PECTIN DEGRADATION IN RIPENING AND WOUNDED FRUITS

DONALD J. HUBER*, YASAR KARAKURT, JIWON JEONG

Horticultural Sciences Department, PO Box 110690, Fifield Hall, University of Florida, Gainesville, FL 32611-0690 USA

ABSTRACT - Pectin depolymerization during fruit ripening has been shown to be largely due to pectinolytic enzymes, including polygalacturonases (E.C. 3.2.1.15) and pectinmethylesterases (E.C. 3.2.1.11). Studies have shown that these enzymes are not the primary determinants of softening, although participation in texture changes during the late stages of ripening seems evident. Pectin depolymerization differs significantly between various fruit types, notably avocado and tomato, even though levels of extractable PG activity in these fruits are similar. Collective evidence indicates that the activities of some cell wall enzymes are restricted *in vivo*, with maximum hydrolytic potential expressed only in response to tissue disruption or wounding. In contrast, other enzymes reported to participate in pectin degradation, notably β-galactosidases/exo-galactanases, exhibit *in vitro* activity far below that anticipated to be required for the loss of cell wall galactosyl residues during ripening. Factors controlling *in vivo* hydrolysis have not been fully explored but might include apoplastic pH, cell wall inorganic ion levels, non-enzymic proteins including the noncatalytic β-subunit and expansins, wall porosity, and steric hindrances. Recent studies of cell wall metabolism during ripening have demonstrated an orderly process involving, in the early stages, cell wall relaxation and hemicellulose degradation followed, in the later stages, by pectin depolymerization. A limited number of studies have indicated that radical oxygen species generated either enzymically or non-enzymically might participate in scission of pectins and other polysaccharides during ripening and other developmental processes. Similar mechanisms might also occur in response to wounding, an event typically followed by an oxidative burst. Cell wall degradation as influenced by physical wounding could be of particular relevance to the deterioration of lightly processed fruits.

ADDITIONAL INDEX TERMS: apoplast, lipids, membranes, oligogalacturonides, pectin fragments, polygalacturonase, radical oxygen species.

DEGRADAÇÃO DE PECTINA DURANTE O AMADURECIMENTO E EM FRUTOS INJURIDADOS

RESUMO – A despolimerização de pectina durante o amadurecimento de frutos tem sido apresentada como ação das enzimas pectinolíticas, incluindo polygalacturonases (EC 3.2.1.15) e pectinamethylesterases (EC 3.2.2.22). Estudos tem mostrado que essas enzimas não são as causadoras primárias do amolecimento , no entanto, sua participação nas mudanças da textura durante os estádios finais do amolecimento parecem evidente. A despolimerização difere significativamente entre vários tipos de frutos, notadamente abacate e tomate, mesmo que níveis de atividade de PG nesses frutos sejam similares. Evidências coletivas indicam que as atividades de algumas enzimas de parede celular são restritas *in vivo*, com o máximo de potencial hidrolítico expresso apenas em resposta ao rompimento do tecido ou ferimentos. Em contraste, outras enzimas participam da degradação de pectina, notadamente β. galactosidase/exo-galactamases, que exibem *in vitro* atividades bem abaixo do valor mínimo para a

* corresponding author: gjh@mail.ifas.ufl.edu

perda de resíduos de galactosyl da parede celular durante o amadurecimento. Fatores que controlam a hidrólise *in vivo* não tem sido totalmente estudados mas podem incluir o pH apoplástico, níveis de íons inorgânicos na parede celular, proteínas não enzimáticas, incluindo a β-subunidade não catalítica e expansina, porosidade da parede e impedimento estérico. Estudos recentes sobre o metabolismo de parede celular durante o amadurecimento tem mostrado ser um processo ordenado, envolvendo nos estádios iniciais, relaxamento da parede celular e degradação de hemicelulose seguida, nos estádios finais, pela despolimerização da pectina. Um limitado número de estudos tem indicado que espécies que geram radicais de oxigênio por meios enzimáticos ou não enzimáticos podem participar da excisão de pectinas e outros polissacarídeos durante o amadurecimento e outros processos de desenvolvimento. Mecanismos similares podem também ocorrer em resposta a ferimentos, um evento tipicamente seguido por um incremento em vias oxidativas. A degradação da parede celular como aquela oriunda do ferimento físico poderá ter particular relevância para a deterioração de frutos ligeiramente processados.

TERMOS ADICIONAIS PARA INDEXAÇÃO: Apoplasto, lipídeo, membranas, oligogalacturonídeos, fragmentos, de pectina, polygalacturonase, espécies com radical de oxigênio.

INTRODUCTION

The process of ripening, a form of programmed organ death, continues to attract the attention of many researchers. Our understanding of the biology of ripening has been aided considerably by the development and application of molecular biological approaches. Perhaps the most widely studied aspects of ripening include ethylene biosynthesis and signal transduction (Bleecker and Kende, 2000), and softening (Brownleader *et al.*, 1999; Wakabayashi, 2000). Whereas specific cell wall changes contributing to softening remain unknown, it is increasingly evident that the process is complex and involves the sequential, orderly participation of a number of cell wall components, including structural polysaccharides, and enzymic and non-enzymic proteins (Rose et al., 1998; Brummel et al., 1999). The objective of this report is to address the mechanisms and control of pectic depolymerization in fruits during ripening and in response to mechanical wounding.

