PAPAYA FRUIT SOFTENING: ROLE OF HYDROLASES

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ABSTRACT

Hydrolases have been detected during fruit softening and may play an important role in papaya softening. Variation in softening of papaya varieties and the incomplete softening of papaya treated with 1-methylcyclopropene (1-MCP) present an opportunity to determine the factors causing fruit softening. To determine whether six hydrolases and pH are involved in papaya softening, comparison of cell wall modification, hydrolases' activities, and mesocarp pH between papaya that differ in softening rates, and normal softening versus softening modified by 1-MCP were used.

Mesocarp of 'Line 8' and 'Sunset' papayas showed significant losses in cell wall mass during normal softening, but papaya did not lose the mass when softening was restrained by 1-MCP treatment. Solubilizations and dissociations of pectic polysaccharides and loosely bound matrix polysaccharides that consisted of xylosyl and galactosyl residues at high concentration were noticeable during normal softening. Although the major changes in galactosyl components of cell wall in 1-MCP-treated papaya were comparable to those in control papaya, the 1-MCP-treated papaya did not soften completely. The comparison of cell wall modification between 1-MCP-treated papaya and control papaya confirmed that the modification of xylosyl component of cell wall was involved in the abnormal papaya softening when treated with 1-MCP.

Activities of β-galactosidase, endoglucanase, endoxylanase, and β-xylosidase were correlated with fruit softening. When softening was modified by 1-MCP treatment, a rise in activity of these hydrolases was delayed; but only endoxylanase activity was completely suppressed throughout ripening. During softening, a change in mesocarp pH was implicated. Mesocarp pH of 'Line 8' and 'Sunset' papayas declined when fruit

started to soften with no reduction in 1-MCP-treated papaya. Applied low pH affected mesocarp firmness, but the effect varied with the stage of fruit ripeness and specific fruit tissue.

Papaya softening is a complex event that involves many cell wall hydrolases, such as endoxylanase, β-xylosidase, β-galactosidase, and endoglucanase. These hydrolases may play their roles in concert, to provide the unique texture of a particular fruit. Endoxylanase appears to play a major role in papaya softening. A regulated decline in mesocarp pH during ripening may regulate these hydrolases and impact papaya mesocarp softening.

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LIST OF ABBREVIATIONS

Ara	Arabinose
CMC	Carboxymethylcellulose
CWM	Crude cell wall material
FWt	Mesocarp fresh weight
Gal	Galactose
Glc	Glucose
LSD	Mean comparison by Fisher's Least Significant Difference
Man	Mannose
MCP or 1-MCP	1-Methylcyclopropene
mRNA	Messenger ribonucleic acids
PG	Endopolygalacturonase
PME	Pectin methylesterase
PMSF	Phenylmethylsulphonyl fluoride
Prob	Probability
r	Correlation coefficient
Rha	Rhamnose
SE	Standard error of mean
Xyl	Xylose

Units

A_{415}	Absorbance value at wavelength 415 nanometer
c-	Centri-, $x10^{2}$
g	Gram
hr	Hour
KDa	Kilo Dalton
L	Liter
m	Meter
m-	Milli-, $x10^{-3}$
M	Molar
min	Minute
n-	Nano-, $x10^{-9}$
N	Normal
ppb	Part per billion
ppm	Part per million
rpm	Round per minute
°C	Degree Celsius
(v/v)	Volume by volume
(w/v)	Weight by volume
μ-	Micro-, $x10^{-6}$

CHAPTER 1

INTRODUCTION

Fruit softening influences not only palatability but also shelf life, wastage, infection by postharvest pathogens, frequency of harvest, transportation, and storage (Brummell and Harpster, 2001; Carey *et al.*, 2001). Over-ripening associated with softening is one of the primary causes of postharvest loss (FAO, 1981; FFTC, 2005). Postharvest losses of fruit such as papaya, mango, banana, citrus, and pineapple can be high as 35% (FFTC, 2005).

Changes in cell wall structure are closely linked to fruit softening (Carey et al., 2001). Enzymatic and non-enzymatic factors play roles in cell wall modification (Almeida and Huber, 1999; Fry, 2004). Many cell wall degrading enzymes have been reported during fruit ripening and softening: polygalacturonase (PG) (Fischer and Bennett, 1991; Seymour and Gross, 1996; Hadfield and Bennett, 1998), pectin methylesterase (PME) (Harriman et al., 1991; Tieman et al., 1992), glucanase (Awad and Young, 1979; Abeles and Takeda, 1990), galactosidase (Pressey, 1983; Carey et al., 1995; Smith et al., 1998), and xylanase (Labavitch and Greve, 1983; Pauli and Chen, 1983). Reduction in cell turgor pressure reduces expansionary pressure on the cell wall contributing in alterations of fruit textural properties (Carey et al., 2001; Brummell, 2006). Expansins, cell wall localized proteins, cause cell wall loosening (McQueen-Mason *et al.*, 1992; Cosgrove, 2000a) and are thought to be associated with wall disassembly during fruit ripening (Cosgrove, 2000a). Apoplastic ionic strength and pH possibly regulate both non-enzymatic and enzymatic cell wall modification (Chun and Huber, 1998; Almeida and Huber, 1999). Hydroxyl radicals (*OH) are capable of oxidative cleaving of wall

polysaccharides nonspecifically (Fry, 1998; Cosgrove, 1999; Liszkay *et al.*, 2003). Also, cations can regulate cell wall modification (Javis, 1982; O'Neill *et al.*, 1996; Molhoj *et al.*, 2003; Fry, 2004). Fruit softening seems to involve many factors. However, the precise roles of the different factors in a particular cell wall alteration and on fruit softening are still unclear. It is also unclear whether all fruit use the same complement and activities of cell wall hydrolases.

Papaya (*Carica papaya* L.), an exotic tropical fruit is usually consumed fresh. The fruit has an important economic value in many tropical countries. In Hawaii, the sale value of papaya was over 11 million dollars, ranking second only to pineapple in 2005 (NASS, 2006). Quality and shelf life of fresh papaya are compromised by rapid softening, as with other fruit. USDA reported in 1992 that over 40% of 59,638 cartons of Hawaiian papaya shipped to US mainland had postharvest defect as soft fruit, and over 50% of the cartons showed postharvest defects such as bruising (Paull *et al.*, 1997). Among papaya cultivars in Hawaii, 'Sunrise' and line 'Line 8' are graded as having a shorter shelf life than 'Kapoho' and 'Sunset'. The shorter posthavest life of 'Sunrise' and 'Line 8' may be associated with the fruit continuing to soften and becoming exceedingly soft and translucent, while the other varieties such as 'Kapoho' and 'Sunset' do not show this very soft translucent stage.

The ethylene reception inhibitor, 1-methylcyclopropene (1-MCP), is used for inhibiting ethylene responses. 1-MCP applications extend the postharvest shelf life and quality of numerous fruits, vegetables, and floricultural crops (Serek *et al.*, 1994; Golding *et al.*, 1998; Ku and Wills, 1999; Hofman *et al.*, 2001; Jiang *et al.*, 2002; Blankenship and Dole, 2003; Huber *et al.*, 2003). Fumigation with 100 nL L^{-1} 1-MCP for 12 hours delays ethylene production, climacteric respiration, skin color development, and softening of

papaya without affecting TSS and fruit weight loss (Manenoi, 2005). The papaya treated with 1-MCP at the color break stage shows an incomplete softening, causing rubbery texture when ripe (Manenoi *et al.*, 2007).

The variation in softening of papaya varieties and the incomplete softening of papaya treated with 1-MCP present an opportunity to determine the factors causing fruit softening. The alteration of softening by 1-MCP could also eliminate some factors whose involvement during ripening may not contribute to fruit softening. The objective of this study was to determine whether particular hydrolases and pH are involved in softening of papaya fruit. The comparison was between papayas that differ in softening rates, and normal softening versus softening modified by 1-MCP was used.

CHAPTER 2

LITERATURE REVIEWS

2.1 Physiology of Fruit Ripening

Fruit ripening marks the completion of development of a fruit (Giovannoni, 2001), the commencement of senescence (Hadfield and Bennett, 1997), and is normally irreversible (Rose and Bennett, 1999; Klee, 2004). A ripening fruit undergoes complex physicochemical changes after harvest, and these changes determine the quality of the fruit (Giovannoni, 2001). Major changes occurring during fruit ripening include changes in respiration, ethylene production, color, flavor, storage materials, and texture (Tucker, 1993).

Fruit have been classified into two major groups by changes in their respiration rate (Biale, 1960; Giovannoni, 2001). Climacteric fruit, such as papaya, tomato, avocado, cherimoya, apple, peach, banana, and mango, show a climacteric peak of respiration during ripening; while non-climacteric fruit, such as pineapple, strawberry, and citrus, exhibit a gradual decline in respiration (Biale *et al.*, 1954; Eaks, 1970; Chalmers and Rowan, 1971; Tucker, 1993; Lelievre *et al.*, 1997; Giovannoni, 2001). Fruit respiration normally uses two major substrates: sugars (Duque *et al.*, 1999) and organic acids such as malic acid (Moreau and Romani, 1982; Bruinsma and Paull, 1984). Both sugars and organic acids are found largely within vacuoles and are major contributors of overall fruit flavor (Tucker, 1993).

All fruit produces basal quantities of ethylene during development (Lelievre *et al.*, 1997; Alexander and Grierson, 2002). However, climacteric fruit show an increase and

produce much larger amounts of ethylene during ripening than non-climacteric fruit (Tucker, 1993; Lelievre *et al.*, 1997). Applied ethylene can accelerate respiration and ripening of mature climacteric fruit (McGlasson and Pratt, 1964). Although applied ethylene causes an increase in respiration of non-climacteric fruit (Eaks, 1970), ethylene is not required for their ripening (Lelievre *et al.*, 1997). In contrast, ethylene is necessary for the coordination and completion of ripening in climacteric fruit (Yang, 1985; Oeller *et al.*, 1991; Picton *et al.*, 1993; Giovannoni, 2001).

Color is the most obvious change in many fruit during ripening. In general, color changes are associated with ripening (Tucker, 1993). Therefore, fruit color is often the major criterion used by consumers to determine whether the fruit is ripe or unripe. The degree of skin color development is also used as a harvesting index (Paull *et al.*, 1997). The most common change in fruit color is the loss of green color. The green color loss is due to chlorophyll degradation. The principal factor responsible for chlorophyll degradation is chlorophyllase, an enzyme discovered by A. Stoll in 1912 that degrades chlorophyll to colorless catabolites (Matile *et al.*, 1996; Pruzinska *et al.*, 2005). The disappearance of chlorophyll is often associated with the synthesis (Jeffery *et al.*, 1984; Fraser *et al.*, 1994) or exposure of other pigments (Miller and Winston, 1939) as found in tomato and orange, respectively. Yellow, orange, and red colors are provided by carotenoids that accumulate in chromoplasts (Kato *et al.*, 2004). Anthocyanins providing red to blue colors in plants are found in cell vacuoles, often in epidermal layers (Moskowitz and Hrazdina, 1981).

Flavor is based on taste and odor. Sense of smell of an individual fruit is due to production of specific volatiles. Volatile profile of any fruit is usually very complex (Tucker, 1993; Moya-Leon *et al.*, 2006). However volatile compounds play an important

role in the optimum eating quality in fruit (Predieri *et al.*, 2005; Moya-Leon *et al.*, 2006). Sugars and organic acids contribute to the fruit taste (Alexander and Grierson, 2002). Carbohydrate degradation, especially conversion of starch to sugar, is the largest quantitative change associated with ripening in some fruit such as banana (Beaudry *et al.*, 1989; Hubbard *et al.*, 1990). Increase in sugar makes fruit much sweeter and more acceptable to consumers (Predieri *et al.*, 2005). The sugar may be derived from phloem transport to the fruit such as papaya, rather than from the breakdown of fruit starch reserves (Chen *et al.*, 2001). Organic acids usually decline during ripening (Ayaz *et al.*, 2001; Medlicott and Thompson, 2006) as they are respired (Duque *et al.*, 1999; Davie and Maw, 2006) or converted to new compounds. There are exceptions, such as bananas, where the highest acid level is attained when the banana is fully ripe (Wyman and Palmer, 1964).

Most fruit softening is a major quality attribute and dictate shelf life (Tucker, 1993). Softening rate differs with fruit types and varieties. Fruit softening may arise from one of three mechanisms: loss of turgor (Shackel *et al.*, 1991; Saladie *et al.*, 2007), degradation of starch (Kojima *et al.*, 1994), and breakdown of the fruit cell walls (Tucker, 1993; Brummell, 2006). Loss of turgor may result from dehydration of the fruit by transpiration, or a change in partitioning of solutes between symplastic and apoplastic compartment without any change in whole tissue osmotic potential (Shackel *et al.*, 1991; Brummell, 2006; Saladie *et al.*, 2007). Degradation of starch probably results in a pronounced textural change, especially in banana where starch accounts for a high percentage of the fresh weight (Kojima *et al.*, 1994). However, texture change during ripening of most fruit is thought to be largely the result of cell wall degradation (Tucker, 1993; Brummell, 2006).

2.2 Physiology and Biochemistry of Fruit Softening

2.2.1 Cellular Characteristics of Fruit

Plant cells are bounded by a relatively rigid cell wall. The cell wall is permeable to water and solutes of up to 60 KDa (Tepfer and Taylor, 1981). The main function of cell wall is to contain cell components and to give structural support to the cell and plant tissue. Within the plasma membrane, the plant cell is comprised of the cytoplasm including a nucleus, a few mitochondria, plastids, and usually one or more large vacuoles. The vacuoles contain various solutes such as sugars, organic acids, and salts (Moskowitz and Hrazdina, 1981). Vacuole is surrounded by a semipermeable membrane, the tonoplast.

Fruit cells are mainly parenchymous (Huber, 1983). Cell strength is determined by bonding between neighboring cells of fruit tissues, mechanical and physical properties of cell wall, and internal pressure (Harker *et al.*, 1997; Edwards, 1999). During fruit softening, cell wall thickness becomes uneven. Cell walls are cemented together by middle lamella, imparting a degree of rigidity to tissues (Seymour and Gross, 1996). The tonoplast and plasma membrane are responsible for maintaining the hydrostatic pressure of the cell and imparting turgidity. Turgidity of the cell maintains the shape of plant tissue (Edwards, 1999). When turgor pressure is lost, the cell structure collapses. Larger cells and more intercellular space are commonly considered signs of softening tissue (Harker *et al.*, 1997). Ripe, softened apple tissues show an increase in cell separation and have rounder cell shape and larger intercellular space than non-softened apple tissues (Lapsley *et al.*, 1992; De Smedt *et al.*, 1998).

2.2.2 Plant Cell Wall

A principal difference between plants and animals is that plant cells are surrounded by a rigid cell wall. Therefore, the form and function of plants depend on properties of their cell walls (Cosgrove, 1997). The complex structure and unique wall of particular cell reflex suitability to the cells function (Carpita and McCann, 2000).

Although plant cell walls vary in their structures and components, they are generally classified into two major types, primary cell wall and secondary cell wall. Primary cell walls are characteristic of growing cells (Cosgrove, 1997). Primary cell walls are typically thin and are born when the cell plate forms during cell division (Cosgrove, 1997). The primary cell wall is capable of increasing in surface area during cell expansion. Secondary cell walls are thicker than primary cell walls, and extra cell wall material is deposited when most cell enlargement has ceased (Cosgrove, 2005). The middle lamella occurs at the interface between the primary cell walls of neighboring cells and cements neighboring cells together (Cosgrove, 2005).

Primary cell walls of land plants generally have a similar fundamental structure and are composed mainly of neutral and acidic polysaccharides, and a small amount of structural proteins (Cosgrove, 1999). The cell wall polysaccharides can be classified into three main groups: celluloses, hemicelluloses, and pectins.

Celluloses are the principal scaffolding component of cell walls. They account for 15-30% of the dry weight of all primary cell walls and a larger percentage of secondary cell walls (Carpita and McCann, 2000). Celluloses are (1→4) β-D-glucan chains that are held together by hydrogen bonding to form crystalline microfibrils (Brummell and

Harpster, 2001). The cellulose microfibrils provide both strength and flexibility and are embedded in a highly hydrated matrix of hemicelluloses and pectins.

Hemicelluloses are now referred to as matrix polysaccharides. Other names include cross-linking glycans and matrix glycans. Matrix polysaccharides bind cellulose microfibrils together into a cohesive network. These polysaccharides also act as a slippery coating to prevent direct microfibril-microfibril contact (Cosgrove, 1997). Matrix polysaccharides include xyloglucan, (galacto)glucomannan, glucurono-arabinoxylan, and xylan (Brummell and Harpster, 2001). Xyloglucan has a (1→4) β-D-glucan backbone like cellulose, with in a regular fashion on three out of four consecutive glucose residues having α-D-xylose branches. Xylose side chains occasionally are extended with β-D-galactosyl-α-L-fucose or α-L-arabinose (Brummell and Harpster, 2001). (Galacto)glucomannan contains a backbone composed of regions of (1→4) β-D-glucan and (1→4) β-D-mannan in approximately equal amounts, with occasional side chains of single units of α-D-galactose (Brummell and Harpster, 2001). Glucuronoarabinoxylan has a backbone of (1→4) β-D-xylan, with side chains of single units of non-reducing terminal α-L-arabinose and α-D-glucuronic acid (Brummell and Harpster, 2001). Xylan is a (1→4) β-D-xylan without side chains (Chanda *et al.*, 1951; Ahmed, 1978).

Pectins act like a hydrophilic filter that prevents aggregation and collapse of the cellulose networks (Javis, 1992). They also determine the porosity of cell walls to macromolecules (Cosgrove, 2002). Pectins include homogalacturonan, rhamnogalacturonan I (RG I), and rhamnogalacturonan II (RG II) (Brummell and Harpster, 2001). Homogalacturonan is composed of a long chain of (1→4) α-D-galacturonic acid. When synthesized, it is initially highly methyl-esterified (Brummell and

Harpster, 2001). RG I is made of alternating α-D-rhamnose and α-D-galacturonic acid residues. Long side chains attached to the rhamnose residues are either unbranched (1→4) β-D-galactan, branched α-L-arabinans, or type I arabinogalactans. RG II is made of a backbone of (1→4) α-D-galacturonic acid like homogalacturonan, but with complex side chains of several neutral sugars. RG II is a minor cell wall component, but RG II monomers can dimerize together as boron di-esters and may affect the porosity of the wall (Brummell and Harpster, 2001).

Wall structural proteins may add mechanical strength to the wall and assist in the proper assembly of other cell wall components (Cassab, 1998). Four major classes of wall structural proteins are recognized: hydroxy-proline-rich glycoproteins, proline-rich glycoproteins, glycine-rich proteins, and proteoglycans (Showalter, 1993; Cosgrove, 1997; Carpita and McCann, 2000).

Degradation of wall polysaccharides and alteration of bonding between these polymers, cause an increase in cell separation and a softening and swelling of the wall. These wall modifications, combined with alterations in turgor of cells, bring about cell elongation, fruit softening, and textural changes during fruit ripening (Brummell, 2006).

2.2.2.1 Cell Wall Modification during Fruit Ripening

During ripening, cell wall architecture and the polysaccharides are modified progressively (Brummell and Harpster, 2001). The amount and types of changes vary between species (Brummell and Harpster, 2001). For many fruit species, ripening is generally accompanied by an increase in pectin solubility and depolymerization, and a loss of non-glucose neutral sugars side chains, principally galactose and arabinose of RG I pectin (Gross and Wallner, 1979; Gross and Sam, 1984; Tucker, 1993; Redgwell *et*

al., 1997a; Brummell, 2006). Depolymerization of pectins may begin during early to mid-ripening but usually is most distinct late in ripening tomato (Brummell, 2006). Depolymerization of pectins occurs predominantly at the late ripening of melon (Rose *et al.*, 1998) and suddenly at the late ripening of peach (Brummell *et al.*, 2004), when softening has developed.

Homogalacturonans, one component of pectin, are firstly synthesized and secreted into the cell wall with a high degree of methyl esterification (Carpita and McCann, 2000). A reduction in degree of pectin methyesterification is notable during fruit ripening (Brummell, 2006). Mature green tomato wall prepared by heat inactivation in ethanol had over 90% of galacturonic acid residues of cell walls methylesterified, but the percentage of methylesterified galacturonic acid residues was greatly reduced at the mid-ripe and full ripe fruit (Koch and Nevins, 1989). Pectin demethylation modifies pH, ion balance, and charge of the apoplast. These modifications also possibly affect pectin solubilization and hydration status (Brummell, 2006).

Cell wall structure becomes increasingly hydrated as the cohesion of the pectin gel changes (Javis, 1984). This is a main factor influencing how easily cell can be split open or separated from one another, a major determinate of fruit texture (Javis, 1984). A reduction in cell-to-cell adhesion is caused by a breakdown and dissolution of the pectin-rich middle lamella. The break down of middle lamella has been shown to begin early in the ripening of soft fruit such as tomato (Crookes and Grierson, 1983) and at late softening in crisp fruit such as apple (Ben-Arie *et al.*, 1979). The breakdown of middle lamella in a tuber has been believed to be due to the breaking of hydrogen bonds between pectins and other cell wall polysaccharides (Sterling, 1966).

In fruit such as strawberry and avocado that develop a soft melting texture during ripening, swelling of cell wall is evident, but in fruit such as apple that ripen to a crisp texture, cell wall swelling is not observed (Redgwell *et al.*, 1997b). Cell wall swelling may be related to a loosening of xyloglucan-cellulose network and to pectin solubilization (Redgwell *et al.*, 1997b; Brummell, 2006). The loosening of xyloglucan-cellulose network and pectin solubilization, combined with the pectic side chain loss, increase wall porosity. Sequentially, the increase in wall porosity may allow greater accessibility of wall-degrading enzymes to wall substrate polymers (Brummell, 2006).

Depolymerization of matrix polysaccharides is observed throughout ripening of many fruit, including strawberry (Huber, 1984), peach (Brummell *et al.*, 2004), and melon (Rose *et al.*, 1998). Depolymerization of matrix polysaccharides is believed to be a major contributor to the reduced rigidity of cell walls that leads to fruit softening (Brummell, 2006). The amount of cellulose in grape berries decreases during softening (Yakushiji *et al.*, 2001), whereas depolymerization of cellulose is not evident during ripening of tomato (Maclachlan and Brady, 1994). However, depolymerization of outer glucan chains of a microfibril, to which cross-linking glycans are attached, can have a large effect on wall properties (Brummell, 2006).

Cell wall biosynthesis continues through fruit ripening (Mitcham *et al.*, 1989; Brummell, 2006). Exogenous radioactive-labeled sugars are incorporated into cell wall of both tomato and kiwi fruit during ripening (Greve and Labavitch, 1991; Redgwell, 1996). Tong and Gross (1988) also observed an increase in synthesis of low molecular weight glucomannan. The role of wall biosynthesis during fruit ripening, and its effect on fruit softening, remains unclear (Brummell, 2006).

2.2.2.2 Enzymatic Cell Wall Modification

Many enzymes have been detected in the cell walls at various stages of fruit development (Wallner and Walker, 1975; Awad and Young, 1979; Ahmed and Labavitch, 1980b; Crookes and Grierson, 1983; Hadfield *et al.*, 1998; Chen and Paull, 2003; Itai *et al.*, 2003; Nakamura *et al.*, 2003). The enzymes found include glycoside hydrolases (glycosidases), glycoside transferases (glycosyltransferases), lyases, and oxidoreductases. However, glycosidases are the most enzyme found in plant cell walls (Fry, 2004). *In vitro*, presence of many cell wall degrading enzymes has been reported, though their role *in vivo* has not been confirmed (Fry, 2004).

Polygalacturonase: Polygalacturonase (PG) is one of glycosidases that hydrolyzes *O*- and *S*-glycosyl compounds (International Union of Biochemistry and Molecular Biology, 2006). They can be the exo- or endo-acting types. Exopolygalacturonase (EC. 3.2.1.67), also called galacturan 1,4-α-galacturonidase, removes a single galacturonic acid unit from the non-reducing end of pectates and other galacturonans. Whereas endopolygalacturonase (EC 3.2.1.15), commonly called polygalacturonase, cleaves demethylated pectate and galacturonans at random (Brummell and Harpster, 2001). The fruit ripening-specific enzyme, usually referred to as PG, is of the endo-acting type; however, both endo and exo types of these enzymes are found in fruit (Hadfield and Bennett, 1998) including papaya (Chan and Tam, 1982). The substrate for PG in the cell wall is mainly homogalacturonans. Homogalacturonans are secreted to the cell wall in a highly methyl-esterified form and must be de-esterified before they can become a substrate for PG (Javis, 1984; Carpita and Gibeaut, 1993).

PG is thought to be the key enzyme involved in the large changes in pectin structure that accompany the ripening of many fruit (Fischer and Bennett, 1991). Pectin disassembly is particularly extensive in tomato and is associated with the later stages of ripening and with fruit deterioration in the overripe stages (Dawson *et al.*, 1992; Huber and O'Donoghue, 1993). In tomato, PG activity is highly correlated with softening in normal ripening fruit (Seymour and Gross, 1996). Studies on expression of PG gene in transgenic *rin* (ripening inhibitor) tomato fruit indicated that PG was the primary determinant of cell wall polyuronide degradation, but degradation was not sufficient for the induction of softening (Giovannoni *et al.*, 1989). Antisense polygalacturonase transgenic tomato, in which the PG level was reduced to 1% of wild type, showed a reduced pectin depolymerization (Smith *et al.*, 1990) but had only a small reduction in fruit softening (Kramer *et al.*, 1992). However, it had enhanced fruit quality and resistance to mechanical damage and delayed occurrence of *Alternaria* rot (Schuch *et al.*, 1991; Kramer *et al.*, 1992). Additionally, studies in the rapidly ripening melon, which exhibits flesh softened and substantial disintegration during the overripe stage, indicated that depolymerization of poiyuronides occurred predominantly at the late ripening stage (Rose *et al.*, 1998). This depolymerization occurred after the expression of PG mRNAs, suggesting the existence of PG-dependent pectin degradation in the later stage (Rose *et al.*, 1998). Hadfield and Bennett (1998) reported that PG-mediated pectin disassembly occurred after the early stages of fruit softening and likely contributed at the overripe stage and during deterioration. The disassembly of pectins may increase the pore size of the pectin network, resulting in cell wall swelling, or increase accessibility of substrates to other enzymes (Hadfield and Bennett, 1998).

Pectin methylesterase: Pectin methylesterase (PME; EC 3.1.1.11) is a carboxylic ester hydrolase (International Union of Biochemistry and Molecular Biology, 2006). It de-esterifies pectin by removing methyl groups from the C6 position of uronic acid residues of pectin, producing methanol and pectate. Demethylation of pectins to their free carboxyl groups, changes the pH and charge in the cell wall, allows the aggregation of polyuronides into a calcium-linked gel structure, and makes the polyuronides susceptible to degradation by PG (Pressey and Avants, 1982; Javis, 1984; Seymour *et al.*, 1987; Carpita and Gibeaut, 1993).

In tomato, extracted PME protein and activity are present during fruit development, increasing from the early green fruit stage to the mature green stage and then during ripening (Harriman *et al.*, 1991; Tieman *et al.*, 1992). Reduction of PME activity in antisense transgenic tomato is associated with a decrease in bound Ca^{2+} and Mg^{2+} in the tomato pericarp and an increase in soluble Ca^{2+} (Tieman and Handa, 1994). The transgenic tomato shows a complete loss of tissue integrity during fruit senescence but little effect of fruit firmness during ripening (Tieman and Handa, 1994). PME may play a role in influencing the degree of pectin solubility by reducing cation binding including the number of sites available for calcium cross-linking (Tieman and Handa, 1994).

β-galactosidase: β-galactosidase (EC 3.2.1.23) is one of glycosidases that hydrolyzes of *O*- and *S*-glycosyl compounds by removing terminal non-reducing β-D-galactose residues from β-D-galactosides (International Union of Biochemistry and Molecular Biology, 2006). Since endo-galactanases have not been detected in the higher plants, the enzyme activity most likely to be responsible for degradation of cell wall β-galactan is exo-β-D-galactosidase (Brummell and Harpster, 2001).

Total β-galactosidase activity is high and does not change during tomato fruit development (Wallner and Walker, 1975; Pharr *et al.*, 1976; Carey *et al.*, 1995). However, when the activities of individual β-galactosidase isoforms are determined, β-galactosidase II (with exo-galactanase activity) shows low activity in green tomato fruit but the activity increases 7-fold during ripening (Pressey, 1983; Carey *et al.*, 1995; Carrington and Pressey, 1996; Smith and Gross, 2000). In *rin* and *nor* (non- ripening) tomato mutants, the loss of galactose in cell wall and the rise of free galactose are much reduced (Gross, 1983; Gross, 1984) and β-galactosidase II activity is not observed (Carey *et al.*, 1995). In tomatoes, antisense β-galactosidase 4 (*TBG4*) plants have shown early in ripening to have reduced galactose loss and reduced softening by up to 40% later in ripening (Smith *et al.*, 2002). The higher galactosyl-containing side chains of cell wall polysaccharides might decrease cell wall porosity and prevent the entry of other enzymes (Carpita and McCann, 2000).

Endoglucanase: Endoglucanase (EC 3.2.1.4) is a glycosidase that catalyzes endo-hydrolysis of *O*- and *S*-glycosyl compounds with 1,4-β-D-glucosidic linkages. Those linkages occur in cellulose, lichenin, and β-D-glucan (International Union of Biochemistry and Molecular Biology, 2006). Endoglucanase also hydrolyzes 1,4-linkages in β-D-glucans containing 1,3-linkages (International Union of Biochemistry and Molecular Biology, 2006). Enzymes causing matrix polysaccharide depolymerization in fruit have not been clearly identified, but may include endoglucanase (Brummell and Harpster, 2001).

Endoglucanases are often referred to as cellulases, but in higher plants, most endoglucanases lack the cellulose binding domains found in microbial cellulases

(Brummell and Harpster, 2001). The exception is a protein sequence encoded *FaEG3* gene of strawberry containing a putative cellulose binding domain (Trainotti *et al.*, 1999). This predicted binding domain has not been shown to function *in vivo*. In most cases endoglucanases are probably inactive against crystalline celluloses (Tucker, 1993; Brummell *et al.*, 1994). *In vitro*, endoglucanases are active against xyloglucan, cello-oligosaccharides, non-crystalline cellulose, and the model substrate CMC (Wong *et al.*, 1977; Hayashi *et al.*, 1984; Hatfield and Nevins, 1986; Nakamura and Hayashi, 1993; Ohmiya *et al.*, 1995). In the cell wall, their substrates probably include xyloglucan, integral and peripheral regions of non-crystalline cellulose, and glucomannan where sufficient consecutive (1→4) β-D-linked glucan residues occur for substrate binding (Brummell and Harpster, 2001).

At least two isoforms of endoglucanases occur during peach fruit development. Only the isoform with a pI of 9.5 is abundant during ripening. The rise in peach endoglucanases activity coincides with ethylene evolution (Bonghi *et al.*, 1998). When treated with 2,5-norbornadiene (a reversible inhibitor of ethylene action), peach fruit shows a strong inhibition in softening, suppression of endoglucanase activity and transcript accumulation, and suppression of ethylene evolution (Bonghi *et al.*, 1998). However, suppression of Le*Cel2* gene encoding endoglucanase in transgenic tomatoes does not affect fruit softening (Brummell *et al.*, 1999). Over-expression of *CaCel1* gene from pepper in transgenic tomato alters other matrix-glycan polymers rather than xyloglucan, and causes slightly firmer fruit than that of wild type at all stages of ripening (Harpster *et al.*, 2002).

Endoxylanase: Endo-(1→4)-β-D-xylanase (EC 3.2.1.8) is a glycosidase that catalyzes endohydrolysis of 1,4-β-D-xylosidic linkages in xylans, arabinoxylans, and

4-MeO-glucuronoxylan (Ward and Moo-Young, 1989). Highly purified endoxylanases have been reported to degrade cellulose (Dekker and Richards, 1976) though this has not been confirmed. Some fungal endo-(1→4)-β-D-xylanase can also cleave (1,3)-α-L-arabinofuranosyl side chains from arabinoxylans and arabinoglucurono-xylan (Dekker, 1985).

Endoxylanases have been studied intensively in cereal crops (Simpson *et al.*, 2003). Although there are limited studies of xylanase during fruit ripening, endoxylanases have been detected in fruit including papaya, pear, avocado, and banana (Paull and Chen, 1983; Yamaki and Kajiura, 1983; Ronen *et al.*, 1991; Prabha and Bhagyalakshmi, 1998; Chen and Paull, 2003; Manenoi, 2005).

