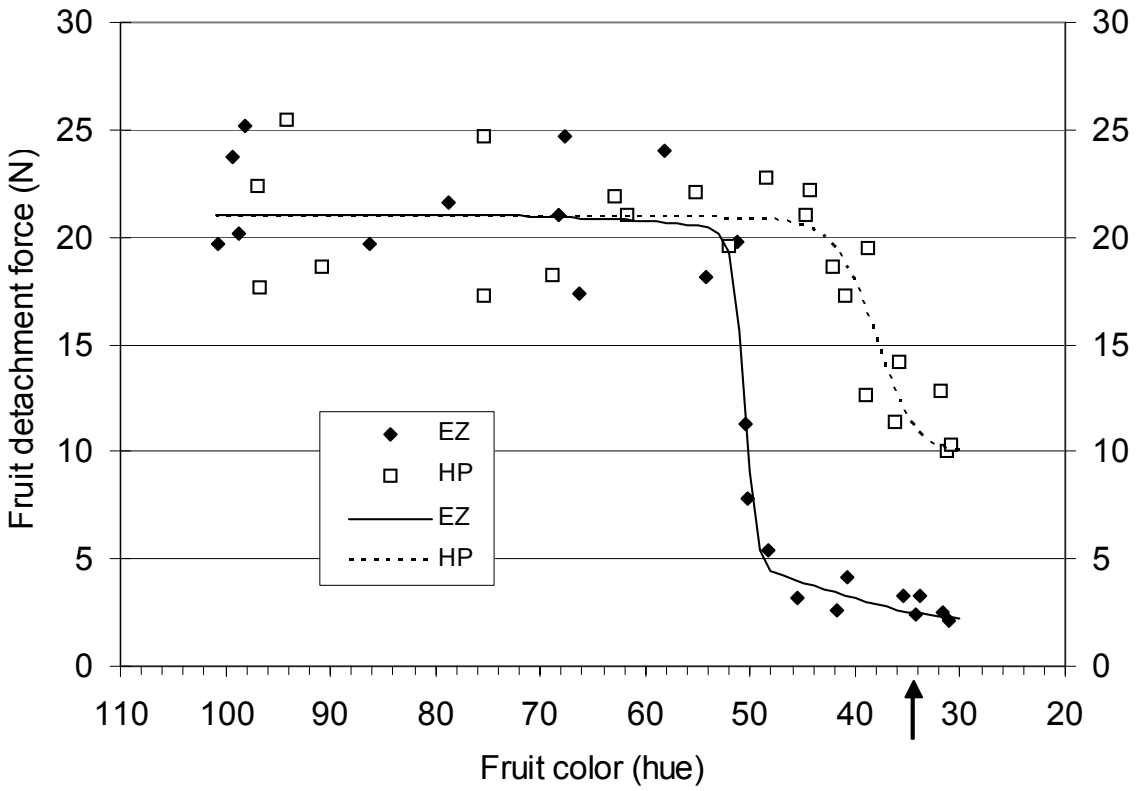

Fig. 3.1. Fruit detachment force during fruit ripening in 'Easy Pick' (EZ) and 'Hard Pick' (HP) tabasco pepper. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Arrow indicates harvest index at hue 34. Coefficient of determination: r^2=0.95 (solid line) and r^2=0.72 (dash line).

Endo-$-1,4-Glucanase (EGase). Activity of EGase in protein extracts was measured by viscosity reduction and the results are shown in Fig. 3.2. Activity was expressed in relation to amount of tissue (Fig. 3.2 A) and in relation to protein content in the extract (Fig. 3.2 B). Based on the data, activity was low or undetected in the early ripening stages (hue < 60). After hue 60, activity was higher in both tabasco genotypes and increased steadily until the end of the ripening process (hue 30). Although, there was a correlation (r = -0.67) between FDF and EGase activity there was no difference in EGase activity between the genotypes.

Polygalacturonase (PG). Activity of PG in protein extract was measured by viscosity reduction and the results were expressed in relation to amount of tissue (Fig. 3.3 A) and to protein content in the extract (Fig. 3.3 B). Based on the data, activity was low or undetected in the early ripening stages (hue < 60). After hue 60, activity was higher in both tabasco genotypes and the highest activity in relation to protein content was detected between hue 50 and hue 40. Thereafter, activity decreased toward the end of the ripening process (hue 30). Although, there was a correlation (r = -0.58) between FDF and PG activity, there was no difference in PG activity between the genotypes.

DISCUSSION

The results of this study indicated that pepper fruit separation at the fruit-calyx junction is associated with fruit ripening. The difference in FDF of ripe fruit between the EZ and HP tabasco genotypes was similar to previously reported studies (Motsenbocker, 1996). The ripening stage when the FDF decreased, however, was more accurately described by using objective measurement of the fruit external color (hue) as a ripening indicator (Fig. 3.1). Color changes during pepper fruit ripening are associated with degradation of chlorophyll, which

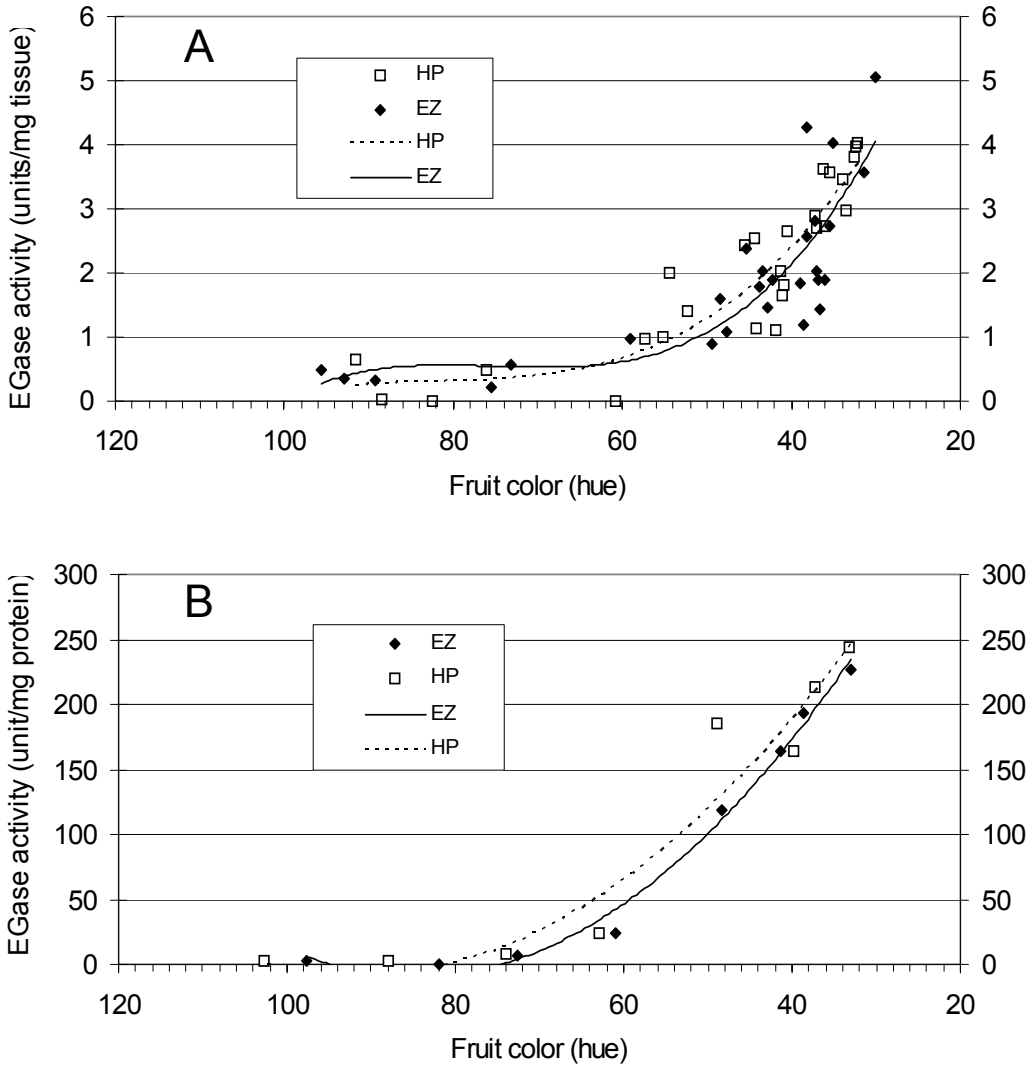

Fig. 3.2. Endo-$-1,4,-glucanase (EGase) activity during fruit ripening in 'Easy Pick' (EZ) and 'Hard Pick (HP) tabasco pepper. Activity in fruit detachment zone extracts was determined by viscosity reduction in relation to fruit tissue (**A**) and to protein content (**B**). One activity unit caused the flowing time to decrease 1% in 1h. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Coefficient of determination in **A:** r^2= 0.85 (dash line) and r^2= 0.70 (solid line), and in **B**: r^2= 0.93 (dash line) and r^2= 0.99 (solid line).

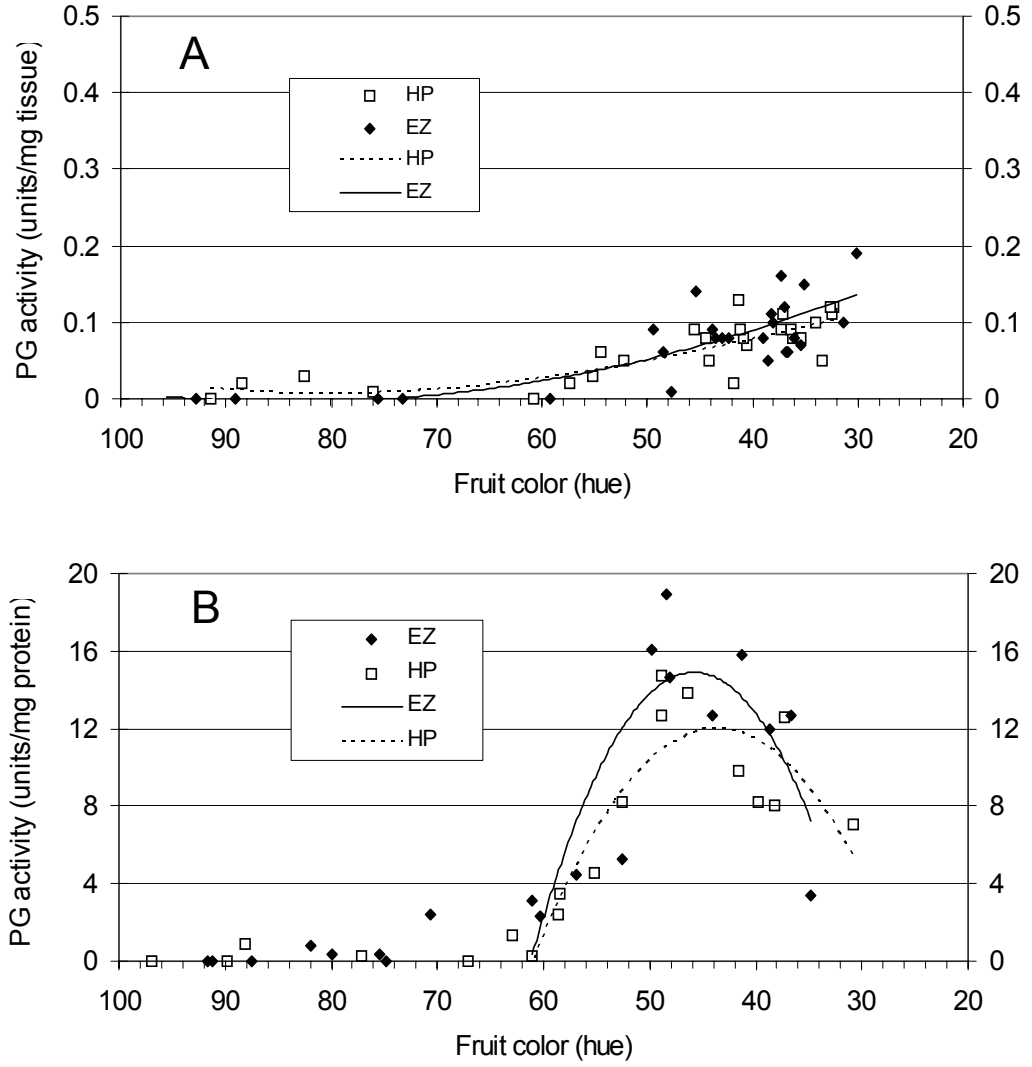

Fig. 3.3. Polygalacturonase (PG) activity during fruit ripening in 'Easy Pick' (EZ) and 'Hard Pick' (HP) tabasco pepper. Activity in fruit detachment zone extracts was determined by viscosity reduction in relation to fruit tissue (**A**) and to protein content (**B**). One activity unit caused the flowing time to decrease 1% in 1h. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Coefficient of determination in **A:** r^2= 0.66 (dash line) and r^2= 0.60 (solid line), and in **B**: r^2= 0.77 (dash line) and r^2= 0.70 (solid line).

unmasks previously present pigments, along with biosynthesis of one or more pigments, usually anthocyanins or carotenoids (Tucker, 1993). In pepper, capsanthin is the most quantitatively important color compound (Davies et al., 1970). Classical tomato fruit ripening stages have been defined according to visual determination of fruit external color (Huber, 1983). Verbal description of colors can be difficult and confusing because two people may describe the same color in very different terms. Description of the color by comparison to standardized color tables have been used also to determine the precise ripening stage (Motsenbocker, 1996). Other researchers used days after harvest as a quantitative representation of fruit ripening in tomato and bell pepper (DellaPenna et al., 1990, Sethu et al., 1996). Fruit ripening is a dynamic physiological process that is affected by several factors such as temperature, ethylene, stress, variety, etc., hence it is difficult to define uniform ripening stages in time. Because color development is closely associated with fruit ripening, objective measurement of the fruit external color (hue)(McGuire, 1992) resulted in a more precise determination of the FDF decline (Fig. 3.1). The ripening stage when the FDF decreased differed between the EZ and the HP genotypes suggesting that different mechanisms may be involved.

Activity of PG and EGase in protein extract increased in both genotypes with fruit ripening. The pattern of PG and EGase activity in tabasco pepper throughout ripening was consistent with the pattern reported in bell pepper (Ferrarese et al., 1995; Sethu et al., 1996) and tomato (Huber, 1983). The level of EGase activity in ripe tabasco pepper was similar to the level reported in peach and PG activity in tabasco was twice as much as peach (Bonghi et al., 1993). EGase in bell pepper, however, was 40 times higher than the results with tabasco pepper (Ferrarese et al., 1995). Activity levels reported in other studies were different because of

differences in the assays (Huber, 1983; Sethu et al., 1996). Although PG and EGase activities in protein extracts were associated with FDF, they were the same in both tabasco pepper genotypes. These results were not consistent with the anatomical differences of parenchyma cell between the EZ and HP genotypes described previously (Sundberg et al., 2003).

EGases have been isolated in several plant species including tomato and bell pepper, and associated with fruit ripening, and organ abscission, in addition to cell elongation (Brummell and Harpster, 2001). In pepper, different EGases were isolated from leaf abscission zone and ripening fruit (Ferrarese et al., 1995). Only one fruit EGase has been reported in pepper and its expression was enhanced by ethylene, although pepper is considered a non-climacteric fruit (Ferrarese et al., 1995; Harpster et al., 1997). In addition, suppression of this EGase resulted in undetectable activity levels but it had no effect on depolymerization of cell wall matrix glycans (Brummell and Harpster, 2001). These results and the results from tabasco pepper suggest that there is only one EGase gene associated with fruit ripening in pepper, but it is not a primary determinant of fruit softening. In tomato however, several EGases were isolated from fruit and individual antisense suppression of LeCel1 and LeCel2 genes reduced mRNA expression to less than 5% in relation to normal fruit (Brummell et al., 1999; Brummell and Harpster, 2001). Although mRNA reduction of these genes had no effect on fruit softening, reduced mRNA of either of these genes in abscission zones increased the force required to cause its breakage.

Similar inconsistences were reported in studies with PG transgenic tomatoes (Giovannoni et al., 1989; DellaPenna et al., 1990; Smith et al., 1990). The mutant *rin* tomato which blocks many aspects of ripening, including PG transcription, was transformed by introducing a chimeric PG gene (Giovannoni et al., 1989; DellaPenna et al., 1990). Expression of PG resulted in

accumulation of active PG and the degradation of fruit cell wall polyuronide. No significant effect on fruit softening was detected though. Similarly, PG activity reduction to 1% in tomato by antisense PG transformation inhibited pectin depolymerization, but pectin dissolution was not affected (Smith et al., 1990). In conclusion, these results indicate that PG is responsible for pectin degradation, but suggest that degradation is not sufficient to induce softening. Also, these results suggest that an additional factor(s) may be involved in cell wall degradation that leads to the ease of fruit detachment in tabasco pepper.

CHAPTER 4. PECTIN ULTRA-DEGRADATION AFFECTS THE EASE OF FRUIT DETACHMENT IN TABASCO PEPPER

INTRODUCTION

Easy detachment of ripe fruit from the calyx is a desirable trait in pepper (*Capsicum* spp.) because it expedites the harvest and increases the yield of mechanically harvested pepper (Davis, 1980). This characteristic was later designated "soft-flesh" since it is the consequence of fruit tissue disintegration. Degradation of cell wall components has been attributed primarily to PG and EGase activity (Brummell and Harpter, 2001). These activities were detected in extracts from EZ and HP tabasco pepper fruit detachment zone and associated with FDF (Chapter 3). The level of activity however, was the same in fruit of both genotypes, so the difference in FDF could not be explained by PG or EGase activity.

Pectin degradation attributed to the action of PG has been studied extensively in tomato and has been associated with fruit softening (Brummell and Harpter, 2001). Pectin degradation is characterized by uronide depolymerization and dissolution during fruit ripening (Gross and Wallner, 1979; Huber, 1983; DellaPenna, 1990). Studies with isolated cell wall however, showed that pectin degradation and dissolution by PG is more effective in de-esterified cell wall (Pressey and Avants, 1982).

The texture of fresh and processed fruit products is affected by pectin metabolism (Jarvis, 1984; Hurtado et al., 2002). The capacity of pectin to form gels and influence the viscosity of solutions depend on the integrity of pectin polymers. Therefore, the pectin integrity in the harvested fruit destined for processing is an important factor that influences the quality of the processed product and every effort is made to preserve the desired pectin characteristics.

In this study, differences in pectin characteristics of fruit detachment zone were analyzed during ripening in the EZ and HP tabasco pepper genotypes (Motsenbocker, 1996). Residual fruit tissue that remains in the calyx after separation suggests that fruit separation depends on fruit tissue degradation instead of a classical abscission layer. In addition, the reduced cell to cell adhesion (Sundberg et al., 2003) and the loss of tissue integrity in ripe deciduous fruits (soft-flesh), but not in non-deciduous ones, suggest that the ease of fruit detachment depends on the level of pectin degradation associated with fruit ripening. Therefore, the objective of this study was to associate pectin degradation to the ease of fruit detachment in tabasco pepper.