PECTIN SOLUBILITY DURING RIPENING

Increased solubility of pectic polysaccharides is one of the most universal features of ripening fleshy fruits. The mechanisms contributing to this process have not been fully elucidated, though the magnitude of solubility increases varies greatly among different fruits. Water-soluble pectins range from 10% of total cell wall uronic acid content in ripe grapefruit (Hwang *et al.*, 1990) to 35 to 40% in ripe cherries (Fils-Lycaon and Buret, 1990) and strawberries (Goto *et al.*, 1996), to as high as 85% in ripe avocados (Wakabayashi *et al.*, 2000). As noted, the processes contributing to the increases in pectin solubility during ripening are not clear. Transgenic tomato fruit with reduced levels of polygalacturonase (PG) showed a significant reduction in the quantity of water-soluble pectins compared with untransformed fruit (Carrington *et al.*, 1993). Initial increases in pectin solubility in the rapid-ripening Charentais melon were associated with a loss in pectin-associated galactose, prior to the appearance of PG (Rose *et al.*, 1998). Redgwell et al. (1992) concluded that the initial solubilization of pectins in ripening kiwifruit required neither depolymerization (PG) nor degalactosylation. The low levels or absence of PG in some fruits, including strawberry and grape, support the view that the enzyme is not a ubiquitous requirement for pectin solubilization. The expression of a putative ripening-related pectate lyase gene in strawberry fruit (Medina-Escobar *et al.*, 1997) raises the

possibility that other, as yet uncharacterized enzymes, are involved in pectin solubility changes. Still other mechanisms may be responsible for pectin solubilization in fruits displaying double-sigmoidal growth kinetics in which ripening and softening occur concomitantly with resumption of rapid cell expansion (Davies and Robinson, 2000).

The levels of pectins soluble in solutions containing chelators, often termed 'ionically bound' pectins, also vary considerably between different fruits. Interpretation of the changes in this pectin fraction is difficult, however, since the solubilization of these polymers is dependent on the removal of calcium. The use of calcium chelators may negate the prior influence of enzymes or other factors on the solubility of these pectins *in vivo.* Pectin solubility is also influenced by cell wall-isolation protocol (Huber, 1991).

CELL WALL HYDROLASES AND PECTIN DEGRADATION

Evidence from a number of labs has shown that reducing pectin depolymerization via molecular silencing of PG (Smith *et al.*, 1990; Giovannoni *et al.*, 1989) has little influence on tomato fruit softening until the late stages of ripening (Kramer *et al.* 1992; Carrington *et al.*, 1993). Consistent with these observations, pectin depolymerization during tomato ripening is restricted compared with *in vitro* potential (Seymour *et al.*, 1987; Huber and O'Donoghue, 1993; Brummel and Labavitch, 1997). Indeed, accelerated degradation of tomato fruit pectins upon tissue disruption was noted over 60 years ago by Kertesz (1938), who observed a rapid (5 to 10 minutes) change in viscosity of cold-pressed tomato fruit that he attributed to the action of 'pectinase' enzymes. Since the studies of Kertesz and others, the participation of specific pectinases, notably PG and PME, in the rheological properties of tomato fruit juice and paste products has been demonstrated (Tucker *et al.*, 1999). Mol mass distributions of pectins derived from mature-green and ripe tomato fruit, and from intact versus homogenates of ripe tomato fruit are shown in Figures 1 and 2, respectively. The mol mass downshifts in pectins from homogenates (Fig. 2) resulted from holding a freshly homogenized ripe tomato for 5 minutes at room temperature prior to sample processing. As with pectin release from enzymically active cell walls incubated under conditions optimized for PG activity (Huber and Lee, 1988), the low mol mass products recovered from homogenates included low DP (degree of polymerization) pectin fragments.

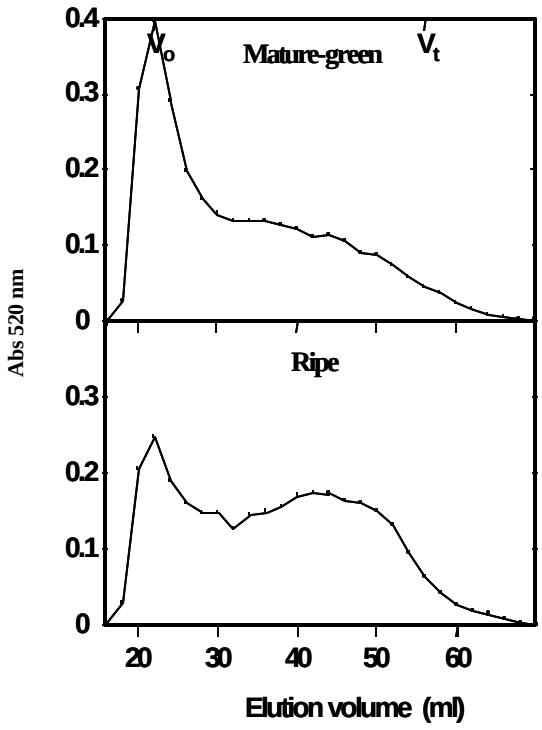

FIGURE 1 - Mol mass distribution of pectins from mature-green (A) and ripe (B) tomato fruit. Combined water-and chelator-soluble pectins (0.5 mg galacturonic acid equivalents) were applied to a Sepharose CL-4B column (29 cm length, 1.5 cm diameter) operated with a mobile phase of 200 mM ammonia acetate, pH 5.0. Fractions of 2 ml were analyzed for uronic acids. V_o= Void volume; V_T = Total Volume