Xyloglucan endotransglycosylase/hydrolase: Endoxyloglucan transferase (EXGT) or xyloglucan endotransglycosylase/hydrolase (XTH, XETs, EC 2.4.1.207) is a hexosyl-transferase. The enzyme hydrolyzes a β-(1→4) bond in the backbone of a xyloglucan and transfers the xyloglucanyl segment on to O-4 of the non-reducing terminal glucose residue of an acceptor, which can be a xyloglucan or an oligosaccharide of xyloglucan (International Union of Biochemistry and Molecular Biology, 2006). Xyloglucan endotransglycosylases are highly specific for xyloglucan both as donor and substrate and acceptor substrate (Brummell and Harpster, 2001). During the reaction, xyloglucan endotransglycosylase forms a relatively stable glycosyl-enzyme intermediary complex which decomposes by transfer of the attached glycosyl preferentially to a xyloglucan acceptor (transglycosylation) or more slowly to water (hydrolysis) (Sulova *et al.*, 1998).

Endo-xyloglucan transferase has a close relation to cell growth, but its role in fruit softening is unclear. Tomato fruit with antisense *LeEXGT1* encoding endo-xyloglucan transferase are smaller in size, while over-expressed transgenic fruit are larger (Asada *et al.*, 1999). Suppression of *LeEXTB1* in transgenic tomato does not affect the fruit softening (De Silva *et al.*, 1994).

β-xylosidase: β-xylosidase (EC 3.2.1.37) is also called xylan-1,4-β-xylosidase, β-D-xylopyranosidase, and xylobiase. β-xylosidase catalyzes hydrolysis of 1,4-β-D-xylans, to remove successive D-xylose residues from the non-reducing termini (International Union of Biochemistry and Molecular Biology, 2006). During avocado ripening, β-xylosidase activity reaches a peak at the same time as the peak of ethylene evolution and coincides with the decline in fruit firmness (Ronen *et al.*, 1991).

2.2.2.3 Non-Enzymatic Cell Wall Modification

Enzymatic and non-enzymatic factors play roles in cell wall modification, possibly leading to fruit softening. The non-enzymatic factors include expansins, ionic strength, pH, active oxygen species, cations, and hydration.

Expansins are cell wall localized proteins that were originally identified by their ability to cause cell wall loosening in *in vitro* assays (McQueen-Mason *et al.*, 1992). Purified expansins lack detectable hydrolase or transglycosylase activity. They do not bring about observable depolymerization of carboxymethylcellulose (CMC), cell wall matrix polysaccharides, or pectins (McQueen-Mason *et al.*, 1992; McQueen-Mason and Cosgrove, 1994; McQueen-Mason and Cosgrove, 1995). However, they do cause mechanical weakening of paper that is essentially pure cellulose (McQueen-Mason and Cosgrove, 1994). Expansins bind weakly to crystalline cellulose *in vitro* but more

strongly to cellulose coated with matrix polysaccharides. They probably act by causing a reversible disruption of hydrogen bonding between cellulose microfibrils and matrix polysaccharides, particularly xyloglucan. This disruption results in a loosening of the wall and allows the turgor-driven slippage of microfibrils relative to one another (McQueen-Mason and Cosgrove, 1995; Cosgrove, 2000b; Whitney *et al.*, 2000). Two families of expansins, α- and β- expansins have been recognized. α-Expansins, small proteins, usually lack glycosylation and are the major expansins in dicots but are also present in monocots (Cosgrove, 1999; Cosgrove, 2000b). β-Expansins are glycoproteins that are more divergent in sequence from but structurally related to α-expansins (Cosgrove *et al.*, 1997). β-Expansins are found at high levels in the pollen of graminaceous monocots but also in vegetative monocots. The α- and β-expansins are likely act on different components of wall (Cosgrove *et al.*, 1997).

During tomato fruit ripening, apoplastic pH declines, while there is an increase in ionic strength and the concentrations of some ions, such as K^+, anions of P, and Cl^- (Almeida and Huber, 1999). Ionic strength and pH changes may regulate both non-enzymatic and enzymatic cell wall modification (Chun and Huber, 1998; Almeida and Huber, 1999). Pectin undergo non-enzymatic degradation due to changes in ionic strength and pH of the cell wall solution (Seymour and Gross, 1996). Partial dissociation of pectin subunits occurs in the presence of sodium chloride or changes in pH (Fishman *et al.*, 1989). Protonation of carboxylate groups of pectins lowers the amount of cations fixed in the cell wall (Cutsem and Gillet, 1983) and has been shown to influence ionic strength and composition, pH, and proton excretion of plasma membrane (Holland and Barr, 1982; Cutsem and Gillet, 1983). Ionic strength and pH may regulate enzymatic cell

wall modification via determination of binding property and activity of wall-modifying enzymes (Chun and Huber, 1998; Almeida and Huber, 1999).

Hydroxyl radicals (*OH) are capable of oxidative cleaving wall polysaccharides nonspecifically (Fry, 1998; Cosgrove, 1999; Liszkay *et al.*, 2003). The oxidative mechanism generating *OH in cell walls of plants may be due to an enzymatic reaction catalyzed by apoplastic peroxidase (Liszkay *et al.*, 2003). L-ascorbate can also non-enzymatically reduce O_2 to H_2O_2 and Cu^{2+} to Cu^{+}, which later react to form *OH (Fry, 1998; Fry *et al.*, 2002). Ascorbate, H_2O_2, and Cu^{2+} are likely to be found in the wall at concentrations that can be effective for *OH generation. The *OH can potentially act to loosen walls if it is produced very close to appropriate targets (Cosgrove, 1999). In tomato fruit cell walls, *OH radicals have been shown to cause pectin solubilization (Fry *et al.*, 2002). It also has been found that apoplastic *OH is enhanced and reacts on polysaccharides during pear (*Pyrus communis*) softening (Fry *et al.*, 2001; Fry *et al.*, 2002). Although H_2O_2 plays a role in wall polymers cleavage, H_2O_2 is also thought to function in oxidative cross-linking of wall phenolic substances causing wall stiffening (Brisson *et al.*, 1994; Schopfer, 1996). Cosgrove (1999) mentioned that there might be a precise balance between cleavage and cross-linkage activities resulting from H_2O_2 production in the wall.

Cations can regulate cell wall modification. Calcium and boron can bind to acidic domains of pectic polysaccharides and form a cross-link between the pectic polysaccharides (Javis, 1982; O'Neill *et al.*, 1996; Molhoj *et al.*, 2003; Fry, 2004). Divalent cations such as Ca^{2+} and Mn^{2+} induce conformational modification of cell wall pectic polymers allowing some site-specific association with monovalent ions (Gillet *et al.*, 1998). Addition of CDTA, a chelating agent that captures metal ions, to unripe

tomato tissue causes cell separation (Tibbits *et al.*, 1998). A decrease in the concentration of free calcium ions causes marked swelling of the tomato pectin gel as a result of dissociation of calcium cross-linkage (Tibbits *et al.*, 1998).

A reduction in turgor pressure occurs during fruit ripening. Turgor pressure reduction may be a result of an increasing concentration of solutes in the cell wall space, wall loosening, and water loss (Brummell, 2006; Saladie *et al.*, 2007). Turgor reduction reduces expansion pressure on the cell wall contributing in alterations of fruit textural properties (Brummell, 2006).

2.3 Botany and Fruit Structure of Papaya

2.3.1 Botany of Papaya

Papaya (*Carica papaya* L.), 'Papaw' or 'Pawpaw', belongs to the family Caricaceae and is the only species in the genus *Carica*. Papaya is a rapid growing herbaceous, but short-lived perennial (Yee *et al.*, 1970). All parts of papaya have latex vessels (Samson, 1986). The plant usually has a single hollow stem and can attain height of 7.5 meters or more. The stem bears a crown of palmate-lobed leaves. The base of stem may grow up to a 30 centimeters in diameter (Yee *et al.*, 1970). Papaya root system is extensive and dense on good soil, but roots stay near the surface on a wet or compact soil (Samson, 1986).

Papaya is a dioecious plant. However, hermaphrodite (bisexual) flowers and trees also occur (Ali and Lazan, 1998). Female flowers are formed alone or in small groups at the leaf axils. The female ovary is 2-3 cm long and has 5 fan-shaped stigmas. Male flowers are located in long hanging panicles. A male flower has 10 stamens, while

a hermaphrodite flower has either 5 or 10 stamens (Samson, 1986). Papaya fruit is hollow fleshy berry but varies in shape and size. Fruit formed from female flowers are oblong to nearly spherical. Whereas fruit formed from hermaphrodite flowers are pear-shaped, spherical, or cylindrical (Paull *et al.*, 1997). Fruit sizes range from 0.3 kg to as large as 10 kg (Nakasone, 1986; Paull *et al.*, 1997). Flesh thicknesses range from 1.5 to 4 cm (Paull *et al.*, 1997). Fruit skin turns from green to yellow at the bottom when mature. Fruit is normally harvested when 10 to 15% of the skin has turned yellow to ensure the ability to ripen and adequate sugars. Flesh of ripe fruit is yellow to orange or reddish (Samson, 1986). Flesh total soluble solids range from 5 to 19% (Paull *et al.*, 1997). The fruit cavity has numerous of dark gray or black seeds, but seedless fruit may occur.

Papaya is native to Tropical America. The first papayas introduced to the Hawaiian Islands were the large-fruit types. The small-fruit papaya called 'Solo' were introduced from Barbados and Jamaica to Hawaii in 1911 and replaced the earlier large-fruit papaya in Hawaiian papaya industry (Yee *et al.*, 1970). 'Solo' papayas in Hawaii are propagated from largely self-pollinated seeds. The seeds of 'Solo' varieties produce a ratio of 66.7% hermaphrodite plants with pear-shaped fruit and 33.3% female plants with round-shaped fruit (Yee *et al.*, 1970).

Various strains of 'Solo' have been selected in Hawaii. Hawaii's 'Solo' papayas have 0.5-1.4 kg fruit with "red" or "yellow" flesh (Nishina *et al.*, 2000). One of the improved 'Solo' strains selected on Oahu, Hawaii, by the University of Hawaii is 'Line 8' released to growers in 1954. 'Line 8' is highly inbred with fruit of high quality and uniformity. The fruit weight ranges from 0.5 to 1 kg. The fruit have a star-shaped seed cavity and have excellent flavor and flesh quality. However, the soft-fleshed fruit is only

suited to the local market (Yee *et al.*, 1970). 'Sunset' has salmon-pink flesh and is a hybrid 'Solo' papaya between 'Line 9' Solo strain with red flesh and 'Kariya' Solo strain with yellow flesh. 'Sunset' has a distinctive aroma and flavor. Its fruit has a star shaped seed cavity and weights from 0.4 to 0.6 kg. Fruit picked at color break stage takes about 15 days to reach 100% skin yellow (Hamilton *et al.*, 1993).

2.3.2 Papaya Fruit Structure

Papaya fruit is normally composed of five carpels to form one large central cavity. The cavity has numerous seeds attached to placentas by 0.5 to 1-cm stalk (Nakasone, 1986; Paull *et al.*, 1997). Each seed is enclosed in a sarcotesta.

The thin fruit skin is green when immature, and yellow to orange-yellow when ripe. During ripening, the flesh color changes from white to yellow, orange-yellow, or red depending on the cultivar. During the green stage, injury to the skin induces milky latex exudation that contains papain, a proteolytic enzyme. Latex diminishes as fruit ripen, and there is no visible latex at the fully ripe stage (Nakasone, 1986).

The gynoecium (ovary) is less than 1 mm in diameter, and all the tissues are meristematic. As the gynoecium enlarges, the outer epidermis increase in size while the sub-epidermal cells remain meristematic and continue to divide (Nakasone, 1986; Paull *et al.*, 1997). The sub-epidermal cells become oval-shaped with intercellular spaces. Parenchyma cells in this pericarp tissue increase in size and divide in various directions, contributing to diameter growth. During this stage, the placentas begin to form opposite the marginal vascular bundles. The placentas extend throughout the inner surface of the ovary except for a small area about the midrib (Nakasone, 1986).

In older fruit, external epidermis cells are small and their peripheral walls are stretched. Beneath the external epidermis, five to ten layers of cells contain chloroplasts. The inner layer cells increase in size and become rounder causing intercellular spaces. The sub-epidermal layers can no longer be seen in mature fruit. The internal part of pericarp consists of spongy tissues with large intercellular spaces (Nakasone, 1986)

2.4 Papaya Fruit Softening

Papaya fruit softens from the endocarp outward to the epidermis. Modifications of papaya cell wall components and activity of some enzymes related to cell wall hydrolysis begin from the inner pericarp to the outer pericarp, and the increase is coincident with softening (Paull and Chen, 1983; Lazan *et al.*, 1995). Enzymes that modify the cell wall have been suggested to be involved in papaya fruit softening (Lazan *et al.*, 1995; Pauli *et al.*, 1999; Manrique and Lajolo, 2004; Manenoi, 2005).

2.4.1 Papaya Cell Wall Modification

Pectins solubility and depolymerization occur during papaya ripening (Lazan *et al.*, 1995; Paull *et al.*, 1999; Manrique and Lajolo, 2004). Pectin depolymerization begins at the inner mesocarp tissue at about the same time as the papaya firmness begins to decline rapidly (Lazan *et al.*, 1995) and water-soluble pectins dramatically increase during ripening (Pauli *et al.*, 1999; Manrique and Lajolo, 2004). Pectin solubilization in outer and inner mesocarp tissues occur throughout ripening at comparable rates (Lazan *et al.*, 1995). The degree of pectin methylation in the middle lamella and primary cell wall declines during ripening (Pauli *et al.*, 1999; Manrique and Lajolo, 2004), while that of water-soluble pectins increases (Manrique and Lajolo, 2004). Pectic polymers are

degraded into smaller molecules during fruit ripening (Paull *et al.*, 1999; Manrique and Lajolo, 2004). Although water-soluble pectins increase and the pectin molecular weight declines during softening, their changes were not correlated with the loss of firmness during early ripening (Pauli *et al.*, 1999).

Matrix polysaccharides are also modified, synthesized, and degraded during ripening (Lazan *et al.*, 1995; Paull *et al.*, 1999). Although depolymerization occurs, the proportion of matrix polysaccharides increases (Lazan *et al.*, 1995; Paull *et al.*, 1999). Monosaccharide analysis of hemicelluloses fraction from papaya fruit indicated glucose and xylose as the main components (Paull *et al.*, 1999; Manrique and Lajolo, 2004). The xylose proportion in the matrix polysaccharide fraction declines during ripening, causing an increase in the glucose proportion in the ripe fruit. However, at the later stage of ripening the proportion of glucose in the matrix polysaccharide fraction drops (Manrique and Lajolo, 2004).

Cellulose residue fraction from extracted papaya fruit cell wall shows a decline during ripening (Paull *et al.*, 1999; Manrique and Lajolo, 2004). Glucose is the main component of cellulosic residue (Paull *et al.*, 1999; Manrique and Lajolo, 2004). The cellulosic residue also contains non-glucosyl monosaccharides (Paull *et al.*, 1999; Manrique and Lajolo, 2004) and their presence indicates an association between polysaccharides of cell wall matrix and cellulose microfibrils (Carpita, 1987; Talbott and Ray, 1992; Manrique and Lajolo, 2004). The cellulosic residue of papaya cell wall shows a decline in galacturonic acid and galactose, but an increase in glucose during ripening (Manrique and Lajolo, 2004). The changes in the association between the matrix polysaccharides and cellulose polymers may also be involved in softening (Manrique and Lajolo, 2004).

2.4.2 Papaya Cell Wall Enzymes

In papaya, polygalacturonase (PG) activity has been reported at harvest and increases at the same time as the rise in respiration and then declines (Paull and Chen, 1983). PG activity also increases with the depth of the mesocarp as does the softening of papaya fruit (Lazan *et al.*, 1995). During papaya ripening, there is an increase in water-soluble pectins and a decrease in esterified protopectins (Shetty and Dubash, 1974; Arriola *et al.*, 1975). These changes have been attributed by an increase in the PG activity (Chan *et al.*, 1981) and the reduction of fruit softening by hot water treatment is correlated with a decrease in PG activity (Chan and Tam, 1982).

It is possible that PME participates in papaya softening (Manrique and Lajolo, 2004). PME gradually increases from the onset of the climacteric and reaches a peak two days after the respiration peak (Paull and Chen, 1983). The increase in PME activity during papaya fruit ripening (Paull and Chen, 1983) could explain the lower methoxyl content in pectin of the ripe papaya (Paull *et al.*, 1999). Demethylation by PME, resulting in a greater numbers of unesterified carboxyl groups, may facilitate PG activity (Huber, 1983) and cause an increase in pectin solubility or changes of matrix structure (Manrique and Lajolo, 2004). Deesterfication occurs with the loss of galactosyl and arabinosyl side chains of pectins in pears (Ahmed and Labavitch, 1980a), apples (Knee, 1973), and peaches (Reeve, 1959). The greater number of unesterified carboxyl groups on pectins may also facilitate binding of cations to pectins (Burns and Pressey, 1987). However, the importance of PME in papaya fruit softening could be questioned since low levels of PME are always detected in preclimacteric mature green papaya (Pauli and Chen, 1983). These activity levels might be sufficient for any required demethylation (Paull and Chen, 1983).

β-galactosidase appears to be an important hydrolase and may contribute to differential softening in papaya (Lazan *et al.*, 1995). Tissue softening of papaya during ripening shows a closer relationship to changes in β-galactosidase activity than to PG or PME activity (Lazan *et al.*, 1995). Three isoforms of β-galactosldase (β-Gal I, β-Gal II and β-Gal III) have been reported in papaya (Ali *et al.*, 1998). Immunobloting showed that papaya β-Gal I protein accumulation occurs throughout ripening, while β-Gal II protein is detectable in developing fruit and its level decreased as fruit ripen (Ali *et al.*, 1998). These β-galactosidase isoforms are thought to be able to hydrolyze papaya cell wall *in vitro* and enhance pectin solubility and depolymerization and the degradation of alkali-soluble matrix polysaccharides (Lazan *et al.*, 2004).

A close relationship exists between endoxylanase activity and the rise in respiration, ethylene production, and softening (Paull and Chen, 1983). Endoxylanase is not detectable in the preclimacteric stage. Its activity increases during the climacteric but declines during the post climacteric stage (Paull and Chen, 1983). When three papaya lines/varieties (with different softening patterns) start to soften, endoxylanase activities increases at the same time as endoxylanase protein accumulates and endoxylanase gene expressions occurs (Chen and Paull, 2003; Manenoi, 2005). 1-MCP treated papaya show a rubbery texture when ripe and have suppressed endoxylanase activity, protein, and gene expression (Manenoi, 2005).

Endo-glucanase (cellulase) activity increases during papaya ripening and occurs at the same time as softening (Pauli and Chen, 1983). Endogiucanase activity is first detected near the endocarp of the fruit. The endoglucanase activity has been utilized to

show the ripening of papaya from the endocarp placenta area outward (Paull and Chen, 1983).

2.5 1-Methylcyclopropene

1-Methylcyclopropene (1-MCP), an ethylene inhibitor discovered by Edward Sisler and Sylvia Blankenship, is a gas with a molecular weight of 54 (Blankenship and Dole, 2003). It has a planar molecular structure with a methyl group attached at the double bond. All the double bonds have inherent strain (Wiberg, 1987), and the double bond on the planer 1-MCP molecule has a high amount of strain energy (Sisler and Serek, 1997). Generally, strained compounds tend to bind to electron donor compounds, such as copper, to relieve the strain (Sisler and Serek, 1997; Sisler and Serek, 1999). This binding presumably is the mechanism of 1-MCP acting in ethylene receptor and inhibiting responses.

2.5.1 Action of 1-MCP

1-MCP presumably binds to a metal, possibly copper ion (Rodriguez *et al.*, 1999; Sisler and Serek, 1999) on the receptor in plant tissues that normally binds to ethylene. Binding of compounds such as ethylene to metals makes the position *trans* to the bound ethylene (Chatt *et al.*, 1955). The bound ethylene can then withdraw electrons from the metal in the receptor (Chatt *et al.*, 1955; Sisler and Serek, 1997), causing an alteration in coordination chemistry of the metal and conformational change in the binding site (Sisler and Serek, 1997; Bleecker, 1999). Binding of ethylene to this receptor is how plant tissues perceive that ethylene is present in the environment (Huber *et al.*, 2003).

The current model of ethylene receptor signaling, based on several experimental observations, demonstrated that ethylene acts as a negative regulator of receptor signaling (Bleecker, 1999). In the absence of ethylene, receptors activate a serine or threonine protein kinase encoded by *CTR1* gene, which in turn represses ethylene response pathway. Ethylene binding switches off the receptors, resulting in de-repression of the response pathways (Bleecker, 1999).

1-MCP competes with ethylene for the receptor binding site and remains bound for many days (Sisler and Serek, 1997). The binding of 1-MCP to the receptors does not induce the triple responses caused by ethylene. When 1-MCP binds to the receptor, it may act in a similar manner to ethylene, withdrawing electrons from a metal (Sisler and Serek, 1997). However, 1-MCP is so highly strained that its effect would be stronger than that of ethylene. 1-MCP perhaps remains bound to the metal in the receptor, is not lost from the complex, and the formation of the active complex is not completed (Sisler and Serek, 1997). As ethylene binding is prevented by 1-MCP, ethylene no longer promotes ripening, senescence, and other ethylene responses (Sisler and Serek, 1999; Huber *et al.*, 2003).

The affinity of 1-MCP for the receptor is approximately 10 times greater than that of ethylene (Blankenship and Dole, 2003). Thus, 1-MCP is active at a much lower concentration compared with ethylene. Effective concentrations are in the range from 2.5 nL L^{-1} to 1 μL L^{-1}. Lower concentrations of 1-MCP applied for longer period of time may be as effective as higher concentration (Blankenship and Dole, 2003). In most studies, treatment durations ranging from 12 hours (6 hours for floral products) to 24 hours are sufficient to achieve a full response (Ku and Wills, 1999; Beaudry and Watkin, 2001; Reid *et al.*, 2001; Blankenship and Dole, 2003; Manenoi, 2005).

2.5.2 Physiological Responses of Plants to 1-MCP

1-MCP reversibly inhibits ethylene responsiveness and extends the postharvest shelf life and quality of numerous fruits, vegetables, and floricultural crops (Serek *et al.*, 1994; Golding *et al.*, 1998; Ku and Wills, 1999; Hofman *et al.*, 2001; Jiang *et al.*, 2002; Blankenship and Dole, 2003; Huber *et al.*, 2003). Depending on the species being treated, 1-MCP may have a variety of effects on respiration, ethylene production, volatile production, chlorophyll degradation and other color changes, protein and membrane changes, softening, disorders and diseases, acidity, and sugars (Serek *et al.*, 1995; Blankenship and Dole, 2003; Huber *et al.*, 2003).

In apple, 1-MCP maintains critical taste components including firmness, sugar content, and titratable acid (Mattheis *et al.*, 2002; Huber *et al.*, 2003). 1-MCP also reduces ethylene production and respiration rate, causes slower degreening and yellowing, and delays production of ripe aroma (Beaudry and Watkin, 2001; Mattheis *et al.*, 2002).

Treatment of tomato with 1-MCP (1 ppm, 24 hours) greatly delays softening and red color development and doubles shelf life (Huber *et al.*, 2003). This 1-MCP treatment has been applied to tomato at turning stage, indicating that 1-MCP is effective in delaying postharvest ripening even when applied after ripening has started (Huber *et al.*, 2003).

1-MCP treatments of avocado have been shown to markedly inhibit ethylene-induced ripening, causing a great delay in ethylene evolution, softening and loss of green color (Feng *et al.*, 2000; Huber *et al.*, 2003). 1-MCP treated avocado at 30-70 nL L^{-1} delays and shows lower PG and glucanase activities (Feng *et al.*, 2000).

In banana, the application of 1-MCP to mature green fruit significantly delays the onset, and affects the rate and magnitude of several physiological responses. Application of 1-MCP at a pre-climacteric stage or 6 and 12 hours after propylene treatment (to initiate ripening) delays the onset of ethylene evolution and respiration, the peel degreening, and the total volatiles production (Golding *et al.*, 1998).

Treatment of 25 μL L^{-1} 1-MCP at 20°C for 14 hours in papaya delays ripening but increases blemishes slightly (Hofman *et al.*, 2001). The application of 100 nL L^{-1} 1-MCP at 21-22°C for 12 hours delays climacteric respiration, ethylene production, skin color development, and softening of papaya (Manenoi, 2005; Manenoi *et al.*, 2007). Papaya treated with 100 nL L^{-1} at less than 25% skin yellow stage shows an incomplete softening, the mesocarp having rubbery texture when ripe (Manenoi, 2005).

2.5.3 Regeneration of Ethylene Sensitivity after 1-MCP Application

Rate of loss in insensitivity to ethylene depends on plant being treated, condition under which they are held (Reid *et al.*, 2001), and 1-MCP concentration (Pesis *et al.*, 2002). Carnations treated with 1-MCP appear to remain insensitive to ethylene for 12-15 days at 24°C (Sisler *et al.*, 1996). Carnations treated with 1-MCP (50 ppb for 6 hours) then held at 20°C recover 50% of their responsiveness to ethylene after 4 days (Reid *et al.*, 2001). However, at 0°C the time taken for the flowers to recover 50% of their response to ethylene is more than a month (Reid *et al.*, 2001). Although 1-MCP treatment of Pelargonium initially inhibits the shattering caused by ethylene, the effect disappears within 1-2 days (Reid *et al.*, 2001). A single postharvest 1-MCP application in apple can prevent ripening for more than 30 days, even at ambient temperature (25°C) (Beaudry and Watkin, 2001). Banana and tomato fruit treated with 1-MCP at

24°C remain insensitive to ethylene for 12 days then can ripen normally (Sisler *et al.*, 1996).

In avocado, ripening is delayed by 10-12 weeks after a 30-70 nL L^{-1} 1-MCP application for 24 hours, then they ripen normally (Feng *et al.*, 2000). Re-application of 300 nL L^{-1} 1-MCP 10 days apart during 5°C cold storage impairs fruit softening. The fruit with two 1-MCP applications remain firm, while fruit with one 1-MCP application soften normally during shelf life at 20°C after 5 weeks of the cold storage (Pesis *et al.*, 2002). However, re-application of 100 nL L^{-1} 1-MCP 10 days apart during the 5°C cold storage causes only a reduction in mesocarp discoloration associated with the cold storage but does not affect softening process after 5 weeks of cold storage (Pesis *et al.*, 2002).

The proposal that 1-MCP binds permanently to receptors at the time of application leads to the assumption that regeneration of ethylene sensitivity is due to appearance of new receptors (Sisler and Serek, 1997; Blankenship and Dole, 2003). However, this assumption has little direct supporting data (Blankenship and Dole, 2003). In the presence of ethylene, 1-MCP treated pea seedling continued to grow for 3 days, then stopped (Sisler *et al.*, 1996). Sisler and Serek (1997) explained that ethylene-insensitive cells probably had completed their expansion by that time and may have been insensitive. Any newly-formed cells probably would be sensitive since they would generate new receptors.

2.5.4 Commercialization of 1-MCP

The first commercial use of 1-MCP was for ornamental crops undertaken by Floralife, Inc. It is formulated as solid bound in a α-cyclodextrin ring. When mixed with water, the α-cyclodextrin dissolves and releases 1-MCP gas. This product has been

approved for use on ornamentals by EPA (the United States Environmental Protection Agency) since 1999 and is sold under the trade name EthylBloc®. Commercial 1-MCP for edible crops was marketed by AgroFresh, Inc., under the trade name SmartFresh®. Both EthylBloc® and SmartFresh® are approved for use in the United States (Blankenship and Dole, 2003).

CHAPTER 3

HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Fruit softening is thought to be the result of cell wall modification. Many cell wall hydrolases that degrade polysaccharides are expressed during softening associated ripening. Hydrolases may play an important role in papaya softening. 1-Methylcyclopropene (1-MCP) treatment alters papaya softening and presents an opportunity for testing hypotheses on the role of hydrolases in papaya softening.

The hypotheses were:

1.) Cell wall polysaccharide modification is central to ripening-associated softening;

2.) Cell wall hydrolases expressed during papaya ripening are correlated with fruit softening; and

3.) pH changes in papaya mesocarp regulate hydrolase activity.

3.2 Objectives

1.) Determine cell wall polysaccharide modification in papaya during normal softening and softening modified by 1-MCP treatment.

2.) Determine relationships between papaya softening and activities of fruit hydrolases, including endopolygalacturonase (PG), pectin methylesterase (PME), β-galactosidase, endoglucanase, endoxylanase, and β-xylosidase, during ripening.

3.) Compare changes in papaya hydrolase activities between normal softening and softening modified by 1-MCP treatment.

4.) Determine changes in mesocarp pH of papaya during normal softening and softening modified by 1-MCP treatment.

5.) Determine effects of exogenous pH on papaya mesocarp softening.

CHAPTER 4

FRUIT SOFTENING AND CELL WALL MODIFICATION OF PAPAYA

4.1 Introduction

During ripening, cell wall architecture and polymers are modified progressively. These changes vary among species (Brummell and Harpster, 2001). Ripening is generally accompanied by an increase of pectin solubility and depolymerization, and a loss of non-glucosyl neutral sugars especially galactose and arabinose in side chains of pectins (Gross and Wallner, 1979; Gross and Sam, 1984; Tucker, 1993; Redgwell *et al.*, 1997a; Brummell, 2006).

A reduction in cell-to-cell adhesion is caused by a breakdown and dissolution of pectin-rich middle lamella. The breakdown of middle lamella has been shown to begin early in the ripening of soft fruit such as tomato (Crookes and Grierson, 1983) and late in softening of crisp fruit such as apple (Ben-Arie *et al.*, 1979). In fruit such as strawberry and avocado that develop a soft melting texture, swelling of cell walls is evident, but in fruit such as apple that ripen to a crisp texture, cell wall swelling is not observed (Redgwell *et al.*, 1997b; Brummell, 2006). Cell wall swelling may be related to a loosening of xyloglucan-cellulose network and to a solubilization of pectin (Redgwell *et al.*, 1997b; Brummell, 2006). Loosening of xyloglucan-cellulose network and pectin solubilization combined with losses in pectic side chains, increase wall porosity. The consequent increase in wall porosity may allow greater accessibility of cell wall-degrading enzymes to substrate polymers (Brummell, 2006).

A decrease in the molecular mass of matrix polysaccharides has been observed during ripening in many species (Huber, 1984; Tong and Gross, 1988; Redgwell *et al.*, 1991; Sakurai and Nevins, 1993; Sakurai and Nevins, 1997; Rose *et al.*, 1998; Brummell *et al.*, 2004). Depolymerization of matrix polysaccharides is believed to be a major contributor in reduced rigidity of cell walls that lead to fruit softening (Brummell, 2006).

The amount of cellulose decreases during softening in grape berries (Yakushiji *et al.*, 2001), however, depolymerization of cellulose microfibrils is not evident during ripening of tomato (Maclachlan and Brady, 1994). Depolymerization of outer glucan chains of a microfibril, to which cross-linking glycans are attached, can have a large effect on cell wall properties and fruit softening (Manrique and Lajolo, 2004; Brummell, 2006).

In order to determine softening-related modifications in papaya cell wall polysaccharides, cell wall materials were isolated and sequentially extracted to produce fractions enriched in particular cell wall components and monosaccharide components determined. Two papaya lines reported to have different softening rates were used. Papaya treated with 1-MCP that show an incomplete softening was used to present alterations in cell wall modification.

4.2 Materials and Methods

4.2.1 Plant Materials

'Line 8' and 'Sunset' fruit were harvested at color break stage (less than 10% skin yellow) from the Poamoho Experimental Station in Central Oahu, Hawaii. One subset of 'Sunset' fruit were fumigated with 100 nL L^{-1} of 1-methylcyclopropene

(1-MCP) for 12 hours at room temperature (approximately 22°C). Another subset of 'Sunset' fruit were not treated with 1-MCP. All fruit were allowed to ripen at the room temperature until the full ripe stage (over 90% skin yellow).

Six fruit of each line were randomly sampled at the color break and at the full ripe stage. Four fruit of 1-MCP treated 'Sunset' were sampled at the full ripe stage. Fruit firmness and flesh translucency were determined. Mesocarp tissue was collected and extracted for cell wall material and later used to extract cell wall polysaccharide fractions. Four sets of fractions from each line at both stages and from 1-MCP-treated and untreated fruit were analyzed for neutral sugars composition of each cell wall polysaccharide fractions.