MATERIALS AND METHODS

Plant Material, Fruit Ripening, and Detachment Force. Fruit from EZ and HP tabasco pepper genotypes were grown, harvested, and analyzed for ripening stage and FDF as described previously (chapter 3).

Tissue and Cell Wall Preparation. After removing the pedicel, a 3 mm thick disk (20 to 30 mg) from the fruit DZ was excised. The disk was used fresh (FT), or it was frozen in liquid nitrogen and freeze-dried (DT). Cell wall was extracted from DT by grinding it in 0.5 mL of 100% (CW) or 60% ethanol (CW60), heat-inactivated at 90 ^{o} C for 20 min, and centrifuged at 16000 g for 5 min in a micro-centrifuge (Eppendorf 5415C, Hamburg, Germany). The pellet was rinsed three times by cycles of 0.5 mL acetone and centrifugation, and then air-dried. Samples were kept in vacuum at room temperature until used.

Pectin Dissolution. The excised FT was dipped in 600 ：L water for 10 min and the soluble uronide that diffused into solution was determined as uronic acid (UA) equivalents by the hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) using galacturonic acid

as a standard. In different experiments, pectin solubility in water and chelator was determined in CW from fruit at different ripening stages. Water-soluble UA was tested by mixing 5 mg CW in 1 mL water at room temperature. After 10 min and 1 h in suspension, the sample was centrifuged and an aliquot was taken to determine the soluble UA content by the hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). Chelator soluble UA was tested similarly, but in extraction buffer (50 mM Na acetate, 40mM EDTA, pH 4.5) for 1 h. In addition, pectin solubility in 60 % ethanol was determined in DT after heat inactivation. The supernatant was dried at 55 °C and the residue dissolved in water to determine the UA content (Blumenkrantz and Asboe-Hansen, 1973). The correlation between FDF and soluble UA was analyzed.

Total Uronide Content. Total uronide was extracted from 5 mg DT, CW, and CW60 by digestion and dissolution in 2 mL H_2SO_4 as described by Ahmed and Labavitch (1977). The solution was then diluted with water to a suitable concentration and tested for UA content by the hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) using galacturonic acid as a standard.

Pectin Depolymerization. The degree of depolymerization of EDTA-soluble pectin extracted from CW was determined by size-exclusion chromatography in a Sepharose CL-4B column (30 x 1.5 cm) following the method of DellaPenna et al. (1990) with some modifications. The tissue sample was suspended in extraction buffer (50 mM Na acetate, 40 mM EDTA, pH 4.5) overnight at 4 °C and diluted to give a uronide concentration of 0.5 mg/mL. A 1 mL sample was passed through the Sepharose column equilibrated with elution buffer (100 mM Na acetate, 20 mM EDTA, pH 6.5). A 0.5 mg/mL solution of galacturonic acid was used as the monomer standard. The elution rate was 0.4 mL/min and 2 mL fractions were collected. The UA content of

each fraction was determined by the hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) and expressed as a percentage of recovery from the injected sample.

Degree of Pectin Esterification (DPE). A sample of 5 mg CW was suspended in 600 ：L water overnight at 4 °C. Then, methanol was extracted by saponification with 300 ：L 1.5 N NaOH at room temperature for one hour. The sample was chilled on ice, acidified with 300 ：L of cold 6 N H_2SO_4, and centrifuged to precipitate and separate solid CW material from the liquid phase. The methanol molar content was determined chemically by the pentane-2,4-dione method (Wood and Siddiqui, 1971). Uronide content was determined as described above (Ahmed and Labavitch, 1977; Blumenkrantz and Asboe-Hansen, 1973) and the total molar content was calculated. The DPE was defined as the proportion in percent of the methanol molar content to the UA molar content.

RESULTS

Pectin Dissolution. The level of soluble UA in FT from ripe (hue <50) fruit increased in both lines, but it was 20 times higher in the EZ genotype (Fig. 4.1A) than in the HP genotype (Fig. 4.1B). The phases that comprise the changes in soluble uronide coincided with the phases of FDF for each genotype. In the initial ripening stages soluble UA was very low or undetected and the FDF was high in both genotypes (phase I, chapter 3). Between hue 52 and hue 48, soluble uronide in the EZ genotype increased rapidly as FDF dropped (phase II). Then in phase III, soluble UA in the EZ genotype remained high (average of 5.81 ：g/mg FT) and FDF stayed low until overripe (hue 30). In contrast, water soluble uronide in the HP line increased slightly in phase II (hue <45), reaching a maximum of 0.41 ：g/mg FT. Correlation analysis indicated that

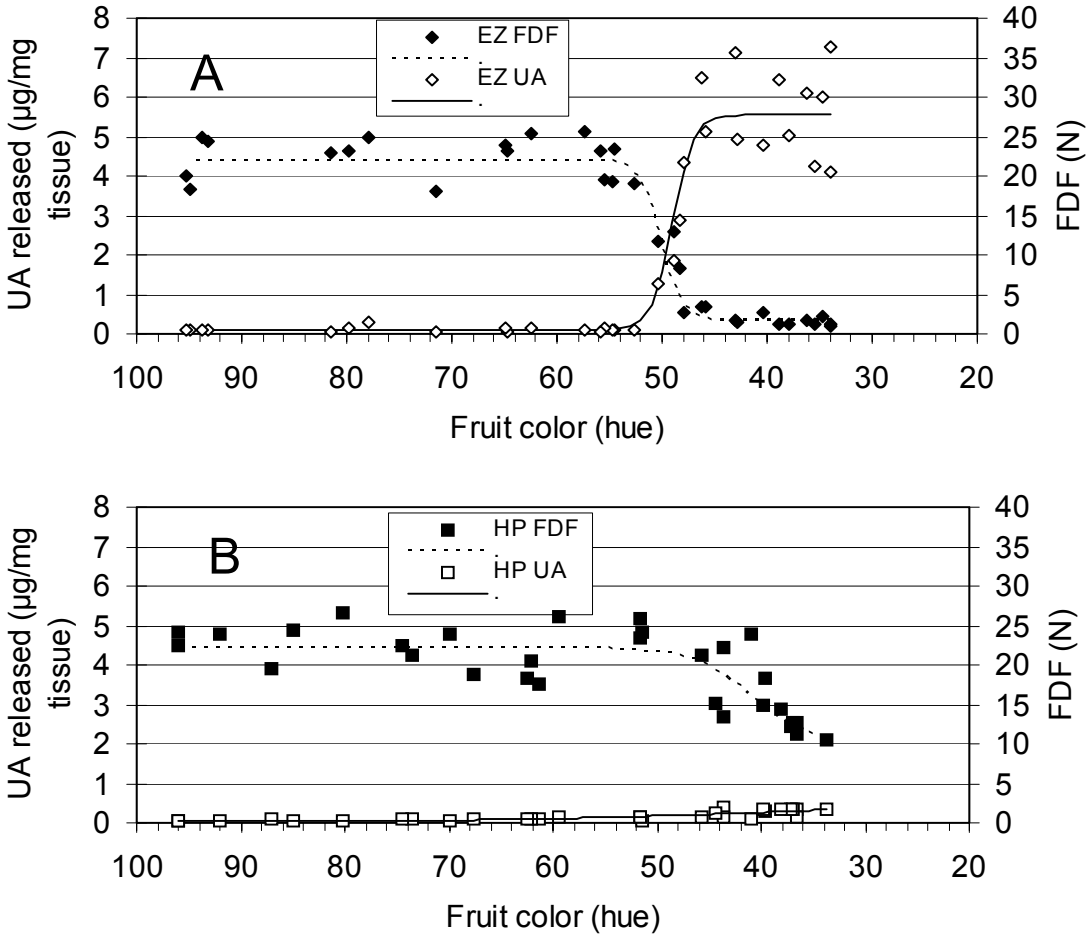

Fig. 4.1. Fruit detachment force (FDF) and water-soluble pectin (as uronic acid (UA)) from fresh fruit tissue throughout ripening in (**A**) 'Easy Pick' (EZ) and (**B**) 'Hard Pick' (HP) tabasco pepper. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Coefficient of determination in **A**: r^2=0.95 (dash line) and r^2=0.93 (solid line), and in **B**: r^2=0.64 (dash line), and r^2=0.70 (solid line).

pectin dissolution from the detachment area is associated (r = -0.92) with FDF which implies that pectin degradation *in vivo* is associated with the ease of fruit separation from the calyx.

Disruption of the cell wall structure and separation of cellular components during CW extraction facilitated the release of soluble pectin. Water soluble uronide extracted from CW throughout fruit ripening (data not presented) followed the same sigmoidal pattern of chelator soluble uronide for each genotype (Fig. 4.2A). There was no difference between extraction for 10 min and for 1 h in either genotype which indicates that most if not all of the water soluble uronide in tabasco CW was released within 10 min.. The level of EDTA-soluble uronide during fruit ripening for both genotypes is shown in Fig. 4.2A. EDTA in the extraction solution is thought to disrupt Ca^{2+} bridges between galacturonic acid residues of adjacent pectin polymers which allows ionically bound uronide to become soluble. This does not appear to be the case in tabasco CW since the level of chelator-soluble UA was the same as water-soluble UA for each genotype throughout ripening. Chelator soluble UA in the early ripening stages up to hue 53 was the same in both tabasco lines. Thereafter, EDTA-soluble uronide increased to an average of 54 ：g/mg in HP CW and 97 ：g/mg in EZ CW or 1.8 times higher. Chelator soluble uronide from CW was also associated (r = -0.84) to the FDF throughout ripening.

Pectin and other CW polymers precipitate in ethanol. Reducing the ethanol concentration to 60 % in the first step of CW extraction was intended to improve the separation of sugars and other soluble components. Because of the unexpectedly low levels of total UA when EZ CW60 was extracted under this condition, 60 % ethanol-soluble UA in DT was determined throughout ripening and the result is shown in Fig. 4.2B for both genotypes. Soluble UA was very low (under 12 ：g/mg DT) in the HP genotype throughout the ripening process and in unripe

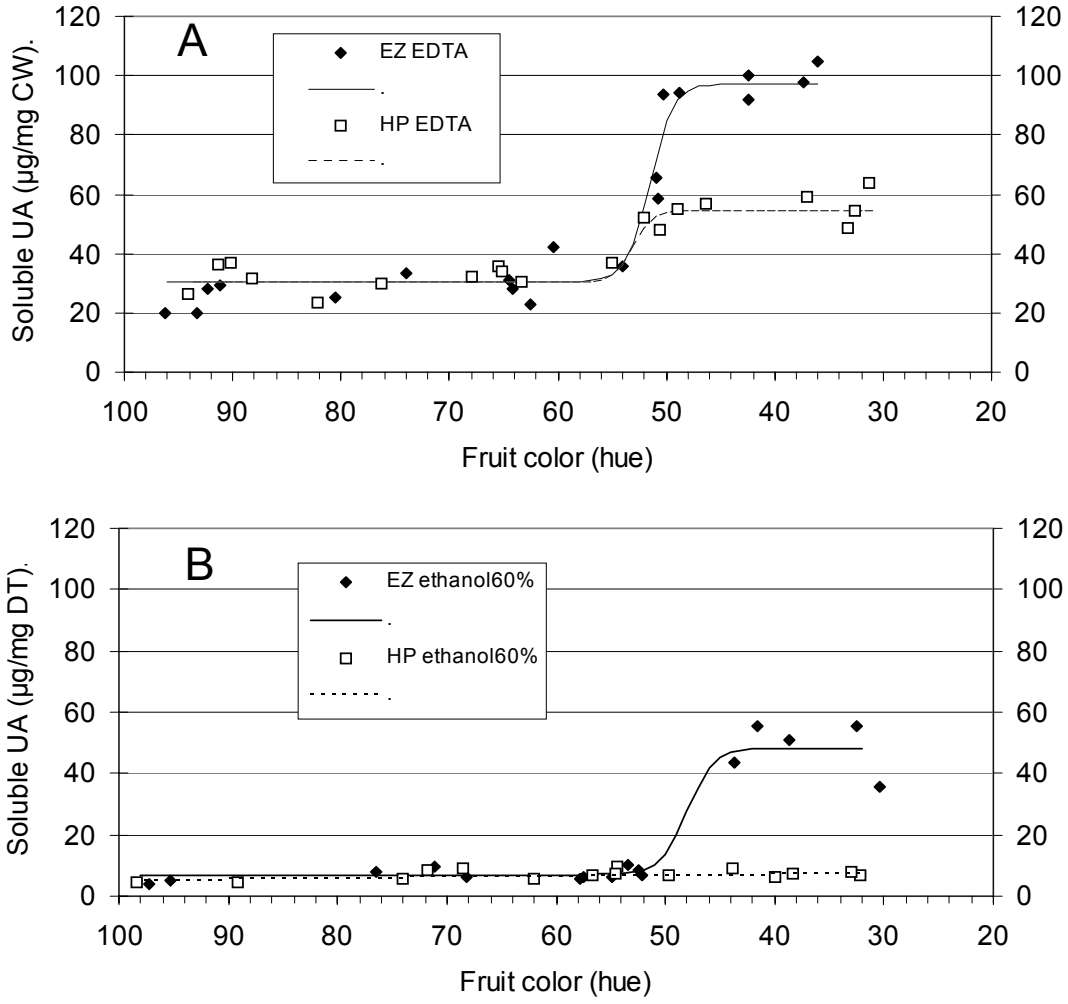

Fig. 4.2. Pectin dissolution throughout fruit ripening in'Easy Pick' (EZ) and 'Hard Pick' (HP) tabasco pepper. (**A**) EDTA-soluble pectin (as uronic acid (UA)) in cell wall extracts (CW). (**B**) UA soluble in 60% ethanol from freeze-dried tissue (DT). Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Coefficient of determination in **A**: r^2=0.87 (dash line) and r^2=0.95 (solid line), and in **B**: r^2=0.24 (dash line), and r^2=0.95 (solid line).

(hue >50) EZ DT. In contrast, the soluble UA from ripe (hue <50) DT of the EZ genotype reached levels above 50 ：g/mg DT indicating that there was a higher proportion of uronide oligomers of low molecular weight than in HP DT. Uronide soluble in 60 % ethanol was associated (r = -0.85) with the FDF throughout ripening. Smaller UA oligomers soluble in 60 % ethanol in ripe fruit of the EZ genotype indicates that higher pectin degradation was associated with the ease of fruit detachment in tabasco pepper.

Uronide Content. Total uronide content in DT, CW60, and CW was analyzed for differences between both tabasco genotypes. Pectin content remained the same in both lines throughout the ripening process when extracted from DT and ranged between 78 ：g/mg to 104 ：g/mg with an average of 88 ：g/mg DT. Ethanol/acetone extraction of CW separated ethanol soluble sugars and acetone soluble lipids increasing the proportion of pectin in comparison to DT. The total uronide content in CW (A) and CW60 (B) from both tabasco genotypes during fruit ripening is shown in Fig. 4.3. Pectin content in CW from both genotypes was the same, but it increased slightly as fruit ripened (Fig. 4.3A). Metabolism and dissolution during ripening of alcohol insoluble carbohydrates may account for this slight increase of the uronide proportion in CW. Reducing the ethanol concentration to 60 % was intended to improve the separation of sugars from CW. In the HP line, extraction in 60 % or 100 % ethanol yielded the same proportion of pectin throughout the ripening process (Fig. 4.3). In contrast, uronide content in CW60 from the EZ line decreased as fruit ripened (Fig. 4.3B). This decrease of uronide content after hue 50 in EZ CW60 indicates that degraded low molecular weight UA became soluble in 60 % ethanol and it was lost during extraction.

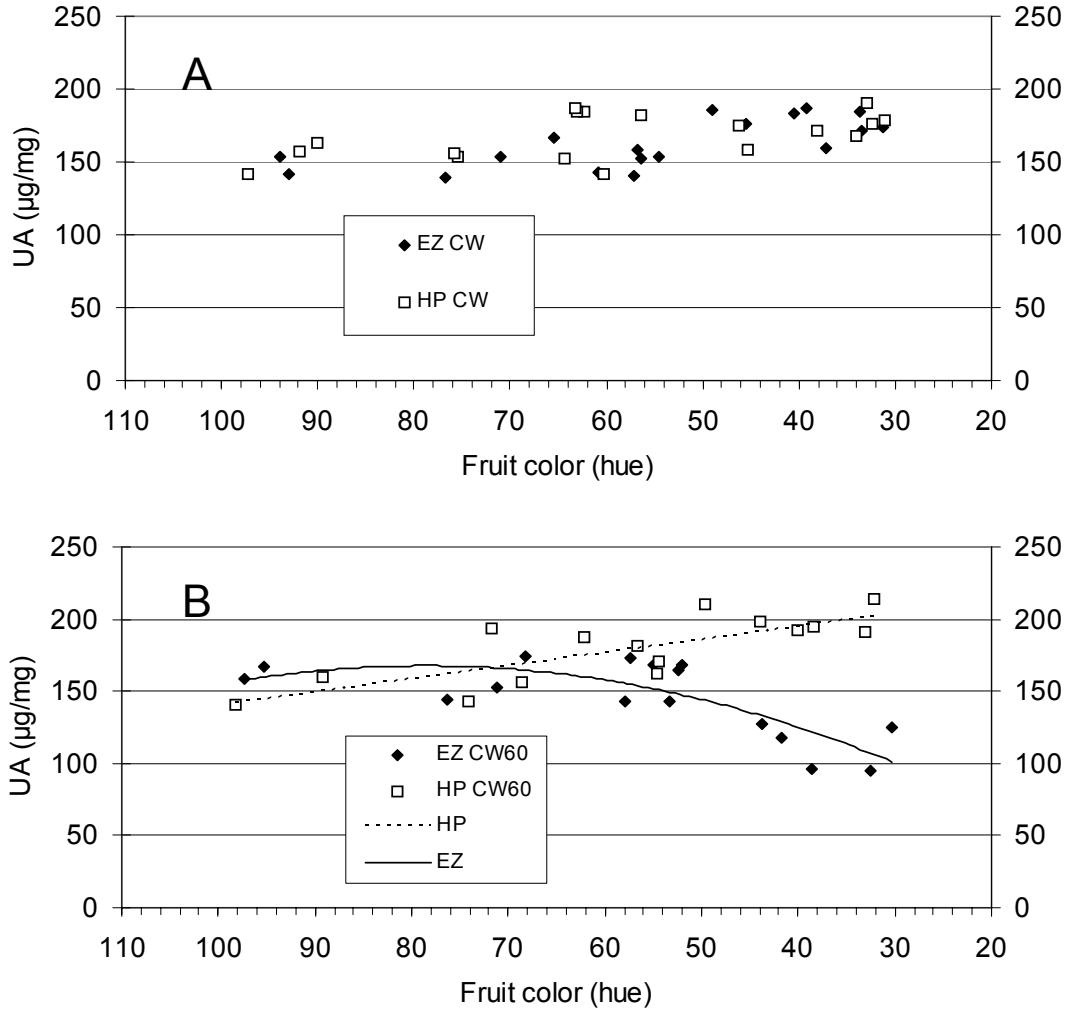

Fig. 4.3. Cell wall pectin content (as uronic acid (UA)) throughout fruit ripening in 'Easy Pick' (EZ) and 'Hard Pick' (HP) tabasco pepper. (**A**) Cell wall (CW) extracted in 100% ethanol. (**B**) Cell wall (CW60) extracted in 60% ethanol. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Coefficient of determination in **B**: r^2=0.59 (dash line), and r^2=0.62 (solid line).