REGULATION OF PECTIN DEPOLYMERIZATION IN RIPENING TOMATO FRUIT

Factors reponsible for restricted PG action *in vivo* are not well understood. Almeida and Huber (1999) observed that the pH of pressure-exuded apoplastic fluid was over 6.0 in mature-green tomato fruit, declining to 4.5 during ripening (Fig. 3). Similar changes in apoplastic pH were reported for ripening peach and nectarine fruits (Ugalde et al. 1988). Tomato PG *in vitro* is catalytically inactive at pH 6.0 (Themmen *et al.*, 1982), the pH of mature-green fruit apoplast, whereas the pH of ripe fruit apoplast is similar to the *in vitro* optimum for the enzyme. The influence of apoplastic pH and mineral levels in the regulation of cell wall metabolism is well recognized for other plant systems (Cosgrove, 1999; 2000; Sakurai, 1998; Soga *et al*., 2000) but has received little attention in fruit tissues. A survey of a number of fruit cell wall enzymes including xyloglucanases (XGase), xyloglucan endotransglycosylase(XET),endo-&- 1,4-glucanases (C_x-cellulases), β-galactosidases and other glycosidases, and pectin-hydrolyzing enzymes reveals pH optima ranging from 4.0 to 7.0 (Almeida, 1999). Although these represent values determined from assays performed *in vitro*, often with non-native substrates and in buffers selected to optimize activity, they offer evidence that dynamic changes in apoplast pH during ripening could strongly influence the sequence and progression of wall disassembly. The expression of aberrant textural conditions in fruits exposed to irradiation (Paull, 1996; Yu *et al*., 1996; Kovacs *et al*., 1997), high or low temperatures (Sozzi *et al.*, 1996; Jackman *et al.*, 1982; Fernandez-Trujillo *et al.*, 1998; Bauchot *et al*, 1999), and the occurrence of other textural disorders including mealiness in nectarines (von Mollendorff *et al.*, 1993) and ethylene-induced watersoaking in watermelon fruit (Elkashif and Huber, 1988) might reflect, in part, altered apoplastic conditions (pH, ion balance) brought about either actively or passively by stress-induced membrane dysfunction.

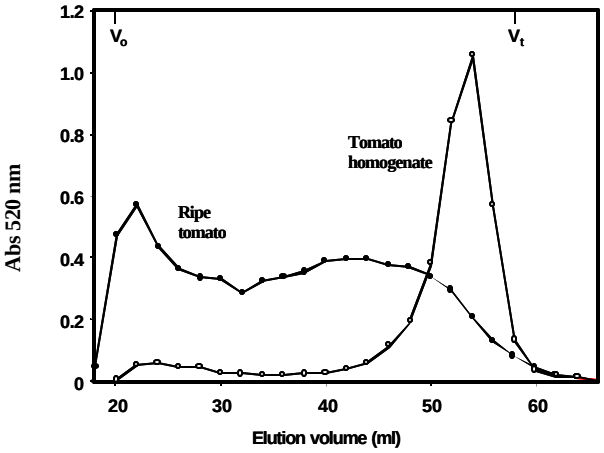

FIGURE 2 - Mol mass distribution of pectins from intact ripe tomato fruit and ripe tomato homogenates. Intact fruit were processed in phenolic solvents to inactivate enzymes and subsequently used for pectin extraction (Huber 1991). Other fruit were homogenized in buffer (50 mM Na-acetate, pH 4.5) and the homogenate permitted to stand at room temperature for 5 minutes. Afterward, the homogenate was processed as for intact fruit. Pectins from the intact and homogenized fruit were applied to a Sepharose 4B-200 column as described for Figure 1.

The trend of decreasing apoplastic pH (and increasing $[K^+]$, Almeida and Huber, 1999) during tomato ripening would be expected to enhance PG activity; however, pectin depolymerization patterns indicate that the activity of the enzyme in healthy, ripe fruit remains well below *in vitro* catalytic potential (Seymour *et al.*, 1987; Huber and O'Donoghue, 1993; Brummel and Labavitch, 1997). The high levels of calcium in tomato fruit apoplast might contribute to the persistent inhibition of pectin hydrolysis during ripening. Calcium levels in apoplastic liquid from tomato fruit remain nearly constant (approximately 4 mM) throughout ripening (Almeida and Huber, 1999), and are more than adequate to strongly suppress PG-mediated pectin release from isolated cell walls (Rushing and Huber, 1987). Additional evidence for regulation of PG by apoplastic

conditions was the observation that incubation of purified tomato PG 2 with cell walls in solutions mimiking the pH and ionic composition of apoplastic fluid of ripe fruit greatly reduced pectin depolymerization compared with that occurring in cell walls incubated at pH 4.5 without added ions (Almeida and Huber, unpublished).