4.2.2 Skin Color, Firmness, and Flesh Translucency Determinations

Fruit skin color was determined as percentage of yellow area on total skin area. Fruit firmness was measured using a force gauge (AccuForce®, Model Cadet) with a 1.6 cm diameter disc and pressed 1 mm into the fruit. The peak forces at two opposite sides of fruit equator were read and averaged. Each fruit was cut into halves. Flesh translucency was recorded as an average percentage of the mesocarp that was translucent on both sides of the half fruit. The mesocarp was sliced, immediately frozen in liquid nitrogen, and stored at -80°C until use.

4.2.3 Cell Wall Material Extraction

Fifty (50) grams of frozen mesocarp was homogenized in 250 mL of chilled 40 mM HEPES-NaOH buffer pH 7.0 at 2°C (using a commercial blender) for three minutes. The homogenate was filtered through Miracloth® (CALBIOCHEM®), and the material

remaining was washed with 1.5 liters of the same buffer for papaya sampled at color break stage. At the full ripe stage, 3 liters of buffer were used. Excess buffer was removed from the remained material by gently squeezing. To inactivate cell wall enzymes, the crude cell wall material was transferred into 125 mL of phenol-saturated Tris (pH 7.0) and the suspension was stirred for 45 minutes as described (Huber, 1991) and again filtered through Miracloth®. The crude inactivated cell wall material was sequentially washed with 0.5 liter of 80% (v/v) ethanol and then with 1 liter of 100% acetone. The cell wall material was transferred into 150 mL of chloroform:methanol (1:1) and the suspension was stirred for 30 minutes. The suspension was filtered through Whatman® GF/C glass filter paper under aspiration. Solid material was washed under aspiration with 0.5 liter of 100% acetone. The material that remained was dried in a fume hood for a few days and later in a desiccator until no further weight change occurred. This washed material was considered as the crude cell wall material (CWM).

4.2.4 Extraction of Cell Wall Polysaccharides

A quarter (0.25) gram of CWM was first extracted with 25 ml of de-ionized water at room temperature for one hour. The suspension was centrifuged at 14,300 rpm for 10 minutes (SA-600 rotor) and the supernatant filtered though Whatman® GF/C glass filter paper under aspiration. Water extraction was repeated and the supernatants combined giving the water-soluble fraction. The following steps of cell wall polysaccharide extraction were conducted as described (Selvendran and O'Neill, 1987) with minor modifications. In brief, the water extracted CWM was sequentially extracted with (1.) 0.05 M CDTA (pH 6.5) twice at the room temperature (giving CDTA_1 fraction and CDTA_2 fraction), (2.) 0.05 M Na_2CO_3 + 20 mM $NaBH_4$ at 1°C (giving Na_2CO_3_1 fraction), (3.) 0.05 M Na_2CO_3 + 20 mM $NaBH_4$ at the room temperature (giving

Na_2CO_3_rm fraction), (4.) 1 M KOH + 10 mM $NaBH_4$ at 1°C (giving 1 M KOH_1 fraction) followed (5.) 1 M KOH + 10 mM $NaBH_4$ at the room temperature (giving 1 M KOH_2 fraction), (6.) 4 M KOH + 10 mM $NaBH_4$ at the room temperature (giving 4 M KOH_1 fraction), and (7.) 4 M KOH + 10 mM $NaBH_4$ + 4% boric acid at the room temperature (giving 4 M KOH_2 fraction). The remaining material was washed with 2 liters of distilled water under aspiration (Appendix I). The white solid material was considered the cellulose fraction. The cell wall polysaccharides fractions were adjusted to pH 5.0, and 2 mL of each cell wall polysaccharide fractions was collected and stored at -20°C for starch, total sugar, and uronic acid analysis. Total volume of each fraction was recorded for calculation of actual mass of each cell wall polysaccharide fraction. All fractions, except for the water-soluble fraction, were dialyzed in a 3,500 MW cut off Spectra/Por® dialysis tubing at 1°C with at least 5 changes of distilled water. Each fraction was lyophilized, weighted, and stored at -20°C.

4.2.5 Total Sugar and Uronic Acid Analysis

Total sugar content was determined by the phenol-sulfuric acid assay (Dubois *et al.*, 1956) with 3 replicates. D-glucose was used as the hexose standard. Cell wall polysaccharide fractions were diluted 5-fold before assay. Four hundred (400) μL of sample or hexose standard was mixed with 400 μL of 5% (w/v) phenol in water and 2 mL of concentrate sulfuric acid added and allowed to stand for 10 minutes before vigorously been shaken. Absorbance at 490 nm was recorded after a further 30 minutes.

Duplicate colorimetric assay of hexouronic acids was conducted on each cell wall fraction using the meta-hydroxydiphenyl-sulfuric assay (Blumemkrantz and Asboe-Hansen, 1973) with minor modifications. Cell wall polysaccharide fractions were diluted

10-fold before assay. Four hundred (400) μL of each sample or D-galacturonic acid standard were added with 2.4 mL of 0.0125 M sodium tetraborate in concentrate sulfuric acid. The mixture was immediately cooled in crushed ice, and then shaken vigorously. The mixture was boiled in a water-bath for 5 minutes and cooled again in a water-ice bath. Forty (40) μl of 0.15% (w/v) *m*-hydroxydiphenyl in 0.5% (w/v) NaOH was added. The mixture was shaken vigorously and allowed to stand for 30 minutes for air bubbles to escape and absorbance at 520 nm recorded. As carbohydrates produce a slight pinkish chromogen with the sulfuric acid/tetraborate at 100°C (Blumemkrantz and Asboe-Hansen, 1973), a blank was run without addition of *m*-hydroxydiphenyl reagent that was replaced by 40 μl of 0.5% (w/v) NaOH. Absorbance of the blank was subtracted from the sample absorbance before calculation of uronic acid content.

4.2.6 Analysis of Neutral Sugar Composition of Cell Wall Polysaccharides

Starch contamination in cell wall fractions was tested. One drop of iodine/potassium iodide reagent was added into 200 μL of each cell wall polysaccharide fraction. All fractions showed a yellow negative result to the starch test.

Dried cell wall fractions were sent to the Produce Quality and Safety Laboratory, USDA, in Maryland where Dr. D. Smith, Dr. K. Gross, and Mr. N. Livsey generously analyzed for neutral sugars composition of cell wall fractions. Cell wall fractions (ca. 5 mg) were weight out and actual total weight recorded. Cell wall fractions were hydrolyzed with 1 mL of 2 N trifluoroacetic acid (TFA) using 50 μg allose as the internal standard. The hydrolysis was carried out in heat sealed tubes at 121°C for one hour. Each tube was opened and the contents pour out through a small ball of glass wool, transferred to vials and dried with nitrogen gas at 37°C. Derivatization was done using

the normal procedures for alditol acetates. To those vials derived from over 5 mg sample, 750 µL methylene chloride ($MeCl_2$) was added and 200 µL transferred to autosample vial inserts. For those vials derived from less than 5 mg sample, 300 µL $MeCl_2$ was added and 200 µL transferred to autosample vial inserts. A 2 µL injection volume was injected twice. Sugar derivatives were separated by gas-liquid chromatography (GLC).

4.3 Results

4.3.1 Cell Wall Modification of 'Line 8' and 'Sunset' Papayas

Ripening, Softening, Flesh Translucency, and Cell Wall Material of Papayas

'Line 8' and 'Sunset' had similar fruit firmness at the color break stage and at the full ripe stage. Both lines did not show any significant difference in days to full ripe stage in this experiment. Only 'Line 8' showed flesh translucency at the full ripe stage (Table 4.1).

Cell wall material (CWM) compared to mesocarp fresh weight (FWt) were not different between both lines at the color break and at the full ripe stage. However CWM compared to mesocarp fresh weight of both lines at the full ripe stage (0.71 – 0.72% of flesh weight) were less than one half of those fruit at the color break stage (1.68 – 1.77% of flesh weight) (Table 4.1).

Changes in Cell Wall Polysaccharide Fractions of Papayas

There was no difference between 'Line 8' and 'Sunset' in the mass of each cell wall polysaccharide fractions at both color break and full ripe stages (Table 4.2). The mesocarp of 'Line 8' significantly declined in the cell wall polysaccharides that were extracted in water, CDTA_1, CDTA_2, Na_2CO_3_1, and Na_2CO_3_rm, and the cellulose fraction during fruit softening. Similar to 'Line 8', 'Sunset' mesocarp 4 M KOH_2 fraction also declined. The major reduction in cell wall polysaccharides during ripening of both lines occurred in the Na_2CO_3_1 and Na_2CO_3_rm polysaccharide fractions. There was a greater than 10-fold reduction in the mass of these cell wall polysaccharide fractions during ripening (Table 4.2)

No difference was observed between 'Line 8' and 'Sunset' for each cell wall polysaccharide fractions proportion compared to the CWM weight (Table 4.3). However, there were differences in proportions of CDTA_1, Na_2CO_3_1, Na_2CO_3_rm, 1 M KOH_1, 4 M KOH_1, and cellulose fractions between the color break stage and full ripe stage of 'Line 8'. 'Sunset' showed the same result between the two stages, except for the proportion of CDTA_2 fraction (Table 4.3).

The total sugar (as glucose) contents of fractions analyzed before dialysis process (Table 4.4), showed similar values to the actual dry weight of matrix glycan fractions (Table 4.2). Although the total sugar content of pectin-rich fractions such as CDTA_1, CDTA_2, Na_2CO_3_1 and Na_2CO_3_rm fraction had similar trends to the actual dry weight of these fractions, they were significantly less than the actual dry weight of these fractions. This possibly occurred as different sugars have different sensitivities in the total sugar assay. Uronic acids, such as galacturonic acid, gave less absorbance

than glucose at the same concentration (data not shown). Analysis of uronic acid contents (as galacturonic acid) of fractions showed a higher mass than the result from total sugar analysis (Table 4.4 and 4.5). Analysis of total sugar and uronic acid contents confirmed that there was no significant loss of polysaccharides during dialysis of each fraction.

No difference in total sugars mass (as glucose) occurred compared to mesocarp fresh weight in all cell wall fractions, except for the water-soluble fraction, between the two lines at the color break stage (Table 4.4). The water-soluble fraction of 'Sunset' was lower in total sugars than 'Line 8'. There was no difference in the total sugar content of each fraction between 'Line 8' and 'Sunset' at the full ripe stage. The water-soluble and 4 M KOH_2 fractions of 'Line 8' papaya at the color break stage showed a higher amount of total sugars than those at the full ripe stage. The CDTA_1, CDTA_2, Na_2CO_3_1, Na_2CO_3_room, and 1 M KOH_1 fractions from both lines at the color break stage had higher amounts of total sugars than those at the full ripe stage. The 1 M KOH_2 and 4 M KOH_1 fractions did not show a significant difference in total sugar content between the color break stage and full ripe stage of both lines (Table 4.4).

Uronic acids (as galacturonic acid) contents of fractions were not different between lines both at the color break and full ripe stages. Similar result was seen between the uronic acid contents of both lines (Table 4.5 and 4.6). At the color break stage, at least 40% of polysaccharides that dissolved in water, CDTA_1, Na_2CO_3_1, and Na_2CO_3_rm were uronic acids. Uronic acid content and the proportion in the uronic acid-rich fractions of both papayas declined during ripening (Table 4.5 and 4.6). Although the uronic acid proportion in the water-soluble polysaccharide fraction was similar between the color break and full ripe stages (Table 4.6), the contents of uronic acids compared to

flesh weight of this fraction in both papaya lines at the full ripe stage were lower than those at the color break stage (Table 4.5). Both papaya lines showed a trace of uronic acids in the 1 M KOH_1, 1 M KOH_2, 4 M KOH_1, and 4 M KOH_2 polysaccharide fractions (Table 4.5). The uronic acid contents and proportions in these fractions of both lines did not change during ripening (Table 4.5 and 4.6).

Table 4.1 Days from color break to full ripe, fruit firmness, flesh translucency, and total crude cell wall material (CWM) compared to mesocarp fresh weight of 'Line 8' and 'Sunset' fruit

Variety	Fruit Stage	Mean ± SE [x]			
		Days to Full Ripen	Firmness (Kg)	Translucency (%)	CWM Weight (% of FWt)
'Line 8'	Color Break	-	20.2 ± 1.0 b	0 [y]	1.77 ± 0.09 b
	Full Ripe	9 ± 0.5	2.9 ± 0.1 a	17.5 ± 1.7	0.71 ± 0.04 a
'Sunset'	Color Break	-	20.5 ± 1.5 b	0 [y]	1.68 ± 0.03 b
	Full Ripe	11 ± 1.1	2.4 ± 0.2 a	0 [y]	0.72 ± 0.03 a

[x] Mean and standard error (SE) were of 6 replicates (fruit). Different letters following means in the same column show that the mean difference was significant at the 0.05 level by LSD.

[y] Mean was not compared since there was no variation within group

Table 4.2 Dry weight of cell wall polysaccharide fractions compared to mesocarp fresh weight of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages

Variety	Fruit Stage		Dry Weight of Fraction [x] (% of Mesocarp Fresh Weight)									
			Water	CDTA_1	CDTA_2	Na_2CO_3_1	Na_2CO_3_rm	1M KOH_1	1M KOH_2	4M KOH_1	4M KOH_2	Cellulose
'Line 8'	Color Break	Mean	0.100	0.429	0.286	0.320	0.075	0.035	0.011	0.051	0.007	0.615
		SE	0.012	0.030	0.028	0.026	0.009	0.005	0.003	0.004	0.001	0.025
	Full Ripe	Mean	0.030	0.131	0.105	0.023	0.005	0.023	0.005	0.045	0.005	0.420
		SE	0.005	0.010	0.010	0.002	0.001	0.001	< 0.001	0.003	0.001	0.028
'Sunset'	Color Break	Mean	0.074	0.423	0.302	0.265	0.077	0.029	0.012	0.051	0.007	0.627
		SE	0.003	0.046	0.019	0.008	0.003	0.005	0.004	0.008	0.001	0.026
	Full Ripe	Mean	0.035	0.145	0.099	0.022	0.006	0.024	0.006	0.048	0.003	0.398
		SE	0.007	0.007	0.007	0.002	0.001	0.002	0.001	0.004	0.001	0.019
T-Test [y] Significance												
'Line 8' vs. 'Sunset'	Color Break		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Full Ripe		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Color Break vs. Full Ripe	'Line 8'		**	**	**	**	**	NS	NS	NS	NS	**
	'Sunset'		**	**	**	**	**	NS	NS	NS	*	**

[x] Mean and SE were of 6 replicates (fruit).

[y] T-Test for equality of means. NS = Means were not significantly different at the 0.05 level. * = Means were significantly different at the 0.05 level. ** = Means were significantly different at the 0.01 level.

Table 4.3 Dry weight of cell wall polysaccharide fractions compared to cell wall material (CWM) weight of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages

Variety	Fruit Stage		Dry Weight of Fraction [x] (% of CWM Weight)									
			Water	CDTA_1	CDTA_2	Na_2CO_3_1	Na_2CO_3_rm	1M KOH_1	1M KOH_2	4M KOH_1	4M KOH_2	Cellulose
'Line 8'	Color Break	Mean	5.62	24.35	16.21	17.88	4.19	1.98	0.60	2.93	0.39	34.93
		SE	0.61	1.58	1.52	0.79	0.45	0.27	0.17	0.36	0.05	1.59
	Full Ripe	Mean	4.08	18.35	15.15	3.29	0.77	3.26	0.77	6.53	0.65	58.80
		SE	0.48	1.21	2.00	0.42	0.13	0.28	0.07	0.70	0.14	1.37
'Sunset'	Color Break	Mean	4.44	25.08	18.00	15.84	4.61	1.75	0.72	3.01	0.44	37.33
		SE	0.19	2.22	1.07	0.50	0.22	0.31	0.22	0.43	0.09	1.00
	Full Ripe	Mean	4.71	20.37	13.96	3.04	0.87	3.39	0.76	6.78	0.49	55.20
		SE	0.72	1.41	1.32	0.29	0.21	0.28	0.07	0.59	0.09	1.53
T-Test [y] Significance												
'Line 8' vs. 'Sunset'	Color Break		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Full Ripe		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Color Break vs. Full Ripe	'Line 8'		NS	*	NS	**	**	**	NS	**	NS	**
	'Sunset'		NS	NS	*	**	**	**	NS	**	NS	**

[x] Mean and SE were of 6 replicates (fruit).

[y] T-Test for equality of means. NS = Means were not significantly different at the 0.05 level. * = Means were significantly different at the 0.05 level. ** = Means were significantly different at the 0.01 level.

Table 4.4 Total sugar content in cell wall polysaccharide fractions compared to mesocarp fresh weight of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages

Variety	Fruit Stage		Total Sugar [x] (mg glucose/ 100 g Mesocarp Fresh Weight)								
			Water	CDTA_1	CDTA_2	Na_2CO_3_1	Na_2CO_3_rm	1M KOH_1	1M KOH_2	4M KOH_1	4M KOH_2
'Line 8'	Color Break	Mean	26	70	16	114	30	34	9	82	11
		SE	2.9	9.6	3.3	19.1	4.1	1.4	0.6	5.0	1.6
	Full Ripe	Mean	10	13	2	4	2	25	7	66	6
		SE	1.9	2.3	0.5	0.5	0.2	2.7	0.7	8.5	0.9
'Sunset'	Color Break	Mean	17	64	12	93	31	30	9	76	11
		SE	1.0	6.8	1.4	12.6	2.6	1.4	0.4	3.7	1.3
	Full Ripe	Mean	12	12	2	3	2	25	8	71	8
		SE	2.4	2.0	3.8	0.5	0.3	1.6	0.7	6.5	0.7
T-Test [y] Significance											
'Line 8' vs. 'Sunset'	Color Break		*	NS	NS	NS	NS	NS	NS	NS	NS
	Full Ripe		NS	NS	NS	NS	NS	NS	NS	NS	NS
Color Break vs. Full Ripe	'Line 8'		**	**	**	**	**	*	NS	NS	*
	'Sunset'		NS	**	**	**	**	*	NS	NS	NS

[x] Mean and SE were of from 6 replicates (fruit).

[y] T-Test for equality of means. NS = Means were not significantly different at the 0.05 level. * = Means were significantly different at the 0.05 level. ** = Means were significantly different at the 0.01 level.

Table 4.5 Uronic acid content in cell wall polysaccharide fractions compared to mesocarp fresh weight of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages

Variety	Fruit Stage		Uronic Acids [x] (mg Galacturonic acid/ 100 g Mesocarp Fresh Weight)								
			Water	CDTA_1	CDTA_2	Na_2CO_3_1	Na_2CO_3_rm	1M KOH_1	1M KOH_2	4M KOH_1	4M KOH_2
'Line 8'	Color Break	Mean	53	190	31	239	38	4	1	5	1
		SE	11.0	22.9	7.3	29.4	4.4	0.5	0.2	1.0	0.5
	Full Ripe	Mean	12	28	3	6	1	3	1	4	1
		SE	2.2	4.2	0.6	1.2	0.2	0.5	0.2	0.4	0.3
'Sunset'	Color Break	Mean	33	180	21	186	34	3	1	4	1
		SE	5.8	5.7	2.2	12.3	1.8	0.7	0.3	0.8	0.3
	Full Ripe	Mean	16	28	2	5	1	3	1	4	1
		SE	4.6	6.2	0.3	0.7	0.3	0.4	0.2	0.2	0.2
T-Test [y] Significance											
'Line 8' vs. 'Sunset'	Color Break		NS	NS	NS	NS	NS	NS	NS	NS	NS
	Full Ripe		NS	NS	NS	NS	NS	NS	NS	NS	NS
Color Break vs. Full Ripe	'Line 8'		*	**	*	**	**	NS	NS	NS	NS
	'Sunset'		*	**	**	**	**	NS	NS	NS	NS

[x] Mean and SE were of 6 replicates (fruit).

[y] T-Test for equality of means. NS = Means were not significantly different at the 0.05 level. * = Means were significantly different at the 0.05 level. ** = Means were significantly different at the 0.01 level.

Table 4.6 Proportion of uronic acids to mass of cell wall polysaccharide fraction in 'Line 8' and 'Sunset' fruit at the color break and full ripe stages

Variety	Fruit Stage		Proportion of Uronic Acids [x] (g Galacturonic Acid/ 100 g Cell Wall Fraction)								
			Water	CDTA_1	CDTA_2	Na_2CO_3_1	Na_2CO_3_rm	1M KOH_1	1M KOH_2	4M KOH_1	4M KOH_2
'Line 8'	Color Break	Mean	51.0	44.3	11.4	73.4	50.9	12.4	14.7	9.2	13.6
		SE	5.7	4.8	2.8	5.0	4.2	1.6	4.0	2.4	5.5
	Full Ripe	Mean	40.5	21.4	2.6	24.3	26.5	13.3	14.8	9.6	13.6
		SE	4.1	2.0	0.8	3.8	4.6	2.3	3.1	0.8	7.7
'Sunset'	Color Break	Mean	44.1	44.4	7.1	70.3	44.0	11.6	5.8	8.2	16.6
		SE	7.3	3.8	0.9	4.4	0.8	2.0	2.1	2.6	6.6
	Full Ripe	Mean	40.8	19.4	2.2	23.5	26.8	12.3	19.0	9.5	30.7
		SE	7.0	4.0	0.4	2.7	6.0	2.0	5.8	1.1	7.6
T-Test [y] Significance											
'Line 8' vs. 'Sunset'	Color Break		NS	NS	NS	NS	NS	NS	NS	NS	NS
	Full Ripe		NS	NS	NS	NS	NS	NS	NS	NS	NS
Color Break vs. Full Ripe	'Line 8'		NS	**	*	**	**	NS	NS	NS	NS
	'Sunset'		NS	**	**	**	*	NS	NS	NS	NS

[x] Mean and SE were of 6 replicates (fruit).

[y] T-Test for equality of means. NS = Means were not significantly different at the 0.05 level. * = Means were significantly different at the 0.05 level. ** = Means were significantly different at the 0.01 level.

Changes in Neutral Sugars Component of Cell Wall in 'Line 8' and 'Sunset' Papayas

Water-soluble cell wall polysaccharides showed xylosyl and galactosyl residues as the major components at the color break stage in both 'Line 8' and 'Sunset' fruit (Figure 4.1 A). The contents of arabinose, mannose, glucose, and especially galactose declined in water-soluble polysaccharides of mesocarp cell wall during ripening (Figure 4.1 B). Xylosyl residues became the major neutral sugar in the water-soluble polysaccharides of ripe fruit cell walls (Figure 4.1 A).

The CDTA_1-soluble polysaccharides consisted mainly of galactosyl, rhamnosyl, and xylosyl residues in cell wall of both lines at the color break stage (Figure 4.2 A). Greater than 60% of galactosyl component dissociated from this fraction during fruit ripening (Figure 4.2 B). The arabinosyl component also declined during ripening (Figure 4.2 B), leading to a higher proportion of rhamnosyi and glucosyl residues in this fraction (Figure 4.2 A).

The CDTA_2-soluble polysaccharides consisted mainly of galactosyl residues in both lines at color break stage (Figure 4.3 A) but the CDTA_2-soluble fraction had an equal amount of glucosyl to rhamnosyl residues. The content of all neutral sugars in this fraction, except for xylose, decreased during ripening (Figure 4.3 B). However, the galactosyi residues decreased to a greater extent relative to other neutral sugars (Figure 4.3 B), causing the other neutral sugars such as mannose and glucose to be a greater proportion of this fraction at the full ripe stage (Figure 4.3 A).

At the color break stage for both lines, over 60% of neutral sugars in Na_2CO_3-soluble cell wall polysaccharides (Na_2CO_3_1 and Na_2CO_3_rm fractions) was galactose

(Figure 4.4 A and 4.5 A). There was a very low amount of arabinosyl, xylosyl, manosyl, and glucosyl residues in those fractions. All the neutral sugars except for galactose and rhamnose showed a small decrease in the Na_2CO_3_1-soluble and Na_2CO_3_rm-soluble polysaccharides during ripening (Figure 4.4 B and 4.5 B). Galactosyl residues showed a significant decline in these cell wall fractions. Ripe fruit had similar proportions of rhamnosyl and galactosyl residues in the Na_2CO_3_1 fraction (Figure 4.4 A) and glucosyl, galactosyl, and rhamnosyl residues in Na_2CO_3_rm fraction (Figure 4.5 A).

Cell wall polysaccharides that dissolved in 1 M KOH solution (1 M KOH_1 and 1 M KOH_2 fractions) were enriched in xylosyl residues and had glucosyl residues as the second major component at both the color break and the full ripe stage (Figure 4.6 A and 4.7 A). The relative proportions of xylosyl residues to the total neutral sugars in these fractions remained stable during ripening but the amount showed a decline (Figure 4.6 B and 4.7 B).

Cell wall polysaccharides that dissolved in 4 M KOH solutions (4 M KOH_1 and 4 M KOH_2 fractions) contained mainly glucosyl and xylosyl residues, at the color break and full ripe stages in both lines (Figure 4.8 A and 4.9 A). Most of the neutral sugars in the 4 M KOH_1-soluble polysaccharides maintained their relative proportions from color break stage to the full ripe stage. There were small changes in the proportions of rhamnosyl residue in the 4 M KOH_2-soluble fraction of both lines. The content of rhamnosyl, mannosyl, and galactosyl residues in 4 M KOH_2-soluble polysaccharides showed a small decline during ripening in 'Sunset' fruit (Figure 4.9 B).

The cellulose fraction contained a similar proportion of glucosyl to galactosyl residues in both 'Line 8' and 'Sunset' fruit at the color break stage (Figure 4.10 A). The

fraction also had a significant proportion of other non-glucosyl neutral sugars. The non-glucosyl neutral sugars, except for mannose, declined during ripening while the content of glucosyl residues was maintained (Figure 4.10 B). In the later stage of fruit ripening, glucose was dominant in the cellulose fraction (Figure 4.10 A).

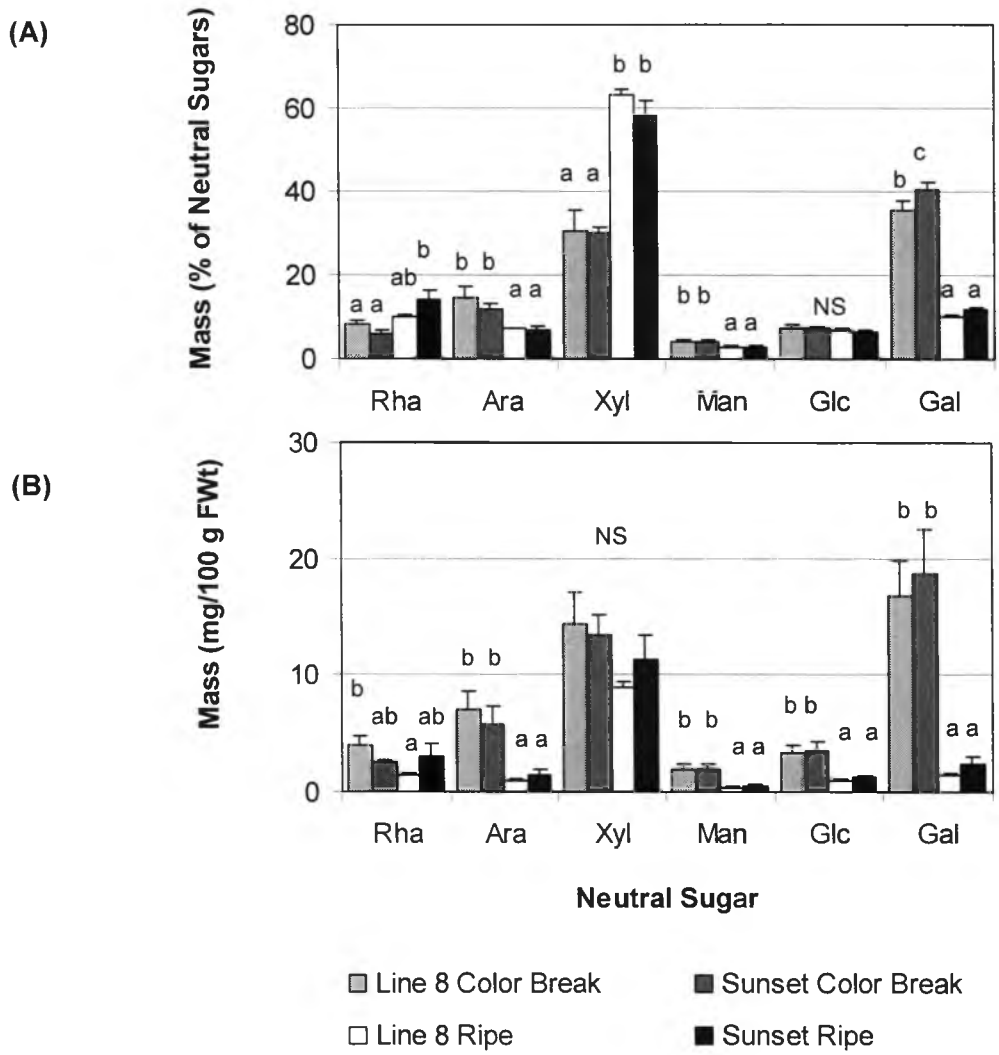


Figure 4.1 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in water-soluble cell wall polysaccharides of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between lines and stages.

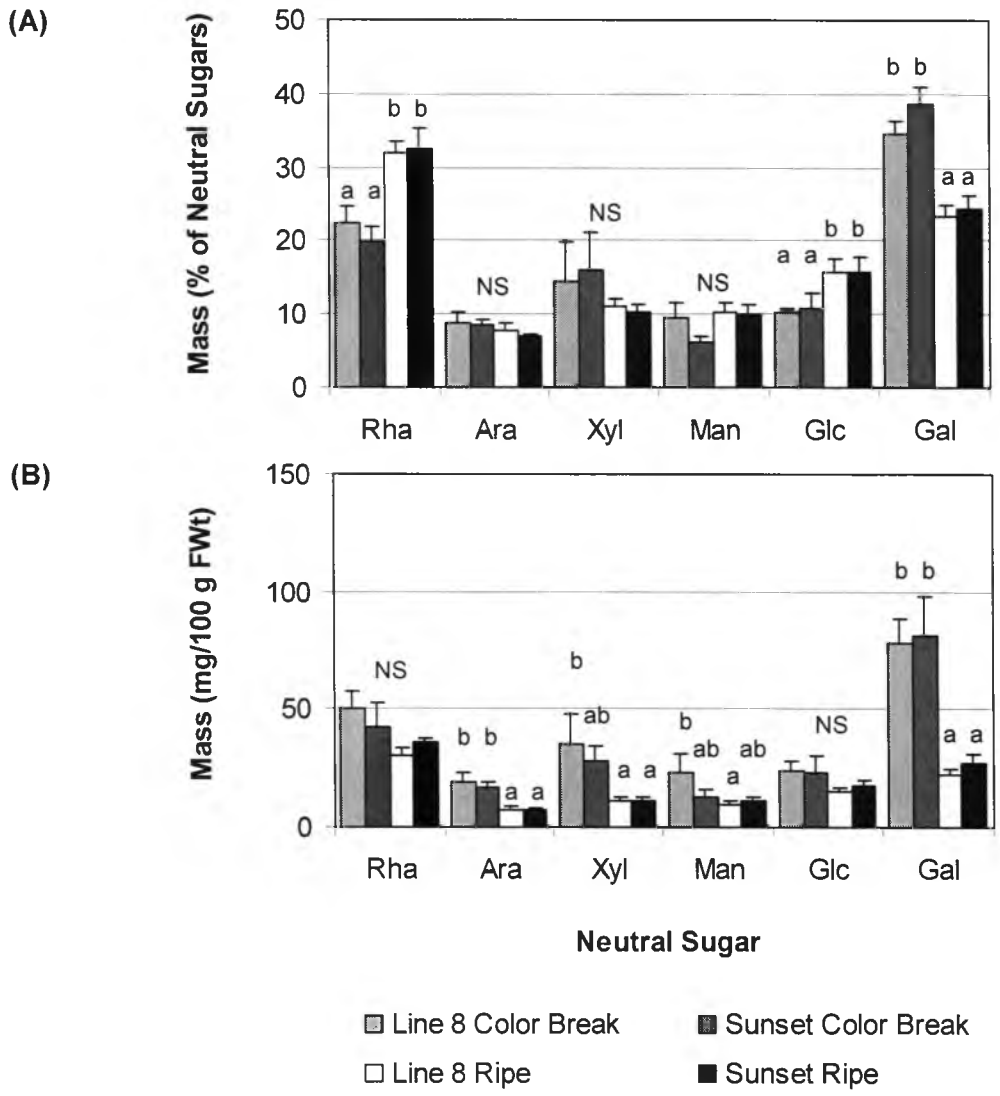


Figure 4.2 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the first CDTA-soluble cell wall polysaccharides of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between lines and stages.