Pectin Depolymerization. The effect of PG activity *in vivo* was evaluated by the degree of pectin degradation determined by molecular-size exclusion chromatography. The molecular size profiles of EDTA-soluble polyuronide extracted from CW of two ripening stages are shown in Fig. 4.4. The elution profile of mature-green fruit (hue 94 and 97 for EZ and HP respectively) was the same in both lines (Fig. 4.4A). Uronide polymers of large molecular size constituted the larger proportion of EDTA-soluble pectin that eluted first in the void volume followed by a tail of polyuronide of decreasing molecular size that extended up to the end of the separation range. In contrast, the elution profile of red-mature tissue was different between the genotypes (Fig. 4.4B). The elution profile of uronide from ripe (hue 40) EZ CW shows an almost complete downshift of the uronide molecular size to oligomers of a few galacturonic acid residues as a consequence of extensive depolymerization. In the case of ripe (hue 44) HP CW, the elution profile shows that uronide was distributed throughout a wide range of large to medium size UA polymers. This indicates that the degree of pectin depolymerization was not as extensive as in the EZ line. This assay was performed also with heat inactivated DT of similar ripening stages and the results were the same (data not presented). Therefore, higher pectin dissolution in the EZ genotype (Fig. 4.1 and 4.2) may be associated with extensive pectin depolymerization. Also, the higher depolymerization into galacturonic acid and UA oligomers soluble in 60 % ethanol may explain the decrease in total uronide content in ripe EZ CW (Fig. 4.3B).

Degree of Pectin Esterification. The association of the DPE with pectin depolymerization *in vivo* and therefore with the ease of fruit detachment was evaluated. The DPE in CW extracted from fruit detachment zones throughout fruit ripening is shown in Fig. 4.5. In the early ripening stages (hue > 60), DPE was the same in both genotypes ranging between

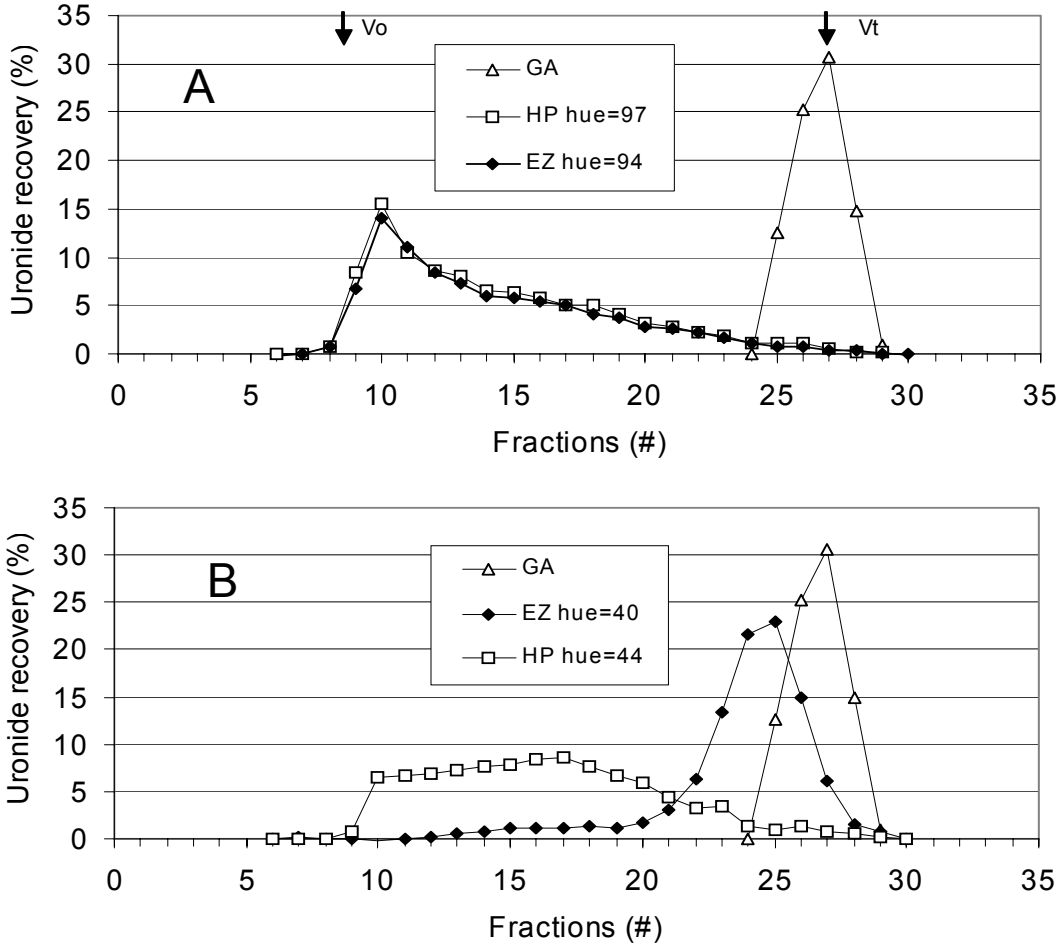

Fig. 4.4. Size-exclusion chromatography profile of EDTA-soluble polyuronide from‘Easy Pick’ (EZ) and ‘Hard Pick’ (HP) tabasco pepper. (**A**) mature-green fruit and (**B**) red-mature fruit. The column (30 x 1.5 cm) was filled with Sepharose CL-4B. Arrows indicate void (Vo) and total (Vt) volume. Uronide content in fractions is expressed as percentage of the total uronic acid (UA) assayed. Galacturonic acid (GA) was used as monomer standard. Fruit ripening stage was determined by the fruit external color (hue).

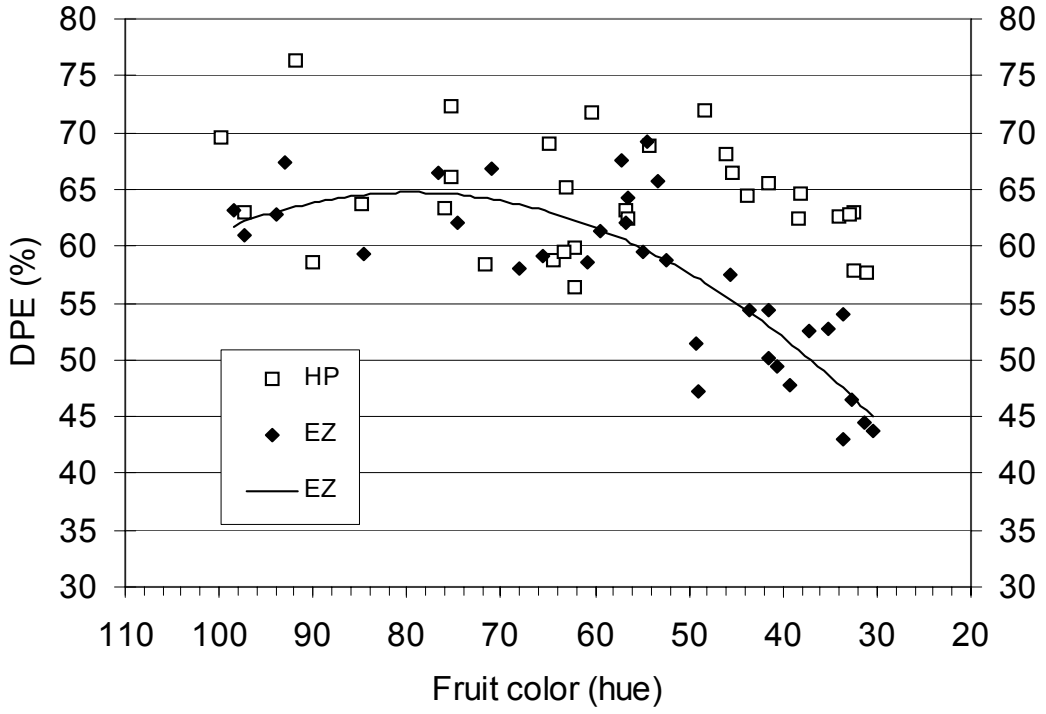

Fig. 4.5. Degree of pectin esterification (DPE) throughout fruit ripening in 'Easy Pick' (EZ) and 'Hard Pick' (HP) tabasco pepper. The DPE was defined as the proportion (%) of methanol to uronide molar content. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Coefficient of determination: r^2=0.69 (solid line).

55 % and 78 %. As fruit ripened, DPE in the HP genotype stayed within the same range until the end of the ripening process. In contrast, the DPE in the EZ genotype decreased after hue 52 down to 43 % in overripe tissue. DPE was correlated (r = 0.78) also with the FDF indicating that PME activity *in vivo* in the EZ genotype had an effect on the DPE that was not detected in HP fruit tissue. Furthermore, the lower DPE indicates increased substrate available for PG action, which may explain the extensive pectin depolymerization in the EZ genotype.

DISCUSSION

The ease of pepper fruit detachment from the calyx when ripe is characterized by a dramatic decrease in FDF which results in clean fruit separation during harvest while the pedicel remains on the plant (Smith,1951; Motsenbocker, 1996). This characteristic may not always be a desirable trait as modern cultivars have been bred for larger and heavier fruit tightly attached to the plant. Similar phenomena have been reported in tomato (Rick and Sawant, 1955) and in peach (Scorza and Sherman, 1996). The results of this study indicates that pepper fruit separation at the fruit-calyx junction was the result of pectin degradation intrinsic to fruit ripening instead of a classical abscission layer. This is supported by the lack of a distinct layer of cells identifiable as abscission zone in the fruit-calyx junction of cayenne pepper (Gersch et al., 1998) and tabasco pepper (Sundberg et al., 2003). The differential reduction of the force required to remove ripe fruit from the pedicel between EZ and HP tabasco pepper (Fig 4.1) was attributed to a differential degree of pectin metabolism as indicated by solubility studies (Figs. 4.1 and 4.2). Pectin degradation and FDF reduction occurred in both genotypes, but the earlier and pronounced FDF drop in the EZ tabasco genotype (Fig. 4.1A) was the result of pectin ultra-depolymerization (Fig. 4.4B) that causes the fruit tissue disintegration. The ripening stage

between hue 52 and hue 48 was the critical stage for the FDF decline (Fig. 4.1). At this same stage, pectin dissolution increased (Fig. 4.1) and the DPE decreased (Fig. 4.5), resulting in a correlation with FDF and hence with the ease of fruit detachment. Pectin ultra-degradation in EZ fruit detachment zone was consistent with the reduced cell to cell adhesion found at the fruit-calyx junction of EZ tabasco pepper (Sundberg et al., 2003) and peach (Bonghi et al., 1992). These results support the relationship between PG-mediated pectin degradation and fruit softening, and establish the association between the ease of fruit detachment and pectin ultra-degradation in ripe tabasco pepper.

Pectin metabolism during fruit ripening in tabasco pepper was characterized by an increase in soluble uronide (Figs. 4.1 and 4.2) and a decrease in polyuronide molecular size (Fig. 4.4), while total pectin content remained the same as determined in DT or increased slightly as determined in CW (Fig. 4.3). Pectin degradation by PG in tomato during fruit ripening exhibited these same characteristics, although the level of pectin dissolution and the degree of pectin depolymerization varied according to the methodology used and genotype evaluated (Gross and Wallner, 1979; Huber, 1983; Koch and Nevins, 1990; DellaPenna et al., 1990; Watson et al., 1994; Brummell and Labavitch, 1997). Chelator-soluble pectin extracted from CW is considered to be ionically bound and part of the cell wall structure (Jarvis 1984). This seems not to be the case in tabasco pepper fruit since the profile of EDTA-soluble UA in extracted CW throughout ripening (Fig. 4.2A) was similar to the profile of water soluble UA in both genotypes (data not presented). Similar levels of EDTA-soluble UA (DellaPenna et al., 1990; Watson et al., 1994) and water-soluble UA (Gross and Wellner, 1979) were reported in tomato. Nevertheless, water-soluble uronide that diffused out from fresh detachment area (Fig. 4.1) and soluble UA in 60 %

ethanol (Fig. 4.2B) presented a more dramatic difference between ripe and unripe CW, and between EZ and HP genotype in ripe fruit. Similar differences in water-soluble UA that diffused from cell wall of tomato pericarp discs during fruit ripening were reported between normal and non-softening tomato lines (Koch and Nevins, 1990), although the soluble UA was extracted by centrifugation.

Pectin dissolution depends on the size of the uronide polymer. In contrast to galacturonic acid and UA oligomers, dissolution of larger pectin polymers is difficult because they are ionically bound and form part of the cell wall structure (Jarvis 1984). In mature-green tabasco fruit, the size exclusion profile of EDTA-soluble uronide was the same for both genotypes (Fig. 4.4A) and it was indistinguishable from those reported for tomatoes (DellaPenna et al., 1990; Huber and O'Donoghue, 1993; Brummell and Labavitch, 1997). At this stage, large pectin polymers were tightly bound so they were not released from fresh tissue into water (Fig. 4.1), unless the cell wall structure was disrupted allowing some dissolution (Fig.4.2A). In red-ripe HP tabasco fruit instead, there was a limited degree of pectin depolymerization comparable to levels found in ripe wild-type tomatoes (DellaPenna et al., 1990; Huber and O'Donoghue, 1993; Brummell and Labavitch, 1997). Pectin dissolution from fresh tissue however, increased slightly (Fig. 4.1B) indicating that most of the pectin was still ionically bound and maintaining the integrity of the cell wall structure. In contrast, pectin ultra-depolymerization detected in ripe fruit of the EZ genotype was similar to that found in ripe 'Hass' and 'Lula' avocado, and after incubation of enzymatically active alcohol-insoluble solids from tomato (Huber and O'Donoghue, 1993), and in tomato paste (Brummell and Labavitch, 1997). Consistent with the reduced cell to cell adhesion in this genotype (Sundberg et al., 2003), highly degraded

oligouronide could no longer hold the cell wall structure leading to its disintegration. Consequently, oligouronide easily diffused out from fresh tissue (Fig. 4.1A). These results suggest that pectin ultra-degradation exhibited by the EZ line is the consequence of an enhanced PG activity *in vivo* not detected in the HP line, despite the fact that PG activity extracted from the detachment zone was the same in both genotypes (chapter 3).

The association between the DPE and the FDF found in this study strongly suggests that the DPE has an effect on PG-mediated pectin degradation and hence on the ease of fruit separation of tabasco pepper. Higher PME activity could result in enhanced PG activity by increasing the amount of substrate available. The level of pectin esterification in the cell wall is thought to be the result of PME activity during growth (Jarvis, 1984), but the DPE during ripening varies according to the genotype and differential expression of specific PME isozymes may be involved (Pressey and Avant, 1972; Tucker et al., 1982). In tomato, PME activity extracted from disrupted fruit tissue increases during fruit ripening (Pressey and Avant, 1982; Harriman et al., 1991; Tieman et al., 1992;), but DPE either decreases dramatically (Koch and Nevins, 1989), decreases slightly (Tieman et al., 1992), or remains the same (Koch and Nevins, 1990). Similarly, the DPE in CW extracted from ripe detachment zones was different between EZ and HP tabasco pepper genotypes (Fig. 4.5). It is known that PME enhances PG activity *in vitro* (Pressey and Avant, 1982, Koch and Nevins 1989). Thus, in the EZ tabasco genotype, pectin de-esterification by higher PME activity *in vivo* may have increased the proportion of polygalacturonic acid susceptible to PG depolymerization. In contrast, pectin degradation in HP peppers appears to be limited to those de-esterified areas with their origin during fruit growth and therefore resulting in larger uronide polymers.

Total pectin content in CW varied according to the method of CW extraction and the level of pectin degradation (Fig 4.3). Metabolism and dissolution during ripening of ethanol-insoluble carbohydrates may be accounted for by the slight increase in CW uronide content during ripening. Previous studies reported a decrease in CW pectin content during fruit ripening and it was attributed to degradation (Gross and Wallner, 1979; Gross et al., 1986). The results of this study indicate that total pectin content was not reduced by PG-mediated depolymerization, but ultra-degraded oligouronide was lost during CW extraction in 60 % ethanol (Fig. 4.3).

Finally, the results obtained in this study revealed a discrepancy in the usefulness of the easy detachment characteristic in pepper. Fruit harvest is facilitated with easily detachable fruits. In fact, hand harvest of EZ tabasco fruit went faster and mechanical harvest yield was three times higher than HP fruit (Davis, 1980). In contrast, the quality of processed product in relation to textural characteristics depends directly on the integrity of pectin (Hurtado et al., 2002). Even though pectin is degraded to some extent during processing (Brummell and Labavitch, 1997; Hurtado et al., 2002), pectin metabolism in the fruit before processing preconditions its integrity. Therefore, the easy detachment characteristic of the fruit shows potential to improve mechanical harvest toward processing, but pectin ultra-degradation may affect the textural characteristics of the processed product.

CHAPTER 5. PECTIN METHYL-ESTERASE ACTIVITY *IN VIVO* IS ASSOCIATED WITH THE EASE OF FRUIT DETACHMENT IN TABASCO PEPPER

INTRODUCTION

The ease of fruit detachment from the calyx in tabasco pepper fruit has been attributed to pectin ultra-degradation (chapter 4). In addition, a lower degree of pectin esterification in cell wall extract from ripe EZ fruit was associated with the ease of fruit separation suggesting that PME activity may enhance pectin depolymerization *in vivo* (chapter 4). In tomato, PME activity has been detected at all developmental stages and several isozymes have been reported (Hall et al., 1994; Gaffe et al., 1994; Warrilow et al., 1994). PME activity was also detected in bell pepper (*C. annuum*) and was associated with fruit softening (Sethu et al., 1996).

Pectin depolymerization and dissolution is attributed to PG activity (Gross and Wallner, 1979; Huber, 1983; DellaPenna, et al., 1990). In studies with isolated cell wall or disrupted tissue, however, PG-mediated pectin depolymerization and dissolution was enhanced by PME (Pressey and Avants, 1982, Koch and Nevins, 1989). PME catalyzes the cleavage of the ester bond between the methyl and the C6 carboxyl group of galacturonosyl residues of the polyuronide chain. As a result, methanol is released and the de-esterified polygalacturonic acid is exposed to the action of PG (Brummell and Harpster, 2001). Enzyme activity studies of proteins extracted from disrupted tissue don't take into consideration that regulatory mechanisms may have been disrupted also resulting in unrestricted enzymatic activity (Brummell and Labavitch 1997). To overcome this, the effect of PME activity *in vivo* on pectin characteristics was studied using transgenic tomato plants (Tieman et al. 1992). Antisense PME suppression increased slightly the degree of pectin esterification and slightly reduced the level of pectin

depolymerization. The fruit firmness, however, was not affected. Therefore, the mechanism of pectin degradation and fruit softening remains unclear.