PECTIN DEPOLYMERIZATION IN RIPENING AVOCADO FRUIT

The pattern of pectin depolymerization in ripening avocado fruit (Huber and O'Donoghue, 1993) provides a sharp contrast to that noted for tomato and other fruits including apple (Fischer *et al.*, 1994), kiwifruit (Gallego and Zarra, 1997; Soda *et al.*, 1987), Japanese and Chinese pear (Moriguchi *et al.*, 1998), plum (Boothby, 1983), carambola (Chin *et al.*, 1999), and papaya (Paull *et al.*, 1999). Nectarine (Lurie *et al.*, 1994) and mango (Muda *et al.*, 1995) fruits exhibit more extensive hydrolysis than the above examples, yet the mol mass downshifts do not involve a large proportion of cell wall pectins as is evident for avocado. As illustrated in Figure 4, water-soluble pectins from avocado undergo marked mol mass downshifts during ripening, eluting as a symmetrical peak near the V_t (total column volume). Sakurai and Nevins (1997) reported a similar trend for pectin mol mass downshifts during avocado ripening. As evident from Figure 4, the mol mass distribution of pectins from ripe avocado was quite similar to that of pectins from tomato homogenates (Fig. 2). Wakabayashi et al. (2000) have shown that the extensive hydrolysis of avocado pectins requires the prior or concerted action of PME. In addition to the mol mass downshifts, nearly 85 to 90 % of the total uronic acid in cell walls from ripe avocado fruit were recovered from cell wall isolates extracted in water. We are aware of no other fruit in which such large quantities of pectins are readily solubilized from cell wall isolates (ethanol-insoluble solids) under mild, nondestructive conditions.

The biochemical basis for the comparatively extensive hydrolysis of pectins during ripening of avocado compared with tomato fruits is not clear. The presence of only low mol mass PG (46 and 48 kD) isoforms in avocado (Wakabayashi and Huber, 2001) compared with the low (45 to 46 kD) and high mol mass (100 kD PG1, a heterodimer of PG2 plus the &-subunit protein) isoforms in tomato suggests that &-subunit-type proteins may be absent or less influential in avocado fruit. As discussed by DellaPenna et al. (1996), the β-subunit, a novel, aromatic amino acid-rich glycoprotein first characterized in tomato fruit, may function to tether PG isozyme 2 to strategic sites in the cell wall, limiting enzyme mobility. The molecular silencing of the β-subunit protein in tomato fruit (Watson *et al.*, 1994), however, was more influential at increasing pectin solubility rather than the extent of depolymerization (Watson *et al.*, 1994; Chun and Huber, 2000). Although interaction of the &-subunit protein and PG *in vivo* has been questioned (Pressey, 1988; Moore and Bennett, 1994), tomato fruit expressing a β-subunit-antisense gene (Watson *et al.*, 1994) are significantly softer when ripe than wild-type fruit (Chun and Huber, 2000).

Another notable distinction between the tomato and avocado PGs is the considerably higher pH optima (6.0) for the avocado (Wakabayashi and Huber, 2001) compared with the tomato (3.5 to 4.5) isoforms (Pressey and Avant, 1973). Although the pH of avocado fruit apoplast is not known, a high pH would favor the activity of not only PG, but also PMEs, many of which possess relatively high pH optima (5 to 7). Additional diversity in fruit endo-PGs was evident from studies of banana (Pathak and Sanwal, 1998; Pathak *et al.*, 2000), shown to contain 2 isoforms with pH optima of 3.3 and 4.3. As in tomato, one of the isoforms

from banana is of high mol mass (130 kD) and heat stable. In contrast to tomato PGs (Ali and Brady, 1982), the banana (Pathak *et al.*, 2000) and avocado (Wakabayashi and Huber, 2001) isoforms are strongly inhibited by mercury, indicating a requirement for sulfhydryl groups.

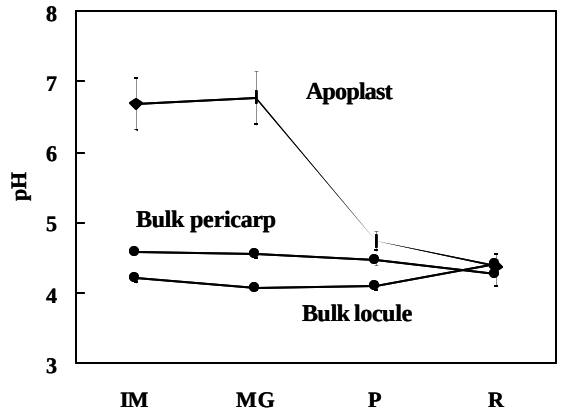

FIGURE 3 - pH of the apoplastic and bulk pericarp and locule tissues at different maturity stages. I, immature; MG, mature-green; P, pink; R, ripe. Error bars represent SE of six observations. (Almeida and Huber 1999) *Used with permission*.

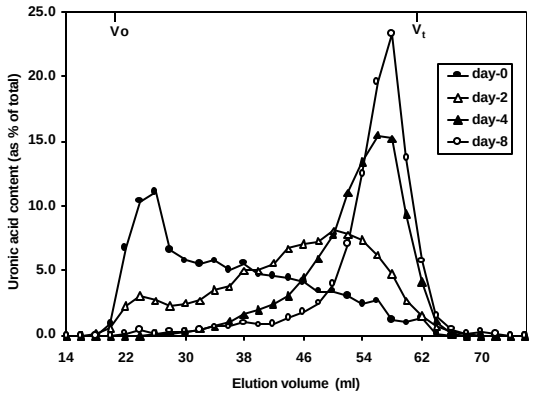

FIGURE 4 - Mol mass distribution of water-soluble pectins from 'Hass' avocado fruit during ripening. Details as described for Figure 1. 0 days, fruit at harvest and after 2, 4, 6, and 8 days (full-ripe, average firmness 10 N) storage at 25°C.