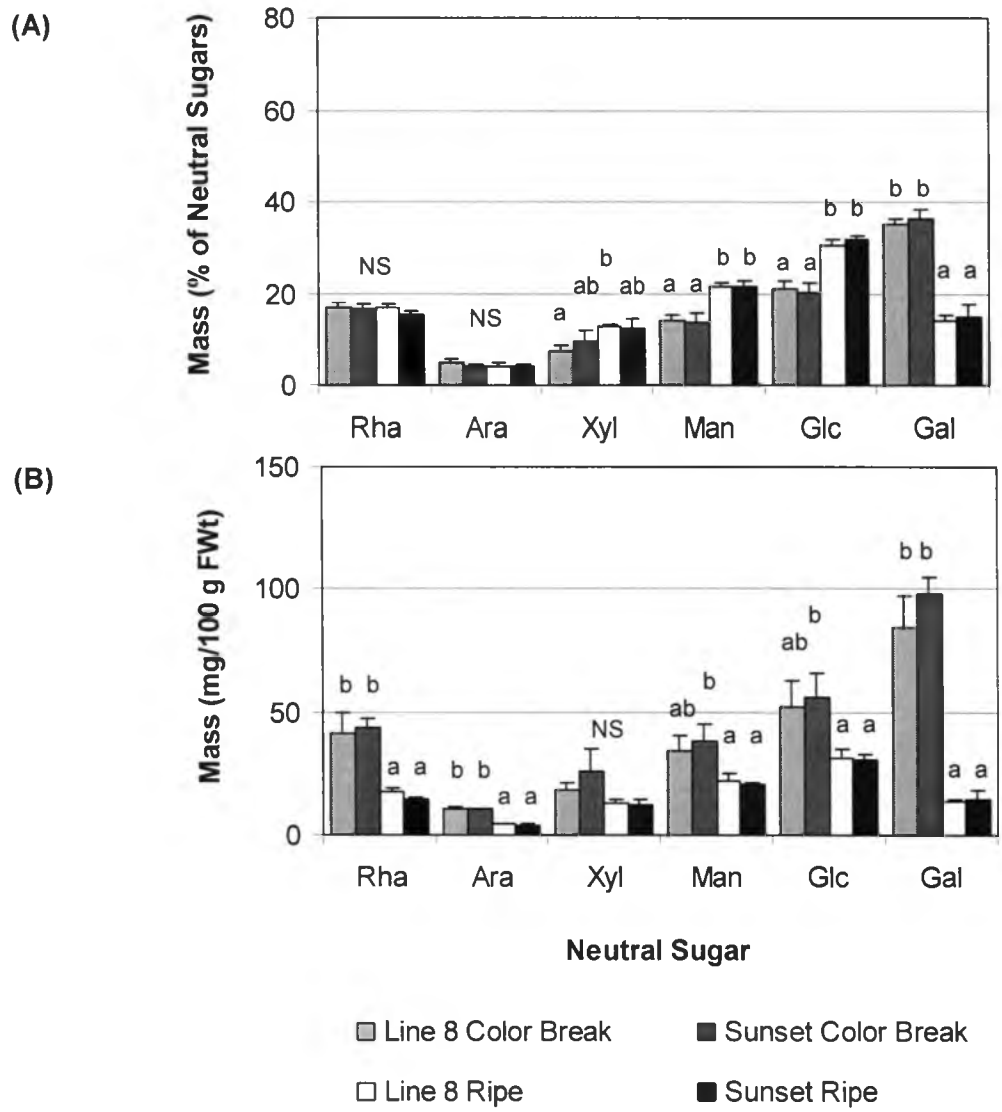


Figure 4.3 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the second CDTA-soluble cell wall polysaccharides of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between lines and stages.

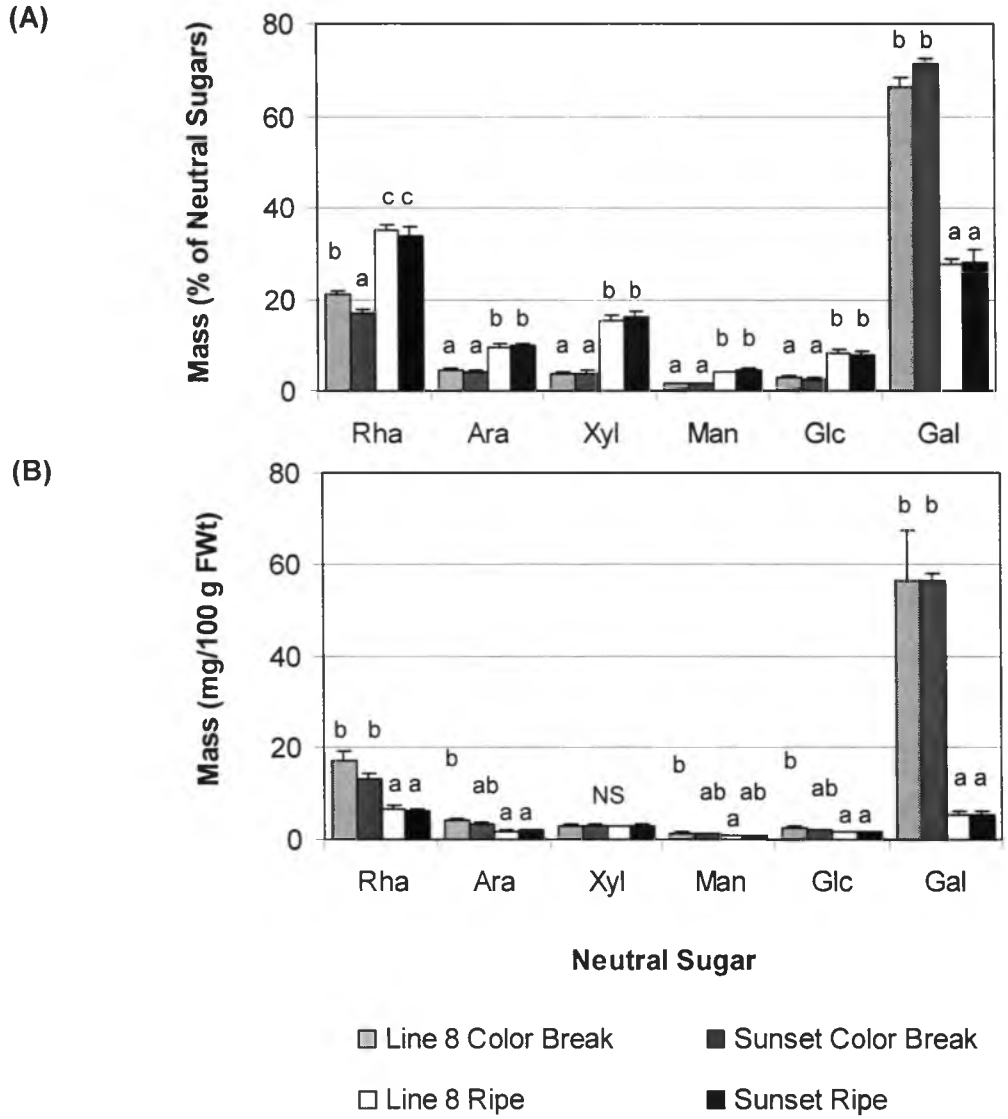


Figure 4.4 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in 1°C Na_2CO_3-soluble cell wall polysaccharides of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between lines and stages.

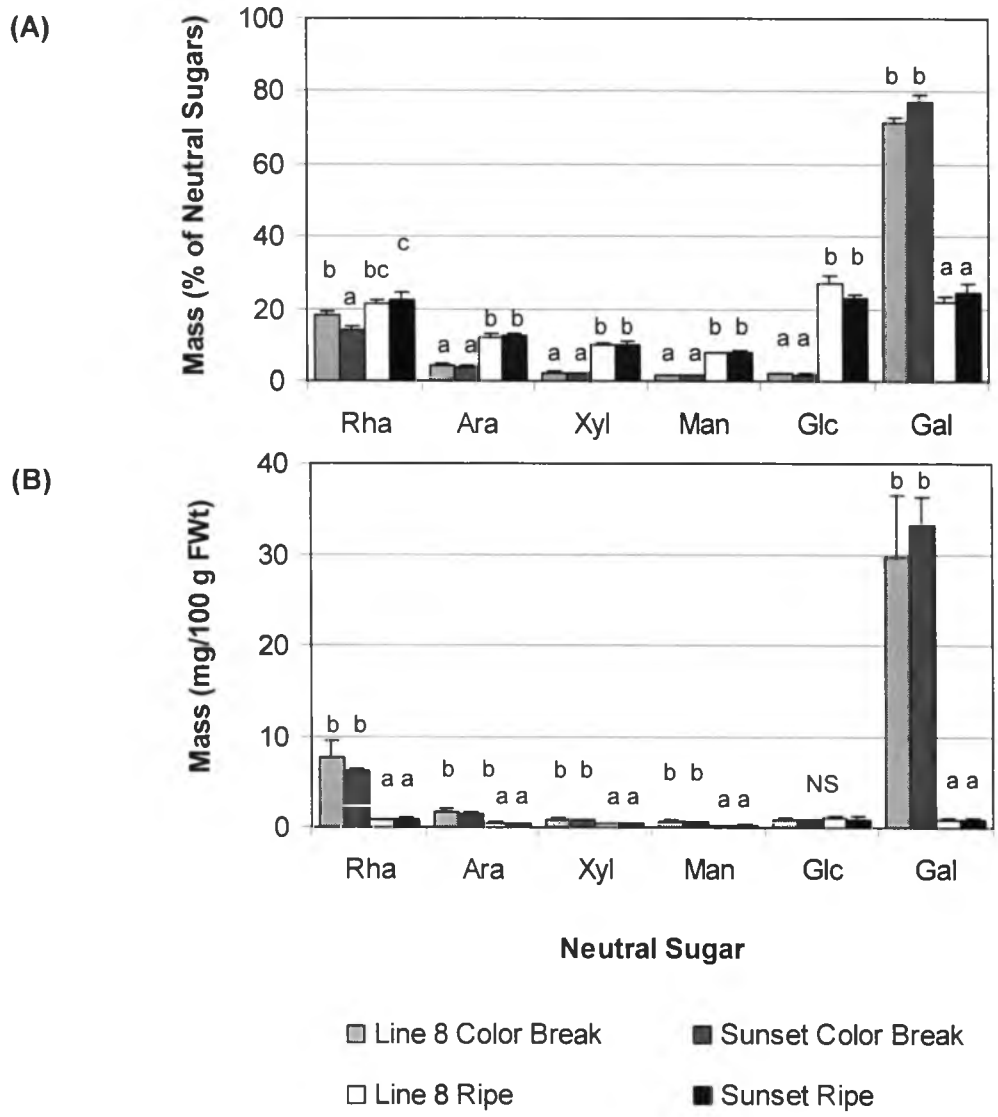


Figure 4.5 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in 22°C Na_2CO_3-soluble cell wall polysaccharides of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between lines and stages.

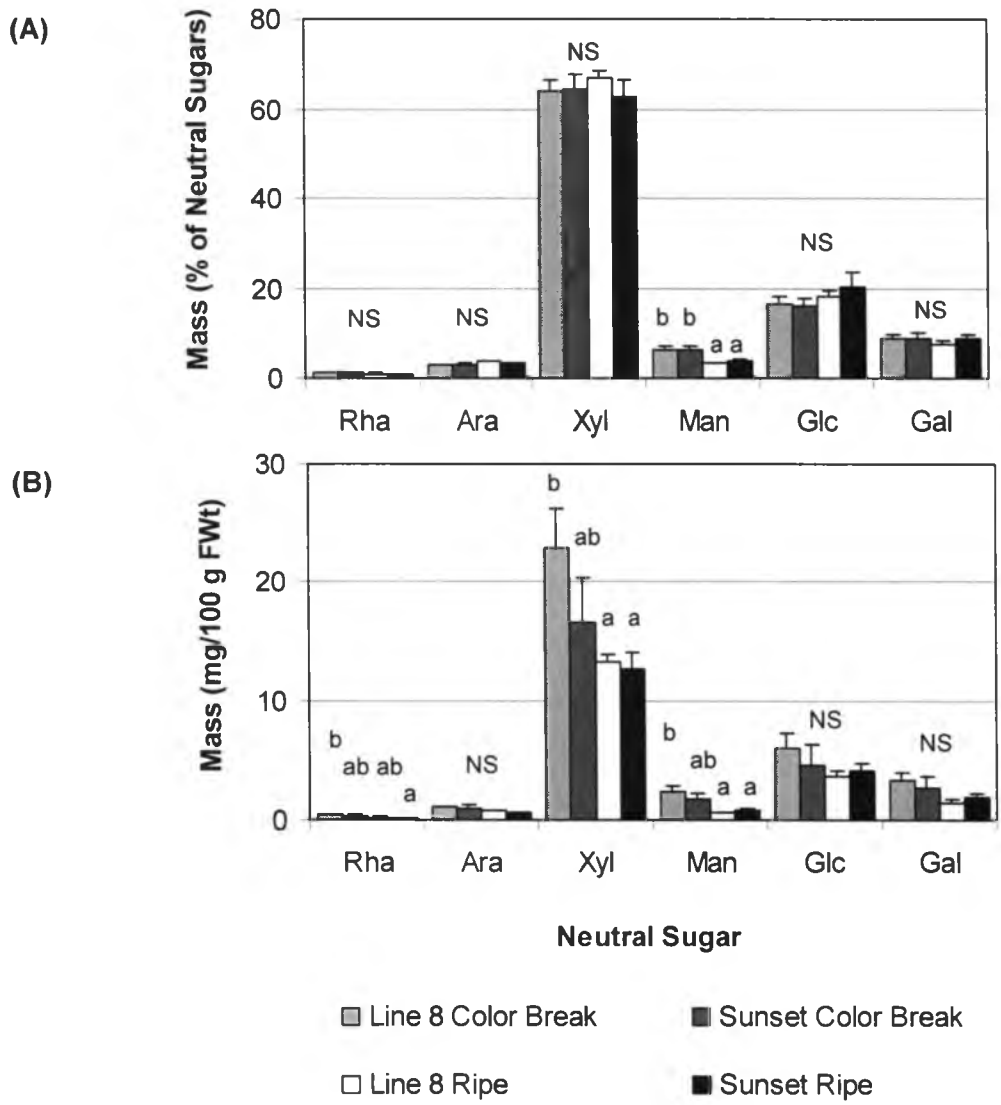


Figure 4.6 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the first 1 M KOH-soluble cell wall polysaccharides of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between lines and stages.

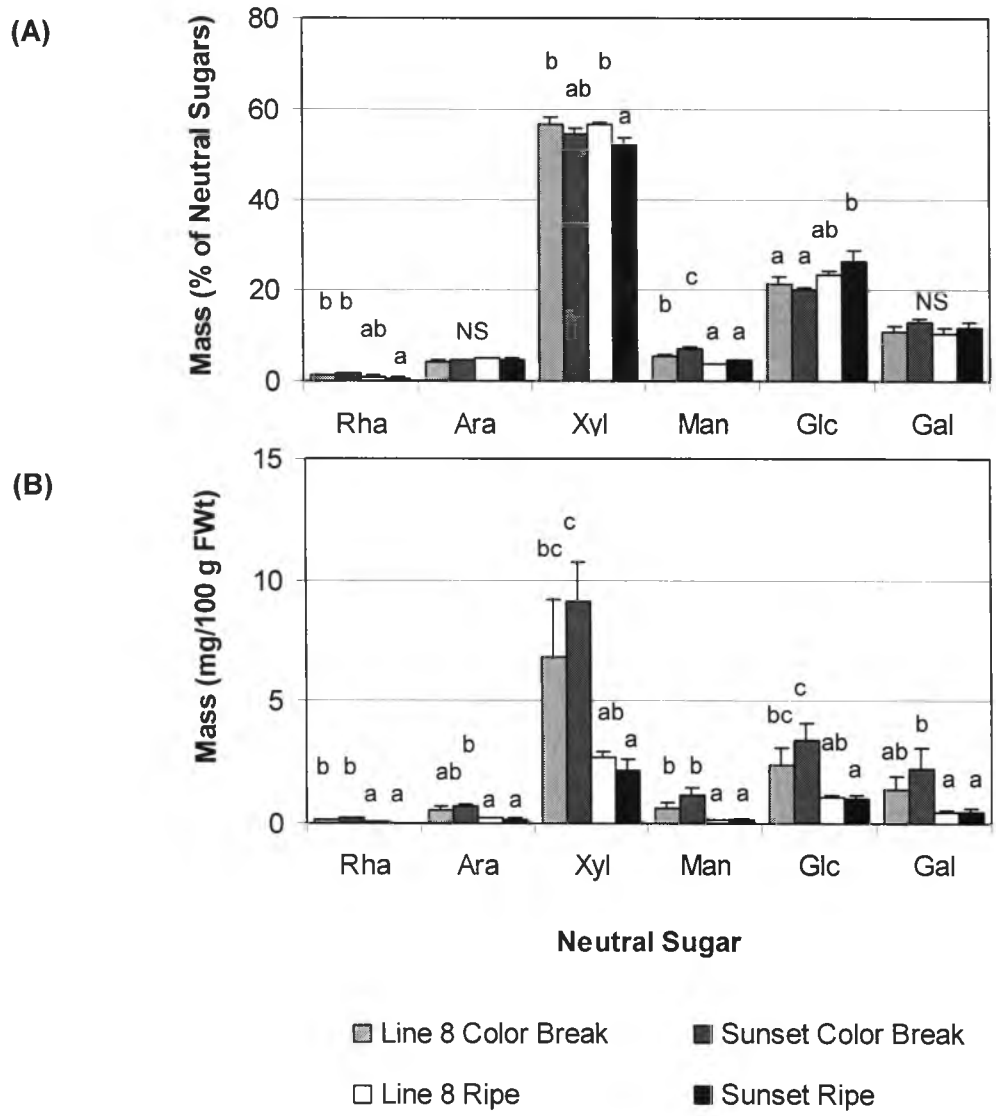


Figure 4.7 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the second 1 M KOH-soluble cell wall polysaccharides of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between lines and stages.

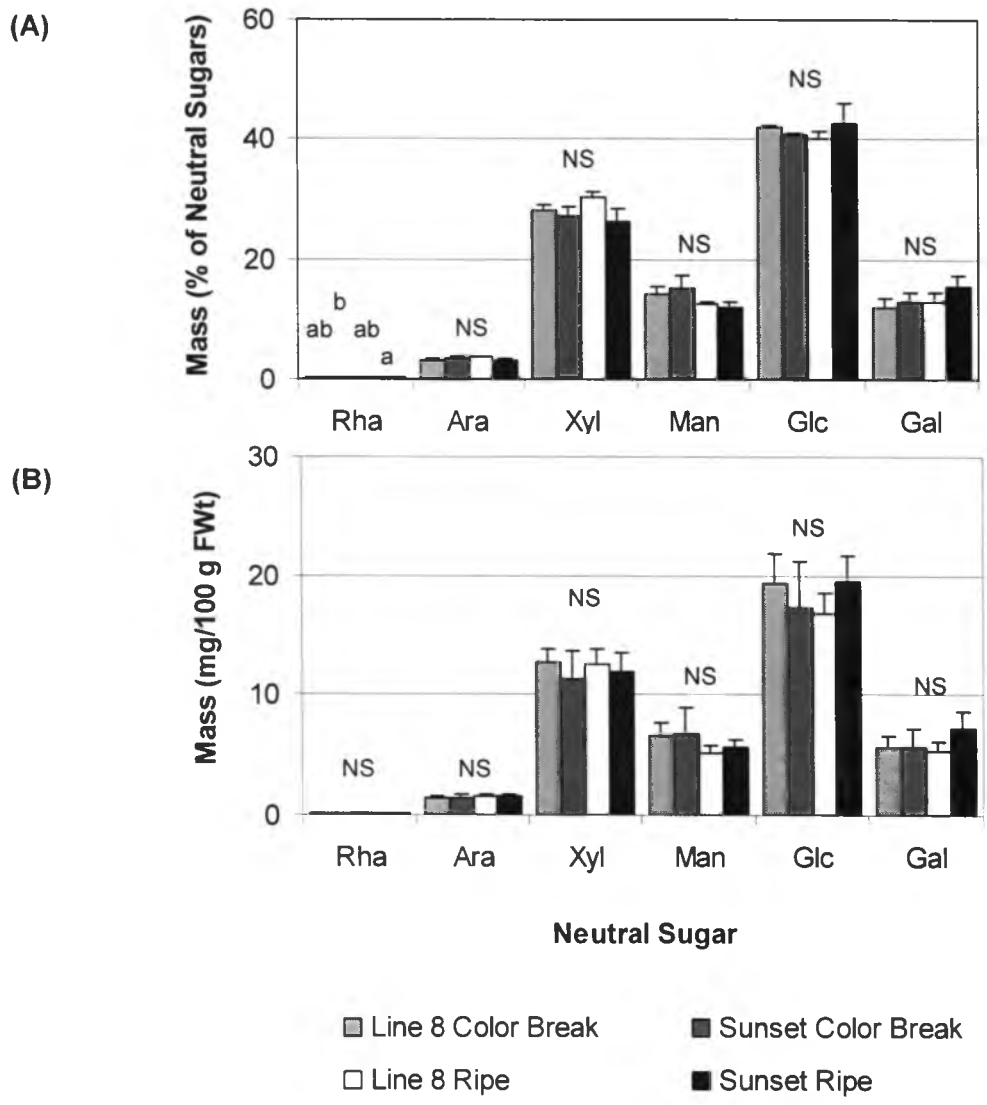


Figure 4.8 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the first 4 M KOH-soluble cell wall polysaccharides of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between lines and stages.

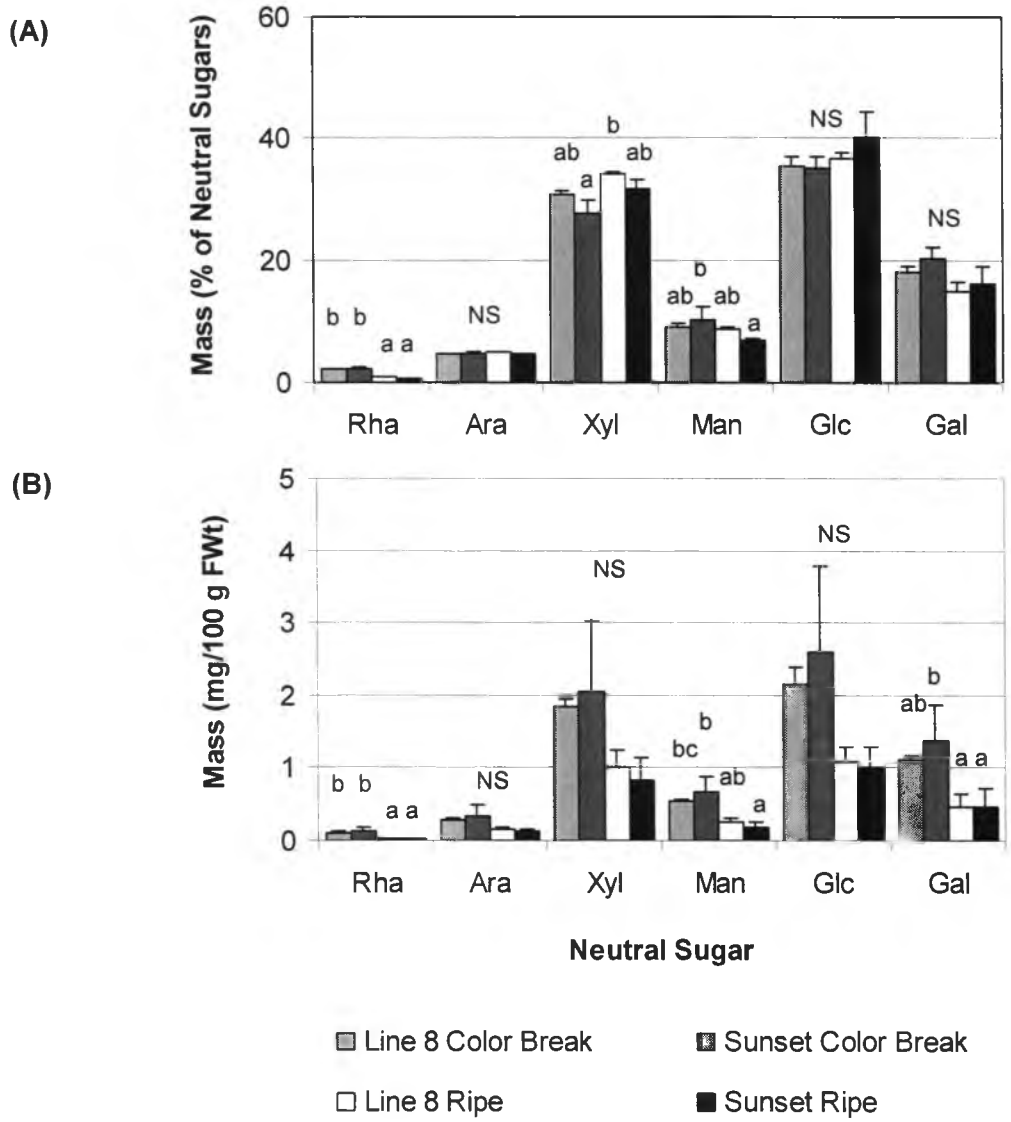


Figure 4.9 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the second 4 M KOH-soluble cell wall polysaccharides of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between lines and stages.

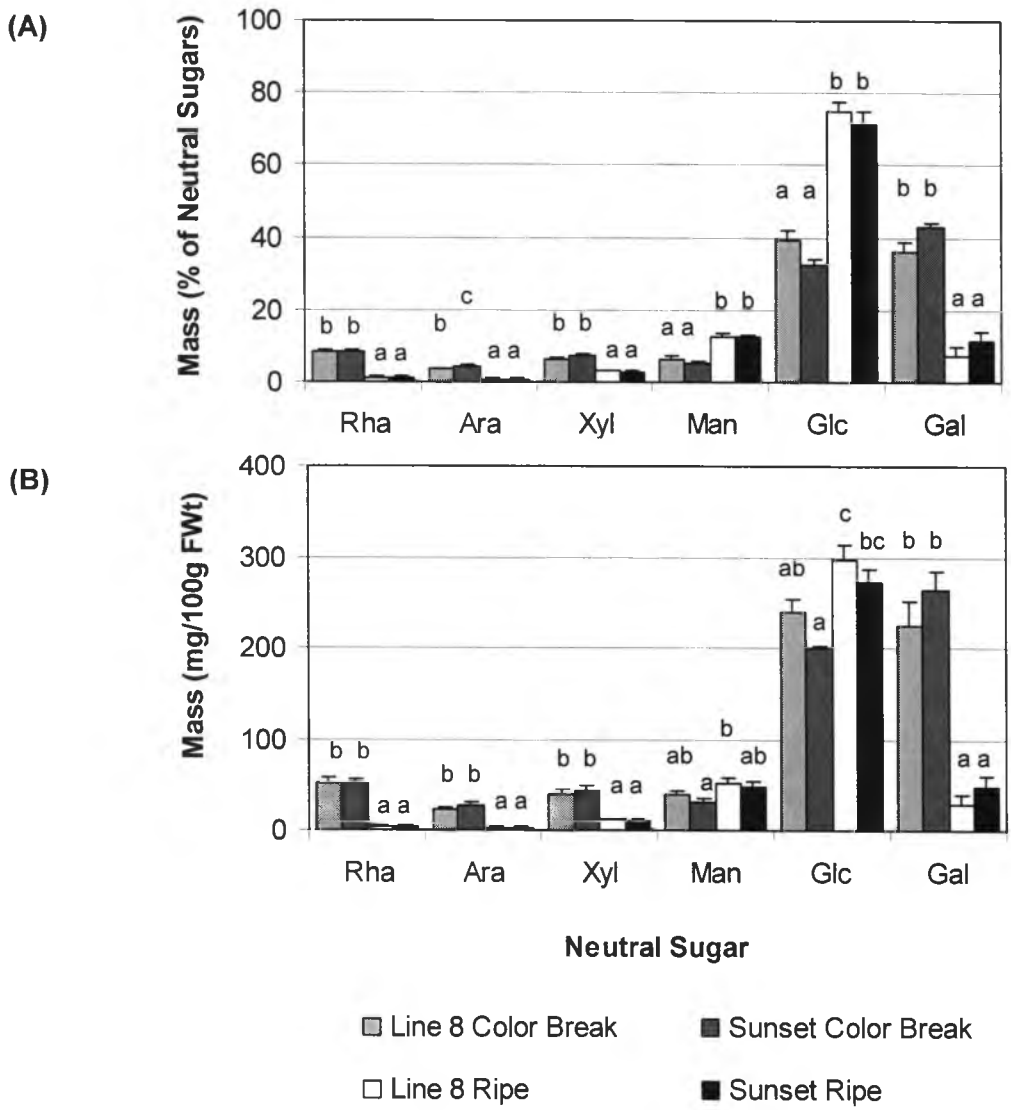


Figure 4.10 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in cellulose fraction of 'Line 8' and 'Sunset' fruit at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between lines and stages.

4.3.2 Cell Wall Modification of 'Sunset' Papaya after 1-MCP Treatment

Alteration in Ripening, Softening, and Cell Wall Material of 1-MCP-treated Papaya

'Sunset' fruit treated with 1-MCP showed no significant delay in ripening (skin color formation) compared to the untreated control fruit but it showed a reduction in softening (Table 4.7). All fruit treated with 1-MCP showed a "rubbery" texture when ripe. The mesocarp of 1-MCP-treated fruit did not lose CWM mass to the same extent as the non-treated fruit during ripening. CWM per mesocarp fresh weight of ripe 1-MCP-treated fruit was similar to that of 'Sunset' fruit at the color break stage (Table 4.7).

Table 4.7 Days from color break to full ripe, fruit firmness, and cell wall material (CWM) of 'Sunset' fruit with and without 1-MCP treatment

Treatment	Fruit Stage	Mean ± SE [x]		
		Days to Full Ripe	Firmness (Kg)	CWM Weight (% of FWt)
Control	Color Break	-	20.5 ± 1.5 c	1.68 ± 0.034 b
	Full Ripe	11 ± 1.1	2.4 ± 0.2 a	0.72 ± 0.028 a
1-MCP	Full Ripe	13 ± 0.9	16.1 ± 0.3 b	1.78 ± 0.016 b

[x] Means and SE were of 6 replicates (fruit) for non MCP-treated 'Sunset' fruit, and from 4 replicates for MCP-treated 'Sunset' fruit. The different letters following means in the same column showed that the mean difference was significant at the 0.05 level by LSD.

Changes in Cell Wall Polysaccharides of Papaya after 1-MCP Treatment

The mesocarp of ripe 1-MCP-treated 'Sunset' fruit had less cell wall polysaccharide mass that dissolved in water, Na_2CO_3_1, and Na_2CO_3_rm than 'Sunset' fruit at the color break stage. During ripening, 1-MCP-treated fruit did not lose cell wall polysaccharides content from the CDTA_1, CDTA_2, Na_2CO_3_1, Na_2CO_3_rm, 4 M KOH_1, and cellulose fraction to the same extent as the control fruit. Fruit treated with 1-MCP also accumulated a higher content of cell wall polysaccharides that dissolved in CDTA_2 and 1 M KOH_1 than the fruit at the color break stage (Table 4.8).

The mass of cell wall polysaccharide fractions to CWM mass (Table 4.9) in ripe 1-MCP-treated fruit showed a lower proportion of water-soluble, Na_2CO_3_1-soluble, and Na_2CO_3_rm-soluble cell wall polysaccharides and cellulose than fruit at the color break stage. However, ripe 1-MCP-treated fruit had a higher proportion of CDTA_1, CDTA_2, Na_2CO_3_1, Na_2CO_3_rm fractions than the ripe control fruit (Table 4.9).

Ripe 1-MCP treated fruit showed less total sugar contents in the water-soluble, CDTA_2-soluble, Na_2CO_3_1-soluble, and Na_2CO_3_rm-soluble cell wall fractions than fruit at the color break stage. However, 1-MCP-treated fruit had a higher total sugar contents in the CDTA_1-soluble, CDTA_2-soluble, Na_2CO_3_1-soluble, and Na_2CO_3_rm-soluble cell wall fractions than the ripe control fruit. Ripe 1-MCP treated fruit also had higher total sugar content in 1 M KOH_1-soluble fraction than control papaya at both stages (Table 4.10) which agreed with data for cell wall fraction content (Table 4.8).