This study was conducted to determine the role of PME activity on the ease of fruit detachment from the calyx in tabasco pepper. A novel method to assess PME activity *in vivo* was developed because of the discrepancies between PME activity in protein extracts and the expected DPE decline (Koch and Nevins, 1989 and 1990; Tieman et al. 1992). Methanol released from fresh fruit tissue and pH in the fruit-calyx junction opening were analyzed to determine their association with the DPE decline in the EZ genotype (chapter 4) and with the ease of fruit separation. Finally, the role of PME activity on tissue integrity and on the ease of fruit separation is discussed.

MATERIALS AND METHODS

Plant Material, Fruit Ripening, and Detachment Force. Fruit from EZ and HP tabasco pepper genotypes were grown, harvested, and analyzed for ripening stage and FDF as described previously (chapter 3)

Fruit-Calyx Junction pH. Changes in pH at the fruit-calyx junction opening were determined during ripening using a micro-probe (Lazar Research Labs., Inc.. Los Angeles, CA) attached to a pH-meter (Corning Science Products, Corning NY). This probe was recommended to measure tissue surface pH and pH of small volumes (5 ：L). The pH in the fruit opening was measured immediately after the pedicel was removed. Alternatively, a drop of water (10 ：L) was placed on the fruit junction opening and the pH was measured after 10 min. The placement of a drop of water improved the contact avoiding further disruption of the tissue.

Protein Extraction. De-seeded fruit pericarp tissue was ground in liquid nitrogen and homogenized in 1mL extraction solution (1 mL of 20 mM EDTA, 2 M NaCl, pH 5). The suspension was stirred for 3h (RotoMix type 50800, Thermolyne, Dubuque, IA) and centrifuged at 14000 rpm in a microcentrifuge for 10 min. The supernatant was filtered through a 0.45 um nylon filter unit (Nalgene, Rochester, NY) and desalted through an Econo-pac® 10 DG column (Bio-Rad Lab., Hercules, CA) into 1.5 mL of eluting solution (1 mM EDTA and 10 mM NaCl, pH 5), and then tested for PME activity. All extraction steps were conducted at 5 °C. Protein content was determined by the method of Bradford (1976) using BSA as standard.

Enzyme Assay by Titration. Activity of PME was determined by titration of the carboxyl groups produced (Harriman et al., 1991). Protein extract (1 mL) was added to 30 ml substrate [0.4 % apple pectin (Sigma Chemical Co.) in 150 mM NaCl, pH 7.5]. The DPE of the apple pectin was 52 % which is equivalent to 7 % w/w of methoxy groups. The reaction solution was incubated at room temperature and titrated with 20 mM NaOH to maintain the pH. A PME activity unit was defined as to produce 1 ：mol of carboxyl groups per minute under these conditions. Control for all activity tests was performed with heat-inactivated protein samples.

Tissue Preparation. After removing the pedicel, a 3 mm long fruit DZ was used for assays unless indicated otherwise. The tissue was analyzed for methanol production either fresh (FSH) with no further disruption, or frozen (FZN) at -20 °C overnight and thawed during assay. Also, methanol production was analyzed in active freeze-dried tissue (ADT) which was frozen in liquid nitrogen and freeze-dried, and inactive freeze-dried (IDT) which was inactivated at 90°C for 20 min. in alcohol and then vacuum dried again.

Methanol Production *In Vivo*. Methanol production *in vivo* from tabasco fruit tissue was determined by gas chromatography. FSH DZ was incubated at room temperature in a 1.8 mL sealed vial. Intact whole fresh fruit was incubated in 8 mL vial fitted with a serum cap. An air sample (100 ：L) from the head space was taken periodically for three days and analyzed by gas chromatography (Varian model 3700, Walnut Creek, CA). The analyzer was set up with an Alltech 6 ft long by 1/8 inch ID stainless steel column packed with 5 % carbowax 20 M on graphpack GBAW 80-120 mesh. The carrier gas was helium, the column pressure was kept at 33 psi, and the oven temperature was 107 °C. Methanol concentration in the air space was determined by the area of the peak in comparison to those of methanol standards.

Methanol Production *In Vitro*. Methanol production *in vitro* was assessed in FZN and ADT DZ. The tissue was frozen overnight in a 1.8 mL sealed vial and then incubated at room temperature for 8 h. Air samples (100 ：L) were taken from the air space and analyzed for methanol content as described above. To evaluate methanol production from re-moistened ADT DZ, tissue was incubated in a 1.8 mL vial with 200 ：L of water at 35 °C for 20 h. IDT DZ was used as control. Similarly, methanol production from ADT DZ was evaluated in 200 ：L of 50 mM NaOAc buffer at pH 5 and pH 6.0, and in 200 ：L of 50 mM Na_2HPO_4 buffer at pH 7.0. Methanol content of the solutions were determined based on the methanol content in air samples of standard solutions.

RESULTS

Fruit-Calyx Junction pH. The pH at the fruit-calyx junction area was measured to determine variations in the apoplastic environment during fruit ripening. The pH in HP fruit was maintained throughout ripening and ranged between pH 5.6 and pH 6.3 (Fig 5.1). In contrast, the

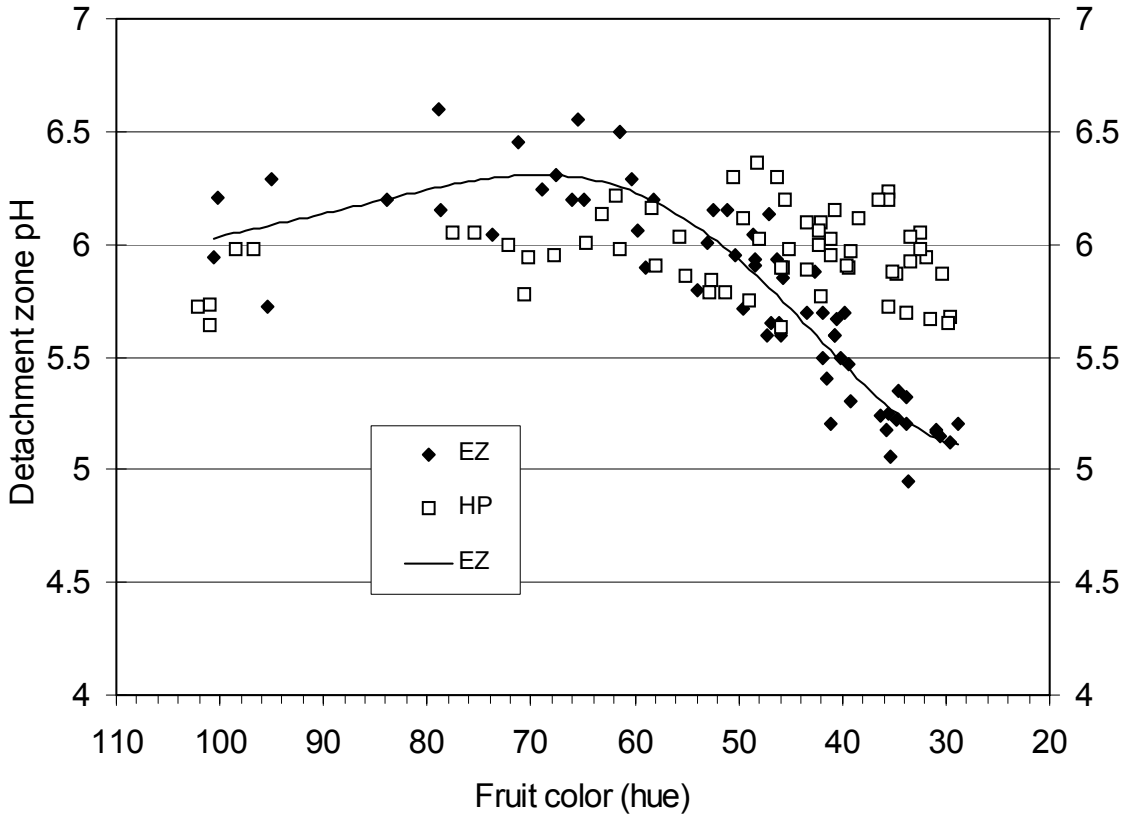

Fig. 5.1. Fruit detachment zone pH throughout fruit ripening in 'Easy Pick' (EZ) and 'Hard Pick' (HP) tabasco pepper. The pH was determined in the fruit opening immediately after pedicel removal. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Coefficient of determination: r^2=0.85 (solid line).

pH in the EZ genotype decreased to an average of pH 5.2 in ripe fruit (hue < 40). The average pH of EZ fruit with hue less than 40 was 0.6 units lower than the pH of HP fruit. Acidification of the apoplastic environment in ripe EZ fruit was considered to be the result of PME mediated pectin de-esterification that freed carboxyl groups in the polyuronide chain.

Enzyme Assay by Titration. Activity of PME was detected in protein extracts from EZ and HP fruit pericarp throughout ripening (Fig. 5.2). PME extract and detection in ripe tissue was increased by maintaining the pH below pH 5.0 and desalting. Activity extracted from DZ was unreliable because of the little sample available. In immature-green tissue (hue >100), PME was high in both genotypes but dropped drastically in mature-green fruit (hue <100) at the beginning of ripening. PME activity stayed low in HP fruit throughout the ripening process. In contrast, PME activity in the EZ genotype increased in ripe fruit (hue between 55 and 35) and decreased in purple-red tissue (hue <35). There was a negative correlation (r = -0.74) between FDF and PME activity during fruit ripening (between hue95 and hue 30) with data from green fruit excluded. This association suggests that there was a ripening specific PME activity in the EZ genotype that had an effect on the ease of fruit detachment from the calyx.

Methanol Production *In Vivo*. Pectin de-esterification by PME activity in vivo was assessed by the methanol released from FSH DZ (Figs. 5.3 and 5.4). A significant amount of methanol released from EZ FSH DZ was detected within 20 hours when the ripening stage was between hue 52 and hue 40 (dark orange to red) (Fig. 5.3A) . Methanol production was not detected in immature-green and early ripening stages (hue >55) (mature-green to orange) and in purple-red fruit tissue (hue <38). Fruit tissue in early ripening stages (hue 80 to 54) incubated for 72 hours reached the adequate ripening stage and released significant amounts of methanol (Fig.

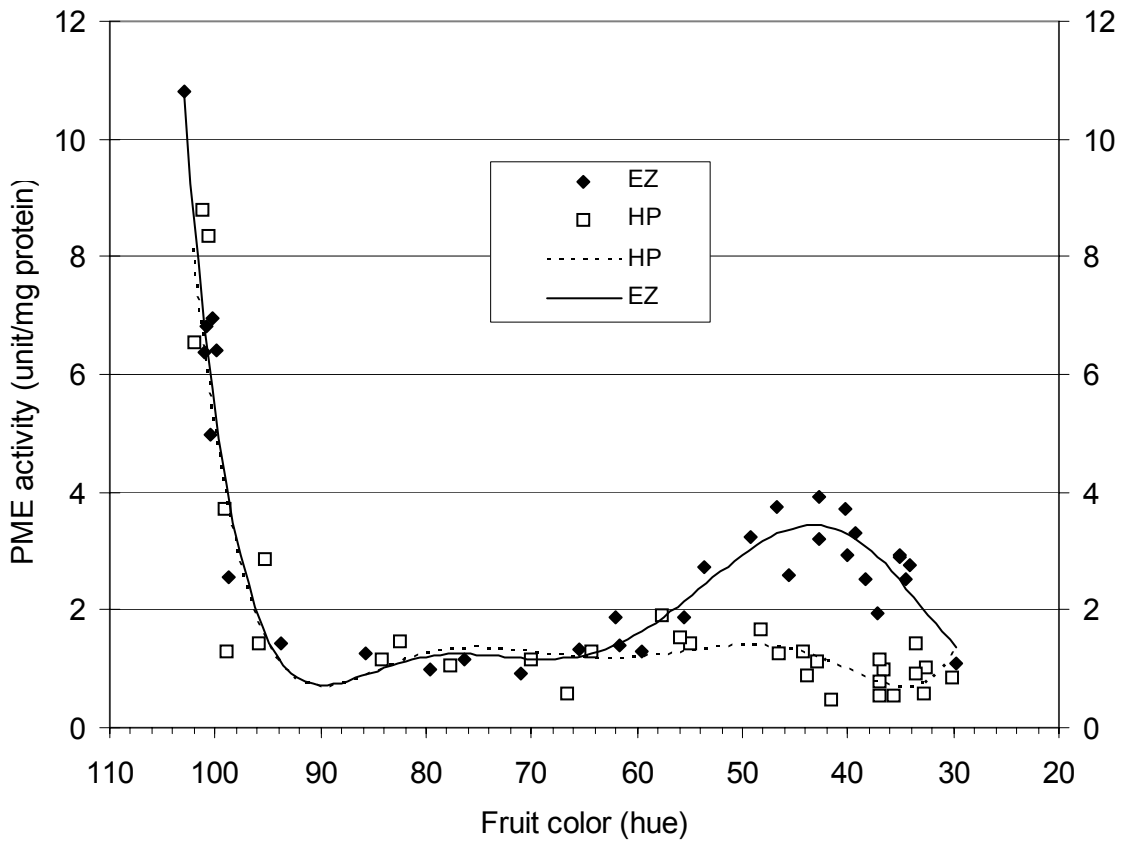

Fig. 5.2. Pectin methyl-esterase (PME) activity during fruit ripening in 'Easy Pick' (EZ) and 'Hard Pick (HP) tabasco pepper. Activity in protein extracts from fruit pericarp was tested by titration of carboxyl group produced. One activity unit releases 1 ：mol H^{+}"min^{-1}. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Coefficient of determination: r^2= 0.83 (dash line) and r^2= 0.93 (solid line).

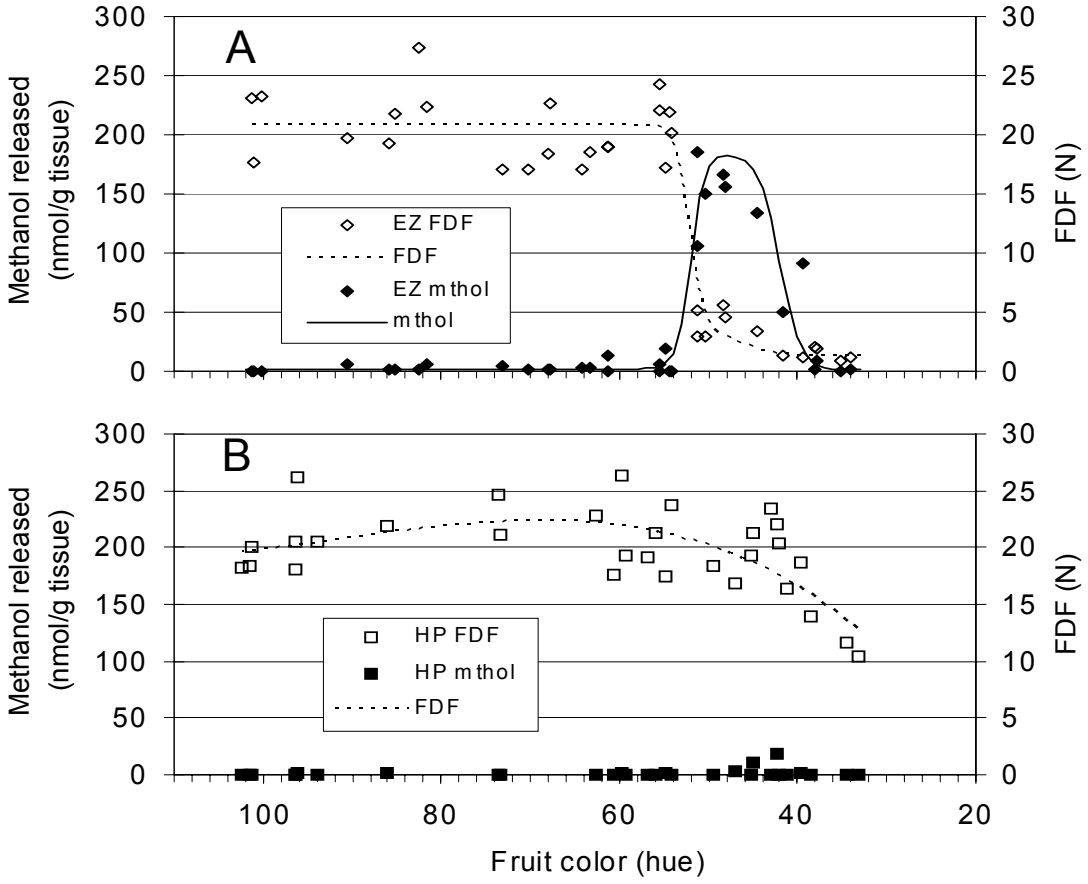

Fig. 5.3. Methanol released from fresh tissue (black symbols) and fruit detachment force (FDF) (open symbols) during fruit ripening in 'Easy Pick' (EZ) (**A**) and Hard Pick (HP) (**B**) tabasco pepper. Methanol content in the air space was determined by gas chromatography after 20 h. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Coefficient of determination in **A**: r^2= 0.92 (dash line) and r^2= 0.91 (solid line), and in **B**: r^2= 0.45 (dash line).