AVOCADO PECTIN DEPOLYMERIZATION AND FRUIT SOFTENING

The extensive hydrolysis of pectins in ripening avocado relative to tomato fruits raised the question as to whether PG might be more influential in the softening of avocado. In an effort to suppress the accumulation of PG in avocado, fruit were treated following harvest with 1-MCP (1-methylcyclopropene), a potent inhibitor of ethylene action (Sisler and Serek, 1997; 1999). As shown in Figure 5 A, the firmness of control (no 1-MCP) fruit declined from nearly 250 N at harvest to about 10 N over a 2-week period at 13 ° C. Fruit treated with 0.9 µl l^{-1} 1-MCP required nearly 4 weeks to reach firmness values of 10 N. PG levels were significantly affected by 1-MCP treatment (Figure 5 B), remaining at harvest levels for up to

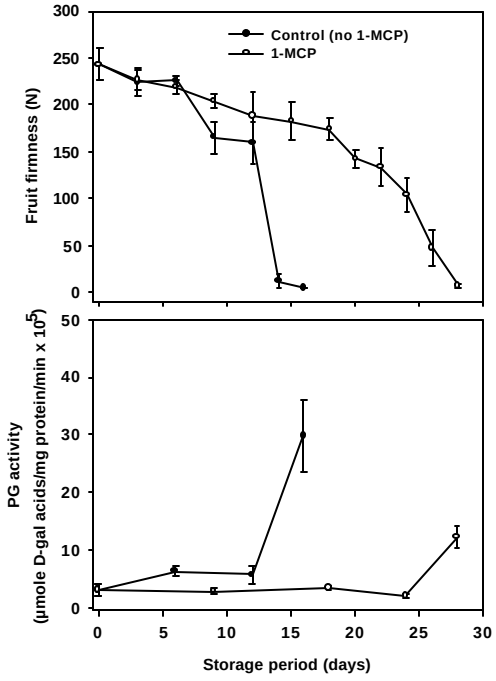

FIGURE 5 - Fruit firmness (A) and PG activity (B) of 'Booth 7' avocados treated with 1-MCP (0.9 µl l^{-1} for 12 h at 20 °C) and then stored at 13 °C. Vertical bars represent standard deviation. (*Jeong and Huber, unpublished*.)

25 days. During this period, the firmness of 1-MCP-treated fruit declined from 250 N to 100 N. In both control and 1-MCP-treated fruit, PG accumulation was temporally correlated with the final trend of softening, during which time firmness decreased to 10 N. These data indicate that significant changes in avocado firmness do occur in the absence of appreciable PG activity, and that the accumulation of the enzyme parallels the decline in firmness occurring during the late stages of ripening. These observations are consistent with interpretations of the role of PG in tomato fruit softening (Kramer *et al.*, 1992; Carrington *et al.*, 1993). The influence of reduced PG levels on pectin solubility and depolymerization patterns in 1-MCP-treated avocado fruit is currently under investigation.

PRODUCTION OF PECTIC FRAGMENTS DURING FRUIT RIPENING

In spite of the extensive hydrolysis of pectins occurring in ripening avocado, low-DP oligomers (< 10) are minor reaction products (Huber and O'Donoghue, 1993). Since PGs from avocado (and tomato) are capable of producing low-DP products, including monomer and dimer, from homogalacturonan (eg. polygalacturonic acid) substrates (Patel and Phaff, 1960; Reymond and Phaff, 1965), the generation of only trace levels of endogenous oligomers during ripening suggests that structural properties of the products impart resistance to exhaustive hydrolysis. The neutral sugar/galacturonic acid mol ratio of the low mol mass, water-soluble pectins in ripe fruit was nearly 1.0 (Jeong and Huber unpublished). This indicates that high glycosylation, as well as methylesterification, which persists at 20 % in ripe fruit (Wakabayashi *et al.*, 2000), may be involved in the arrest of hydrolysis. We have also noted a lack of endogenous, low-DP-oligomer production in ripe tomato fruit (Huber and O'Donoghue, 1993). In contrast, Melloto et al. (1994) and Dumville and Fry (2000) reported the presence of low-DP oligouronides in tomato fruit. The latter report expressed the view that oligouronides were not typically produced in healthy plant tissues, with the notable exception of tomato fruit. In our experience, however, tomato fruit do not appear to represent an exception. Excised (wounded) tomato pericarp discs incubated in buffer at 23°C released significant quantities of pectic oligomers, and this release was proportional to cut surface area (Huber and Lee 1989). Discs maintained in buffer at 1°C released only trace levels of pectins, most of which were of high mol mass. This indicates that endogenous oligouronides were not present at quantitatively significant levels prior to wounding.