Although the mesocarp of ripe 1-MCP-treated fruit showed less uronic acid content in Na_2CO_3_1-soluble and Na_2CO_3_rm-soluble cell wall polysaccharides than

color break fruit (Table 4.11); ripe 1-MCP-treated fruit maintained a high proportion of uronic acids in these fractions (Table 4.12). The mesocarp of ripe 1-MCP-treated fruit had a higher proportion of uronic acids to fraction mass and a higher uronic acid content in CDTA_1-soluble, CDTA_2-soluble, Na_2CO_3_1-soluble, and Na_2CO_3_rm-soluble cell wall polysaccharides than the mesocarp of ripe control fruit. However, ripe 1-MCP-treated fruit had a smaller content of uronic acids in water-soluble polysaccharides than the color break fruit and maintained the same uronic acid proportion to fraction mass in this fraction as the control fruit (Table 4.11 and 4.12).

Table 4.8 Dry weight of cell wall polysaccharide fractions compared to mesocarp fresh weight of 'Sunset' fruit with and without 1-MCP treatment

Treatment	Fruit Stage		Dry Weight of Fraction [x] (% of Mesocarp Fresh Weight)									
			Water	CDTA_1	CDTA_2	Na_2CO_3_1	Na_2CO_3_rm	1M KOH_1	1M KOH_2	4M KOH_1	4M KOH_2	Cellulose
Control	Color Break	Mean	0.074 b	0.423 b	0.302 b	0.265 c	0.077 c	0.029 a	0.012	0.051	0.007 b	0.627 b
		SE	0.003	0.046	0.019	0.008	0.003	0.005	0.004	0.008	0.001	0.026
	Full Ripe	Mean	0.035 a	0.145 a	0.099 a	0.022 a	0.006 a	0.024 a	0.006	0.048	0.003 a	0.398 a
		SE	0.007	0.007	0.007	0.002	0.001	0.002	0.001	0.004	0.001	0.019
MCP-Treated	Full Ripe	Mean	0.043 a	0.497 b	0.371 c	0.172 b	0.050 b	0.044 b	0.013	0.060	0.008 b	0.580 b
		SE	0.008	0.047	0.025	0.006	0.004	0.005	0.002	0.008	0.001	0.027

[x] Means and SE were of 6 replicates (fruit) for non MCP-treated 'Sunset' fruit, and of 4 replicates for MCP-treated 'Sunset' fruit. Different letters following means in the same column showed that the mean difference was significant at the 0.05 level by LSD.

Table 4.9 Dry weight of cell wall polysaccharide fractions compared to cell wall material (CWM) weight of 'Sunset' fruit with and without 1-MCP treatment

Treatment	Fruit Stage		Dry Weight of Fraction [x] (% of CWM Weight)									
			Water	CDTA_1	CDTA_2	Na_2CO_3_1	Na_2CO_3_rm	1M KOH_1	1M KOH_2	4M KOH_1	4M KOH_2	Cellulose
Control	Color Break	Mean	4.44 b	5.08 ab	18.00 b	15.84 c	4.61 c	1.75 a	0.72	3.01 a	0.44	37.33 b
		SE	0.19	2.22	1.07	0.50	0.22	0.31	0.22	0.43	0.09	1.00
	Full Ripe	Mean	4.71 b	20.37 a	13.96 a	3.04 a	0.87 a	3.39 b	0.76	6.78 b	0.49	55.20 c
		SE	0.72	1.41	1.32	0.29	0.21	0.28	0.07	0.59	0.09	1.53
MCP-Treated	Full Ripe	Mean	2.42 a	27.97 b	20.88 b	9.65 b	2.83 b	2.45 ab	0.71	3.37 a	0.47	32.60 a
		SE	0.43	2.77	1.49	0.25	0.26	0.24	0.11	0.43	0.04	1.59

[x] Means and SE were of 6 replicates (fruit) for non MCP-treated 'Sunset' fruit, and of 4 replicates for MCP-treated 'Sunset' fruit. Different letters following means in the same column showed that the mean difference was significant at the 0.05 level by LSD.

Table 4.10 Total sugar content in cell wall polysaccharide fractions compared to mesocarp fresh weight of 'Sunset' fruit with and without 1-MCP treatment

Treatment	Fruit Stage		Total Sugar [x] (mg glucose/ 100 g Mesocarp Fresh Weight)								
			Water	CDTA_1	CDTA_2	Na_2CO_3_1	Na_2CO_3_rm	1M KOH_1	1M KOH_2	4M KOH_1	4M KOH_2
Control	Color Break	Mean	17 b	64 b	12 c	93 c	31 c	30 a	9	76	11 b
		SE	1.0	6.8	1.4	12.6	2.6	1.4	0.4	3.7	1.3
	Full Ripe	Mean	12 ab	12 a	2 a	3 a	2 a	25 a	8	71	8 a
		SE	2.4	2.0	3.8	0.5	0.3	1.6	0.7	6.5	0.7
MCP-Treated	Full Ripe	Mean	11 a	53 b	7 b	30 b	13 b	37 b	8	77	9 ab
		SE	0.7	1.9	0.5	1.0	0.8	2.8	0.9	9.9	0.4

[x] Means and SE were of 6 replicates (fruit) for non MCP-treated 'Sunset' fruit, and of 4 replicates for MCP-treated 'Sunset' fruit. Different letters following means in the same column showed that the mean difference was significant at the 0.05 level by LSD.

Table 4.11 Uronic acid content in cell wall polysaccharide fractions compared to mesocarp fresh weight of 'Sunset' fruit with and without 1-MCP treatment

Treatment	Fruit Stage		Uronic Acids [x] (mg galacturonic acid/ 100 g Mesocarp Fresh Weight)								
			Water	CDTA_1	CDTA_2	Na_2CO_3_1	Na_2CO_3_rm	1M KOH_1	1M KOH_2	4M KOH_1	4M KOH_2
Control	Color Break	Mean	33 b	180 b	21 b	186 c	34 c	3	1	4	1 b
		SE	5.8	5.7	2.2	12.3	1.8	0.7	0.3	0.8	0.3
	Full Ripe	Mean	16 a	28 a	2 a	5 a	1 a	3	1	4	1 ab
		SE	4.6	6.2	0.3	0.7	0.3	0.4	0.2	0.2	0.2
MCP-Treated	Full Ripe	Mean	13 a	178 b	17 b	84 b	27 b	3	<1	2	<1 a
		SE	1.3	23.4	1.1	4.6	0.8	0.2	0.4	0.7	0.5

[x] Means and SE were of 6 replicates (fruit) for non MCP-treated 'Sunset' fruit, and of 4 replicates for MCP-treated 'Sunset' fruit. Different letters following means in the same column showed that the mean difference was significant at the 0.05 level by LSD.

Table 4.12 Proportion of uronic acids to mass of cell wall polysaccharide fraction in 'Sunset' fruit with and without 1-MCP treatment

Treatment	Fruit Stage		Proportion of Uronic Acids [x] (g Galacturonic Acid/ 100 g Cell Wall Fraction)								
			Water	CDTA_1	CDTA_2	Na_2CO_3_1	Na_2CO_3_rm	1M KOH_1	1M KOH_2	4M KOH_1	4M KOH_2
Control	Color Break	Mean	44.1	44.4 b	7.1 c	70.3 c	44.0 b	11.6	5.8 ab	8.2	16.6 ab
		SE	7.3	3.8	0.9	4.4	0.8	2.0	2.1	2.6	6.6
	Full Ripe	Mean	40.8	19.4 a	2.2 a	23.5 a	26.8 a	12.3	19.0 b	9.5	30.7 b
		SE	6.9	4.0	0.4	2.7	6.0	2.0	5.8	1.1	7.6
MCP-Treated	Full Ripe	Mean	31.9	37.6 b	4.5 b	49.1 b	55.5 b	7.9	2.0 a	3.8	1.0 a
		SE	2.9	7.6	0.3	2.2	5.3	1.1	3.2	0.7	5.9

[x] Means and SE were of 6 replicates (fruit) for non MCP-treated 'Sunset' fruit, and of 4 replicates for MCP-treated 'Sunset' fruit. Different letters following means in the same column showed that the mean difference was significant at the 0.05 level by LSD.

Changes in Neutral Sugar Component of Cell Wall of Papaya after 1-MCP treatment

Changes in the proportions of neutral sugar to the total neutral sugars and contents of the neutral sugar components in the cell wall polysaccharide fractions during ripening were notably different between control (untreated) and 1-MCP-treated fruit.

The water-soluble polysaccharides of cell wall showed galactosyl and xylosyl residues as the main neutral sugar components at the color break stage of 'Sunset' fruit (Figure 4.11 A). The untreated control fruit showed a dramatic decline in the proportion of galactose to the total neutral sugars and an increase in xylosyl proportion in this fraction during ripening. Ripe 1-MCP-treated fruit did not show a decrease in galactosyl proportion in the water-soluble polysaccharides. Galactosyl residues remained the major component of water-soluble fraction of ripe 1-MCP treated papaya (Figure 4.11 A). Changes in the content of neutral sugars in the water-soluble cell wall polysaccharide showed that the 1-MCP treatment reduced dissolutions of xylosyl and arabinosyl residues from cell walls (Figure 4.11 B).

The CDTA-soluble cell wall polysaccharides (CDTA_1 and CDTA_2 fractions) had galactosyl residues as the major neutral sugar component at the color break stage (Figure 4.12 A and 4.13 A). The proportion of galactosyl residues in these fractions decreased during ripening of untreated fruit. This decline did not occur in the 1-MCP-treated fruit. Ripe 1-MCP-treated fruit also showed a higher proportion of xylosyl residues in both CDTA-soluble polysaccharide fractions (Figure 4.12 A and 4.13 A). Control fruit showed a decline in the content of rhamnosyl, arabinosyl, and galactosyl residues in these fractions during ripening. In contrast, ripe 1-MCP treated fruit

maintained the content of all neutral sugars in these polysaccharide fractions similar to the color break stage, and also showed a marked increase in xylosyl content, and a moderate increase in arabinosyl content (Figure 4.12 B and 4.13 B).

Cell wall polysaccharides extracted by the Na_2CO_3 solution at the different temperatures (Na_2CO_3_1 and Na_2CO_3_rm fractions), were rich in galactosyl residues at color break stage (Figure 4.14 A and 4.15 A). The galactosyl proportions in these fractions decreased over 60% during the ripening of control fruit. Ripe 1-MCP-treated fruit showed a decrease in galactosyl proportions in these fractions but not to the same extent as the control fruit (Figure 4.14 A and 4.15 A). The contents of neutral sugars showed that the control papaya lost a significant amount of galactose and a small amount of rhamnose from these fractions. Ripe 1-MCP-treated fruit also lost a significant amount of the galactosyl content from the Na_2CO_3 fractions though a smaller amount than that in ripe control fruit. A greater content of rhanosyl, arabinosyl, xylosyl, manosyl, and glucosyl residues occurred in these fractions from the ripe 1-MCP-treated fruit than from the ripe control fruit (Figure 4.14 B and 4.15 B).

The 1 M KOH-soluble cell wall polysaccharides (1 M KOH_1 and 1 M KOH_2 fractions) were rich in xylosyl residues at both the color break and full ripe stage (Figure 4.16 A and 4.17 A). During ripening of control fruit, all neutral sugars, especially xylose, were dissociated from these matrix polysaccharides. Ripe 1-MCP-treated papaya did not show the same loss of most neutral sugars including xylose from these fractions; however, the mesocarp did lose galactosyl content during ripening (Figure 4.16 B and 4.17 B).

At the color break stage, 4 M KOH-soluble cell wall polysaccharides contained mainly glucosyl and xylosyl residues (Figure 4.18 A and 4.19 A). Small changes in the neutral sugar proportions occurred in the 4 M KOH-soluble fractions of the 1-MCP-treated fruit and non-treated fruit during ripening. Ripe 1-MCP-treated fruit had a higher of mannosyl and rhamnosyl proportions in these fractions than ripe control fruit (Figure 4.18 A and 4.19 A). Control fruit lost rhamnosyl and mannosyl residues from these fractions during ripening (Figure 4.18 B and 4.19 B). However, 1-MCP-treated fruit did not lose the glucosyl contents and also had a higher content of glucosyl residues in the 4 M KOH_2 fraction than the ripe control fruit (Figure 4.18 B and 4.19 B).

The cellulose fraction at the color break stage contained mainly glucosyl and galactosyl residues (Figure 4.20 A). A small proportion of other non-glucosyl neutral sugars were also found in this fraction. All non-glucosyl neutral sugars, except for mannose, decreased in proportions in the cellulose fraction during ripening of control fruit (Figure 4.20 A). The contents of all neutral sugars, except for mannose and glucose, declined during ripening of control fruit (Figure 4.20 B). The 1-MCP-treated fruit maintained its contents of rhamnosyl, arabinosyl, xylosyl, and mannosyl residues in cellulose fraction but still lost galactosyl content as occurred in control fruit, and had a higher content of glucosyl residues (Figure 4.20 B).

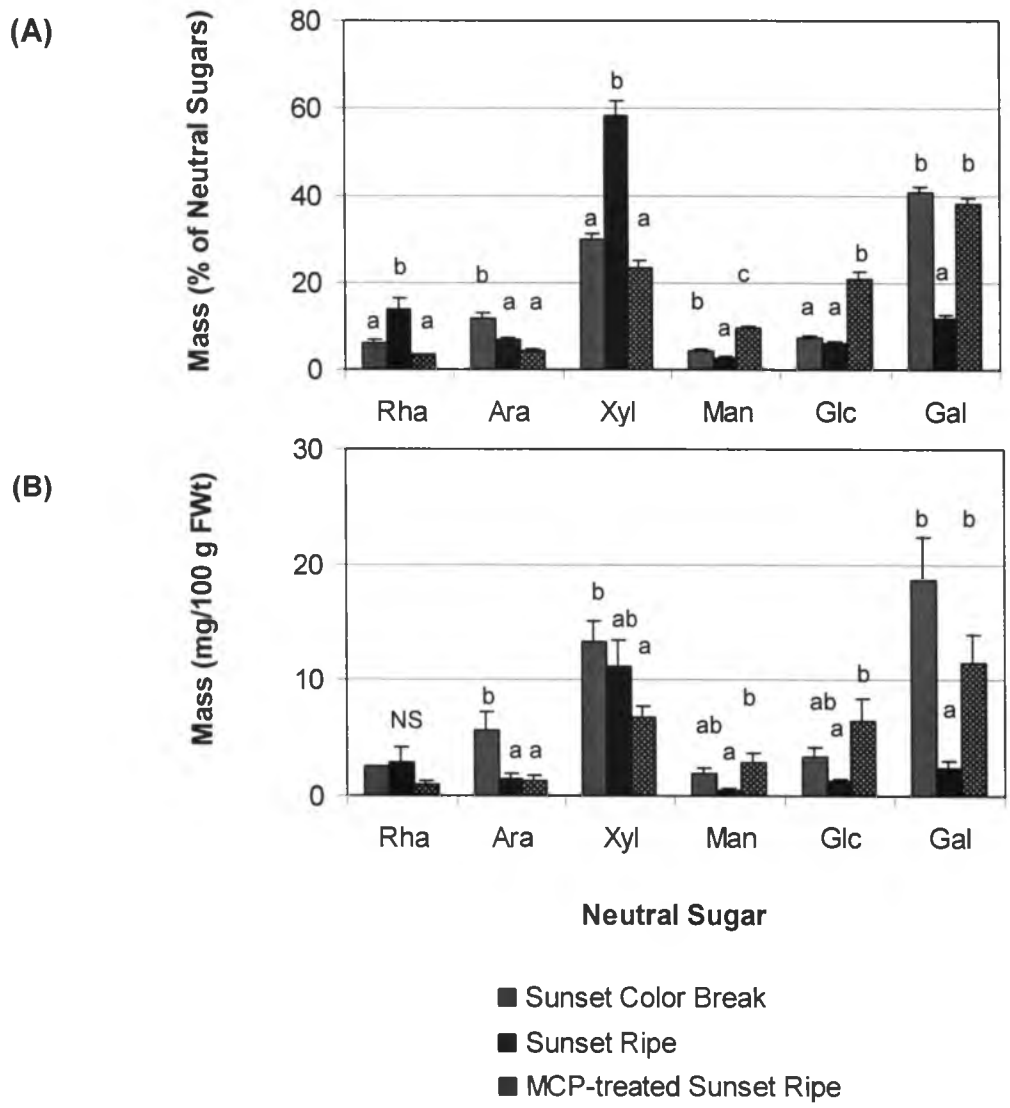


Figure 4.11 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in water-soluble cell wall polysaccharides of 'Sunset' fruit with and without 1-MCP treatment at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between stages and 1-MCP treatment.

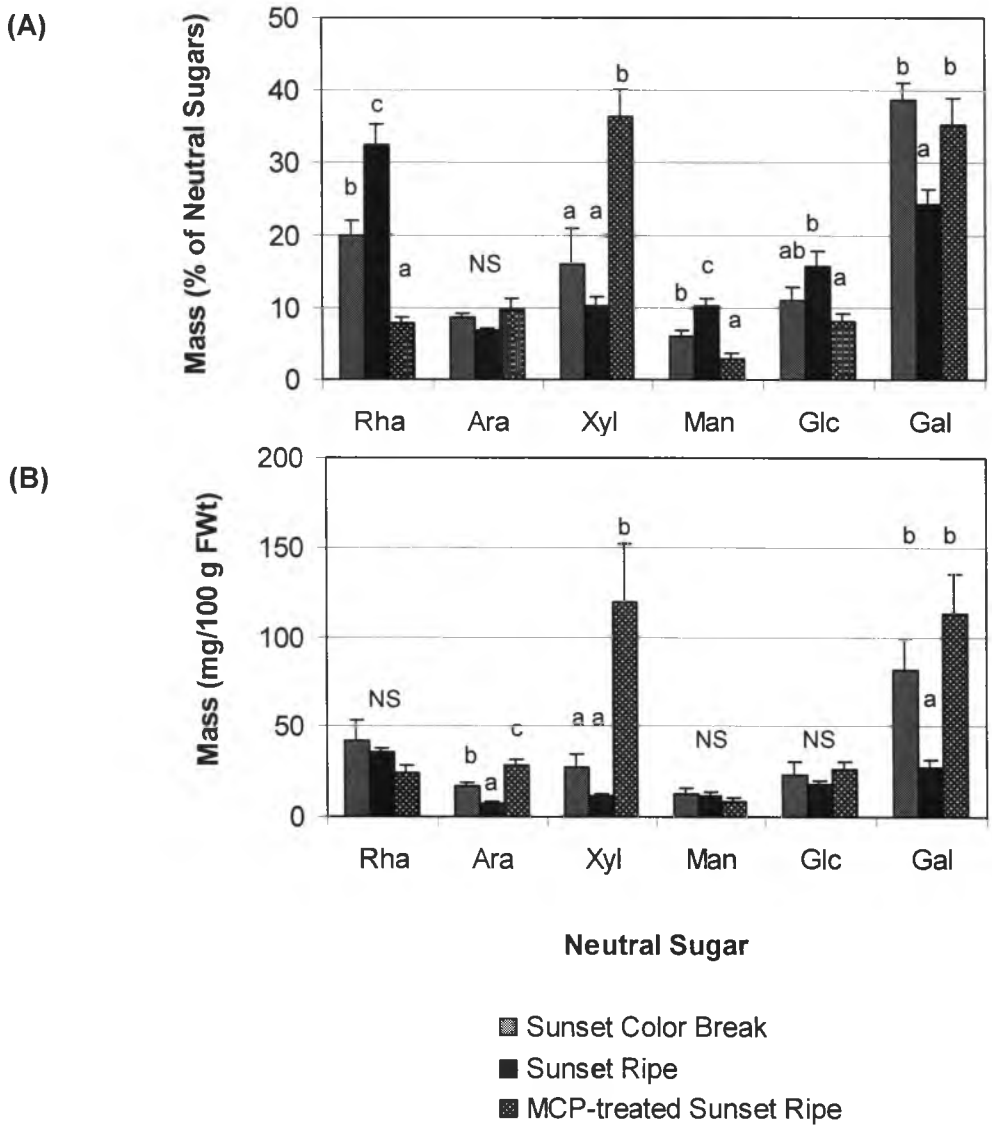


Figure 4.12 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the first CDTA-soluble cell wall polysaccharides of 'Sunset' fruit with and without 1-MCP treatment at the color break and full ripe stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between stages and 1-MCP treatment.

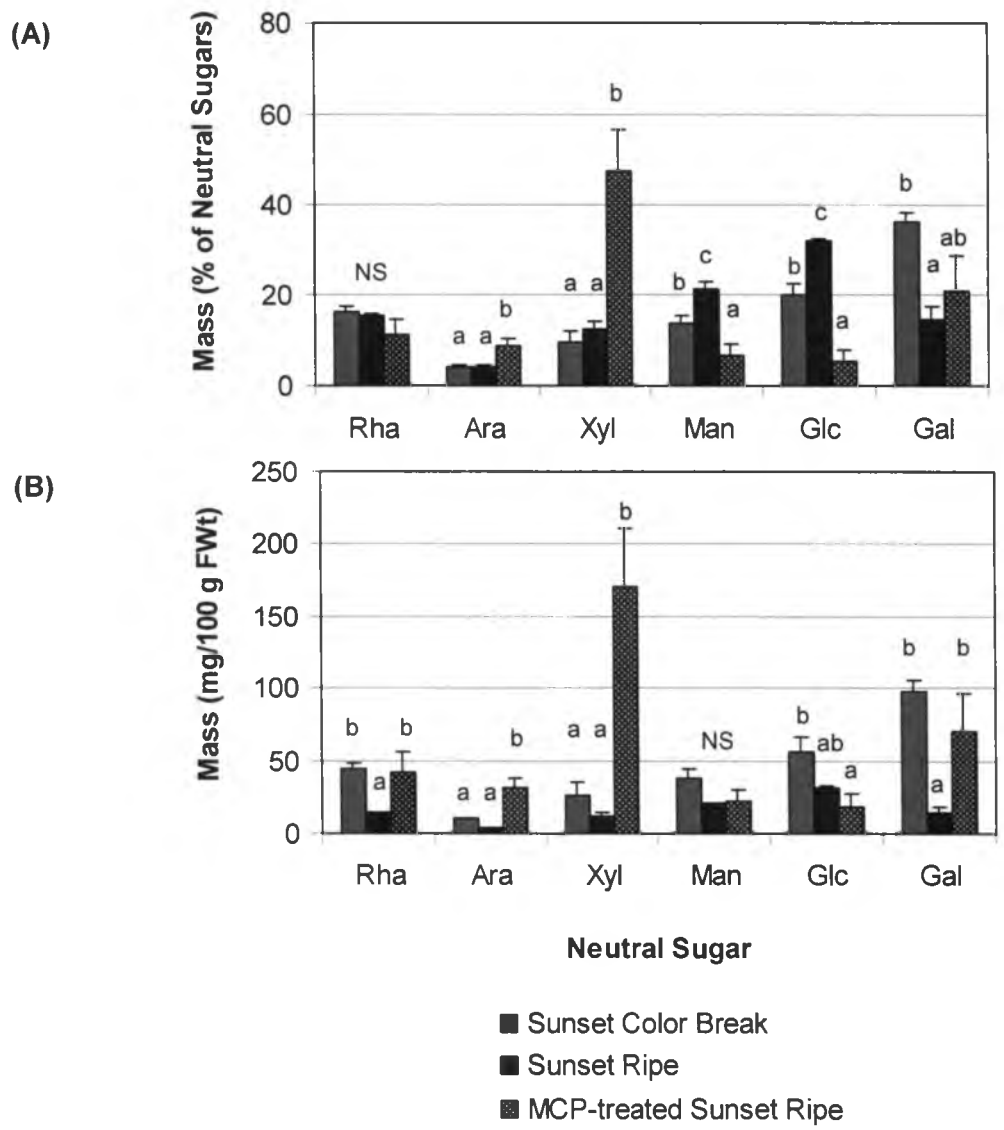


Figure 4.13 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the second CDTA-soluble cell wall polysaccharides of 'Sunset' fruit with and without 1-MCP treatment at the color break and full ripen stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between stages and 1-MCP treatment.

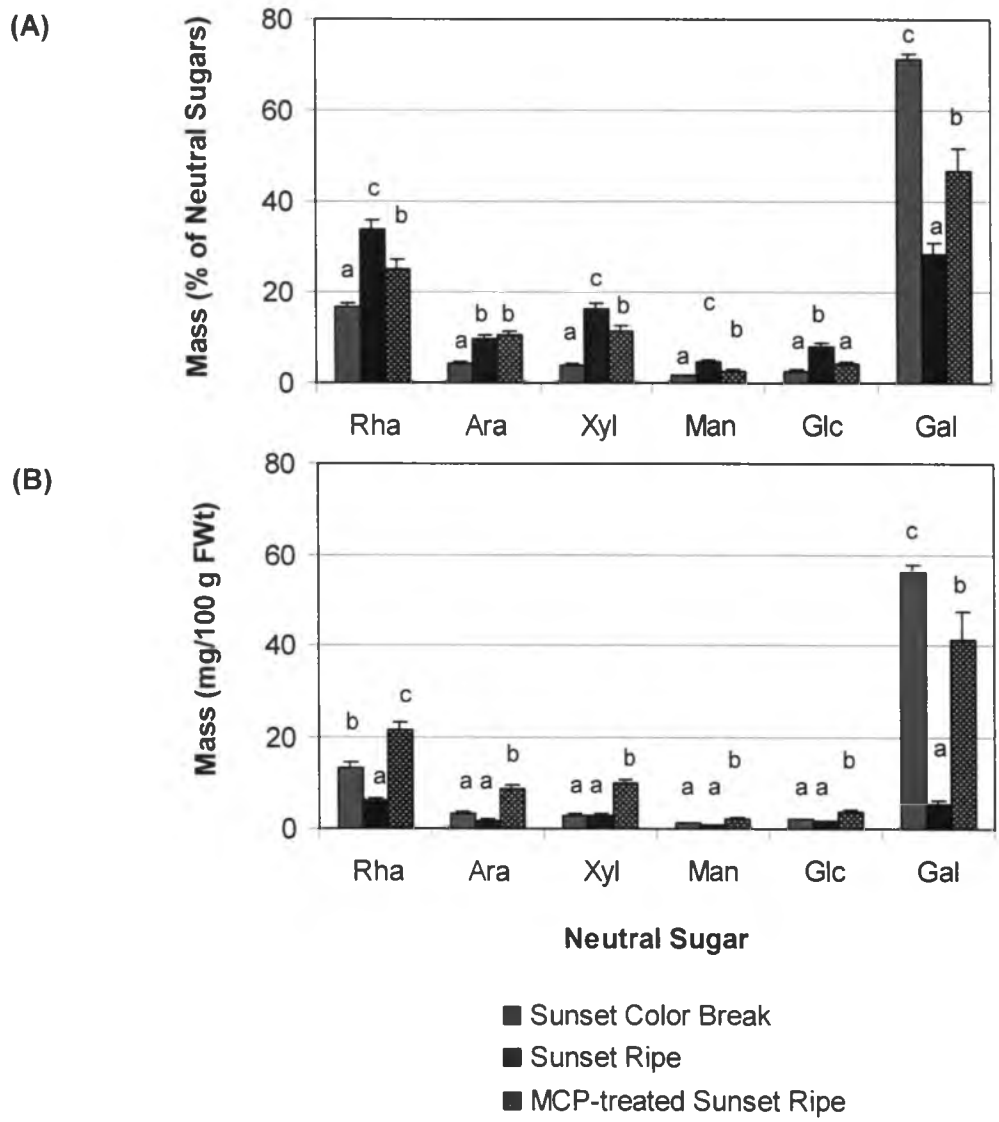


Figure 4.14 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in 1°C Na_2CO_3-soluble cell wall polysaccharides of 'Sunset' fruit with and without 1-MCP treatment at the color break and full ripen stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicates that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between stages and 1-MCP treatment.

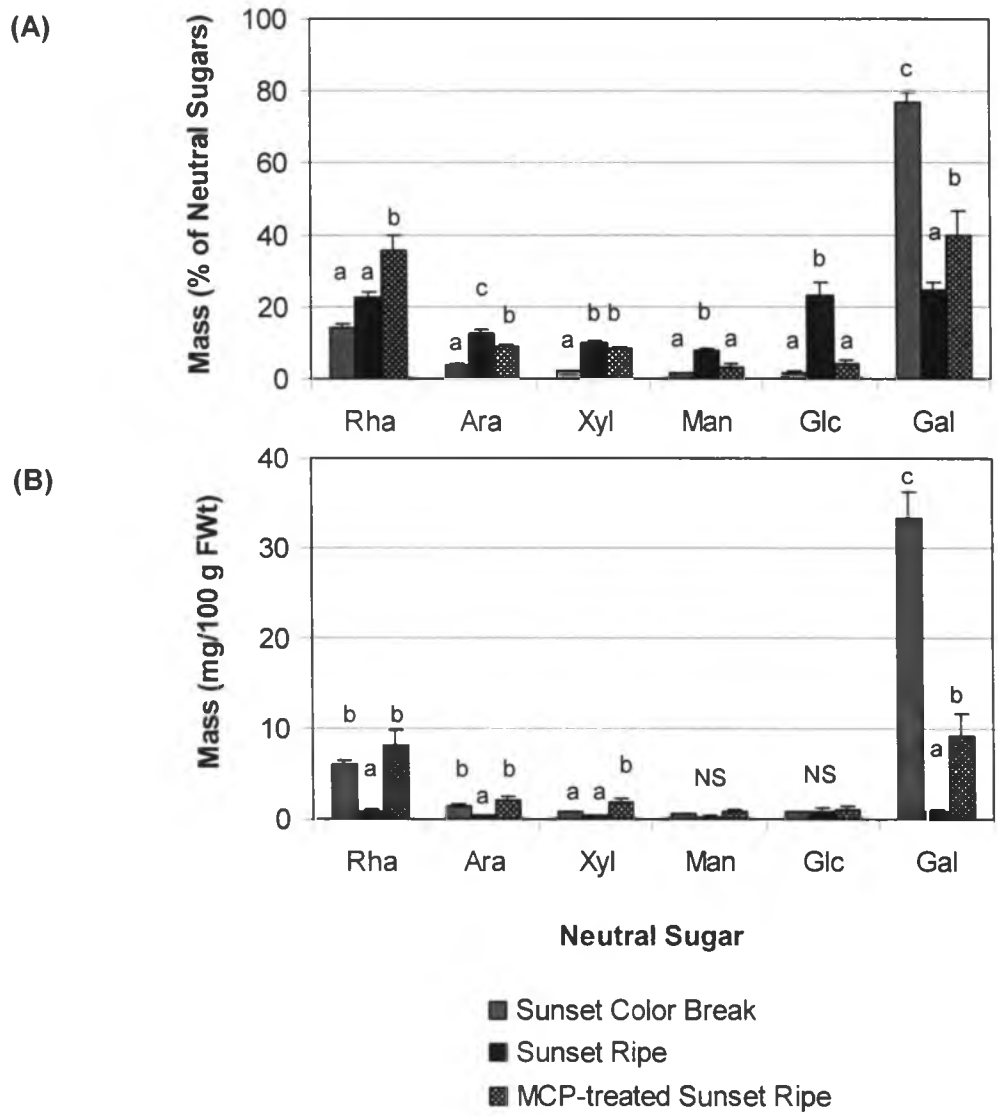


Figure 4.15 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in 22°C Na_2CO_3-soluble cell wall polysaccharides of 'Sunset' fruit with and without 1-MCP treatment at the color break and full ripen stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between stages and 1-MCP treatment.