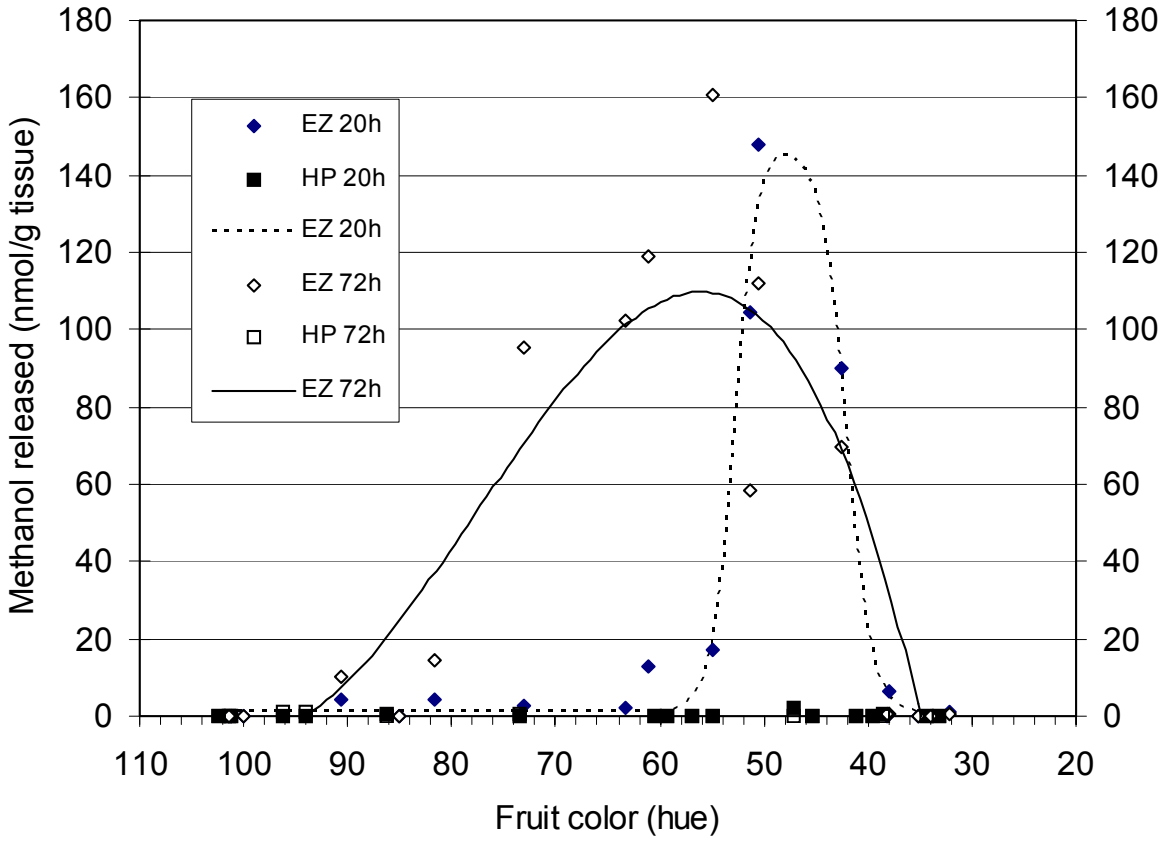

Fig. 5.4. Methanol released from fresh tissue after 20 h and 72 h. Methanol content in the air space was determined by gas chromatography. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). (EZ) 'Easy Pick' and (HP) 'Hard Pick' tabasco pepper genotypes. Coefficient of determination: r^2= 0.98 (dash line) and r^2= 0.79 (solid line).

5.4). In contrast, methanol production was not detected in the HP line throughout ripening even when incubation time was 72 hour (Figs. 5.3B and 5.4). Methanol production between hue 102 and hue 45 (mature-green to methanol peak) followed an inverse relationship (r = -0.94) with FDF in the EZ tabasco genotype (Fig. 5.3A). In the early ripening stages (hue >53) when the FDF was high, methanol production was not detected. Then, between hue 52 and 47 when FDF decreased, methanol production increased reaching its maximum. Although methanol was undetectable or very low after hue 40, FDF stayed low until the end of the ripening process (hue 30). In contrast, no relationship could be found in the HP genotype since methanol was not detected (Fig. 5.3B). The same relationship between methanol production and FDF in the EZ genotype was found with intact fruits and no methanol was detected in HP samples (data not presented).

Methanol Production *In Vitro*. Methanol released from FZN and ADT DZ was analyzed throughout ripening to assess PME activity in disrupted tissue. Both tabasco genotypes produced detectable amounts of methanol from thawed FZN tissue (Fig. 5.5) and from ADT DZ soaked in water, but not from IDT (Fig. 5.6). This indicates that methanol is released by an enzymatically active metabolic process. Methanol released from FZN and ADT DZ varied among plants of the same genotype but there was no difference between genotypes throughout ripening. Methanol production was high in immature-green fruit (hue >95), decreased at the beginning of ripening until the orange stage (hue 90 to 55), and then increased again in ripe tissue (hue 55 to 30). The level of methanol released per amount of tissue differed between FZN and ADT because; for FZN samples, methanol released corresponds to air methanol content, and for ADT samples corresponds to total methanol in solution (Figs. 5.5 and 5.6). The pattern of

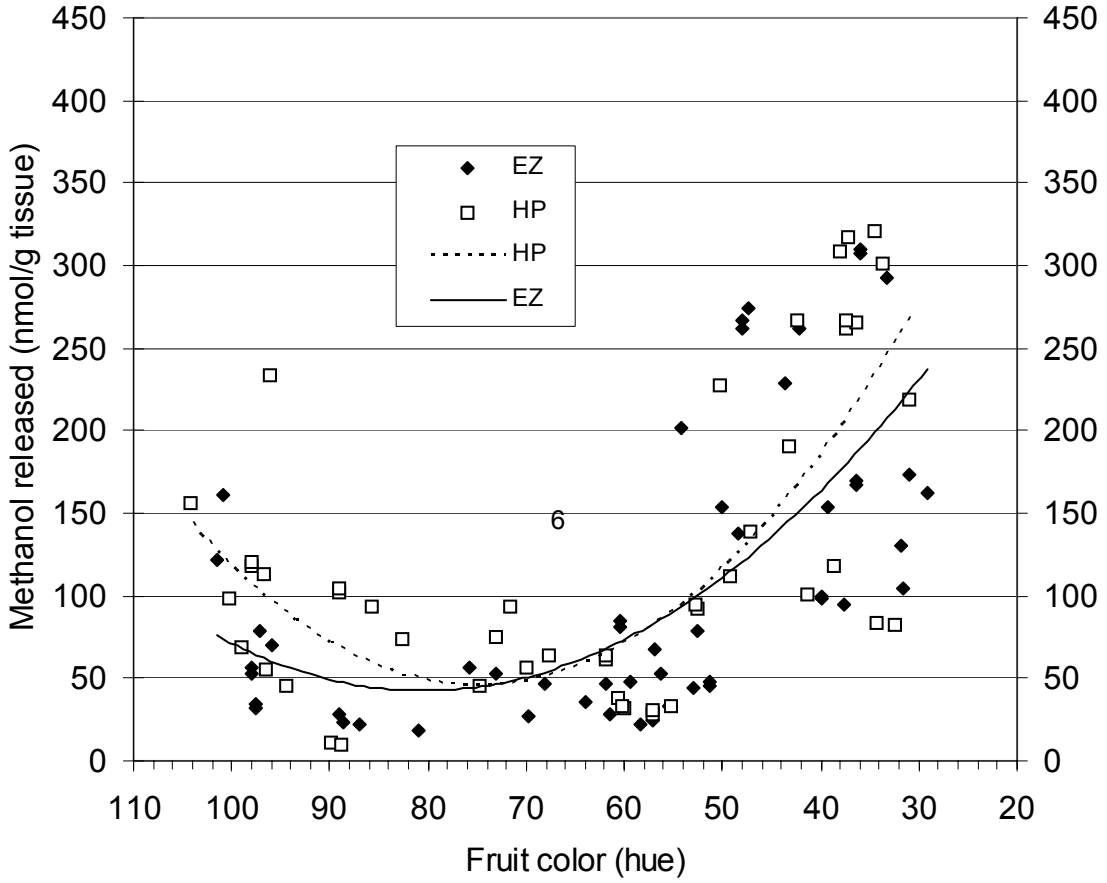

Fig. 5.5. Methanol released from disrupted (frozen (FZN)) tissue during fruit ripening. Methanol content in air space after 8 h was determined by gas chromatography. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). (EZ) 'Easy Pick' and (HP) 'Hard Pick' tabasco pepper genotypes. Coefficient of determination: r^2= 0.52 (dash line) and r^2= 0.43 (solid line).

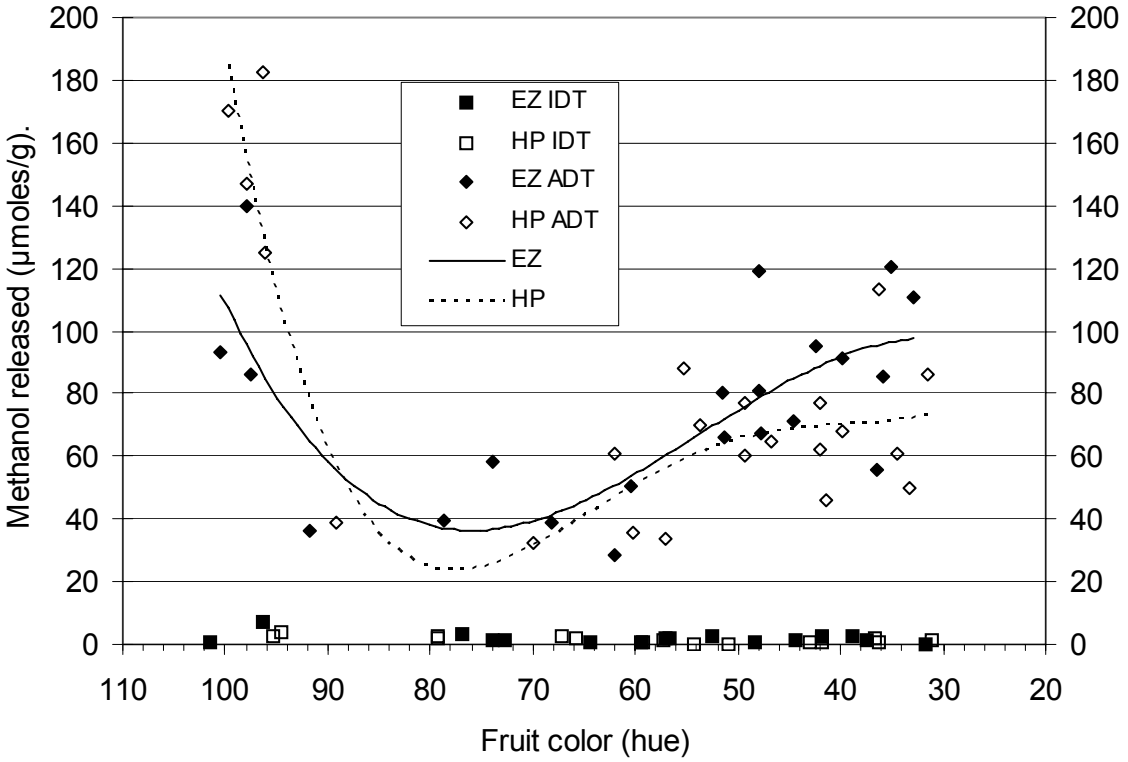

Fig. 5.6. Methanol released from remoistened freeze-dried (ADT) and heat-inactive dried (IDT) fruit tissue during fruit ripening. Total methanol content in solution after 20 h was obtained from the methanol concentration in the air space determined by gas chromatography. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). (EZ) 'Easy Pick' and (HP) 'Hard Pick' tabasco pepper genotypes. Coefficient of determination: r^2= 0.78 (dash line) and r^2= 0.53 (solid line).

methanol released during ripening for ADT was comparable to the pattern of protein extract with exception of HP ripe tissue (Fig 5.2). PME activity increased in disrupted (FZN and ADT) ripe HP tissue to the same level detected in the EZ genotype, which indicates that a PME activity was expressed by a similar amount.

Methanol released from ADT DZ soaked in buffer at pH 5 (Fig. 5.7A), pH 6 (Fig. 5.7B), and pH 7 (Fig. 5.7C) was tested throughout ripening to evaluated PME activity *in situ* at expected apoplastic pH conditions. Methanol released increased in tissue from both genotypes with increasing solution pH. The pattern of methanol production during ripening was the same as in water soaked tissue. The amount of methanol released from ripe (hue <55) fruit tissue at pH 6, however, was higher in the EZ genotype (38 ：mol/g ADT) than in the HP genotype (19 ：mol/g ADT). Methanol released at pH 5 an pH 7 were not significantly different throughout ripening. These results indicates that PME activity was affected by pH conditions and PME activity can be detected at pH found in the apoplast.

DISCUSSION

The ease of fruit detachment from the calyx is caused by pectin ultra-degradation (chapter 4) which is enhanced by a ripening specific PME activity. In the EZ genotype, the FDF drop coincided with and was associated with the peak of methanol production *in vivo* which was attributed to PME activity (Fig 5.3A). These results suggest that in the EZ genotype, PME activity *in vivo* reduced the DPE, thereby resulting in pectin ultra-degradation (chapter 4). Consequently, the cell wall is disintegrated causing the fruit to detach easily from the calyx. In contrast, methanol was not detected in the HP genotype (Fig 5.3B) which requires higher force for fruit detachment. In addition, contrary to the results of PME activity in protein extracts,

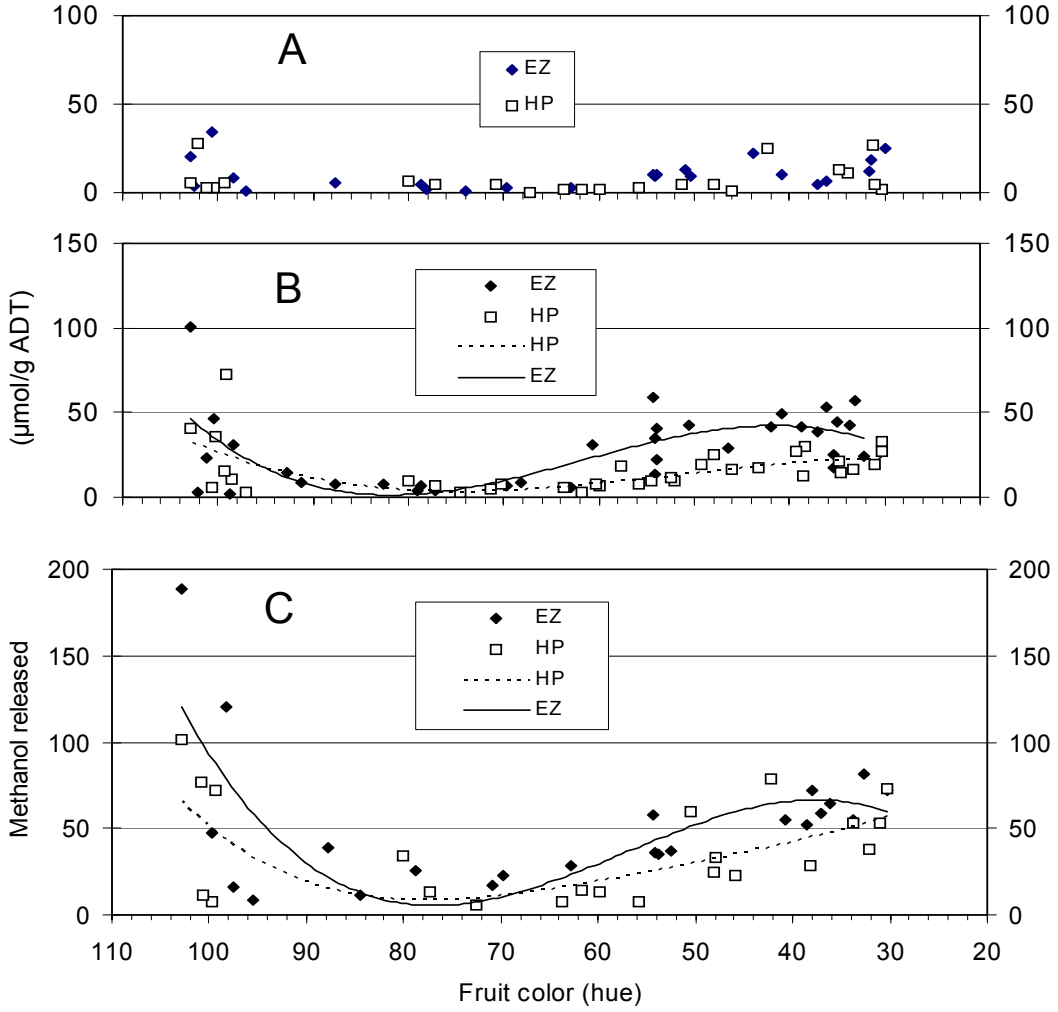

Fig. 5.7. Methanol released at pH 5.0 (**A**), pH 6.0 (**B**), and pH 7.0 (**C**) from remoistened freeze-dried tissue (ADT) during fruit ripening. Total methanol content in solution after 20 h was obtained from the methanol concentration in the air space determined by gas chromatography. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). (EZ) 'Easy Pick' and (HP) 'Hard Pick' tabasco pepper genotypes. Coefficient of determination in **B:** r^2= 0.35 (dash line) and r^2= 0.44 (solid line), and in **C:** r^2= 0.39 (dash line) and r^2= 0.52 (solid line).

methanol was not detected in HP tissue and in mature-green EZ tissue. This suggests that there may be a regulatory mechanism blocking PME activity *in vivo*, and that the ripening specific PME activity in the EZ genotype is not affected.

Methanol production from plant tissue was attributed to PME activity. There is enough evidence indicating that methanol is released by PME which catalyzes the de-methoxylation of galacturonosyl residues in the pectin polymer (Wood and Siddiqui, 1971; Bartolome and Hoff, 1972; Frenkel, et al., 1998; Brummell and Harpter, 2001). In this study, methanol released *in vivo* in the EZ genotype was associated with and may explain the DPE decrease in CW (chapter 4), and the pH decrease in the detachment opening during ripening (Fig 5.1). Decrease of DPE during ripening has been associated with PME activity *in vitro* (Koch and Nevins, 1989 Tieman et al., 1992). Antisense suppression of PME activity *in vivo* though, resulted in a slight DPE increase in tomato fruit tissue (Tieman et al., 1992). Also, pH in tomato apoplastic fluids decreased during ripening (Almeida and Huber, 1999). There is no previous report of methanol released from live plant tissue associated with PME activity *in vivo*. Free methanol content in crude plant homogenates though, was attributed to PME activity *in vivo* (Bartolome and Hoff, 1972; Frenkel, et al., 1998; Koch et al., 1999). In tabasco pepper, methanol released from disrupted tissue (FZN and ADT) was associated with PME activity *in vitro* throughout ripening (Figs. 5.5 and 5.6). In addition, methanol production was not detected in heat-inactivated IDT which indicates that the reaction responsible for methanol production is mediated by enzymatic activity.

PME activity is present in all ripening stages as determined by *in vitro* assays, although variations throughout ripening were detected (Figs. 5.2, 5.5 and 5.6). The level of PME activity

and pattern during ripening detected in tabasco fruit extracts were similar to those reported for bell pepper (Sethu et al., 1996), but 10 times lower than tomato (Harriman et al., 1991; Warrilow et al., 1994). Methanol production *in vitro* from disrupted tissue developed a similar pattern as PME activity in EZ protein extracts: high in immature-green tissue, decreased in early ripening, and increased again in ripe fruit (Figs. 5.2, 5.5 and 5.6). The high PME activity *in vitro* detected in immature-green fruit of both genotypes appears to have no effect on the apoplastic pH (Fig. 5.1) and on the DPE (chapter 4). In contrast, The higher PME activity *in vivo* detected in EZ ripe fruit (Fig. 5.3) was associated with the lower apoplastic pH and DPE in this genotype (Fig. 5.1 and chapter 4). These results may explain the inconsistency in reports about PME effect on DPE (Koch and Nevins, 1989 and 1990; Tieman et al., 1992). Detection of high PME activity in immature-green fruit though, support the requirement of pectin assembly during growth and development: pectin is synthesized fully esterified so it can be transported in solution and the process of de-esterification occurs at the destination site by PME as required (Jarvis 1984).