GALACTANASES AND PECTIN DEGRADATION

In addition to endo-PGs, other enzymes have been reported to depolymerize or otherwise influence the mol mass distribution of pectins in fruits. Among these enzymes, most attention has focused on &-galactosidases (EC 3.2.1.23), largely because galactosyl residues represent the major cell wall neutral sugar lost during ripening of most fruits (Gross and Sams, 1984). β-Galactosidases from fruit and other sources are similar in showing high activity toward Δ-NO_2-phenyl &-D-galactopyranoside, and β-galactosidases (and other glycosidases) have been reported in all fruits examined. In only a few studies, however, have &-galactosidases, which are typically present in multiple isoforms (Pressey, 1983; Carey *et al.*, 1995; Smith and Gross, 2000; Li *et al.*, 2001), been shown to degrade isolated cell walls or polysaccharides. The cell wall-active isoforms likely represent *exo*-β-D-galactanases rather than oligomer- or dimer-preferring glycosidases. Of three &-galactosidases reported in tomato fruit, only one isoform (β-gal II), the levels of which increased 4-fold during ripening, degraded pectic-derived substrates (Pressey, 1983). More refined analysis of tomato β-gal II (Carey et al. 1995) confirmed the galactan-hydrolyzing activity of the enzyme; however, the purified enzyme exhibited unusual behavior in being active toward isolated cell walls but not purified tomato galactan. Based

on the capacity of the enzyme to release only monomeric galactose from galactan substrates, the authors identified the enzyme as an *exo*-1,4-β-D-galactanase. A β-galactosidase from kiwifruit (Ross *et al.*, 1993) also produced monomeric galactose from a number of substrates, including a pectic fraction; however, the levels of galactose released were considerably lower than the decline in cell wall galactosyl residues during ripening. Extremely low activity toward cell walls or galactan polymers was also evident for β-galactosidases from apple (Ross *et al.*, 1994) and Japanese pear (Kitagawa *et al.*, 1995) fruits. Ross et al. (1993) has stated that if the galactan-hydrolyzing β-galactosidases are solely responsible for the decline in cell wall galactosyl residues during ripening, then the activity of the enzymes must be considerably higher *in vivo*.

The effect of degalactosylation on the physical properties of pectins is not known. β-Galactosidases/exo-galactanases from avocado (de Veau *et al.*, 1993) and muskmelon (Ranwala *et al.*, 1992) fruits were shown to induce mol mass downshifts in isolated pectin fractions, presumably via hydrolysis of pectic galactans. In the latter study, treatment of an EDTA-soluble pectin with a high-saline extractable &-galactosidase resulted in mol mass downshifts far in excess of those noted during muskmelon ripening. Scrutiny of the gel filtration profiles, however, reveals no evidence for monomers, the expected product of β-galactosidase/exo-galactanase action (Carey *et al.*, 1995; Ross *et al.*, 1993). Since the enzymes used by Ranwala et al. (1992) were only partially purified, the participation of enzymes other than &-galactosidases in the pectin mol mass downshifts in ripening muskmelon fruit cannot be discounted. In support of this view, Hadfield et al. (1998) reported that expression of melon cDNA clones with high homology to PG clones from other fruits coincided with the onset of pectin mol mass downshifts and with the accumulation of pectin-degrading activity.

A recent analysis of tomato β-galactosidases has revealed a minimum of seven &-galactosidase genes (Smith and Gross, 2000), 6 of which were suggested to participate in the deglycosylation of tomato cell wall polysaccharides. Moreover, as noted by these authors, differences in expression patterns during fruit development and the reported differences in substrate specificities of β-galactosidases/exo-galactanases (Li *et al.*, 2001) raise the possibility that these enzymes target different substrates and function in a variety of developmental processes. The loss in cell wall galactosyl residues in senescing carnation petals (de Vetten and Huber, 1990) and in harvested asparagus spears (O'Donoghue *et al.*, 1998; Rodríguez *et al.*, 1998), attests to multiple functions for galactanases and galactose turnover. In addition to their effects on pectic polymers, some β-galactosidases are active toward hemicelluloses (Ranwala *et al.*, 1992; Li *et al.*, 2001), resulting in the production of monomeric galactose (Li *et al.*, 2001). That galactosidases and galactanases might play a role in textural properties is supported by a recent report that pea cotyledons containing galactan-rich pectin were significantly firmer than pectic-galactan-depleted cotyledons (McCartney *et al.*, 2000). Tucker et al. (1999) reported that cold-break tomato pastes from fruit expressing a galactanase antisense-gene exhibited higher viscosity compared with pastes from normal fruit, providing indirect evidence for a role for these enzymes in pectin degradation.

NONHYDROLYTIC MECHANISMS CONTRIBUTING TO PECTIN DEPOLYMERIZATION IN RIPENING FRUITS

Reports of expansin-type proteins in a range of vegetative organs including leaves, coleoptiles, hypocotyls and others (Cosgrove, 1999; 2001) have demonstrated a role for these 'nonhydrolytic' proteins in extension growth. Recent studies have shown that structurally related proteins may play important roles in fruit growth and softening, either via direct effects on specific cell wall polymers, promoting cell wall relaxation or creep, or indirectly by increasing the