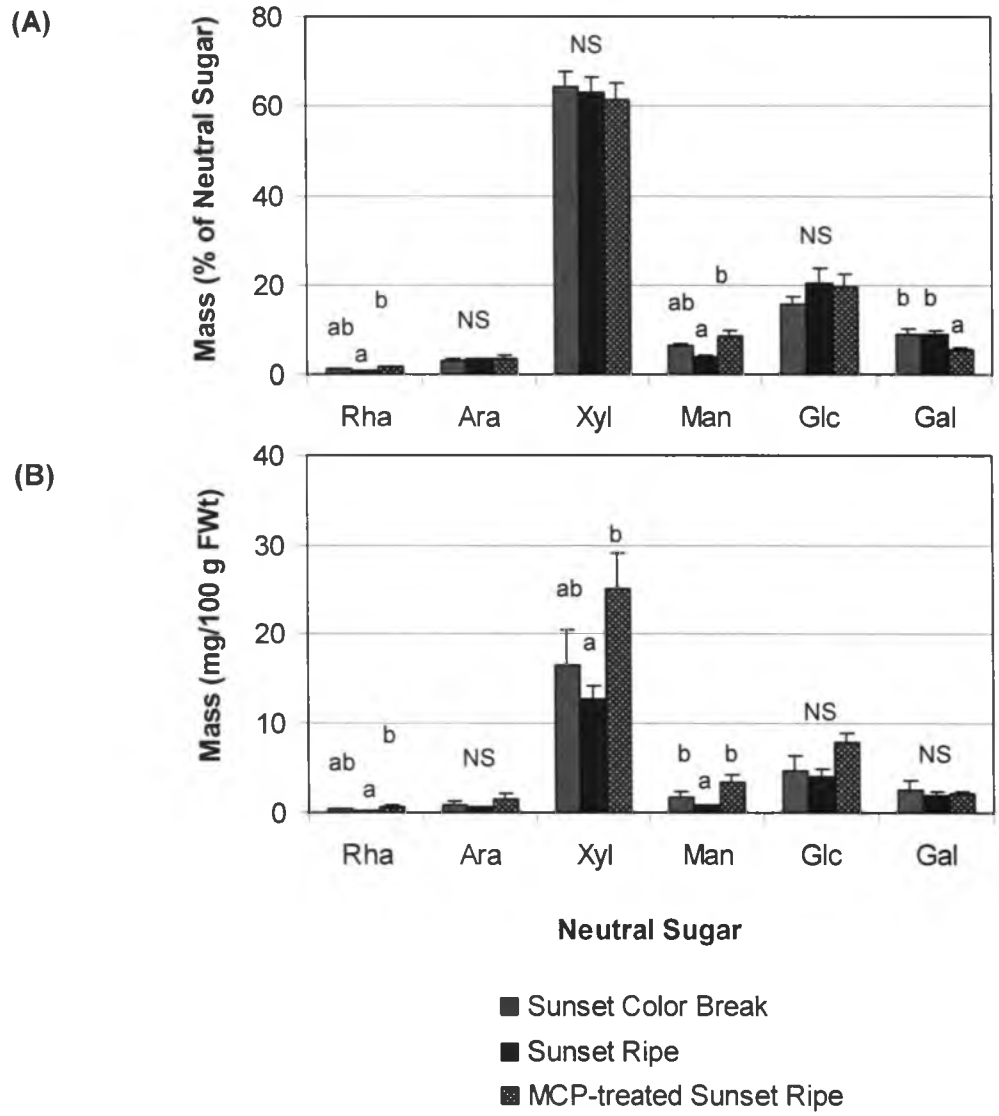


Figure 4.16 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the first 1 M KOH-soluble cell wall polysaccharides of 'Sunset' fruit with and without 1-MCP treatment at the color break and full ripen stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between stages and 1-MCP treatment.

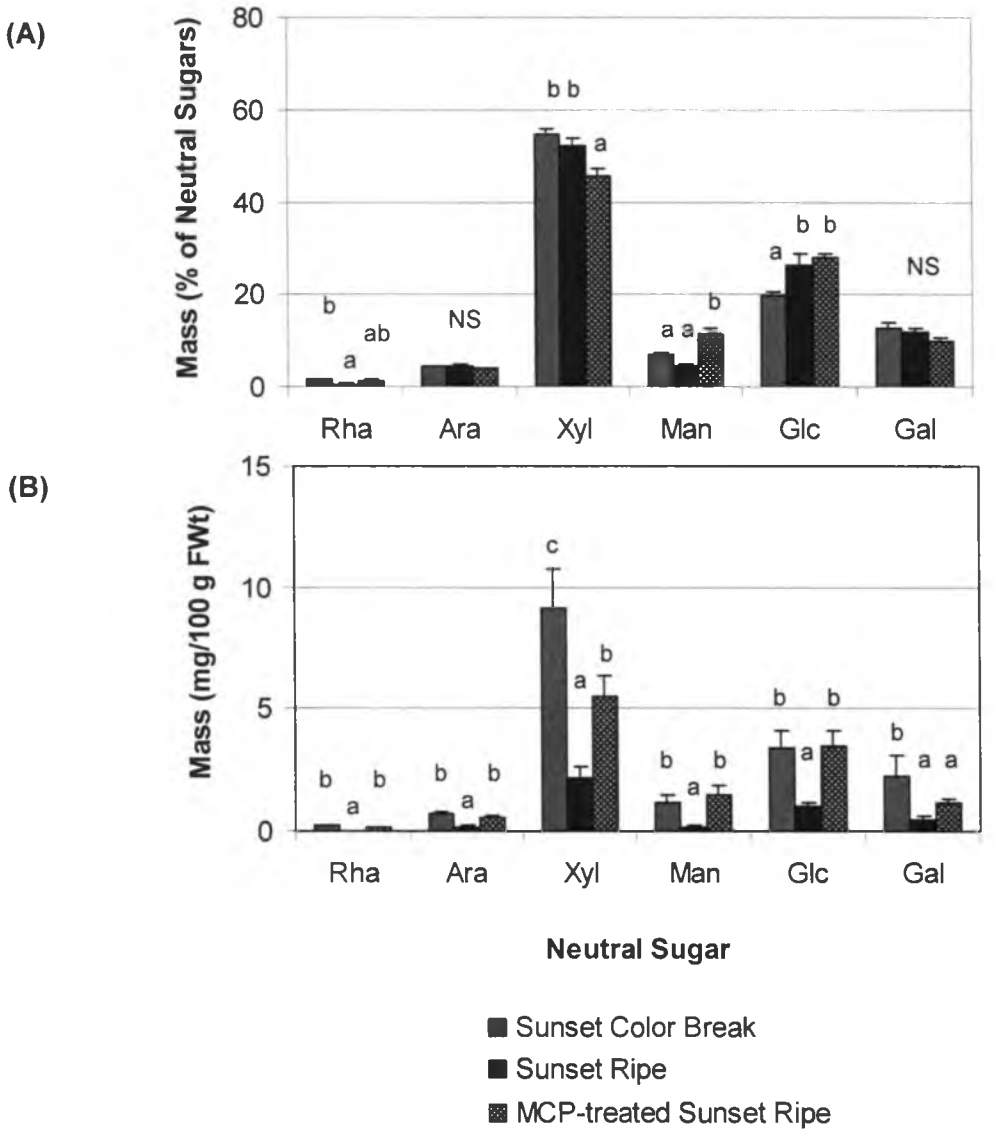


Figure 4.17 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the second 1 M KOH-soluble cell wall polysaccharides of 'Sunset' fruit with and without 1-MCP treatment at the color break and full ripen stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between stages and 1-MCP treatment.

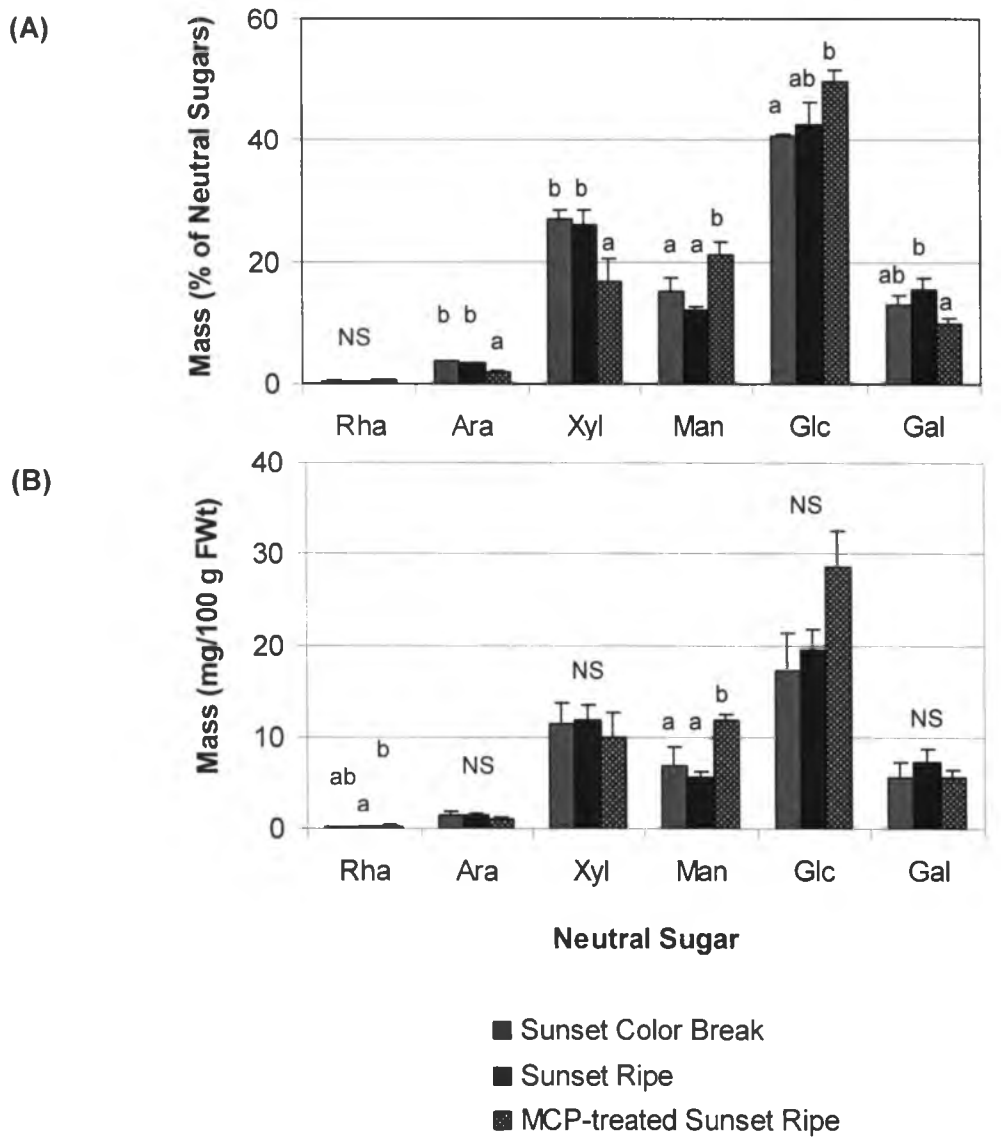


Figure 4.18 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the first 4 M KOH-soluble cell wall polysaccharides of 'Sunset' fruit with and without 1-MCP treatment at the color break and full ripen stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between stages and 1-MCP treatment.

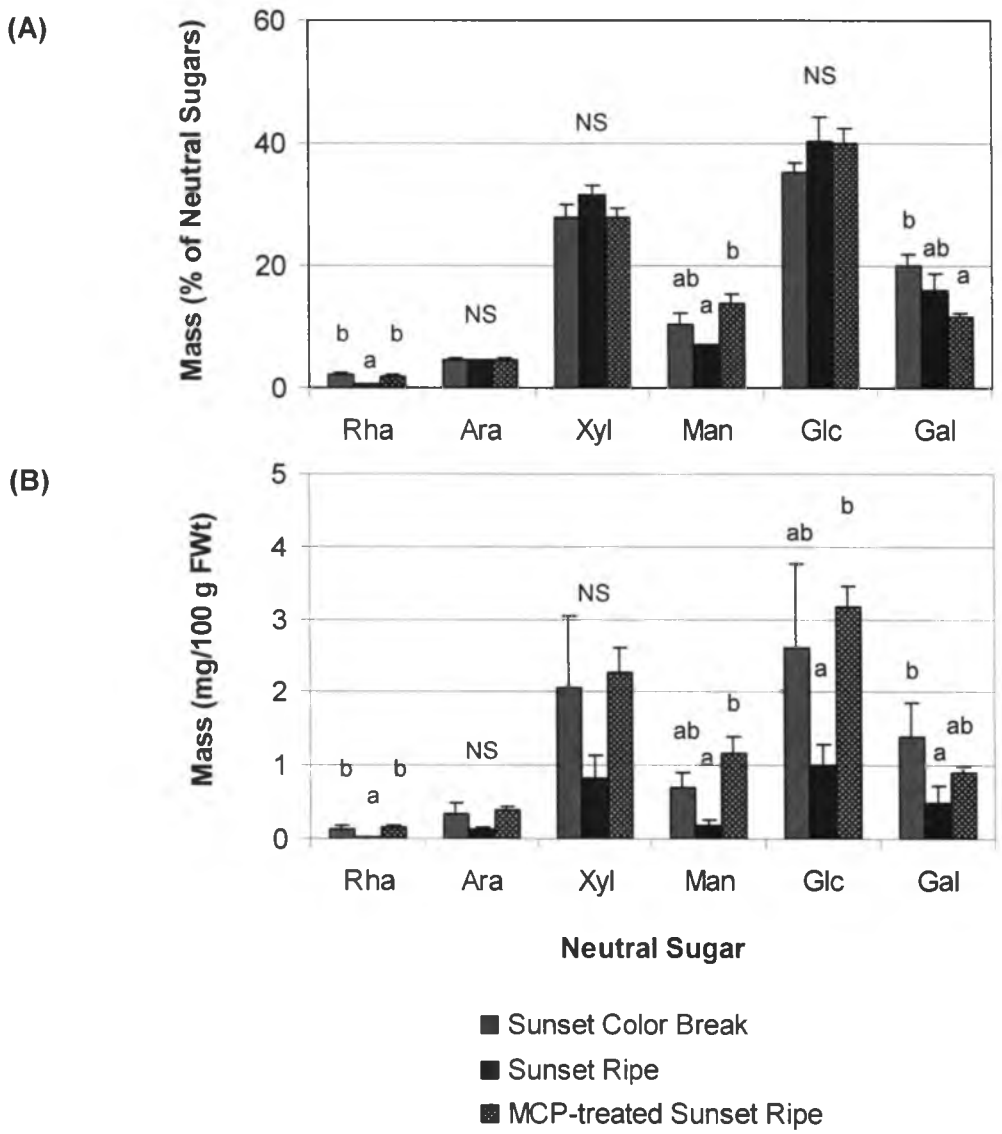


Figure 4.19 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in the second 4 M KOH-soluble cell wall polysaccharides of 'Sunset' fruit with and without 1-MCP treatment at the color break and full ripen stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between stages and 1-MCP treatment.

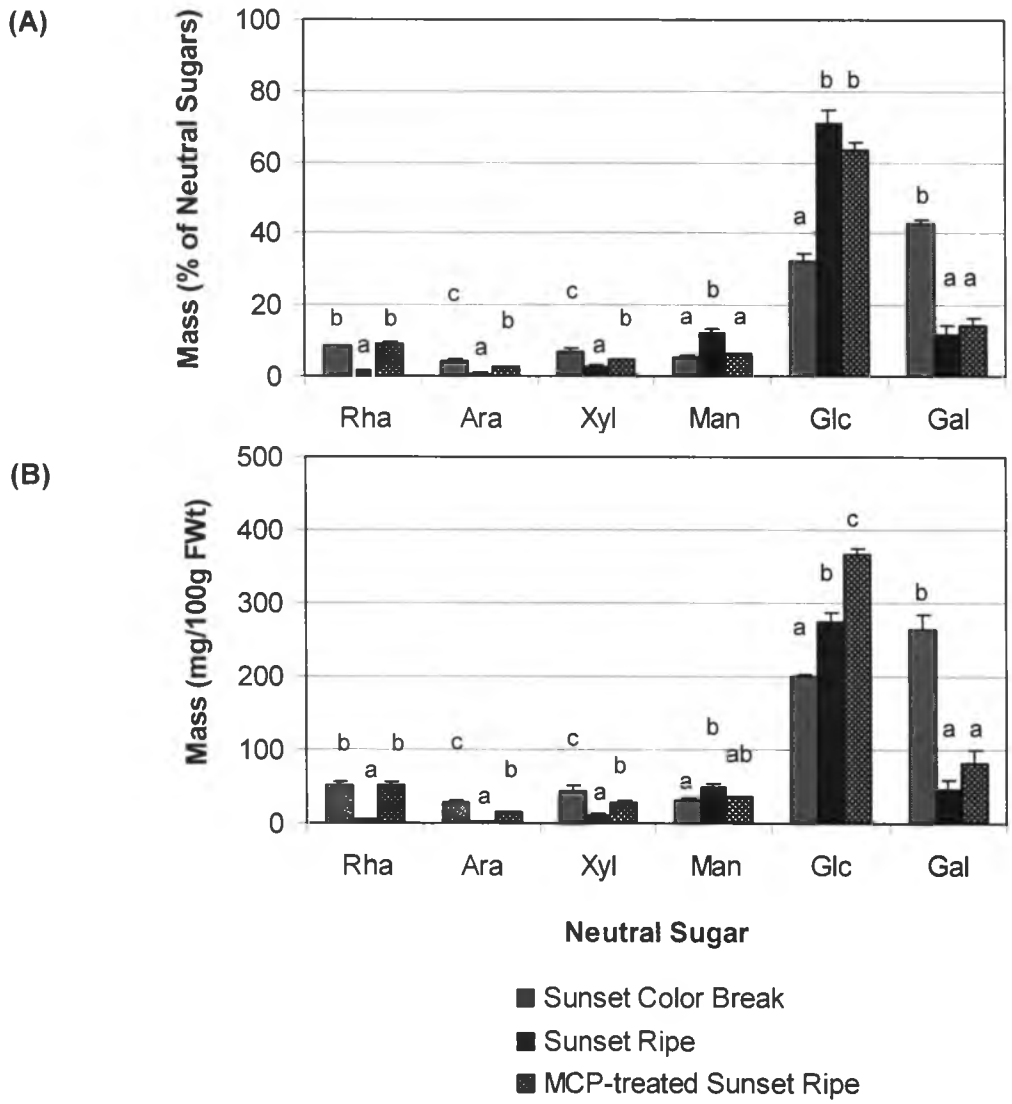


Figure 4.20 The proportion to the total neutral sugars (A) and content (B) of neutral sugars in cellulose fraction of 'Sunset' fruit with and without 1-MCP treatment at the color break and full ripen stages. Rha = rhamnose, Ara = arabinose, Xyl = xylose, Man = mannose, Glc = glucose, and Gal = galactose. Each bar represent mean + SE of 4 replicates. The same letter above bars of the same neutral sugar indicated that proportions or contents of the neutral sugar were not significantly different at 0.05 levels by LSD. NS indicated no significant difference between stages and 1-MCP treatment.

4.4 Discussion

4.4.1 Cell Wall Modification of 'Line 8' and 'Sunset' Papayas

No difference occurred between 'Line 8' and 'Sunset' fruit cell wall material mass during ripening. The mesocarp of both lines lost more than one-half of cell wall masses from the color break stage to full ripe. A significant mass reduction in the mesocarp cell wall occurred in the CDTA-soluble fraction, Na_2CO_3-soluble fractions and cellulose fraction.

Water-soluble polysaccharides are presumably not cell wall associated (Manrique and Lajolo, 2004). Water-soluble cell wall polysaccharides of 'Line 8' and 'Sunset' fruit mesocarp contained more than 40% uronic acids in mass. Xylose and galactose were the major neutral sugar components of these water-soluble polysaccharides at the color break stage. Polysaccharides that consist mainly uronic acids, xylosyl and galactosyl residues were dissociated from mesocarp cell wall by solubilization at the early ripening. At the late ripening stage, the water-soluble cell wall polysaccharides were rich in uronic acids and xylosyl residues.

At the color break stage, the CDTA_1-soluble, Na_2CO_3_1-soluble, and Na_2CO_3_rm-soluble fractions were rich in uronic acids (44-73% in mass) while the CDTA_2-soluble fraction had little uronic acids. CDTA removes Ca^{2+} from cell walls, leading to solulization of pectins held in the cell walls by ionic bonds (Coimbra *et al.*, 1996; Brummell and Harpster, 2001). Na_2CO_3 de-esterifies and releases pectins held in the cell walls by covalent bonds (Selvendran and O'Neill, 1987; Brummell and Harpster, 2001). The CDTA-soluble cell wall fraction is considered to consist mainly structural pectin-rich polysaccharides of middle lamella (Coimbra *et al.*, 1996); and the Na_2CO_3-

soluble cell wall fraction consists mainly pectin-rich polysaccharides of the primary cell wall (Selvendran, 1985; Selvendran and O'Neill, 1987). Pectic polysaccharides of the middle lamella had galactose and rhamnose as the major neutral sugar components in both papaya lines. Galactose was also the major neutral sugar component of Na_2CO_3-soluble cell wall polysaccharides but this fraction had a small proportion of rhamnose. Pectic polysaccharides of these fractions at color break stage possibly included homogalacturonans, and rhamnogalacturonan I with long galactan side chains. The CDTA-soluble fractions, pectins in the middle lamella, had high proportions of xylosyl and glucosyl residues indicating that these fractions possibly included some xyloglucans. The occurrence of covalent linkage between xyloglucan and acidic polysaccharides have been shown (Thompson and Fry, 2000; Popper and Fry, 2005).

The proportions and contents of uronic acids and galactosyl residues in the pectin-rich polysaccharides of the middle lamella and primary cell wall declined significantly during papaya fruit softening. Depolymerization of cell wall-intact pectins at middle lamella and primary cell wall of papaya during softening has been reported (Paull *et al.*, 1999; Manrique and Lajolo, 2004). Gel filtration chromatography profiles of neutral sugars and uronic acids of polysaccharides from these two studies indicated that depolymerization of middle lamella pectins was accompanied by a galacturonic acid release while depolymerization of primary cell wall pectins was probably due to neutral sugar loss (Manrique and Lajolo, 2004).

Loosely bound matrix polysaccharides were extracted by 1 M KOH solutions (Brummell and Harpster, 2001). Matrix polysaccharides contained about 60% xylosyl residues in mass of the total neutral sugars and about 20% glucosyl residues. In most xyloglucans of flowering plants, xylosyl residues are added when three contiguous

glucosyl residues occur in the backbone (Kato *et al.*, 1981; Carpita, 1996). The 3:1 ratio of xylosyl residues to glucosyl residues suggested that loosely bound matrix polysaccharides in cell wall of papaya mesocarp likely were of the xylan type than a xyloglucan. The xylan type of matrix polysaccharides in papaya mesocarp is similar to those of gramineous monocots rather than those of typical dicots (Carpita, 1984; Carpita, 1996). During papaya fruit softening, xylosyl contents in these matrix polysaccharides declined.

Matrix polysaccharides tightly bound to other cell wall polysaccharides were extracted by 4 M KOH solutions (Brummell and Harpster, 2001). The fractions contained mainly glucosyl and xylosyl residues in about the same proportion. A smaller proportion of galactose and mannose were found. The tightly bound matrix polysaccharides probably were of the xyloglucan type, or a mixture of xyloglucan and xylan type. In addition to xyloglucans and xylans, the fractions most likely contained glucomannans and this is consistent with the study of Manrique and Lajolo on 'Sunrise' fruit (Manrique and Lajolo, 2004). Gel filtration chromatography profiles (Manrique and Lajolo, 2004) indicated that there was no evidence of depolymerization of these matrix glycans during papaya fruit ripening. However, we found that the content of rhamnose branch-residues, in these polysaccharides in the second 4 M KOH extraction declined during ripening.

The cellulose fraction contained glucosyl and galactosyl residues and small amount of other non-glucosyl residues. It has been confirmed that cellulose microfibrils are bounded with other cell wall polysaccharides mainly galactans which agreed with the study on 'Sunrise' fruit (Manrique and Lajolo, 2004). However, all non-glucosyl sugars, excluding mannose, were disassociated from cellulose microfibrils during ripening of

both lines. Glucose increased in content and proportion in the cellulose fraction and suggested that cellulose biosynthesis continued during papaya fruit ripening.

4.4.2 Cell Wall Modification of 1-MCP-Treated 'Sunset' Fruit

While the mesocarp of non-treated fruit lost cell wall greater than 50% in mass during ripening, the mesocarp of ripe 1-MCP-treated fruit showing a 'rubbery' texture maintained cell wall mass as at the color break stage. The fruit did lose some mass from Na_2CO_3-soluble polysaccharides, mainly from the primary cell wall during ripening. However, these losses were much less than losses of polysaccharides in the ripe control papaya. The mesocarp of ripe 1-MCP-treated papaya also showed a significant increase in mass of CDTA_2-soluble polysaccharides and 1 M KOH_1-soluble matrix polysaccharides. The losses of Na_2CO_3_soluble fraction mass in ripe 1-MCP-treated fruit coincided with the losses of the uronic acid contents in these fractions. However, the increased mass of CDTA_2-soluble polysaccharides in ripe 1-MCP-treated fruit was not due to an increase of uronic acid content. The increased mass of the CDTA-soluble cell wall polysaccharides in the ripe 1-MCP-treated fruit coincided with the higher association of xylosyl residues to these pectic polysaccharides.

Water–soluble polysaccharides of the non-treated fruit mesocarp contained mainly galactosyl and xylosyl neutral sugars. The mesocarp of ripe 1-MCP-treated fruit still declined to a similar polysaccharides mass compared to the ripe control fruit. Declined contents of uronic acids were similar between the ripe 1-MCP-treated fruit and ripe untreated fruit. However, the mesocarp cell wall of ripe 1-MCP-treated fruit dissociated a lower content of xylosyl residues than mesocarp of untreated fruit. This

change may explain why the ripe 1-MCP-treated fruit had a significantly higher proportion of xylosyl residues in the middle lamella polysaccharides than the control fruit.

During ripening, the mesocarp of control fruit lost neutral sugars from the Na_2CO_3-soluble cell wall polysaccharides with the greatest decline in the galactose. While 1-MCP-treated fruit also significantly lost galactose, though an increase in the content of the minor neutral sugar components including rhamnose, arabinose, xylose, and mannose occurred.

The mesocarp of ripe 1-MCP-treated fruit maintained the contents of neutral sugars in the loosely bound matrix polysaccharides fraction as the fruit ripened. The proportion of xylose, the major neutral sugar component, increased. The mesocarp of 1-MCP-treated fruit did not lose neutral sugar components from the tightly bound matrix polysaccharides (4 M KOH) fractions during ripening, whereas the control fruit did. The content of glucose, the major neutral sugar component, in these cell wall polysaccharides of ripe 1-MCP-treated fruit was higher than that of ripe control papaya.

The 1-MCP-treated fruit still lost galactosyl residues from the cellulose fraction as occurred in the control fruit, but the minor neutral sugar components were maintained during ripening in 1-MCP-treated fruit while in untreated fruit they declined.

Beside uronic acids, galactosyl and xylosyl components of the mesocarp cell wall were modified during ripening and softening, more so, than other monosaccharides. However, the modification of cell wall components in 1-MCP-treated fruit showed that although the major changes in galactosyl component of mesocarp cell wall still occurred as in control fruit, the 1-MCP-treated fruit did not soften completely. Cell wall modification of 1-MCP-trearted fruit still suggested that a modification of the xylosyl

component of cell wall may play an important role during papaya fruit softening. The modification of xylosyl residues involved in papaya softening included solubilization of xylosyl residues, and disassociation of xylosyl residues from the pectic polysaccharides of the middle lamella and from loosely bound matrix polysaccharides.

CHAPTER 5

CHANGES OF HYDROLASES' ACTIVITIES DURING PAPAYA SOFTENING

5.1 Introduction

Fruit softening is one of the major factors that determine fruit quality, shelf life, and handling (Brummell and Harpster, 2001). Fruit softening is thought to be the result of cell wall degradation (Huber, 1983). Some hydrolases that degrade cell wall polymers increase in activity during ripening and the increases is coincident with fruit softening.

Endopolygalacturonase (PG) which cleaves unesterified α-(1→4)-galacturonan linkages of pectates and other galacturonans, has been related to tomato and avocado softening (Crookes and Grierson, 1983; Huber and O'Donoghue, 1993). β-galactosidase removes terminal non-reducing β-D-galactosyl residues of side chain galactans, and in tomato (Pressey, 1983; Carey *et al.*, 1995) and papaya (Lazan *et al.*, 1995) softening has been reported to be closely related to an isoform of β-galactosidase. Cellulase or endoglucanase, catalyse the endohydrolysis of β-(1→4)-glucosidic linkages of glucans, and an increase in endoglucanase activity is highly correlated with increased respiration and softening in avocado (Awad and Young, 1979). Endoxylanase catalyzes endohydrolysis of β-(1→4)-xylosidic linkages of xylans and a close relationship exists between endoxylanase activity and the rise in respiration, ethylene evolution and softening of papaya (Paull and Chen, 1983; Chen and Pauli, 2003). β-xylosidase removes terminal non-reducing D-xylose residues of β-(1→4)-xylans; and in avocado, xylosidase activity reaches a peak at the same time as the climax of ethylene evolution and the highest rate of softening (Ronen *et al.*, 1991).

Although, hydrolases activities increase synchronously with natural softening of fruit, the particular hydrolase may not have a major influence on softening. For example, polygalacturonase had been believed for a long time to be a major hydrolase involved in tomato softening. However, transgenic tomatoes with suppressed expression of polygalacturonase activity showed little effect on softening (Smith *et al.*, 1988; Giovannoni *et al.*, 1989). Transgenic fruit repressed or over-expressed activities of particular hydrolases give a clear picture of those hydrolases' roles on the wall modification and softening of the fruit (Sheehy *et al.*, 1988; Smith *et al.*, 1988; Giovannoni *et al.*, 1989; Tieman *et al.*, 1992; Brummell *et al.*, 1999). However these techniques are time consuming, especially for perennial fruit trees such as papaya.

The inhibitor, 1-methylcyclopropene (1-MCP) blocks ethylene receptor and control ethylene responses (Sisler and Serek, 1997) including fruit ripening. Papaya softening and texture are altered by 1-MCP treatment when the fruit is less than 25% ripe (Manenoi *et al.*, 2007). The alteration of softening pattern and texture by 1-MCP provides a valuable tool to determine the role of hydrolases on papaya softening.

The objective of this study was to determine the relationship between papaya softening and activities of fruit hydrolases, including endopolygalacturonase (PG), pectin methylesterase (PME), β-galactosidase, endoglucanase, endoxylanase, and β-xylosidase, during ripening. Fruit hydrolase' activities were compared during normal softening and when softening was modified by 1-MCP treatment to investigate the relationship to papaya softening.

5.2 Materials and Methods

5.2.1 Plant Materials

'Sunset' fruit were harvested at color break stage (less than 10% skin yellow) on several occasions from the Poamoho Experimental Station in Central Oahu, Hawaii. Fruit were divided into two subsets. One subset was fumigated with 100 nL L^{-1} of 1-methylcyclopropene (1-MCP) for 12 hours at room temperature (approximately 22°C). Another subset was not treated with 1-MCP. All fruit were allowed to ripen at the room temperature. The fruit were sampled when they had reached a specific ripening stages: 10% skin yellow (color break), 25% skin yellow, 50% skin yellow, 75% skin yellow, 100% skin yellow (full ripe), full ripe plus 3 days, and full ripe plus 6 days. Fruit firmness was measured using a force gauge (AccuForce®, Model Cadet) with a 1.6 cm diameter disc and pressed 1 mm into the fruit. Peak forces (Kg) at two opposite equatorial area of each fruit were read and averaged. Mesocarp tissue around fruit equator was sliced and immediately frozen in liquid nitrogen. The frozen tissue was then stored at -80°C until use.

5.2.2 Crude Enzyme Extraction and Hydrolase Assay

Four replicates (fruit) at each stage and in each of the two treatments were extracted to determine hydrolase activity. Duplicate or triplicate assays were conducted on each sample.

Four grams of the frozen mesocarp was homogenized in 8 mL of chilled either 0.5 M sodium chloride + 1 mM dithiopyridine + 1 mM phenylmethylsulphonyl fluoride (PMSF), or 40 mM sodium acetate and 40 mM sodium phosphate buffer (pH 4.6) +

1 mM dithiopyridine + 1 mM PMSF. The homogenate was allowed to stand at ice temperature for 15 minutes and the debris was centrifuged down at 5,000 g 4°C for 10 minutes. The supernatant was then filtered through Miracloth®. The filtrates were considered as crude enzymes. The crude enzyme extracted with sodium chloride solution was used for β-galactosidase and pectin methylesterase assays. The crude enzyme extracted in buffer was used for endopolygalacturonase, endoglucanase, endoxylanase, and β-xylosidase assays. Ten minutes-boiled crude enzyme extract was used in each assay as the blank. Hydrolase activity was reported as the difference between un-boiled and boiled blank.

Endopolygalacturonase Assay

Endopolygalacturonase assay was modified from the method of Boudart *et al.* (2003). Two hundred (200) µL of 50 mM sodium acetate buffer pH 5.2 was mixed with 275 µL of freshly prepared 0.2% (w/v) polygalacturonic acid from orange (Sigma®) in 50 mM sodium acetate buffer pH 5.2. Twenty five (25) µL of crude enzyme extract was added, mixed well, and incubated at 30°C for 30 minutes. The reaction was stopped by adding 1 mL of chilled absolute ethanol and the reaction vial was centrifuged at 10,000 rpm for 10 minutes to precipitate the substrate. The supernatant which contain galacturonic acids product of the reaction was collected for the colorimetric assay of hexouronic acid (Blumemkrantz and Asboe-Hansen, 1973).