The current study suggests that the PME activity *in vivo* in the EZ genotype is a ripening specific PME isozyme. Multiple PME forms were identified in tomato depending on fruit ripening stage and variety (Pressey and Avant, 1972, Tucker et al.,1982, Warrilow et al., 1994). Two iso-forms were present in mature-green and ripe fruit, one form appeared in ripe fruit only, and another form was present in one variety out of three studied (Pressey and Avant, 1972). Also, different physiological functions, such as cell wall growth or degradation during ripening, were suggested for isozymes with different kinetic properties (Warrilow and Jones, 1995). In tabasco pepper, the PME activity *in vivo* was detected in ripe EZ fruit only and coincided with

higher activity in protein extracts from ripe fruit (Fig. 5.2). Serological and molecular studies are necessary to determine the expression characteristic of this PME isozyme.

Pectin ultra-degradation in EZ ripening fruit (chapter 4) is initiated by the ripening specific PME activity. Although pectin depolymerization occurred in both lines during fruit ripening, the uronide molecular size in EZ ripe fruit was much smaller than the HP genotype (chapter 4). The enhancement of PG-mediated pectin depolymerization and dissolution by PME activity *in vitro* was demonstrated with isolated cell wall and attributed to a reduction in the DPE (Pressey and Avant, 1982, Koch and Nevins 1989). Also, antisense suppression of PME in tomato resulted in a slight reduction of PG-mediated pectin depolymerization (Tieman et al., 1992). In tabasco pepper, the DPE reduction occurred in fruits of the EZ line only (chapter 4). These results suggest that the metabolic mechanism leading to the ease of fruit detachment from the calyx begins with the ripening specific PME activity (Fig. 5.3). This activity reduces the DPE (chapter 4) leaving higher amount of polygalacturonic acid available for PG action (Pressey and Avant, 1982, Tieman et al., 1992). Consequently, PG activity is enhanced resulting in pectin ultra-depolymerization and dissolution (chapter 4). In conclusion, this enhanced pectin degradation causes the disintegration of the fruit tissue and decreases the FDF required to separate EZ ripe fruit from the calyx (chapter 3). In contrast, PME activity *in vivo* seems to be obstructed in the HP genotype (Fig 5.3B) and the DPE does not change during ripening (chapter 4). Therefore, PG can act only on pectin de-esterified areas originated during growth resulting in limited depolymerization and low dissolution (chapter 4). The limited pectin depolymerization maintained the integrity of the cell wall in the fruit-calyx junction zone making the separation more difficult (Fig 5.3B).

A regulatory mechanism seems to be involved in PME activity *in vivo* and thus it has a critical role in controlling cell wall integrity. This is supported by the difference in methanol production between live and disrupted tissue (Figs. 5.3, 5.5 and 5.6). When the tissue is disrupted, this mechanism is also disrupted and unrestricted PME activity is detected as assayed by methanol production from FZN and ADT, and titration in protein extracts (Fig 5.2). In tomato, PG-mediated pectin degradation is normally restricted *in vivo*, but in homogenized tissue (Brummell and Labavitch, 1997) or in active CW extract (Koch and Nevins, 1989; Huber and O'Donoghue, 1993) the restriction was removed and pectin ultra-degradation and dissolution occurred. This can be explained by the activation of PME in disrupted tissue enhancing PG-mediated pectin depolymerization. In conclusion, PME and its regulatory mechanism have a critical role in cell wall stability during growth, and in pectin degradation and dissolution during fruit ripening. In addition, the data indicate that PME is the limiting factor for PG-mediated pectin ultra-degradation *in vivo*.

CHAPTER 6. PECTIN METHYL-ESTERASE ISOFORM ASSOCIATED WITH THE EASE OF FRUIT DETACHMENT IN TABASCO PEPPER

INTRODUCTION

In tabasco pepper, the ease of fruit detachment from the calyx is reportedly related to pectin ultra-degradation (Chapter 4) which is induced by ripening specific pectin methyl-esterase (PME) activity (Chapter 5). PME activity *in vivo* was detected only in the 'Easy Pick' (EZ) tabasco genotype by the methanol released from ripe fruit tissue. In tomato, PME protein and activity were detected throughout fruit development, increasing from early fruit development to a peak at the onset of ripening and then declining slightly in ripe fruit (Harriman et al., 1991; Tieman et al., 1992). At least four PME genes were reported in tomato and several of them were highly homologous (Harriman et al., 1991; Hall et al., 1994; Gaffe et al., 1997). Although these isoforms have been detected in ripe tomato, they were expressed mainly in mature-green fruit. The molecular size of these PME proteins ranged between 33k to 44k (Harriman et al., 1991; Warrilow et al., 1994; Gaffe et al., 1997). An additional ripening specific PME isoform was detected in ripe tomato and another was present in one variety out of three investigated (Pressey and Avants, 1972). Molecular characterization of these ripening specific PME isoforms in tomato were not reported. Similarly, characterization of the PME activity detected in pepper has not been previously reported.

The objective of this study was to isolate PME activities in immature-green and ripe tabasco pepper fruit, and to determine the molecular characteristics of the ripening specific PME isoform associated with the ease of fruit detachment from the calyx. To reach these goals, a colorimetric PME-activity test was developed to detect pH decreases in the substrate solution. PME-mediated pectin de-esterification generates free carboxyl groups in each galacturonan

component of the chain reducing the solution pH. Similarly, localized pH reduction in a gel was used to identify PME isoforms after gel electrophoresis.

MATERIALS AND METHODS

Plant Material, Fruit Ripening, and Detachment Force. Tabasco pepper fruit from EZ and HP genotypes were analyzed for color and FDF as described previously (Chapter 3).

Protein Extraction. Cell wall proteins were extracted from immature and mature-green fruit pericarp following the method of Warrilow and coworkers (Warrilow et al., 1994) with modifications. All the steps during extraction were conducted at 5 °C. Tissue (30 g) was pulverized in liquid N_2 with a mortar and pestle and homogenized in two volumes (v/w) of cold homogenization solution (1% Triton X-100 and 0.1% ascorbic acid, pH 3.3). The slurry was centrifuged for 10 min at 23,700 g (Avanti J-25, rotor JA 25.50, Beckman-Coulter, Palo Alto, CA) and the supernatant was discarded after checking for PME activity. The pellet was rinsed once by suspending it in water followed by centrifugation. The second pellet was suspended in an equal volume (v/w initial weight) of extraction solution (2 M NaCl, pH 7.5) and stirred for 4 h. The slurry was centrifuged again and the supernatant was saved as CW-proteins. Proteins were fractionated with ammonium sulfate at 35% and 75% and the pellets were suspended in buffer (10 mM Na_2HPO_4, 10 mM NaCl, pH 7.5). The ammonium sulfate fractions were desalted through Econo-pac® 10 DG columns (Bio-Rad Lab.) into the same buffer and assayed for protein content and PME activity.

A different method was followed to extract protein from red-ripe fruit pericarp because high levels of PME activity was present in the first supernatant (homogenization solution) and the activity was lost rapidly when extracted in 2 M NaCl at pH 7.5. Total proteins were extracted

directly from 20 g of tissue pulverized in liquid N_2 with 2 volumes (v/w) extraction solution (10 mM EDTA and 2 M NaCl, pH 5). The slurry was stirred for 2 h and centrifuged as described above. The supernatant was filtered through glass microfibre filters GF/C (Whatman, England) and then through 0.45 um nylon syringe filter units (Nalgene, Rochester, NY) to separate debri. Samples were desalted as described above into 1 mM EDTA, 150 mM NaCl, pH 5 and assayed for PME activity and protein content.

Separation. PME separation was conducted by ion-exchange chromatography in 1.5 x 15 cm column of CM-Sepharose CL6B. All the steps were conducted at 5 °C. Desalted protein extract (4 mL) from mature-green fruit was applied to the column equilibrated with elution buffer (10 mM Na_2HPO_4 and 10 mM NaCl, pH 7.5) and washed with 30 mL elution buffer. Then, a 400 mL linear gradient from 10 to 500 mM NaCl in elution buffer was applied. The column was washed further with 30 mL of 1 M NaCl. The conditions used to separate PME from ripe fruit were different. A Biologic LP system (Bio-Rad Lab.) equipped with a UV detector and an electrical conductivity cell was used. Desalted protein extract (25 mL) was applied to the CM-Sepharose column equilibrated and run with 10 mM NaOAc, pH 5.0, 1 mM EDTA, and 150 mM NaCl. Then, a 250 mL linear gradient from 150 to 500 mM NaCl in running buffer was applied followed by 30 mL 1 M NaCl. The flow in all separation processes was 0.5 mL$\cdot$min^{-1} and 5 mL fractions were collected. Fractions were monitored for PME activity and the active fractions of each peak were used for further analysis.

Gel Electrophoresis. Gel electrophoresis under denaturing conditions (SDS-PAGE) was conducted with PME active samples (Laemmli, 1970). An aliquot was mixed with an equal volume of 2x dissociation buffer (62.5 mM tris pH 6.8, 2 % SDS, 5 % mercaptoethanol, 10 %

glycerol, 0.002 % bromophenol blue), heated at 100 °C for 4 min, and cooled down in ice. Protein samples from desalted extract (20 ：g) and from active peak fraction (0.5 - 2 ：g) were applied to a discontinuous gel (4 % polyacrylamide for stacking and 12 % for separation) and run at 30 mA until the dye front was 1 cm from the bottom. Because the low protein concentration in the fractions, proteins in 1 mL active fraction were precipitated by adding ethanol to 60 %. The pellet was rinsed with acetone, suspended in 20 ：L 10 mM tris-HCl, pH 6.8 and 20 ：L 2x dissociation buffer, and analyzed by SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R-250, or silver following the procedures of the kit's manufacturer (Bio-Rad). The MW of the PME protein was calculated by the relative mobility (RM) in relation to marker proteins. The marker proteins were bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100), and "-lactalbumin (14,200).

Pectin Methyl-Esterase Activity Assays. PME activity in fractions from ion-exchange chromatography was monitored by a colorimetric assay which assesses pH reduction in the reaction mixture. Fraction samples (20 ：L) were mixed with 200 ：L substrate (0.3 % apple pectin, 150 mM NaCl, 2 mM $NaP0_4$, pH 7.5, and 0.01 % bromothymol blue) in 96-cell micro-plate (one cell per fraction). PME mediated pectin de-esterification reduces the pH by generating free carboxyl groups in each galacturonan component of the chain. As pH decreased in the active samples, bromothymol blue in the solution changed color from blue (pH 7.5) to yellow (pH 6.0). Absorbance at 600 nm was measured in a microplate reader (EL311, Bio-Tek Instruments, Winooski, VT) every 30 min to quantify changes in color. PME activity was expressed as the absorbance reduction in one hour.

PME activity was detected also by colorimetric assessment of pH reduction directly in the gel after SDS-PAGE. After the run, the gel was rinsed three times in 100 mL of cold 10 mM $NaP0_4$ and 10 mM DTT, pH 5.5 for 20 min each. The pH was increased by rinsing in 200 mL 20 mM $NaP0_4$, pH 7.5 for 20 min and then the gel was stained in 200 mL 0.01% bromothymol blue and 2 mM $NaP0_4$, pH 7.5, for another 20 min. The gel was put in a petri dish and warm solution (1 % agarose, 0.3 % pectin, 2 mM $NaP0_4$, pH 7.5, and 0.05% bromothymol blue) was poured to form a three mm thick gel on top. The gel was kept at room temperature in the dark for 24 h and the appearance of a green-yellowish band indicating a localized pH reduction was considered to be PME activity.

RESULTS

Separation. Cell wall proteins extracted from immature and mature-green fruit, and total proteins extracted from red-mature fruit were separated by ion-exchange chromatography and analyzed for PME activity. Only 6% of the cell wall proteins extracted from green fruit pericarp and 2% of the total protein extracted from ripe pericarp were recovered in the fractions with PME activity. The colorimetric method used to monitor fractions detected PME activity in 20 ：L samples containing less than 300 ng protein (Fig. 6.1). Activity peaks were more noticeable by quantitative measurement of the absorbance reduction at 600 nm as the solution pH decreased (Fig. 6.2). Chromatography activity profiles of green fruit tissue revealed three main PME activity peaks (Fig. 6.2). The activity profiles from both tabasco pepper genotypes were similar indicating that the same PME isoforms were present in both genotypes.

CM-Sepharose chromatography profiles of total protein extracted from ripe fruit pericarp are shown in Fig. 6.3. A large protein peak eluted between 30 and 160 min which corresponds to

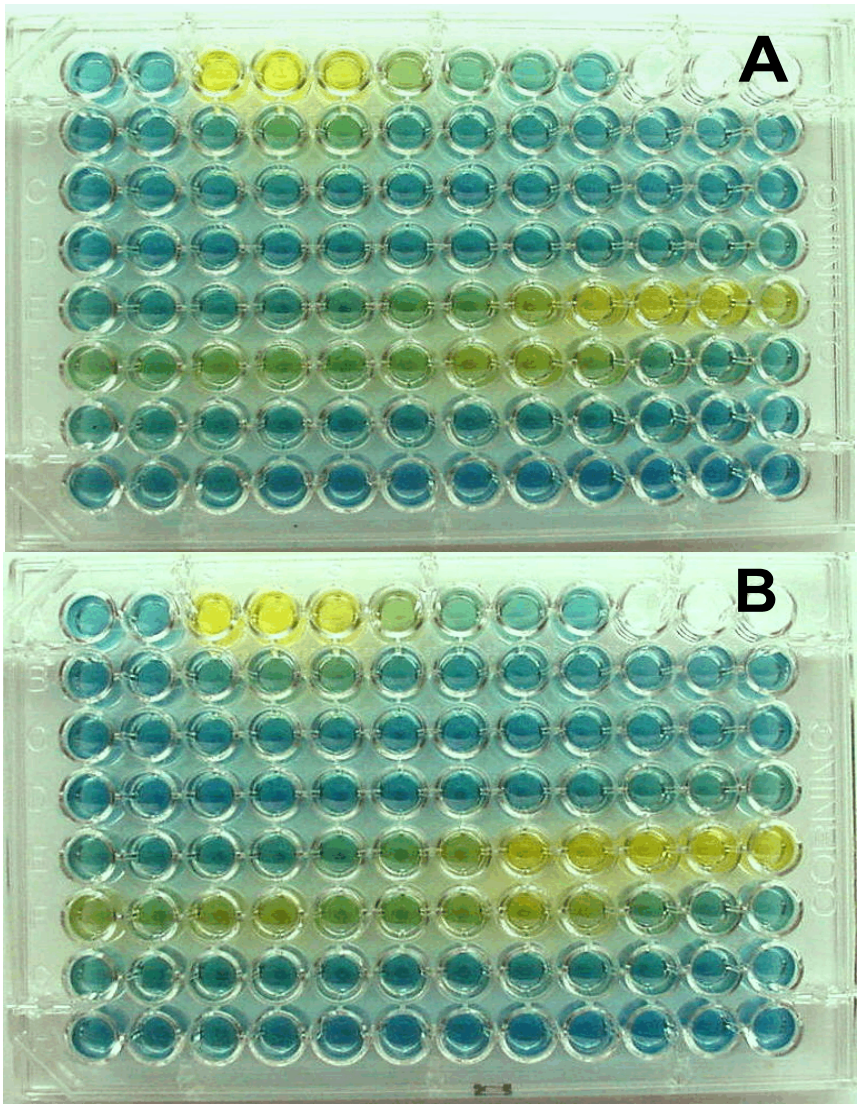

Fig. 6.1. Colorimetric assessment of pH reduction (blue = pH 7.5, yellow = pH 6.0) by pectin methyl-esterase (PME) activity. Cell wall proteins from mature-green fruit were separated by ion-exchange chromatography in a CM-sepharose column (1.5 x 15 cm) and eluted with a gradient from 10 mM to 500 mM NaCl. Cells A-1 and A-2 are blanks. Cells A-3 to A-9 correspond to pH range (pH 6.0 - pH 7.5). Cells B-1 to H-12 correspond to fractions 1 to 88. (**A**) 'Easy Pick' and (**B**) 'Hard Pick' tabasco pepper genotypes.

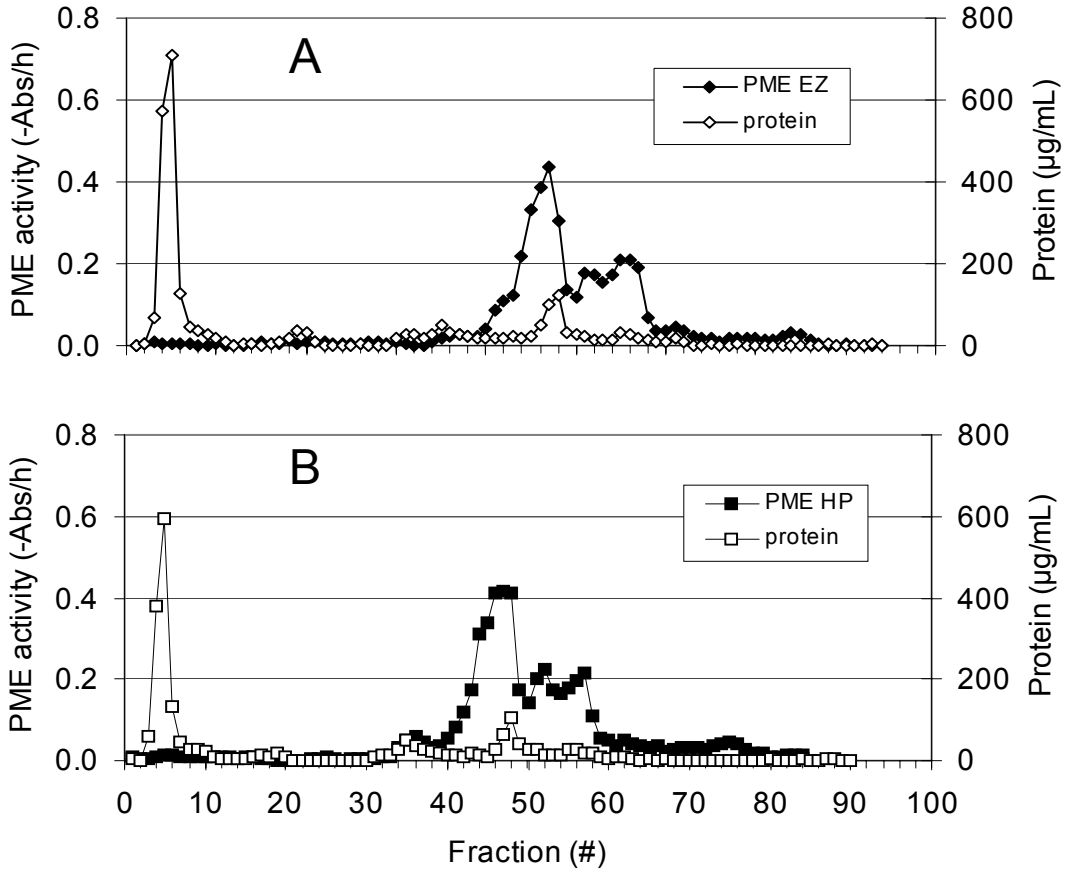

Fig. 6.2. Fractionation of pectin methyl-esterases (PME). Cell wall proteins from mature-green fruit pericarp were separated by ion-exchange chromatography in a CM-sepharose column (1.5 x 15 cm) and eluted with a gradient from 10 mM to 500 mM NaCl. PME activity determined by colorimetric assay: absorbance (600 nm) reduction (-Abs) in 1h. (**A**) 'Easy Pick' (EZ) and (**B**) 'Hard Pick' (HP) tabasco pepper genotypes. Protein content (open symbols) and PME activity (closed symbols).