accessibility of wall polysaccharides to enzymic hydrolysis (Brummel *et al.*, 1999; Rose *et al.*, 2000). Ripening-related, expansin-type activity has been reported in tomato, pepper, avocado and pear (Rose *et al.*, 2000), strawberry (Civello *et al.*, 1999), and peach (Hayama *et al.*, 2000) fruits. Evidence for a role of expansins in softening was observed in studies of tomato fruit with suppressed or over-expressed levels of *Exp1*, a ripening-specific expansin (Brummel *et al.*, 1999). Fruit under-expressing Exp1 protein were firmer than controls, exhibited suppressed pectin depolymerization during late ripening, but showed normal mol mass downshifts in hemicelluloses. Fruit over-expressing Exp1 were softer throughout ripening, and exhibited unaltered pectin metabolism but enhanced breakdown of hemicelluloses. Creep activity, assessed from the addition to cucumber hypocotyls of cell wall protein extracts, demonstrated expansin-like proteins in a variety of fruits, though the abundance or activity differed significantly (Rose *et al.*, 2000). In one contrasting report, Hayama et al. (2000) observed no differences in transcript abundance or immunologically detected levels of a ripening-specific expansin between melting-flesh and stony-hard peach cultivars. Based on models of the function of expansin, however, only one component (creep) is mediated *via* direct action of the protein. This effect may not be evident in firmness determined via puncture analysis of peach mesocarp. Other, indirect effects of expansins, including enhanced susceptibility of cell wall polymers to enzyme hydrolysis, would depend not only on expansin levels *per se* but also on the levels and activities of hydrolytic enzymes. Consequently, that expansin levels are similar between the two peach cultivars does not preclude a role for the protein in softening.

An interesting though not widely examined mechanism of polysaccharide depolymerization in ripening fruit envisions the participation of radical oxygen species (ROS). The first report of the potential involvement of ROS in the degradation of cell wall polysaccharides under physiological conditions appears to be that of Miller (1986), who observed a decrease in viscosity and generation of reducing sugars upon incubation of cell wall polymers, including pectin and polygalacturonic acid, in 1 mM H_2O_2 at pH 6.5. H_2O_2-mediated degradation was also noted with tomato and cucumber fruit cell walls. Fry (1998) observed more extensive hydrolysis of pectin, xyloglucan, and other polysaccharides by including ascorbate and Cu^{2+}, both known components of apoplastic fluid, along with H_2O_2. By examining the effects of free radical scavengers, Fry (1998) concluded that the hydroxyl radical (·OH), generated from a Fenton-type reaction, was responsible. The presence in the apoplast of components required for ·OH generation (Takahama and Oniki, 1997; Zarra *et al.*, 1999) and the rapid occurrence of radical-mediated polymer scission under physiological pH values led Fry (1998) and Schweikert et al. (2000) to conclude that radical-mediated polysaccharide degradation may be relevant in many developmental processes including germination, growth, and ripening. Whereas Miller (1986) and Fry (1998) emphasized a role for ROS generated via non-enzymic reactions, Schweikert et al. (2000) considered the participation of ·OH produced through peroxidase-mediated reactions. Since many forms of peroxidase are tightly wall-associated (Sato *et al.*, 1995; Nair and Showalter, 1996), peroxidase- versus nonenzymic-generated radicals would seem to afford more control over polysaccharide scission by facilitating targeted or site-directed cleavage. While ROS-mediated scission of cell wall polysaccharides offers an interesting adjunct to enzyme-based (hydrolases) or 'creep' (expansins) mechanisms, the apoplast is well endowed with antioxidant enzymes (eg. catalase, reductases) and metabolites (Vanacker *et al.*, 1998) whose effects would tend to reduce the occurrence of radical-mediated degradation. It is possible that the competence of the antioxidant system becomes compromised during ripening and senescence (Bartosz, 1997; Kanazawa *et al.*, 2000), progressively shifting the balance in favor of prooxidative reactions.

POLYSACCHARIDE DEGRADATION IN LIGHTLY PROCESSED FRUITS

If radical-based mechanisms of polysaccharide scission are more operational in wounded or otherwise physically compromised tissues (Bolwell *et al.*, 1995), then they may be of importance in the deterioration of lightly processed (LP) fruits. Fruits destined for processing as LP products are nearly fully ripe and are subjected to a combination of peeling, cutting, slicing, or dicing. Studies have shown that accelerated softening is a prominent feature of LP fruits (O'Connor-Shaw *et al.*, 1994; Watada and Qi, 1999). Since LP fruits are typically held at # 5ºC, low-temperature injury may contribute to the decline in tissue firmness (Jackman *et al.*, 1992), particularly in fruits of tropical origin. Studies of LP fruits, however, typically do not include intact fruit stored under identical conditions, so it is difficult to ascertain the influence of wounding versus low temperature in enhancing firmness decline. As shown in Figure 6, the firmness of LP papaya fruit declined significantly more rapidly and extensively than tissue derived from intact fruit stored under identical conditions, supporting the view that the accelerated softening of LP papaya fruit is not a reflection of low-temperature injury.

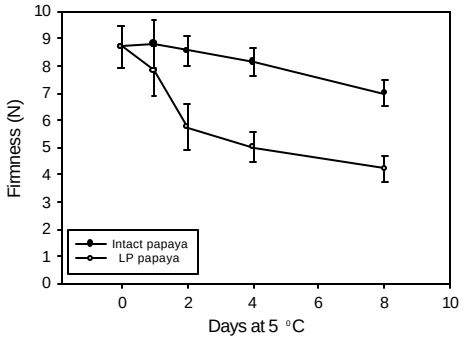

FIGURE 6 - Firmness of intact and lightly processed (LP) papaya fruit during storage at 5 ^{0}C. *(Karakurt and Huber, unpublished).*