Duplicate colorimetric assay of hexouronic acid (Blumemkrantz and Asboe-Hansen, 1973) was conducted on each supernatant. Two hundred (200) µL of the supernatant was diluted with 200 µL of distilled water, and 2.4 mL of 0.0125 M sodium tetraborate in concentrate sulfuric acid added, and the mixture cooled in crushed ice.

The cooled mixture was mixed vigorously and boiled in a water bath for 5 minutes. The mixture was cooled again in a water-ice bath and 40 µL of 0.15% (w/v) *m*-hydroxydiphenyl in 0.5% (w/v) sodium hydroxide was added. The mixture was mixed vigorously and allowed to stand for 30 minutes to allow air bubbles in the mixture to escape, and absorbance at 520 nm recorded.

Pectin Methylesterase Assay

Pectin methylesterase activity was determined by monitoring the color change of bromothymol blue pH indicator (Hagerman and Austin, 1986; Pilatzke-Wunderilich and Nessler, 2001). Eight hundred (800) µL of crude enzyme extract was diluted in 4 mL of chilled distilled water. The diluted crude enzyme was adjusted to pH 7.5 just before the assay. The substrate as 0.2% (w/v) citrus pectin (Sigma®) + 0.002% (w/v) bromothymol blue + 100 mM sodium chloride was fleshly prepared and adjusted to pH 7.5 just before each assay. The absorbance of the substrate at 616 nm was approximately 0.8-0.9 for the highest sensitivity. One hundred (100) µL of diluted crude enzyme was added to 900 µL of the substrate solution and quickly mixed by inversion one time. The mixture was immediately placed in a spectrophotometer with 25°C temperature setting. Change in absorbance at the 616 nm of the mixture was observed for 3 minutes. The pectin methylesterase activity was reported as the difference in absorbance between at the first and second minute of the reaction ($dAbs_{616}$/min).

β-galactosidase Assay

The modified β-galactosidase assay of Stragene® (www.stratagene.com/manuals/200383.pdf) was used. A mixture of 50 mM β-mercaptoethanol 45 µL and 100 mM NaH_2PO_4+10 mM KCl + 1 mM $MgSO_4$ buffer (pH 7.5) 855 µL was prepared fresh before

each assay. One hundred (100) μL of crude enzyme was added to the mixture and the mixture was incubated in a water bath at 37°C for 5 minutes, 200 μL of pre-warmed (37°C) substrate solution (4 mg/mL *p*-nitrophenyl-β-D-galactopyranoside in the previous buffer) was then added. The assay mixture was mixed thoroughly and incubated in the water bath at 37°C for 30 minutes. The reaction was stopped by the addition of 500 μL of 1 M Na_2CO_3 solution. The absorbance of the mixture at 415 nm was recorded.

Endoglucanase Assay

Two hundred (200) μL of crude enzyme extract was thoroughly mixed with 800 μL of 1% (w/v) carboxymethylcellulose (CMC) in 50 mM sodium acetate buffer (pH 5.2). The mixture was incubated in a water bath at 30°C for 1 hour. The viscosity of the mixture was immediately determined by recording time used for the mixture to run out of a 0.1 mL glass pipette from the 0.00 mL mark to 0.05 mL mark. The time after 1 hour incubation was the zero time. The mixture then was returned to the water bath and incubated for a further 23 hours. The mixture was shaken vigorously for 3 seconds and viscosity immediately determined. The activity of endoglucanase was reported as % loss of viscosity per day by the following equation.

$$\% \text{ loss of viscosity/day} = \frac{(\text{Run time at the } 1^{st} \text{ hr})-(\text{Run time at the } 24^{th} \text{ hr})}{(\text{Run time at the } 1^{st} \text{ hr})} \times \frac{24 \text{ hr}}{23 \text{ hr}}$$

Endoxylanase Assay

One hundred (100) μL of crude enzyme extract was mixed with 400 μL of 0.1% RBB (Remazol Brilliant Blue)-xylan + 0.01% timersol in 50 mM morpholinoethanesulfonic acid (MES) pH 6.0. The mixture was incubated at 30°C for 19 hours. The reaction was terminated by adding 1 mL of chilled absolute ethanol. Without mixing, the pellet was

centrifuged down at 10,000 rpm for 10 minutes. The supernatant was taken and the absorbance at 590 nm determined.

β-xylosidase Assay

β-xylosidase assay was determined by a modification of the assays of Ronen *et al.* (1991) and Itai *et al.* (2003). Two hundred and fifty (250) μL of 10 mM *p*-nitrophenyl β-D-xylopyranoside in 50 mM sodium acetate buffer (pH 5.2) was added to 125 μL of the same buffer and 125 μL of crude enzyme. The reaction mixture was mixed well and incubated in a water bath at 37°C for 4 hours. The reaction was terminated by addition of 500 μL of 1 M Na_2CO_3 solution. The mixture was mixed well and the absorbance at 405 nm determined.

Total Protein Assay

Total protein in the crude enzyme extracts was determined using the BioRad® protein assay based on Bradford's method (Bradford, 1976). Fifty (50) μL of the crude enzyme extracts or 200 μL of the diluted crude enzyme were brought to 800 μL with distilled water, and then mixed with 200 μL of concentrate BioRad® reagent. The mixtures were incubated at the room temperature for 5 minutes. Absorbance at 595 nm was recorded with BSA as the standard.

5.3 Results

5.3.1 Ripening and Softening of Papaya Delayed by 1-MCP Treatment

Fruit treated with 1-MCP had a relative constant low rate of ripening compared to the control untreated fruit (Figure 5.1). Control fruit had similar rate of skin color development to the 1-MCP-treated fruit until the 50% skin yellow stage, and had a faster rate after this stage compared to the 1-MCP-treated fruit. The control fruit reached the full ripe stage (100% skin yellow) about 4 days earlier than the 1-MCP-treated fruit.

The 1-MCP treatment changed the pattern of papaya softening (Figure 5.2) and the fruit had an incomplete softening and a 'rubbery' texture when ripe. The control fruit decreased in firmness at a higher rate after 25% through 75% skin yellow stages (4 to 7 days after harvest). Firmness of the control fruit at the 50% skin yellow stage was two tenth of that at the 25% skin yellow stage (5 versus 24 Kg). 1-MCP-treated fruit showed a slow decline in firmness throughout ripening. Firmness of 1-MCP-treated fruit at the full ripe stage (13 days after harvest) was more than 7 times higher (14 versus 2 Kg) than the control at the same stage (8 days after harvest). Even 6 days after the full ripe stage, the 1-MCP-treated fruit were still 4 times higher in fruit firmness than the control at the full ripe stage.

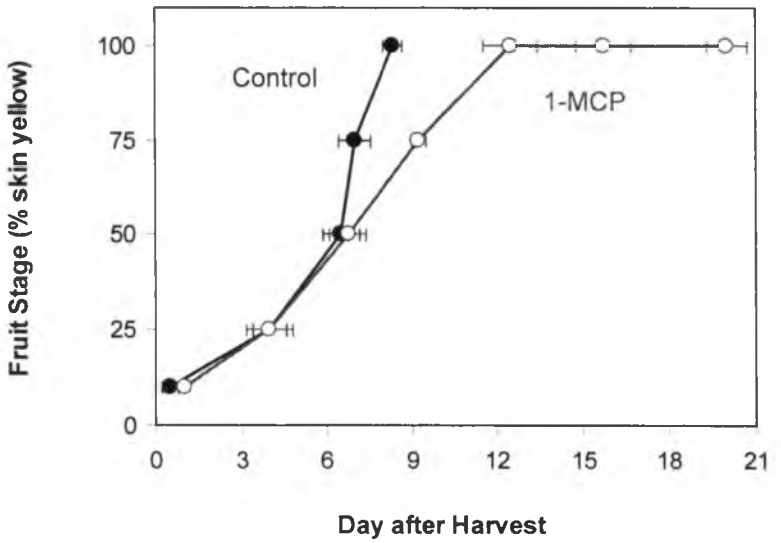


Figure 5.1 Ripening rate of 'Sunset' fruit with and without 1-MCP treatment. Markers were mean ± SE of 4 replicates (fruit).

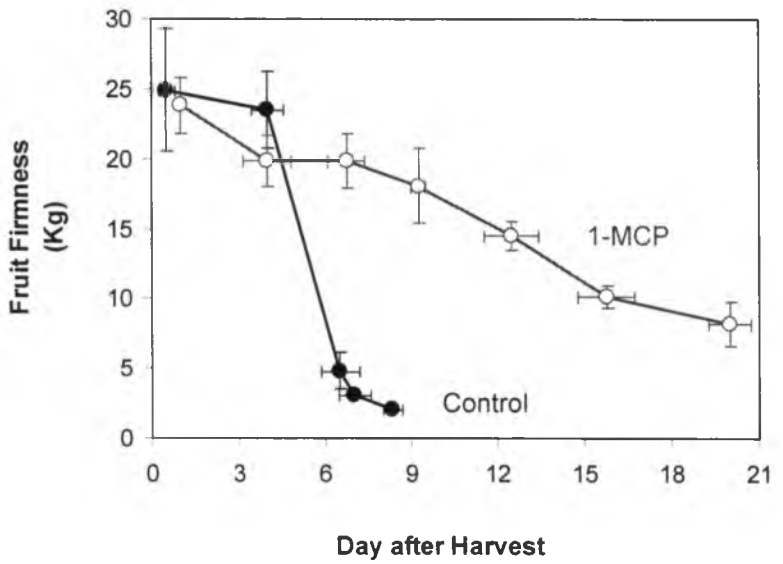


Figure 5.2 Fruit firmness during ripening of 'Sunset' fruit with and without 1-MCP treatment. Markers on each line from left to right were mean ±SE of firmness and ±SE of day after harvest of 4 fruit at 10%, 25%, 50%, 75%, 100%, 100%+3days, and 100%+6days stages, respectively.

5.3.2 1-MCP Treatment Altered Amounts of Proteins in Papaya Crude Enzyme Extracts

The change in the amount of proteins in the crude enzyme extracts which include cell wall hydrolases depended upon the extractant used (Figure 5.3). A higher amount of proteins was extracted by 0.5 M sodium chloride solution than by 40 mM sodium acetate plus 40 mM sodium phosphate buffer (pH 4.6) from color break (10% skin yellow) stage. The amount of proteins extracted by the sodium chloride solution tended to remain unchanged during ripening while the amount of proteins extracted by the buffer increased after the 25% skin yellow stage. At the full ripe stage, the amount of proteins extracted by the buffer was the same as the amount of proteins extracted by the sodium chloride solution (Figure 5.3 C).

Treatment with 1-MCP caused both an increase and decrease in the amount of protein in mesocarp depending on extractant used. MCP-treated fruit tended to have a higher amount of proteins extracted by the sodium chloride solution than the control fruit but a lower amount when extracted by the buffer after the 25% skin yellow stage (Figure 5.3 A and B). As the protein amount in mesocarp changed during ripening and in fruit modified by 1-MCP treatment, the cell wall hydrolases' activities were compared on a mesocarp fresh weight basis. Comparing activities on a protein amount basis in the crude enzyme extracts when no growth, division, or enlargement has occurred could lead to errors in conclusions.

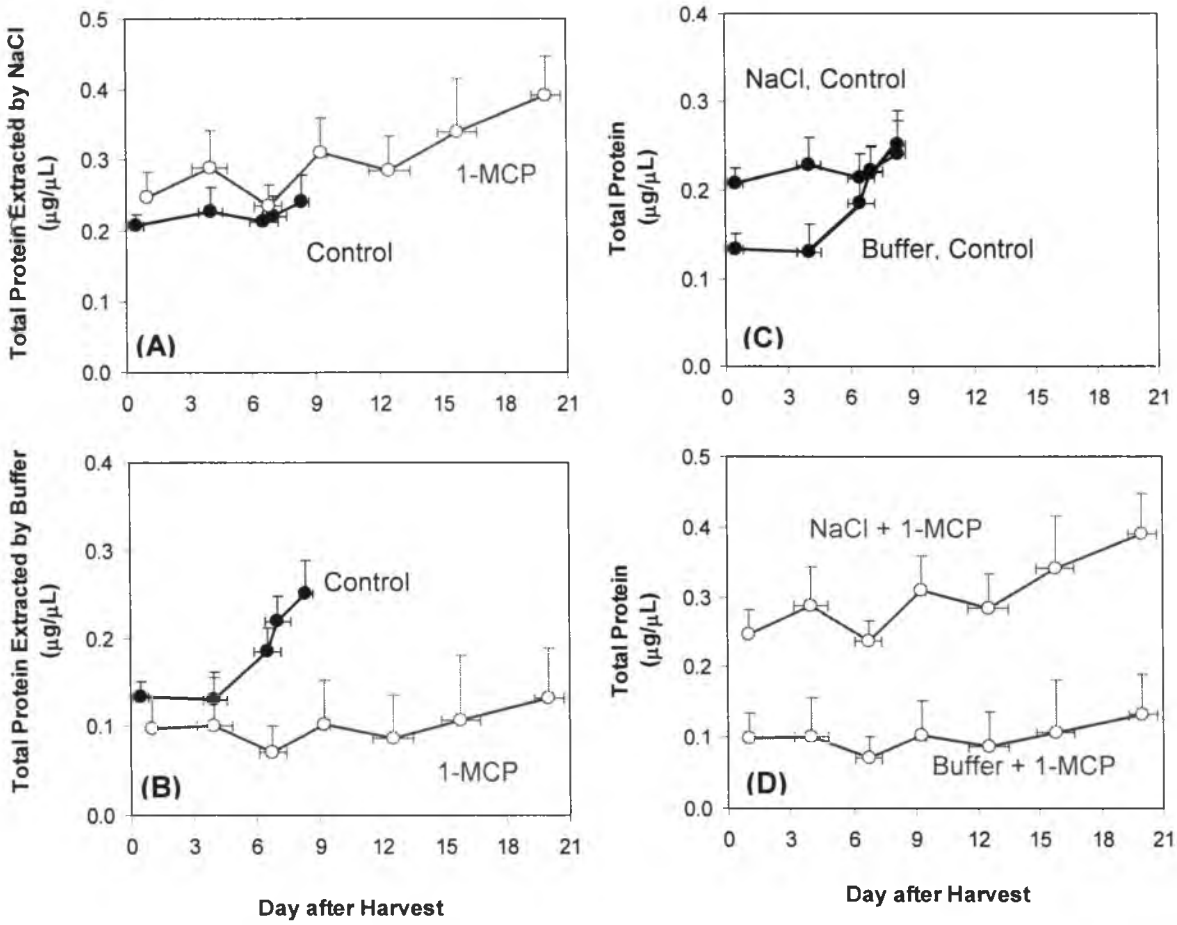


Figure 5.3 Total protein in crude enzyme extracts of 'Sunset' fruit with and without 1-MCP treatment. Crude proteins were extracted from 4 g of mesocarp in 8 mL of 0.5 M NaCl solution (A), or 40 mM sodium acetate, 40 mM sodium phosphate buffer pH 4.6 (B), and comparison of NaCl and buffer extracted protein of untreated fruit (C) and of 1-MCP-treated fruit (D). Markers on each line, from left to right, were mean +SE of total protein and ±SE of day after harvest of 4 fruit at 10%, 25%, 50%, 75%, 100%, 100%+3days, and 100%+6days stages, respectively.

5.3.3 Activities of Hydrolases Altered by 1-MCP Treatment

PG activities varied in both the 1-MCP-treated fruit and control fruit (Figure 5.4) and activity did not show significant correlation with fruit firmness (Figure 5.5). The PME activity of the control fruit increased after the 25% skin yellow stage (4 days after harvest) then remained constant until the full ripe stage (Figure 5.6). The 1-MCP-treated fruit gradually increased in PME activity throughout ripening, and tended to have a higher PME activity than control fruit. Linear correlation coefficients (r) between average PME activity and fruit firmness of the control and 1-MCP-treated fruit were significant (Figure 5.7). However, the correlation coefficients when analyzed from individual data were not significant since each stage showed high variation in PME activity.

The activity of β-galactosidase increased and was coincident with a significant decline in control fruit firmness (Figure 5.8). β-galactosidase activity was delayed by 1-MCP treatment and the activity recovered at a later stage of ripening. β-galactosidase activity of 1-MCP-treated fruit at the later stages was greater than the highest activity in the control fruit. There was a significant correlation between β-galactosidase activity and fruit firmness in both control fruit and 1-MCP-treated fruit (Figure 5.9).

Endoglucanase activity increased slightly before the decrease in fruit firmness. The activity began to increase at 25% skin yellow stage and continued throughout fruit ripening (Figure 5.10). The greatest increase occurred between the 50% to 75% skin yellow stages (6.6-7.0 days after harvest). Treatment with 1-MCP lowered the increase in endoglucanase activity and the activity increased gradually throughout ripening. Endoglucanase activity of the 1-MCP-treated fruit at late stages of ripening (16-20 days after harvest) was greater than the control at the 50% skin yellow stage (6.5 days after

harvest) when the control fruit softened. Endoglucanase activities of the control fruit and MCP-treated fruit correlated with fruit firmness (Figure 5.11).

Endoxylanase activity was very low or not detected at the harvest stage (Figure 5.12) then increased significantly after the 25% skin yellow stage and continued to increase throughout ripening. The endoxylanase activity of the control fruit was highly correlated to fruit firmness (Figure 5.13). Following 1-MCP treatment, endoxylanase activity was highly suppressed for up to 20 days after harvest (Figure 5.12). Because of very low or undetected endoxylanase activity of 1-MCP-treated fruit throughout ripening, the activity did not correlated to the softening (Figure 5.13).

The β-xylosidase activity of control fruit increased from the 25% skin yellow stage onwards (Figure 5.14). However, β-xylosidase activity was detected at harvest as was PME, β-galactosidase, and endoglucanase activities (Figure 5.6, 5.8 and 5.10). β-xylosidase activity of the control fruit was correlated with fruit firmness (Figure 5.15) and 1-MCP treatment altered β-xylosidase activity during ripening. 1-MCP-treated fruit maintained the level of β-xylosidase activity that slightly increased at later stages of ripening (Figure 5.14). β-xylosidase activity was not correlated to the modified firmness of 1-MCP-treated fruit (Figure 5.15).

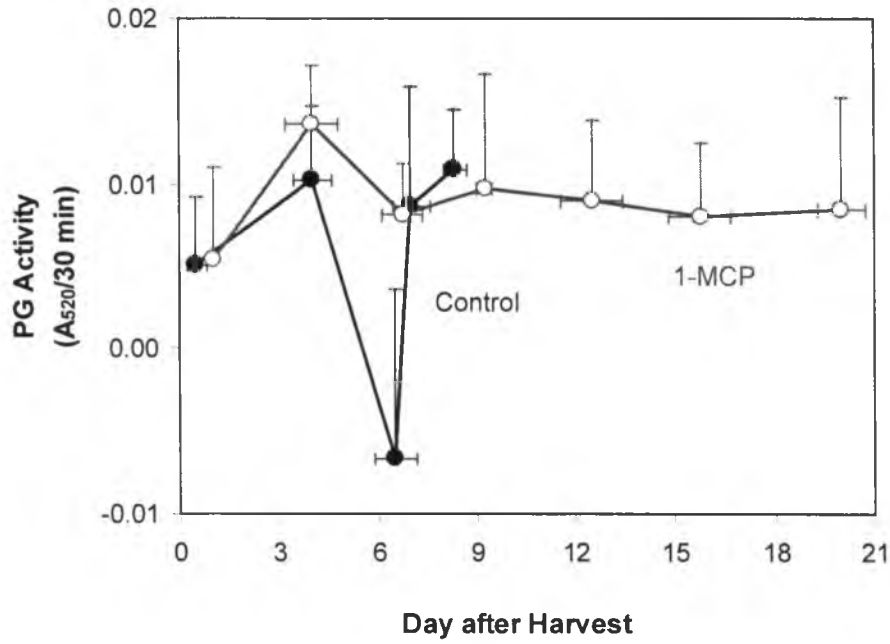


Figure 5.4 Endopolygalacturonase (PG) activities during ripening of 'Sunset' fruit with and without 1-MCP treatment. Markers on each line, from left to right, were the mean +SE of PG activity and ±SE of day after harvest of 4 fruit at 10%, 25%, 50%, 75%, 100%, 100%+3days, and 100%+6days stages, respectively.

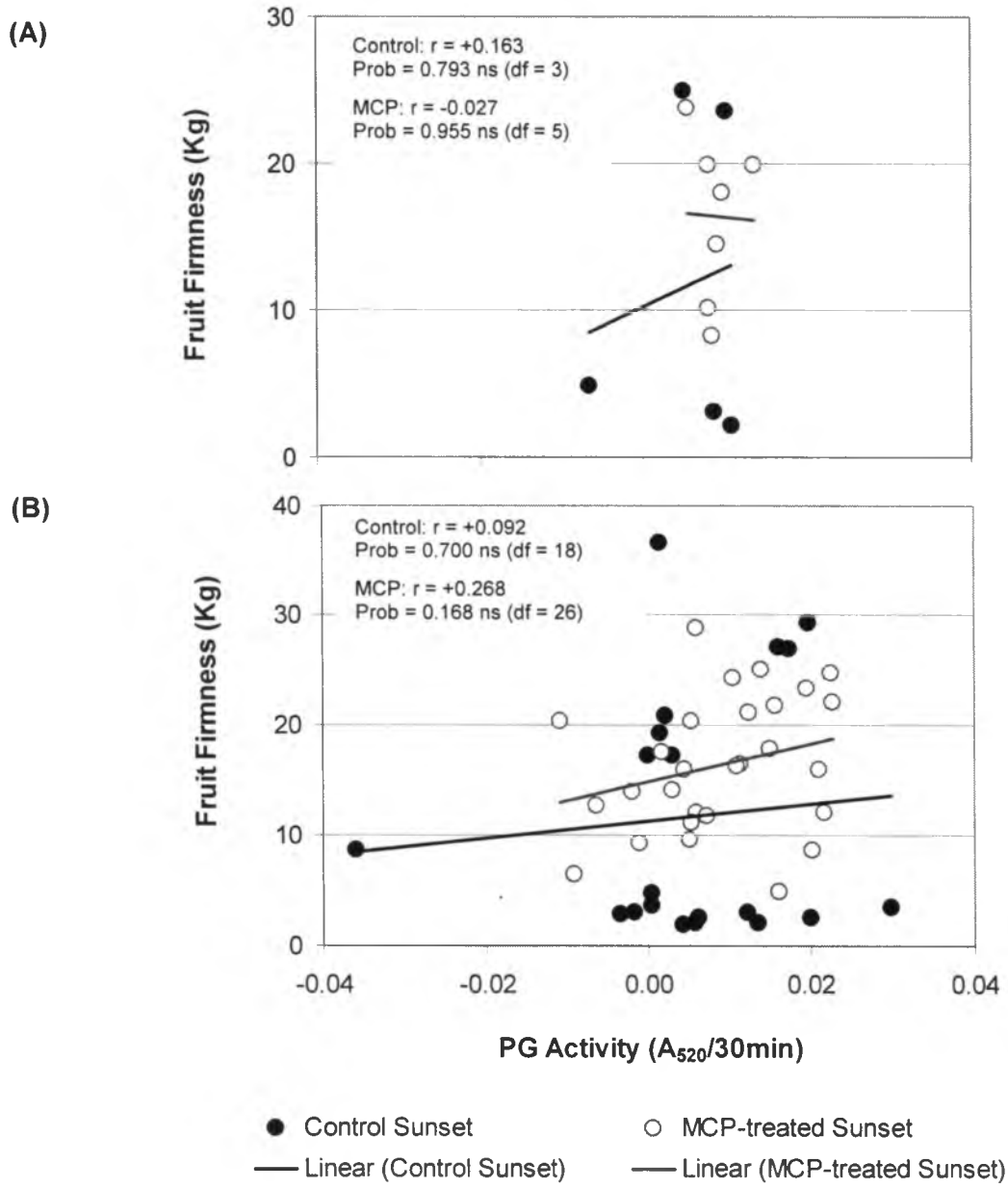


Figure 5.5 Linear correlations between endopolygalacturonase (PG) activity and fruit firmness during ripening of 'Sunset' fruit with and without 1-MCP treatment. (A) Linear correlation between the activities and fruit firmness of average data. (B) Linear correlation between the activities and fruit firmness of individual data.

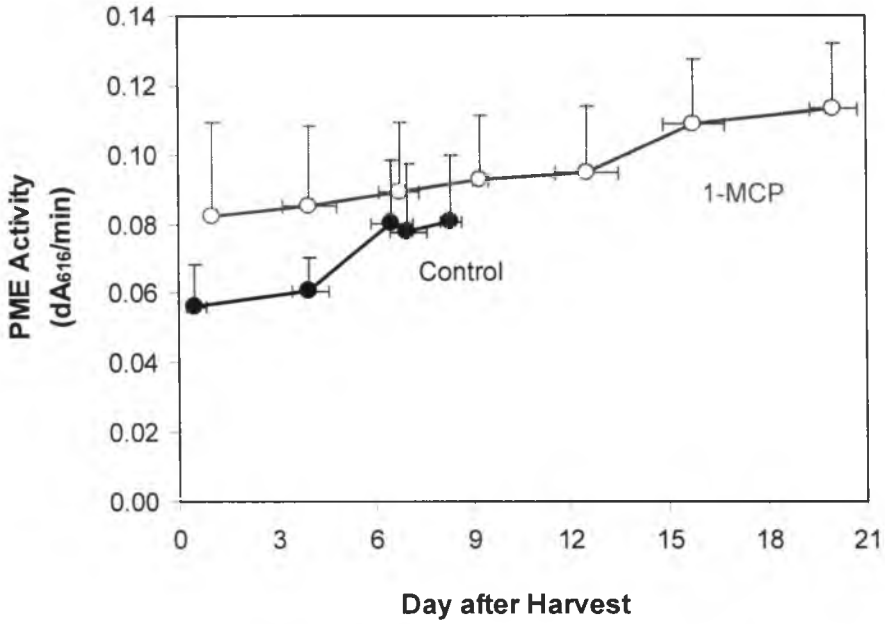


Figure 5.6 Pectin methyiesterase (PME) activities during ripening of 'Sunset' fruit with and without 1-MCP treatment. Markers on each line, from left to right, were the mean +SE of PME activity and ±SE of day after harvest of 4 fruit at 10%, 25%, 50%, 75%, 100%, 100%+3days, and 100%+6days stages, respectively.

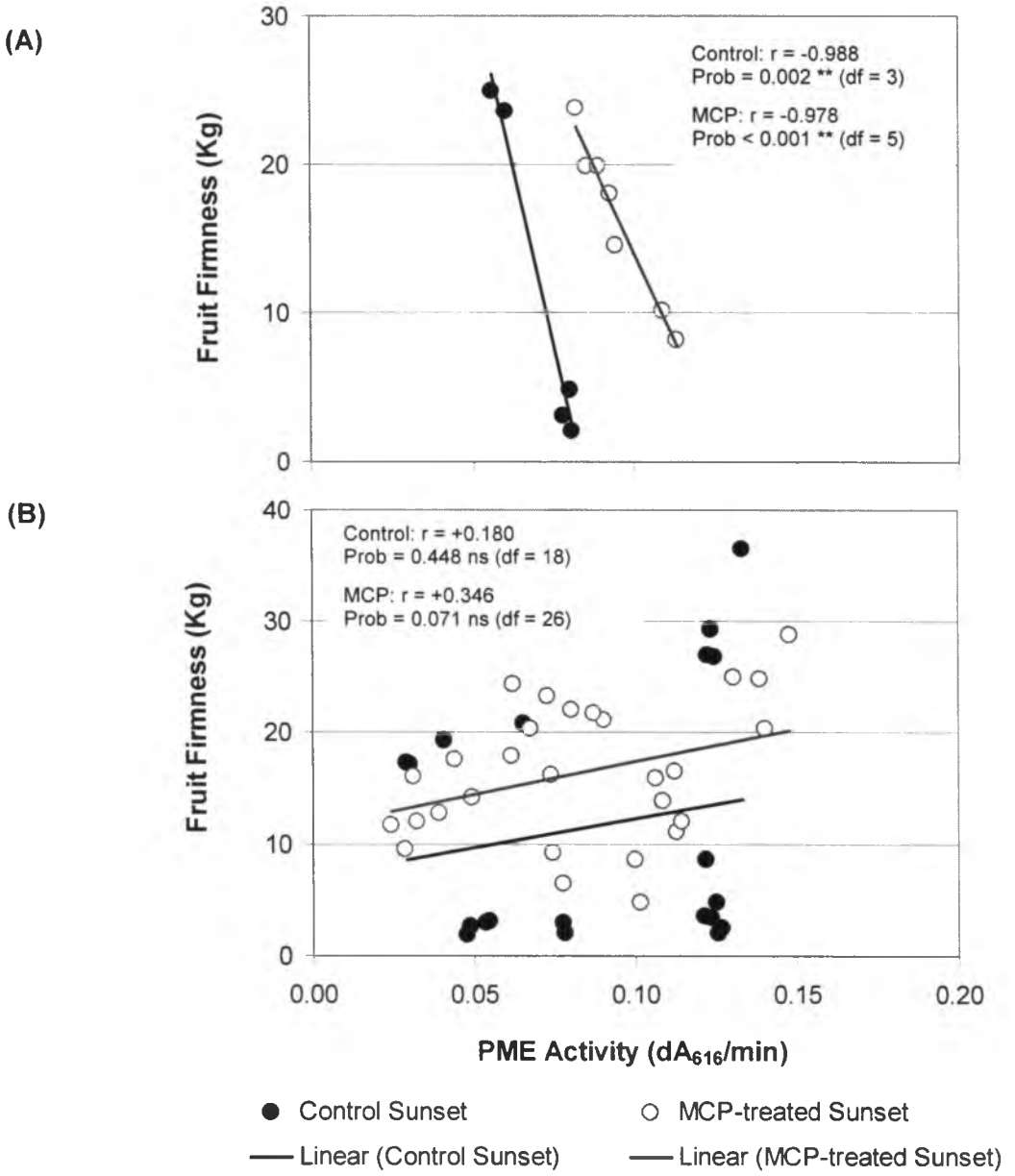


Figure 5.7 Linear correlations between pectin methylesterase (PME) activity and fruit firmness during ripening of 'Sunset' fruit with and without 1-MCP treatment. (A) Linear correlation between the activities and fruit firmness of average data. (B) Linear correlation between the activities and fruit firmness of individual data.

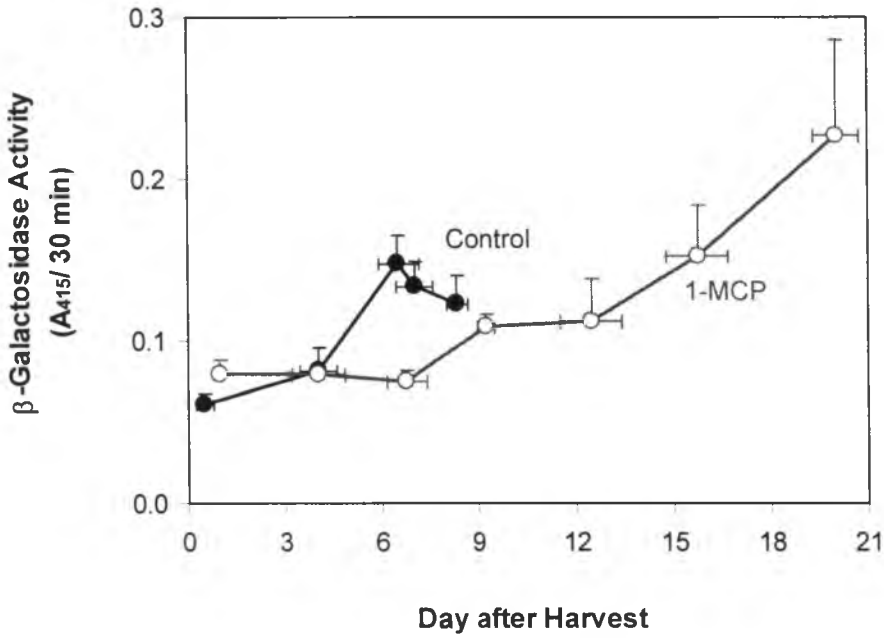


Figure 5.8 β-galactosidase activities during ripening of 'Sunset' fruit with and without 1-MCP treatment. Markers on each line, from left to right, were the mean +SE of β-galactosidase activity and ±SE of day after harvest of 4 fruit at 10%, 25%, 50%, 75%, 100%, 100%+3days, and 100%+6days stages, respectively.