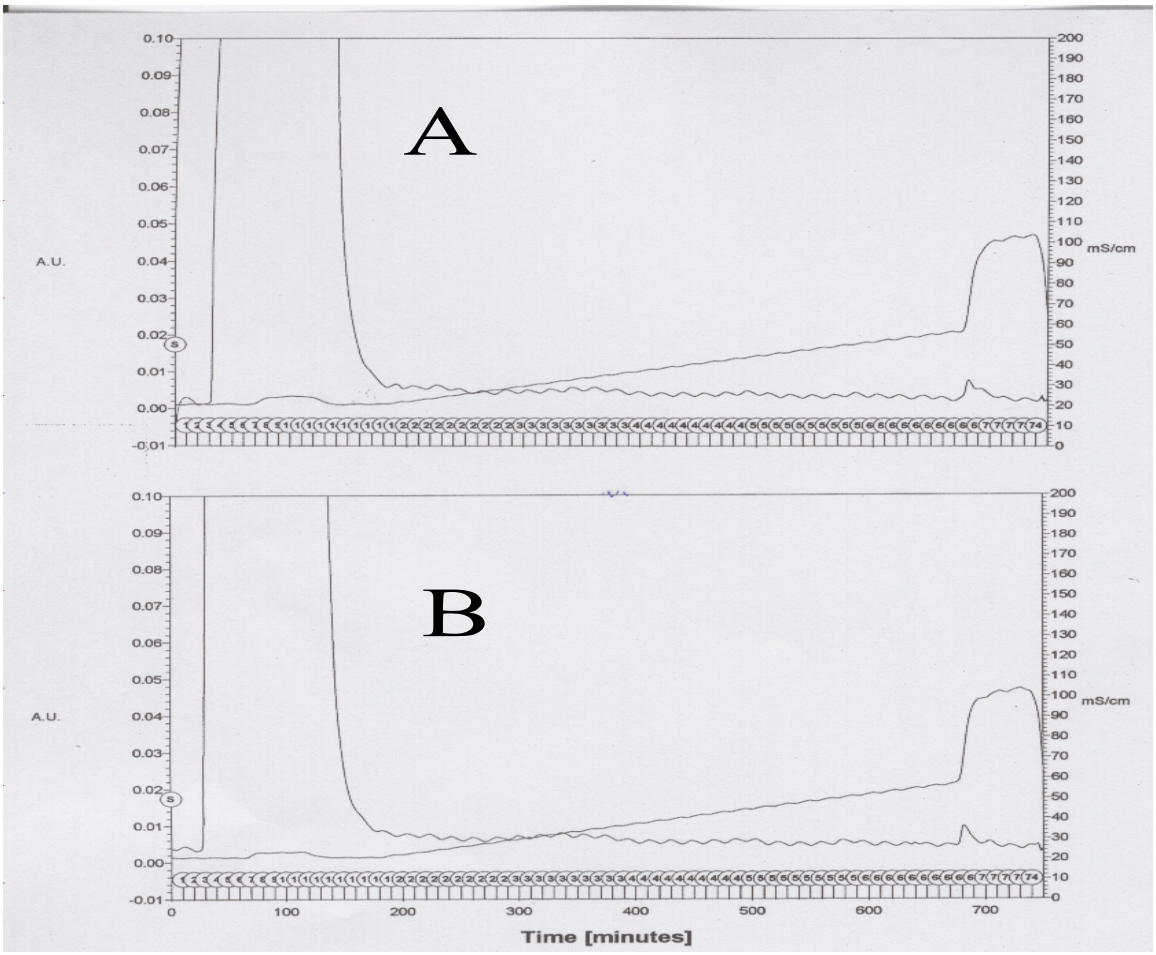

Fig 6.3. Fractionation of pectin methyl-esterases (PME). Total proteins from mature-red fruit pericarp were separated by ion-exchange chromatography in a CM-sepharose column (1.5 x 15 cm) and eluted with a gradient from 150 mM to 500 mM NaCl. Protein content is indicated by UV absorbance (AU) and NaCl concentration is indicated by the electrical conductivity (mS/cm) of the eluting solution. (**A**) 'Easy Pick' and (**B**) 'Hard Pick' tabasco pepper genotypes.

the proteins that were not retained by the gel. This flow-through protein accounted for 98% of total protein assayed. A slight protein dome eluted between 320 and 390 min and another one when the NaCl concentration increased to 1 M (680 min) as indicated by UV light absorbance. There were no apparent differences between the profiles from the tabasco genotypes. In contrast, the activity profiles of the fractions revealed two PME active peaks (named PME-1 and PME-2) in the EZ genotype and only one (PME-2) in the HP genotype (Figs. 6.4 and 6.5). The first PME active peak (PME-1) in the EZ chromatography profile (fractions 27-29) eluted at a NaCl concentration equivalent to an electrical conductivity of 27 $mS \cdot cm^{-1}$ (Table 6.1). The amount of activity recovered for this isoform was variable among the extracted samples (Table 6.1). PME-1 was detected consistently in protein extract from EZ fruit only. PME-2 was present consistently in the HP genotype, however, in the EZ genotype it was present in fruits from two out of three plants used for extraction. PME-2 from both genotypes eluted at a NaCl concentration represented by an electrical conductivity of 33 $mS \cdot cm^{-1}$ under the chromatographic conditions described previously (Table 6.1). The decrease in absorbance detected in fractions 60 to 74 of the EZ activity profile occurred in the first hour and then stopped without reaching the bright yellow color (pH < 6.0) expected with PME activity (Fig. 6.5A). In addition, absorbance reduction in these fractions was not detected in the profiles of other EZ extractions.

Molecular Characteristics. Proteins in PME-1 and PME-2 active fractions were analyzed by SDS-PAGE (Fig. 6.6). EZ PME-1 active fractions were resolved into two protein bands (Fig. 6.6, lane EZ-PME-1) and with heavier loads additional faint bands were resolved. The molecular size of proteins consistently present in PME-1 fractions were 22,500 and 36,700. In gels assayed to for localized pH reduction, a yellow-green band appeared in the PME-1 lanes

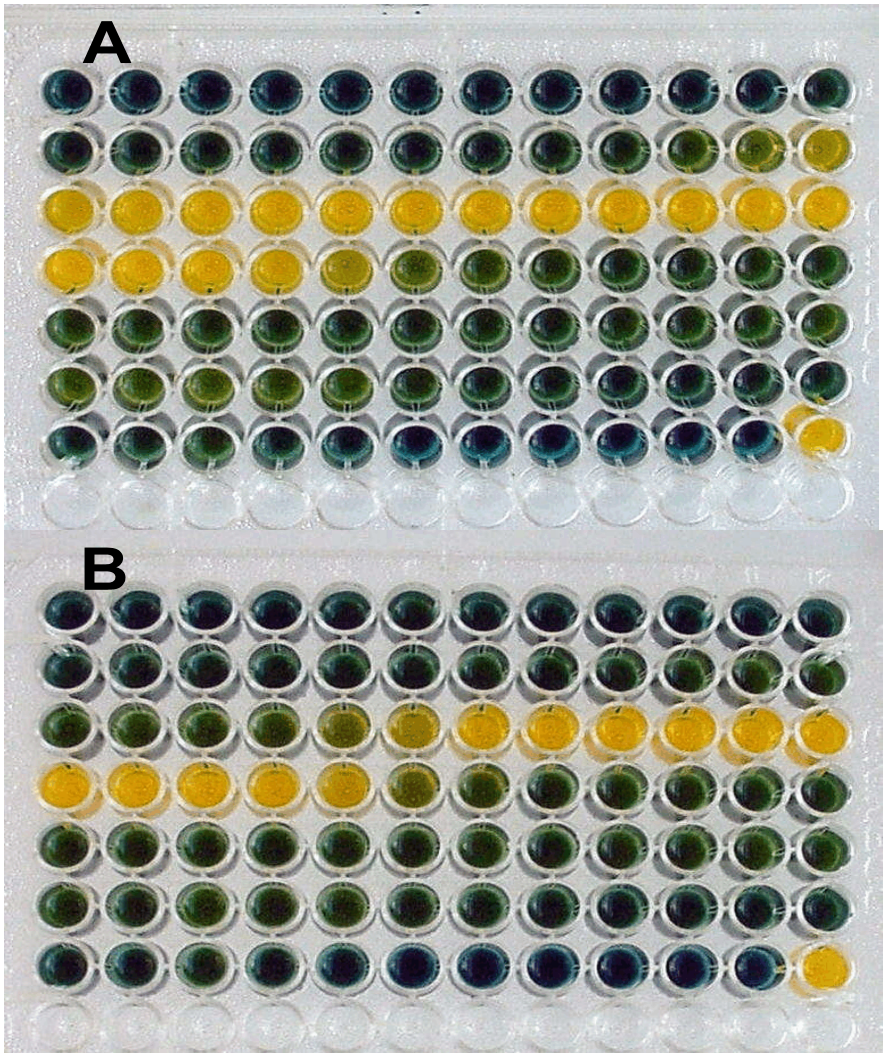

Fig 6.4. Colorimetric assessment of pH reduction (blue = pH 7.5, yellow = pH 6.0) by pectin methyl-esterase (PME) activity. Total proteins from ripe fruit separated by ion-exchange chromatography in a CM-sepharose column (1.5 x 15 cm) and eluted with a gradient from 150 mM to 500 mM NaCl. Cells A-1 to G-2 correspond to fractions 1 to 74. Cell G-3 to G-11 are blank pH 7.5, and cell G 12 is blank pH 6.0. (**A**) 'Easy Pick' and (**B**) 'Hard Pick' tabasco pepper genotypes.

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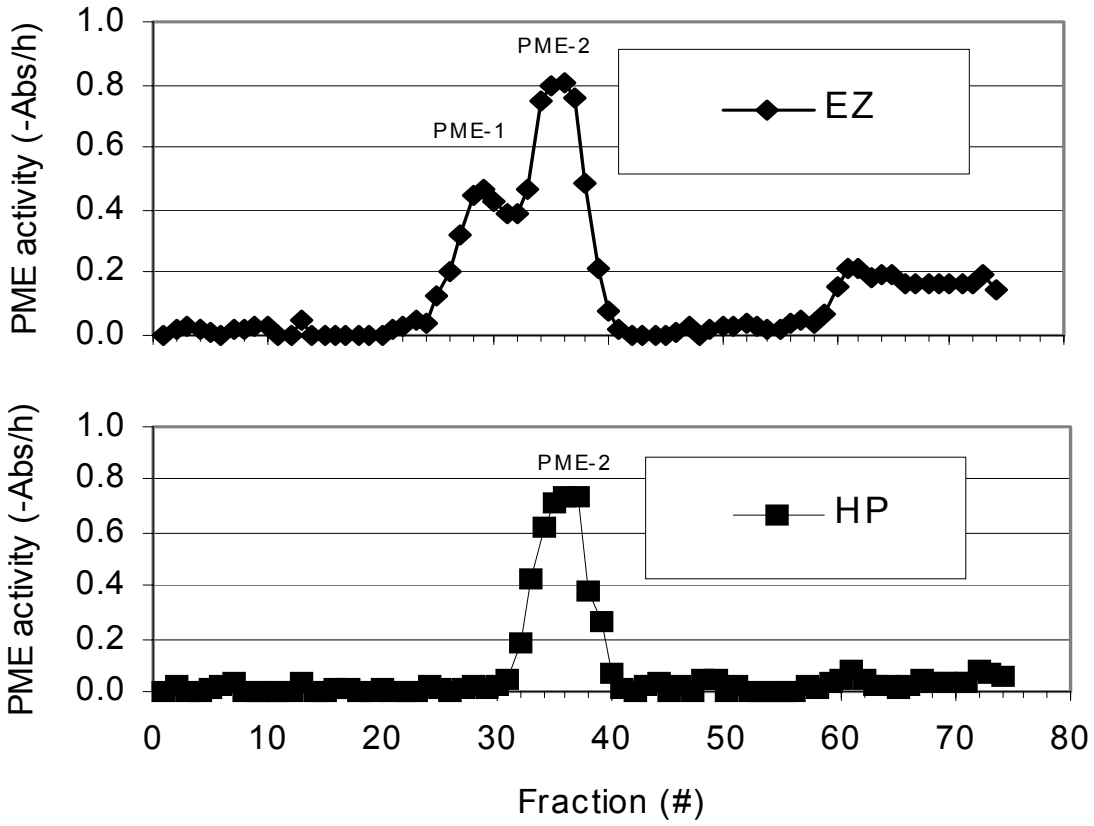

Fig. 6.5. Fractionation of pectin methyl-esterases (PME) from ripe tabasco pepper. Total proteins from mature-red fruit pericarp were separated by ion-exchange chromatography in a CM-sepharose column (1.5 x 15 cm) and eluted with a gradient from 150 mM to 500 mM NaCl. PME activity determined by colorimetric assay: absorbance (600 nm) reduction (-Abs) in 1h. (**A**) 'Easy Pick' and (**B**) 'Hard Pick' tabasco pepper genotypes.

Table 6.1. Characteristics of pectin methyl-esterase isoforms (PME-1 and PME-2) isolated from 'Hard Pick' (HP) and 'Easy Pick' (EZ) ripe tabasco pepper fruit.

Separation step	Molecular size	PME activity[1] (unit•mg^{-1} protein)	NaCl EC[2] (mS•cm^{-1})
Desalted extract			
EZ		0.9 - 1.73	
HP		0.3 - 0.7	
Ion-exchange chromatography			
EZ PME peak-1	22.5 k & 36.7 k	5 - 160	27
EZ PME peak-2	36.7 k & 40.8 k	15	33
HP PME peak-2	40.8 k	8	33

[1] Units of PME activity: moles of carboxyl groups produced in 1 min.
[2] EC: electrical conductivity indicates NaCl concentration when protein was desorbed from the column.

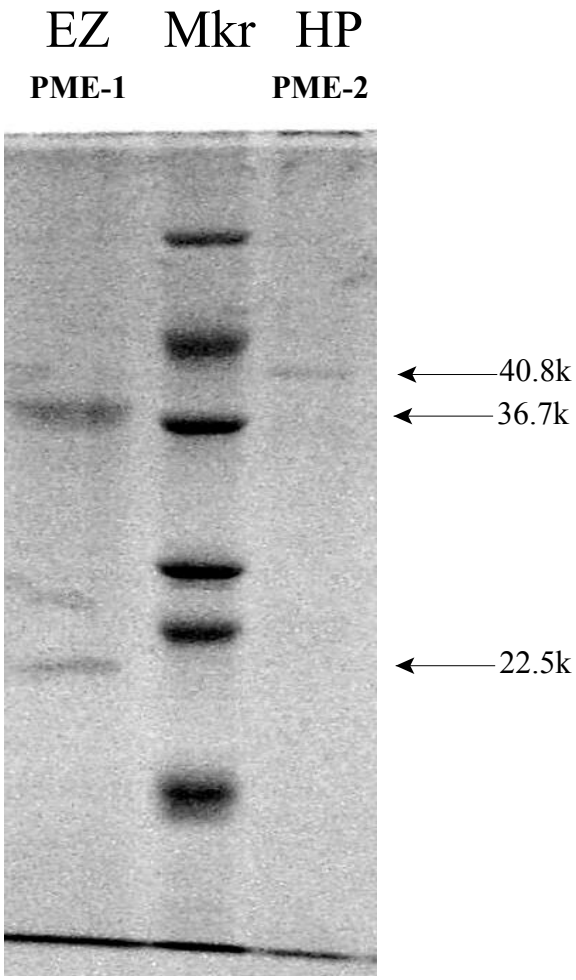

Fig. 6.6. Gel electrophoresis (SDS-PAGE) of fractions containing pectin methyl-esterase isoform 1 (PME-1) and 2 (PME-2). Total proteins from red-mature 'Easy Pick' (EZ) and 'Hard Pick' (HP) tabasco pepper fruit were separated by ion-exchange chromatography. Marker proteins (Mkr) 66k, 45k, 36k, 29k, 24k, and 20.1k. Molecular size of protein bands were calculated by the relative mobility.

which coincided with the large prominent protein band (Fig. 6.7). Color change was not detected in the other protein bands present in PME-1 fractions. Localized color change in the gel was detected when the sample load contained PME activity higher than 0.5 unit. Localized color change was not detected in PME-2 samples (Fig. 6.7). The low protein concentration in PME-2 fractions either from HP or EZ fruit may account for the lack of color change. One protein band with molecular size of 40,800 was resolved consistently from HP PME-2 fractions (Fig. 6.6, lane HP-PME2). Two protein bands, however, were resolved in EZ PME-2 fractions (data not presented). The larger band coincided with the HP PME-2 protein and the smaller band coincided with PME-1 36,700 protein, but the amount of protein was significantly less.