Although there is limited information on the mechanism of deterioration of LP fruits stored at low temperatures, studies of physically wounded tissues (Esquerrϑ-Tugayϑ *et al.*, 2000) support the notion that a multitude of processes, including polysaccharide degradation, are involved. As noted above, pectic oligomers released from wounded tomato pericarp (Huber and Lee, 1989) are in excess of levels recovered from intact fruit. The increased production of oligouronides may involve alleviation of *in vivo* constraints on pre-existing PG, or wound-induced enzyme synthesis. Bergey et al. (1999) reported that PG transcript and activity levels increased in tomato leaves in response to mechanical wounding, or to the application of pectic fragments or systemin. The comparable response in both wounded and non-wounded leaves indicates systemic activation. Moretti et al. (1998) found that extractable PG activity increased nearly 30 % in the wounded tissue of impact-bruised tomato fruit. While the latter observation provides evidence for wound-induced PG accumulation in tomato fruit, the rapid depolymerization of pectins in fruit homogenates indicates that activation of pre-existing PG is sufficient to stimulate oligomer production in wounded tomato fruit. Figure 7 illustrates the mol mass distribution of water-soluble pectins from

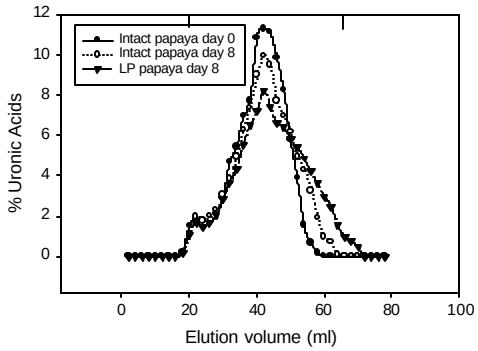

FIGURE 7 - Molecular mass distribution of water-soluble pectins from intact and lightly processed (LP) papaya fruit during storage at 5 ^{0}C. Water-soluble pectins (0.5 mg galacturonic acid equivalents) were applied to a CL 4B-200 column as described for Figure 1. *(Karakurt and Huber, unpublished).*

intact and wounded (LP) papaya fruit stored for 8 days at 5 °C. Although pectins from both intact and LP fruit changed during storage, those from LP fruit were of greater polydispersity and showed more depolymerization than pectins from intact fruit. Consistent with the mol mass profiles, extractable polygalacturonase activity increased nearly 30 % in the LP compared with intact fruit.

Another prominent feature of LP fruit is increased respiration, with LP fruits typically exhibiting a 2- to 3-fold increase compared with the intact commodity (Watada *et al.*, 1996). In the short-term, it is likely that the pheripheral, injured cells have a proportionally greater contribution to enhanced respiration. The respiratory response to wounding may have mechanistic parallels to the 'oxidative burst,' a response of plant tissues to pathogen ingress involving the production of H_2O_2 and ROS (Bolwell *et al.*, 1995; Low and Merida, 1996). As noted above, some authors have proposed that non-enzymically (Miller, 1986; Fry, 1998) and peroxidase- (Schweikert *et al.*, 2000) generated H_2O_2 and/or ROS might contribute to the degradation of pectic and other cell wall polysaccharides in fruits. A contribution of lipoxygenase (LOX) activity to ROS ($O_2^{\cdot -}$) production has also been suggested (Lynch and Thompson, 1984). LOX isoforms are widely distributed in plant tissues, and likely are involved in diverse developmental processes; however, the enzymes are frequently associated with stress and senescence phenomena (Rosahl, 1996). As shown in Figure 8, the total LOX activity of LP papaya fruit increased nearly 2-fold within 24 hours at 5 °C compared with intact fruit and remained significantly higher throughout 8 days of storage. In addition to the possible contribution of LOX to ROS production and membrane and polysaccharide degradation, peroxidative reactions involving membrane-derived fatty acids would generate signal-transduction metabolites (jasmonic acid, traumatin) and, consequently, activate defense responses systemically.

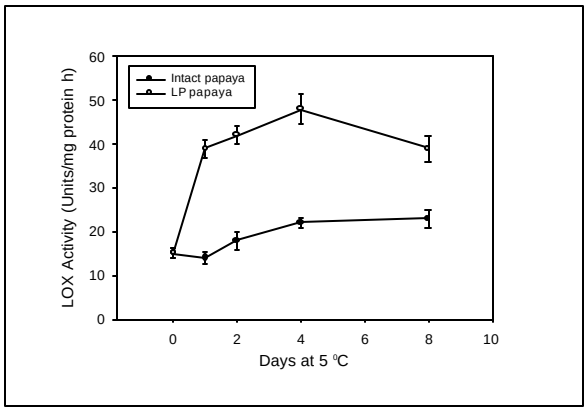

FIGURE 8 - Lipoxygenase activity in intact and lightly processed (LP) papaya fruit during storage at 5 °C. *(Karakurt and Huber, unpublished).*

SUMMARY

There is likely no one scenario that accurately describes the course of pectin metabolism in ripening fruits. On the one hand, pectin depolymerization is a consistent feature of fruit expressing polygalacturonase, though the extent of hydrolysis varies greatly among different fruits. On the other hand, pectin solubilization is characteristic of all fleshy fruits, indicating that factors other than PG contribute to structural modification of pectins. Endogenous levels of PG and PME do not always correlate well with trends of pectin depolymerization, indicating that the activity of these and likely other enzymes is restricted *in vivo.* Mechanical wounding appears to alleviate constraints on hydrolysis, resulting in more extensive pectin degradation. Degalactosylation, and deglycosylation in general, may contribute to pectin changes during the early stages of ripening. Radical oxygen species of various origins have been proposed to participate in the degradation of pectins and other polysaccharides during ripening, and may be of special relevance to LP (wounded) fruits.

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