DISCUSSION

Three main PME activities from immature and mature-green tabasco fruit were separated by ion-exchange chromatography (Fig. 6.1). All isoforms detected in green fruit were present in both the EZ and HP tabasco genotypes. PME activity *in vivo* and DPE reduction, were not detected in unripe tissue (Chapter 4 and 5). Therefore, PME isoforms present in immature and mature-green tabasco pepper fruit were not associated with the ease of fruit separation from the calyx. The presence of multiple PME isoforms extracted from mature-green tomato fruit has been reported in several studies (Pressey and Avants, 1972; Tucker et al., 1982; Gaffe et al., 1994; Warrilow et al., 1994). Several isoforms have been cloned and characterized (Harriman et al., 1991; Hall et al., 1994; Gaffe et al., 1997). The PME isoforms detected in mature-green tabasco pepper may belong to the same group isolated from green tomato since they did not affect DPE during ripening (Chapter 4). The role of PME in plant growth, however, has not been elucidated since several isoforms with different kinetic characteristics seems to be involved (Warrilow and

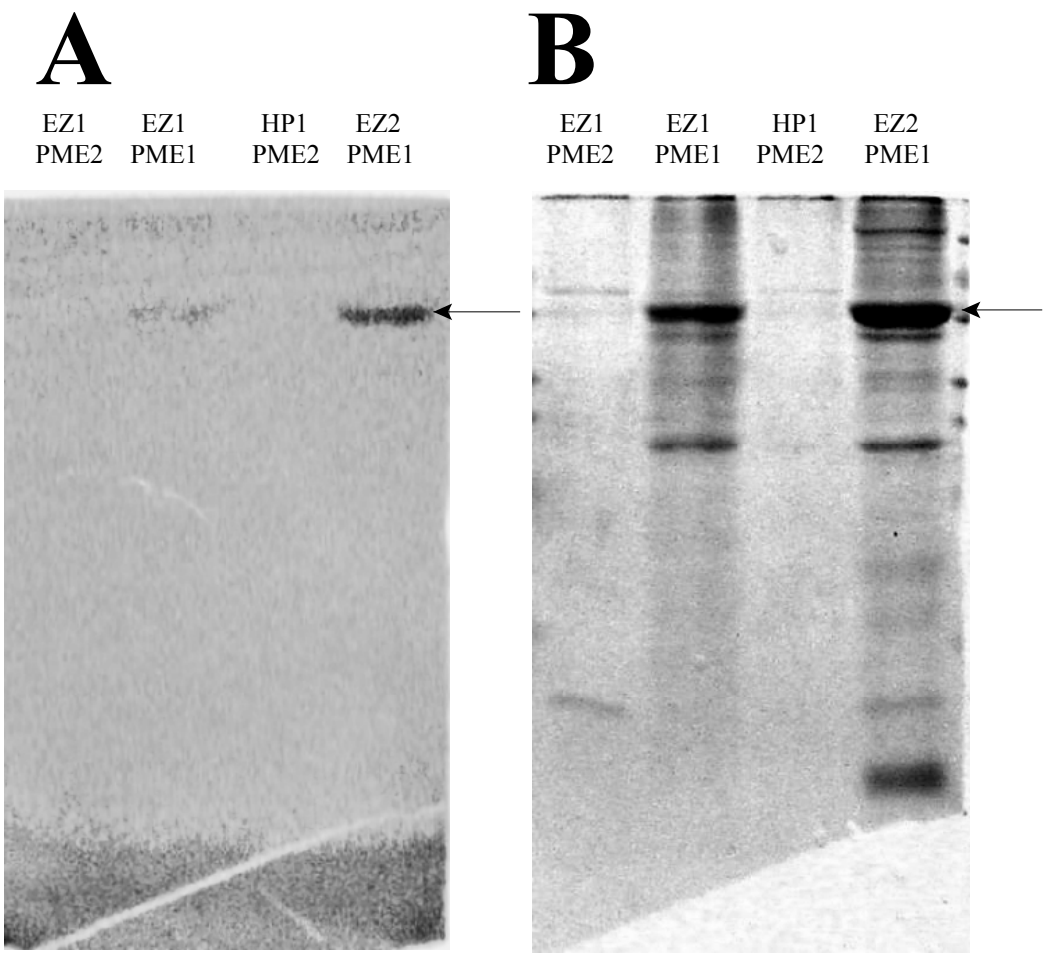

Fig.6.7. Gel electrophoresis (SDS-PAGE) of fractions containing pectin methyl-esterase isoform 1 (PME-1) and 2 (PME-2). (**A**) PME activity detected colorimetrically in the gel after several washes with DTT and (**B**) the same gel after staining with coomassie blue. Total proteins from ripe 'Easy Pick' (EZ) and 'Hard Pick' (HP) tabasco pepper fruit were separated by ion-exchange chromatography. EZ1 and EZ2 correspond to extractions 1 and 2.

Jones, 1995). It was suggested that a free carboxyl group in the pectin molecule may be necessary to serve as an initiation point for PME enzymatic attack (Pressey and Avants, 1972). The function of one of the pectin methyl-esterases may be to create initiation points for the other isoforms. Another possible role for PME may be related to healing of wounded tissue. Cell wall needs to be modified during healing to allow callus to form. Therefore, PME is ready to become active when tissue is disrupted (Chapter 5). This is supported by the report that PME activity was detected in protein extracts from all tissue types (Gaffe et al., 1994; Gaffe et al., 1997).

The significance of the presence of PME isoforms in green fruit, however, is not clear because their activity *in vivo* has not been detected previously and correlation with fruit softening has been difficult (Pressey and Avants, 1982; Tucker et al., 1982; Harriman et al., 1991; Tieman and Handa,1994). Proposed roles for PME in plants include (a) localized reduction in pH due to demethoxylation of pectin that may be associated with extension and growth (Brummell and Harpster, 2001), (b) de-esterification of pectin making it more susceptible to depolymerization by PG and enhancing cell separation during fruit ripening and abscission (Fischer and Bennett, 1991), and (c) generation of free carboxyl groups, which facilitates formation of new Ca^{2+} cross-linkages in cell wall (Fry, 1986). Based on the kinetic properties of tomato PE-A activity *in vitro*, it was suggested that this PME isolated from mature-green fruit was responsible for continued pectin demethoxylation leading to pectin degradation (Warrilow and Jones, 1995). In contrast, antisense suppression of this tomato PME increased slightly the DPE and showed no effect on fruit firmness suggesting that it may be involved in fruit growth and development instead (Tieman et al., 1992). The results obtained with tabasco pepper indicate that PME activities expressed in green tissue and at least one detected also in ripe tissue were blocked *in vivo,* and a ripening

specific PME activity appears to be responsible for pectin demethoxylation during fruit ripening (Chapter 5 and this Chapter).

Two PME activities were detected in ripe tabasco pepper fruit (Fig.6.5). PME-1 was detected in fruit extracts from ripe EZ tabasco pepper only and appears to be responsible for methanol production *in vivo* and the DPE reduction detected in ripe fruit of this genotype (Chapter 4 and 5). The role of PME-1 therefore, seems to be the enhancement of pectin degradation associated with fruit softening and the ease of fruit detachment during ripening (Chapter 4). Suppression or elimination of PME-1 activity in the EZ genotype and expression in the HP genotype, as well as inheritance studies may support this conclusion (Smith, 1951; Tieman et al., 1992). In contrast, PME-2 activity was detected in both genotypes, and therefore, was not associated with methanol production *in vivo*. PME-2 however, appears to be responsible for activity in protein extracts and in disrupted tissue from ripe fruits (Chapter 5). A ripening specific PME activity was separated also from tomato protein extract by ion-exchange chromatography, but its role in fruit ripening was not determined (Pressey and Avants, 1972). This is the first study reporting the association of a ripening specific PME activity with pectin degradation *in vivo* and with the ease of fruit detachment (Chapter 4).

Multiple protein bands were resolved in the fractions containing EZ PME-1 (Fig. 6.6). In contrast, only one protein was resolved consistently in HP PME-2. Previous studies with PME extracted from mature-green tomato reported also several protein bands associated with PME active fractions after ion-exchange chromatography (Warrilow et al., 1994). According to the localized pH decrease in the gel after electrophoresis, the EZ PME-1 active isoform appears to be the 36.7 k protein (Fig. 6.7, lanes EZ1-PME1 and EZ2-PME1). The sensitivity of the localized pH

reduction test in polyacrylamide gel was very low in comparison to a similar test reported previously (Gaffe et al., 1994). It is possible that the low mobility of PME and pectin in polyacrylamide gel has affected the sensitivity of the test. Since the EZ PME-2 36.7 k isoform was not present in HP extracts, PME-2 40.8 k protein seems to be the PME-2 active isoform (Fig.6.6). The molecular size of PME-1 and PME-2 associated protein, with the exception of PME-1 22.5 k, were within the range (30,200 to 43,200) of PME associated proteins isolated from tomato (Harriman et al., 1991; Warrilow et al., 1994). PME-1 36.7 k protein was abundant in the chromatography fractions and well defined in the gels, but PME-1 activity decreased rapidly in high NaCl concentration. In contrast, PME-2 proteins detected in the gels were very low and activity was stable during extraction and analysis. Tomato PME isoforms were previously reported to be difficult to resolve and detect in SDS-PAGE (Warrilow et al., 1994). PME-1 was not totally isolated in this study and the pIs of the tabasco PME isoforms are yet to be determined. Further purification and IEF analysis, therefore, as well as serological and molecular studies are necessary to fully characterize PME-1 and PME-2 isoforms.

Finally, serological and molecular (cDNA) studies are also necessary to characterize the expression pattern of tabasco pepper PME-1 and PME-2 throughout fruit development and ripening (Harriman et al., 1991; Tieman et al.,1992; Gaffe et al., 1994; Hall et al., 1994). Sequence analysis of the PME isoforms are necessary to determine the level of homology with PME expressed in different ripening stages and tissues, and in other species (Hall et al., 1994; Gaffe et al., 1997). Sequence analysis of PME-1 and PME-2 may also elucidate molecular differences associated with the ability to act *in vivo*. A possible explanation for the activity *in vivo* of PME-1 would be the ability to be exported from the cell. PME-2 may have lost the sequence

necessary to be exported and remains in the cytoplasm until tissue is disrupted, or, its role may be related to the healing process after wounding.

CHAPTER 7. SUMMARY AND CONCLUSIONS

Pectin metabolism was investigated in tabasco pepper during fruit ripening to determine the factors associated with the ease of fruit detachment from the calyx when ripe. The ease of fruit separation is a characteristic of wild peppers, but it is not present in most cultivated peppers and the pedicel remains attached to the fruit when harvested. Two tabasco pepper genotypes that differ in the ease of fruit detachment were identified previously (Motsenbocker, 1996) and used in this study: EZ which requires a low force to detach from the calyx and HP which requires higher force. In this study, changes in FDF throughout ripening was measured in association with the fruit external color (hue). In EZ tabasco pepper, the FDF decreased from 20 N to 3 N as the fruit external color changed from hue 52 to hue 48 in 12 h. In the HP genotype, however, the FDF began to decrease at hue 45 down to only 10 N in purple-red fruit (hue 30). Objective color measurement facilitated the determination of the precise ripening stage when the FDF decreased and it was used to analyzed events associated with the ease of fruit detachment from the calyx.

Cell wall degrading enzymes were analyzed during ripening to determine their association with the ease of fruit separation in tabasco pepper. Activity of PG and EGase in protein extract increased in ripe fruit from both genotypes and were correlated with FDF. PG and EGase activity, however, were the same in both the EZ and HP genotypes. This suggests that the difference in FDF between the EZ and HP genotypes depends on an additional factor.

Pectin characteristics of fruit DZ were analyzed to determine differences in pectin degradation associated with the ease of fruit detachment. Pectin dissolution was inversely correlated to the FDF during fruit ripening. The amount of soluble uronide released from fresh ripe fruit tissue and in cell wall extracts was higher in the EZ genotype than the HP genotype.

The higher pectin dissolution was attributed to the enhanced pectin depolymerization detected in the EZ genotype. Size-exclusion chromatography of EDTA-soluble polyuronides showed that pectin was degraded in ripe tissue of both tabasco genotypes, but the degree of depolymerization was more extensive in the EZ genotype. These results suggest that the ease of fruit detachment was caused by the disintegration of the cell wall as the result of pectin ultra-degradation. The results obtained in this study however, indicate that this characteristic may have limited usefulness. Although easy fruit detachment has the potential to improve mechanical harvest, pectin ultra-degradation may affect the textural characteristics of the processed product.

Since EGase and PG activity were the same in protein extracts from both genotypes, and the DPE and the pH at the junction area was lower in ripe EZ fruit, the role of PME activity in pectin degradation was investigated. PME activity was assessed *in vivo* by the methanol released from the de-esterification reaction. Methanol production was detected in ripe EZ fruit only when the fruit external color was between hue 52 and hue 40. The rise in methanol production coincided with the FDF decline. Therefore, the decrease in DPE and pH at the fruit junction area detected in EZ ripe fruit was attributed to PME activity *in vivo*. In contrast, PME activity *in vitro* was detected in protein extracts and in disrupted fruit tissue from both genotypes at all ripening stages. PME activity *in vitro* was high in immature-green fruit, decreased in early ripening stages, and then increased again in ripe fruit. These results suggests that there was a PME regulatory mechanism that blocked PME activity *in vivo*, but this mechanism was lost in disrupted tissue.

PME activity in disrupted mature-green fruit tissue was attributed to three main PME activities separated by ion-exchange chromatography. Because these PME activities appeared

not to have an effect on the DPE during ripening, they were not associated with the ease of fruit detachment in tabasco pepper. Two PME activities in protein extract from ripe fruit were separated by ion-exchange chromatography. PME-1 activity was detected in protein extracts from EZ fruit only. Consequently, PME-1 appears to be responsible for the methanol produced *in vivo* by ripe fruit of the EZ genotype. In contrast, PME-2 was detected in protein extracts from both genotypes. PME-2, therefore, appears to be responsible for PME activity in disrupted ripe fruit tissue from the HP genotype. The PME-1 36.7 k and the PME-2 40.8 k proteins seems to be the active forms for each PME activity. Further purification of PME-1 and PME-2 isoforms and molecular studies are necessary to fully characterize this enzyme and to understand the characteristics associated with the ability to act *in vivo*.

Finally, the suggested model for pectin degradation in tabasco pepper indicates that PME-1 has a role in the ease of fruit detachment from the calyx. A sequence of events conducive to pectin degradation and to the ease of fruit detachment from the calyx is summarized in Fig. 7.1. In the EZ genotype, PME-1 becomes active *in vivo* at hue 52 and reduces the DPE which results in higher proportion of polygalacturonic acid available for PG action. Enhanced pectin depolymerization by PG, which was already present, increases pectin dissolution in fresh tissue and the cell wall structure is disrupted. Consequently, fruit tissue is disintegrated resulting in easy fruit separation. In contrast, the HP genotype lacks PME-1 activity and PME-2 activity appears to be blocked *in vivo.* The DPE is not affected during fruit ripening and PG can act only on pectin de-esterified areas originated during growth resulting in limited depolymerization and low dissolution. Partially degraded pectin is still able to maintain the integrity of the cell wall structure in the fruit-calyx junction zone and the FDF decline is suppressed.

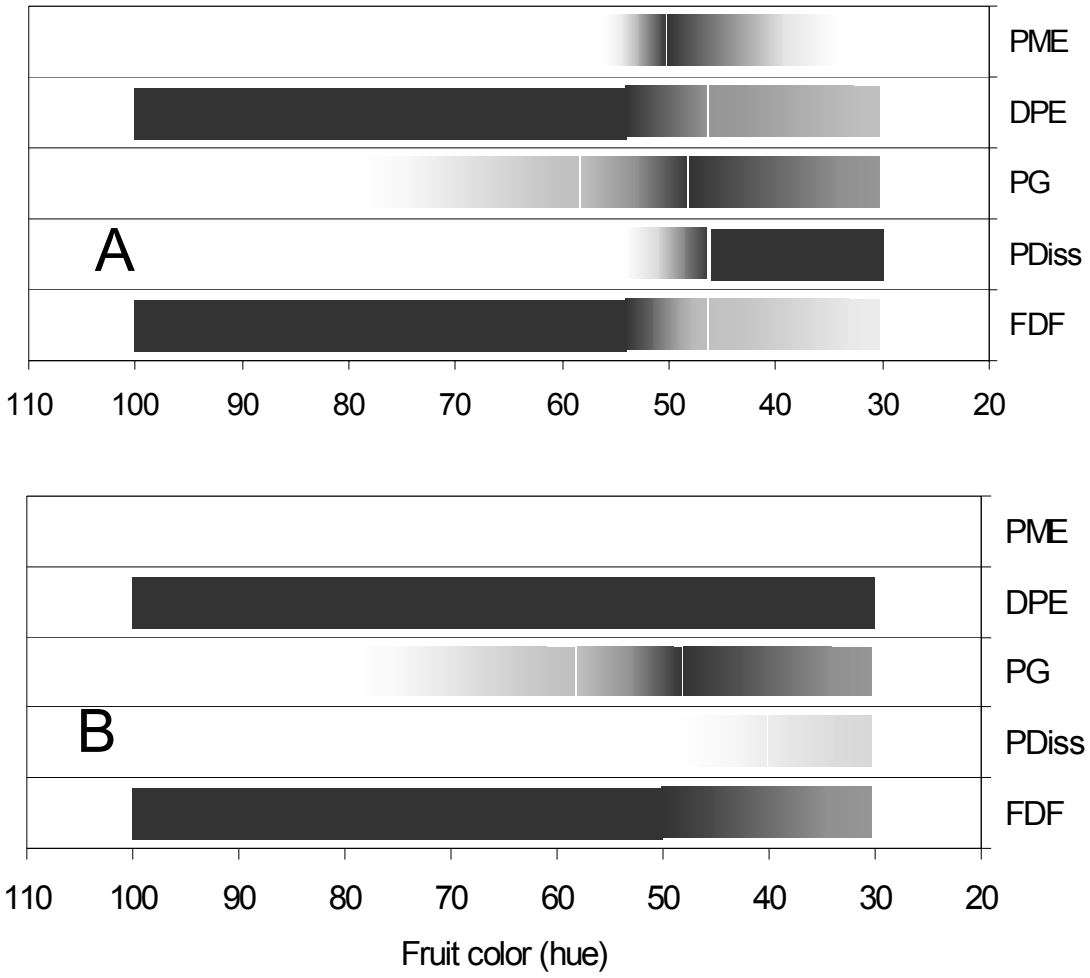

Fig 7.1. Tabasco pepper ripening related events associated with the ease of fruit detachment from the calyx. EZ (**A**) and HP (**B**) pepper genotypes. Fruit ripening is represented by the fruit external color (hue 100 corresponds to immature-green and hue 30 to purple-red). Events are: pectin methyl-esterase activity *in vivo* (PME), degree of pectin esterification (DPE), polygalacturonase activity in protein extracts (PG), pectin dissolution from fresh tissue (PDiss), and fruit detachment force (FDF). Relative detection level is represented by the color gradient. Black corresponds to maximum level and white corresponds to undetected.

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VITA

Ramón was born in Talcahuano, Chile, a few decades ago. He grew up in Santiago, the capital, and attended the National Institute high school. He studied at the University of Chile and graduated as Ingeniero Agrónomo in 1988 with Maximum Distinction. The title of his thesis was 'Characterization of the viruses associated with grapevine leafroll in grapes (*Vitis vinifera* L.) cv. Black Seedless, and recovery of virus free plants by heat-treatment and tissue culture'.

After graduation, he went to the University of California at Davis to investigate the use of sulfite in table grapes (and to taste the good wines of Napa Valley). While he was in California, the cyanide-grape scandal occurred, and he became involved in the subsequent investigation.

Ramón came to LSU and graduated with a master of science degree in plant pathology. The title of his thesis was 'Characterization of a Cryptic Virus Found in Pepper (*Capsicum annuum*) cv. Jalapeño M'. Since his graduation, he has worked as Research Associate in the Department of Horticulture at LSU. While working in vegetable crops, he became a graduate student again to pursue a doctoral degree.

Ramón is married to Lori and they have a six years old daughter, Madeleine. Ramón has also and older son, Adrián, who is 26 years old.