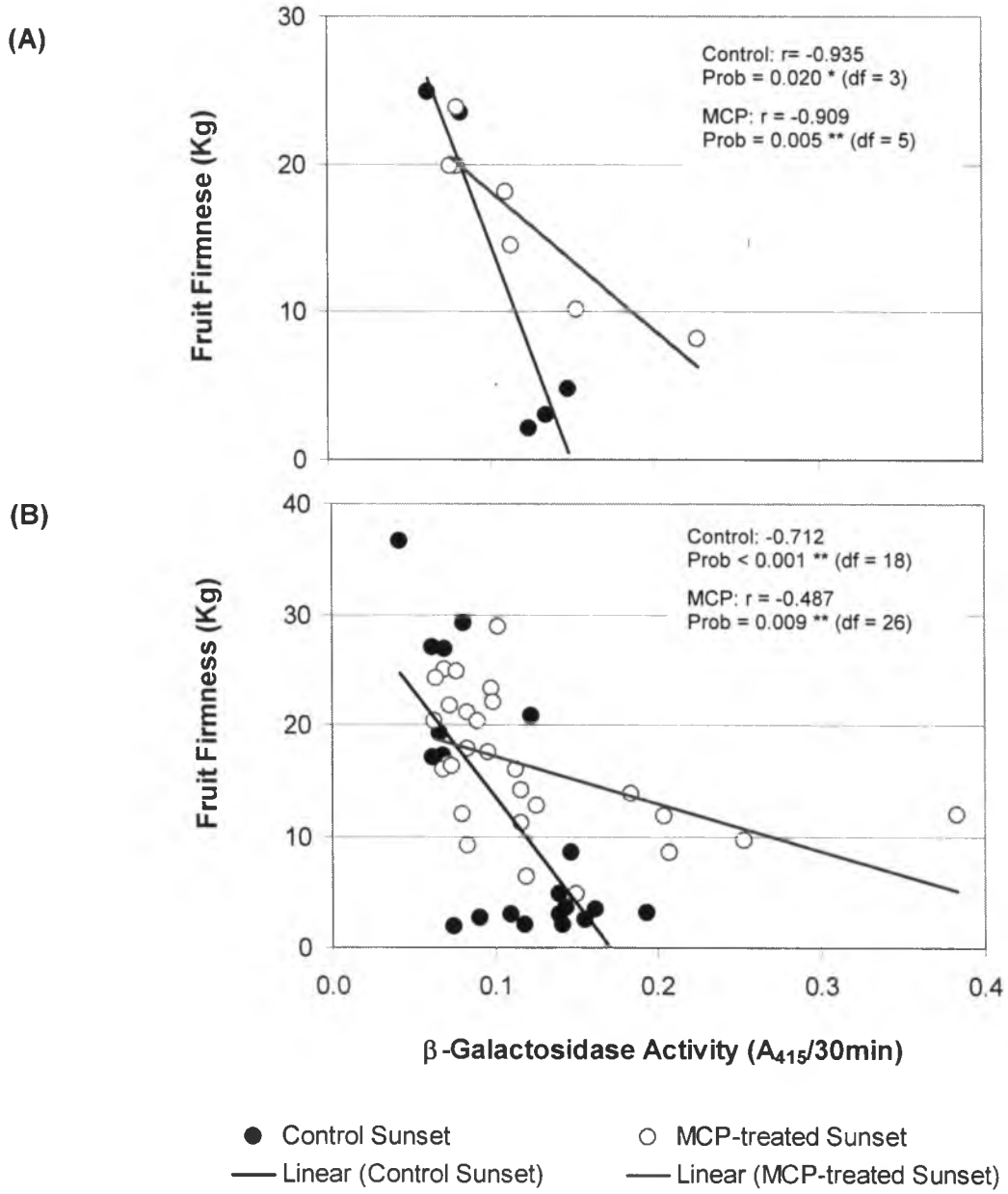


Figure 5.9 Linear correlations between β-galactosidase activity and fruit firmness during ripening of 'Sunset' fruit with and without 1-MCP treatment. (A) Linear correlation between the activities and fruit firmness of average data. (B) Linear correlation between the activities and fruit firmness of individual data.

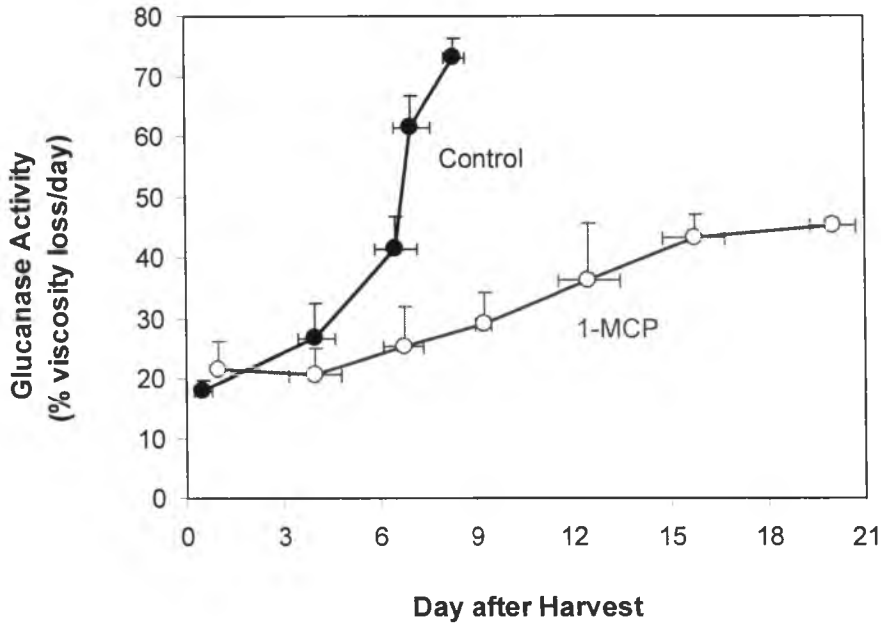


Figure 5.10 Endoglucanase activities during ripening of 'Sunset' fruit with and without 1-MCP treatment. Markers on each line, from left to right, were the mean +SE of endoxylanase activity and ±SE of day after harvest of 4 fruit at 10%, 25%, 50%, 75%, 100%, 100%+3days, and 100%+6days stages, respectively.

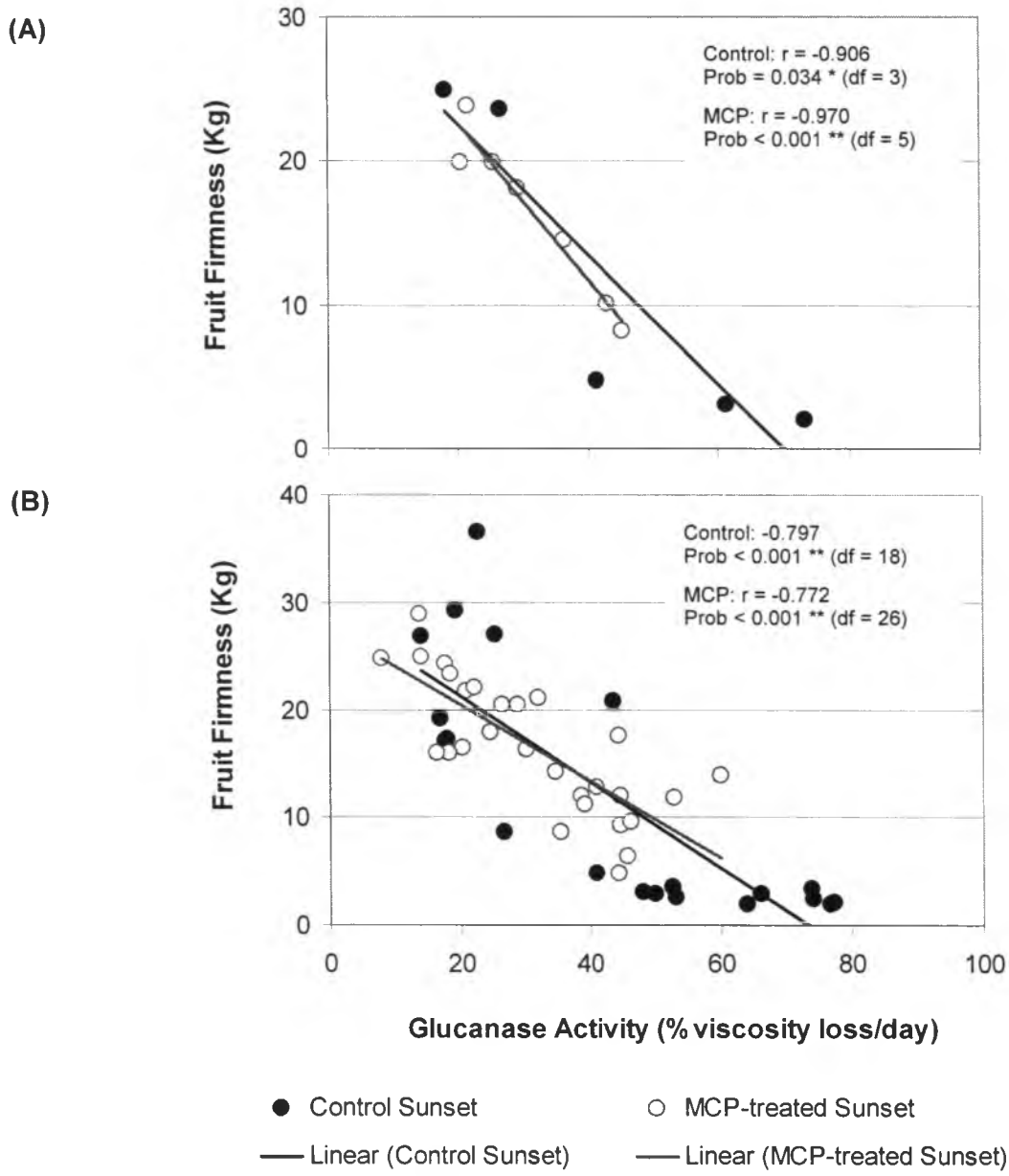


Figure 5.11 Linear correlations between endoglucanase activity and fruit firmness during ripening of 'Sunset' fruit with and without 1-MCP treatment. (A) Linear correlation between the activities and fruit firmness of average data. (B) Linear correlation between the activities and fruit firmness of individual data.

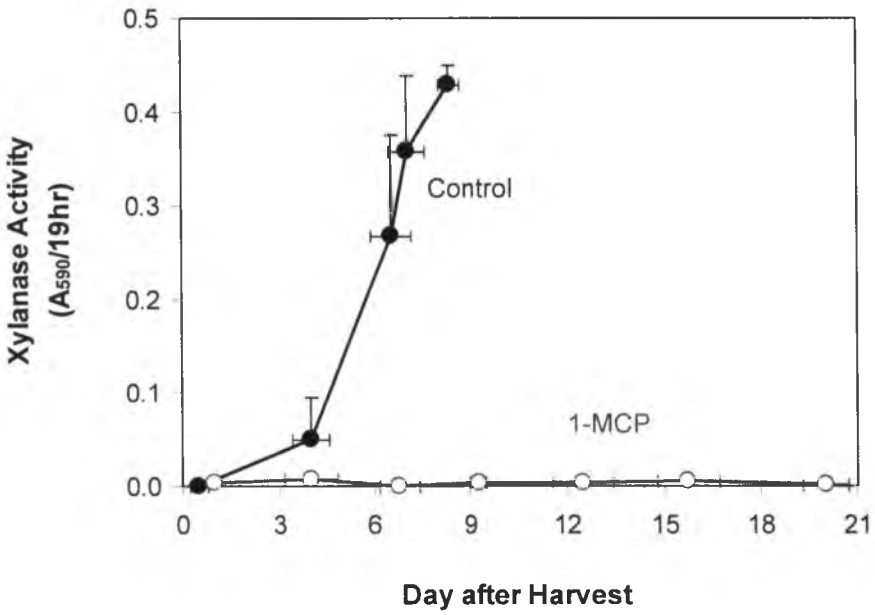


Figure 5.12 Endoxylanase activities during ripening of 'Sunset' fruit with and without 1-MCP treatment. Markers on each line, from left to right, were the mean +SE of xylosidase activity and ±SE of day after harvest of 4 fruit at 10%, 25%, 50%, 75%, 100%, 100%+3days, and 100%+6days stages, respectively.

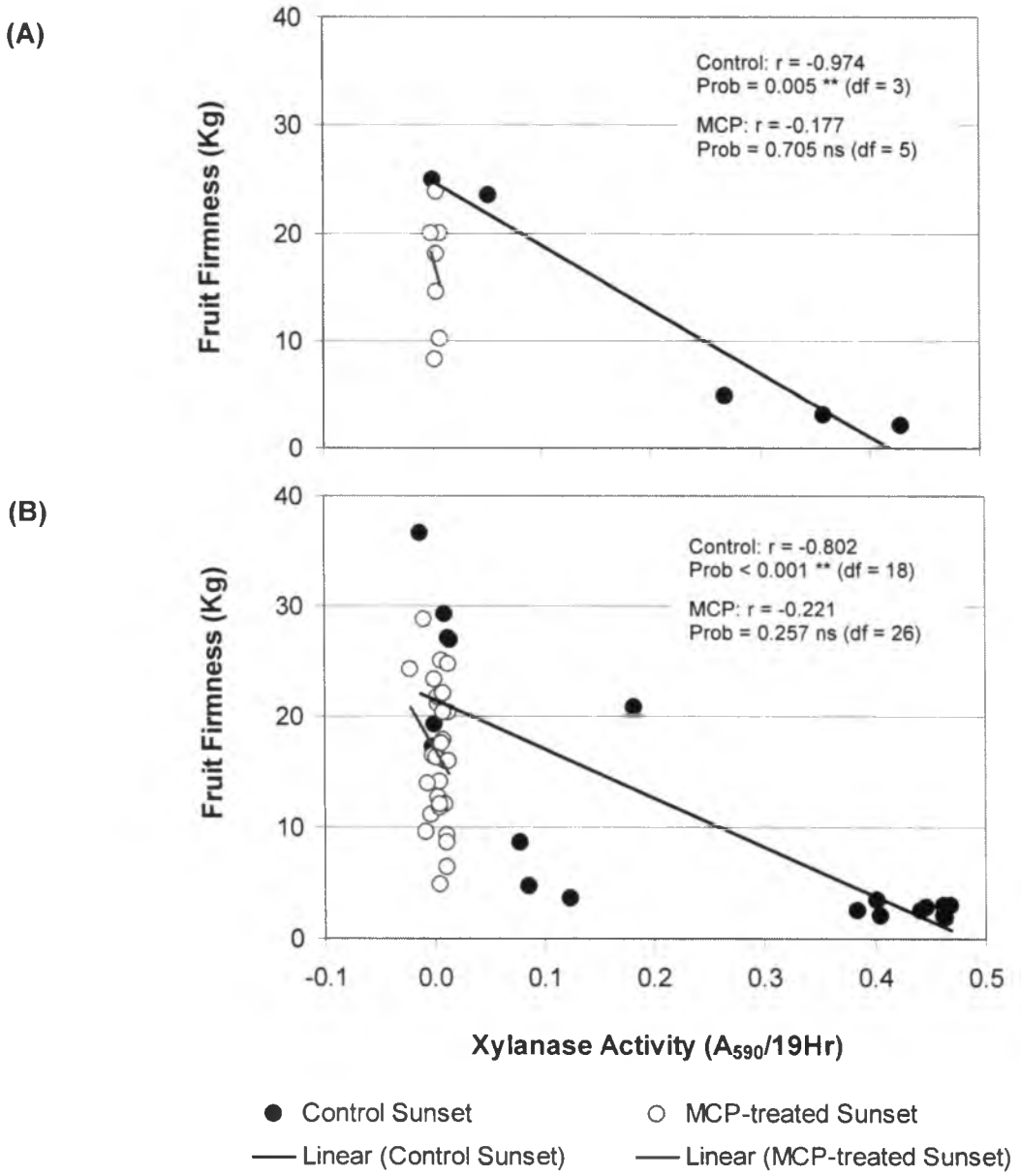


Figure 5.13 Linear correlations between endoxylanase activity and fruit firmness during ripening of 'Sunset' fruit with and without 1-MCP treatment. (A) Linear correlation between the activities and fruit firmness of average data. (B) Linear correlation between the activities and fruit firmness of individual data.

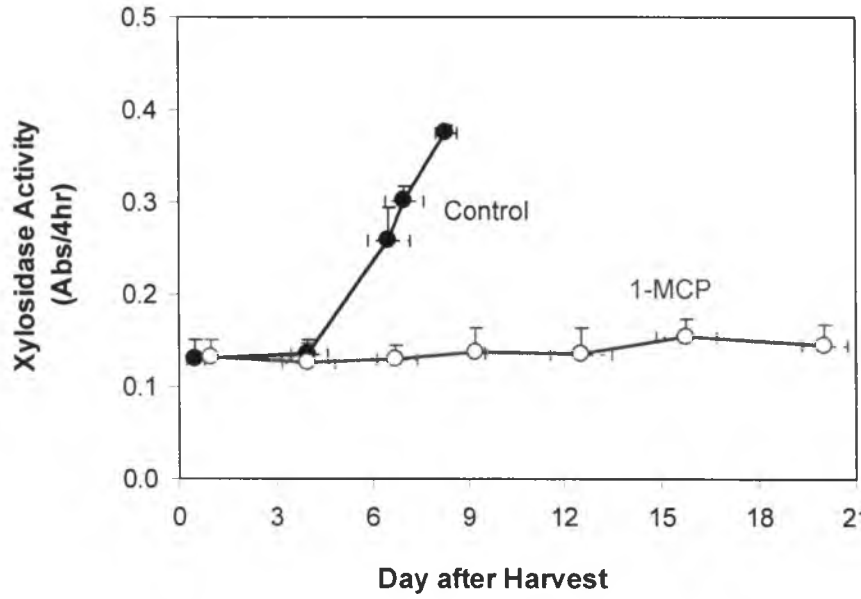


Figure 5.14 β-xylosidase activities during ripening of 'Sunset' fruit with and without 1-MCP treatment. Markers on each line, from left to right, were the mean +SE of β-xylosidase activity and ±SE of day after harvest of 4 fruit at 10%, 25%, 50%, 75%, 100%, 100%+3days, and 100%+6days stages, respectively.

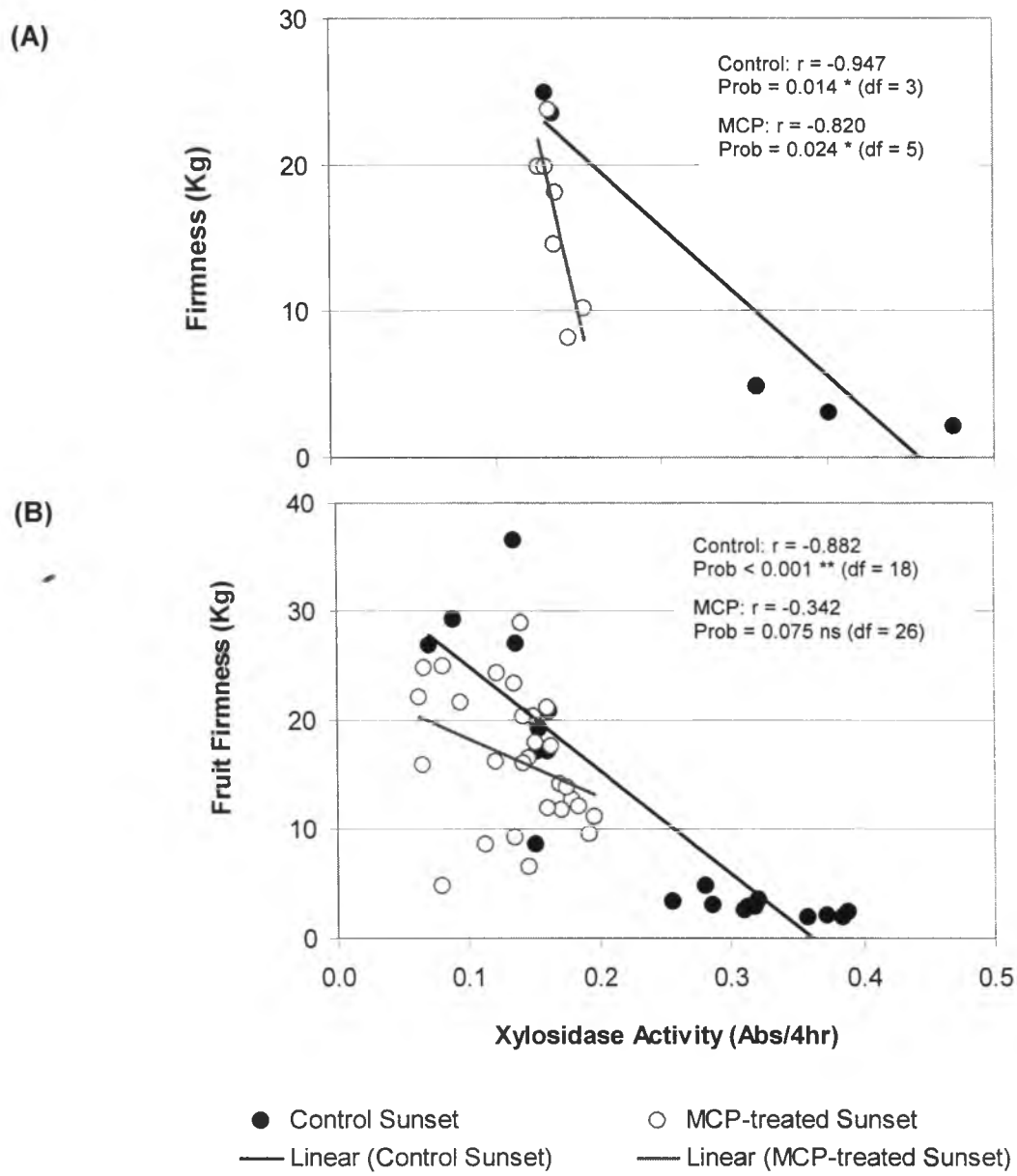


Figure 5.15 Linear correlations between β-xylosidase activity and fruit firmness during ripening of 'Sunset' fruit with and without 1-MCP treatment. (A) Linear correlation between the activities and fruit firmness of average data. (B) Linear correlation between the activities and fruit firmness of individual data.

5.4 Discussion

The treatment of 'Sunset' fruit with 1-MCP delayed softening. The mesocarp of 1-MCP-treated fruit showed a noticeable alteration from the mesocarp of non-treated fruit when ripe. All fruit treated with 1-MCP showed a 'rubbery' texture as mesocarp tissue did not separate when squeezed. This softening disorder caused by 1-MCP treatment at certain stages has been reported in other papaya varieties (Manenoi *et al.*, 2007) and tomato (Hurr *et al.*, 2005). The incomplete softening of papaya following 1-MCP treatment may be due to selective loss of cell wall hydrolase activity. The amounts of protein extracted by different extractants were also different following 1-MCP treatment. It is possible that 1-MCP not only causes down-regulations of some enzymes, but it also causes up-regulations of others. In this study, the 1-MCP-treated papaya tended to have a higher PME activity than that of the control papaya. An up-regulation of *LeARF1* coding α-arabinofuranosidase in tomato was also found after 1-MCP treatment (Itai *et al.*, 2003).

β-galactosidase and endoglucanase activities were correlated with fruit firmness of both the control papaya and 1-MCP-treated papaya. These hydrolases were apparently involved in papaya softening but may not be the major causes of the softening. Although their activities were suppressed by 1-MCP-treatment during early ripening, the activities recovered at later stages of ripening to higher levels than the activity when the control papaya started to soften. If these hydrolases had a major role in fruit softening, the 1-MCP-treated papaya would soften normally. However, they may play a role in fruit softening when the major hydrolases are not expressed in a normal manner such as after 1-MCP treatment.

Endoglucanase may not be a key cell wall hydrolase that causes fruit softening as has been suggested for avocado and tomato. Treatment of avocado with 1-MCP prior to exposure to ethylene delays the increase in endoglucanase activity (Feng *et al.*, 2000) and the level of endoglucanase activity was low throughout the storage period. Though avocado has a lower endoglucanase activity, fruit treated with the 1-MCP ripen and soften normally (Feng *et al.*, 2000). CEL2 gene encodes a tomato endoglucanase that show differential expression in ripening tomato (Lashbrook *et al.*, 1994), and when the mRNA accumulation is repressed, the fruit soften normally (Brummell *et al.*, 1999). Overexpression of a ripening-related pepper endoglucanase in transgenic tomato also does not increase tomato softening (Harpster *et al.*, 2002).

β-galactosidase may play and important role in fruit softening. Our results agree with those of Lazan *et al.* (Lazan *et al.*, 1995) that papaya softening during ripening was more closely related to changes in β-galactosidase activity than PG or PME activity. At least three isoforms of β-galactosidase/galactanase exist in papaya during ripening (Lazan *et al.*, 2004). In tomato, at least seven β-galactosidase genes are expressing during fruit development. The TBG4 coding for β-galactosidase II isoform is present during tomato ripening (Smith *et al.*, 1998; Smith and Gross, 2000). Six antisense lines to TBG4 are 26-40% firmer than controls (17 N in firmness) at red-ripe stage (Smith *et al.*, 2002). However the firmness alteration of TBG6 antisense tomato was less than alteration caused by 1-MCP treatment. Treated at light red stage with 900-1,800 nL/L 1-MCP for 24 hours, 'Falcato' tomato was over 100% firmer than the control tomato (5-6 N in firmness) after 6 to 18 days of storage (Ramin, 2006). In the present study, the 1-MCP-treated papaya was over 700% firmer than the control papaya compared at the full ripen stage. Moreover, the β-galactosidase activity of the 1-MCP-treated fruit

recovered and the activity levels at later stages were greater than the peak activity in the control fruit (Figure 5.8). The alteration in papaya softening and texture following 1-MCP treatment was possibly due to changes in other factors, rather than β-galactosidase activity.

The key hydrolase associated with part of papaya softening appear to be endoxylanase. Endoxylanase activity was not detected at harvest but rose at the start of softening. Activity was highly correlated with firmness of 'Sunset' fruit during ripening. A high relationship also occurs between endoxylanase activity and papaya softening in other lines: 'Sunrise' (Pauli and Chen, 1983), 'Line 8', and 'Line 4-16' (Manenoi, 2005). 1-MCP treatment caused a complete suppression in endoxylanase activity *in vitro* (Figure 5.12) throughout ripening; coincident with the incomplete softening of papaya. 'Sunset' fruit has undetectable endoxylanase (CpaEXY1) mRNA after 1-MCP treatment through to the full ripe plus 12 days stage. Endoxylanase protein accumulation, that was detected by protein blotting and immunodetection, is not present in 1-MCP-treated 'Sunset' papaya during ripening (Manenoi, 2005).

β-xylosidase activity of papaya showed a similar pattern to endoxylanase activity during normal ripening and showed a high correlation with fruit firmness. However, its activity was detected at harvest whereas endoxylanase was not. β-xylosidase activity was also detected in avocado at harvest (Ronen *et al.*, 1991). β-xylosidase activity was not completely suppressed by 1-MCP treatment as was endoxylanase activity. Proteome variations in cherry tomato (Faurobert *et al.*, 2007), and β-xylosidase genes expressions and activity of tomato cv. Alisa Craig (Itai *et al.*, 2003) showed that β-xylosidase is expressed during fruit development and ripening, but the protein

expression and activity were highest during early fruit growth. The activity of β-xylosidase decreases during later fruit development and did not vary during ripening (Itai *et al.*, 2003) and the expressions of two β-xylosidase genes in the tomato appear to be independent of each other (Itai *et al.*, 2003).

In conclusion, papaya softening is a complex event that involves many cell wall hydrolases, such as endoxylanase, xylosidase, β-galactosidase and endogiucanase. These hydrolases may play their roles in concert, to provide a unique texture of particular fruit. Endoxylanase appear to play a role in papaya softening. The failure of 1-MCP-treated papaya to soften completely and to have a 'rubbery' texture was associated with a selective suppression of endoxylanase activity.

CHAPTER 6

PAPAYA ACIDITY CHANGES DURING RIPENING AND MESOCARP FIRMNESS RESPONDING TO EXOGENOUS PH

6.1 Introduction

Apoplatic pH regulates both non-enzymatic and enzymatic cell wall modification (Chun and Huber, 1998; Almeida and Huber, 1999). Enzymes have optimum activities at particular pH *in vitro* (Kanellis *et al.*, 1989; Chun and Huber, 1998) though it is less clear whether *in vitro* activity directly relates to *in vivo* condition. Protons are also exchanged for bivalent cations on carboxylic groups of the cell wall (Cutsem and Gillet, 1983), partially dissociate pectin uronic acid subunits (Fishman *et al.*, 1989), and control interactions between structural carbohydrates and proteins (Rayle and Cleland, 1992).

Apoplastic pH of tomato during ripening has been reported and may have role in tomato softening (Almeida and Huber, 1999). Apoplastic pH of papaya fruit has not been reported. Few reports are available of papaya juice pH. Juice pH of papaya ranges from 4.2 to 6.00 (Nath and Ranganna, 1981; FDA, 2003; Berry and Sargent, 2004). During testing for chilling effect on papaya quality, Proulx *et al.* (2005) found that juice pH of papaya cv. 'Exp.15' at color break stage was 5.3 to 5.6. Papaya fruit stored at 20°C showed a slight decrease in juice pH to below 5.0 after 2-3 days of storage and remained at that pH for a further 6 days of storage. Whereas papaya held at less than 10°C maintained juice pH throughout storage (Proulx *et al.*, 2005). However, we still lack adequate information on the changes in papaya mesocarp pH during ripening and especially its correlation with fruit softening.

The objective of this study was to determine the changes in papaya mesocarp pH during fruit softening and whether mesocarp pH was modified by 1-methylcyclopropene (1-MCP) treatment. Exogenous pH buffer solutions were applied to papaya mesocarp cubes to determine the effects of pH on papaya mesocarp softening.

6.2 Materials and Methods

6.2.1 Determination of Papaya Acidity during Ripening

Fruit of 'Line 8' and 'Sunset' papaya were harvested at the color break stage (less than 10% skin yellow) from Poamoho Experimental Station in Central Oahu, Hawaii. Fruit was fumigated with 100 nL L^{-1} 1- MCP after harvested at the room temperature (21-22°C) for 12 hours. 1-MCP-treated fruit and non-treated control fruit were allowed to ripen at the room temperature. Skin yellow color was used as ripening index. Fruit were randomly collected at certain ripening stages including the color break (10% skin yellow), 25% skin yellow, 50% skin yellow, 75% skin yellow, full ripe (more than 90% skin yellow), full ripe plus 3 days, and full ripe plus 6 days stages. There were 4-5 fruit (replicates) for each papaya lines, ripening stages, and treatments.

Fruit firmnesses were determined using a force gauge (AccuForce® Model Cadet) with a 1.6 cm in diameter disc and pressed 1 mm into the skin. Peak forces at two opposite sides of fruit equator were averaged. Mesocarp tissue at fruit equator was sliced, frozen in liquid nitrogen, and stored at -80°C.

Four grams of frozen mesocarp were homogenized in 8 mL of chilled 0.5 M sodium chloride including 1mM PMSF and 1 mM dithiopyridine. The homogenates were allowed to stand at ice temperature for 15 minutes and centrifuged at 5,000 g 4°C for 10

minutes. Supernatants were then filtered through Miracloth® and the filtrates collected. Eight hundred (800) µL of the filtrates were diluted in 4 mL of distilled water, and the pH determined.

6.2.2 Determination of Papaya Firmness Responding to Exogenous pH

'Line 8' papayas were harvested at mature green and color break stages from Poamoho Experimental Station in Central Oahu, Hawaii. Fruit mesocarp was diced in to cubes 2 cm by 2 cm and 1.5 cm thick and 5-7 fruit were used for each experiment. Mesocarp cubes from each fruit were divided into all treatments. Citrate buffer was used as it has a wide range in buffering capacity. Mesocarp cubes were submerged with 30 mL of 25 mM citrate buffers at various pHs (4.50, 5.00, 5.50, and 6.00) at room temperature and at 1°C. Mesocarp cubes submerged in 30 mL distilled water were used as the control. One cube from each fruit in each treatment was sampled for determination of firmness at different times after the start of incubation. Firmness, as peak force, was read at the outer and inner sides of the mesocarp cube using a force gauge (AccuForce® Model Cadet) with a cone-shaped tip (0.7 cm diameter pressed 0.5 cm into the cube). The pH changes of the buffers and distilled water were recorded using a pH meter (Accumet® Model Basic AB15).

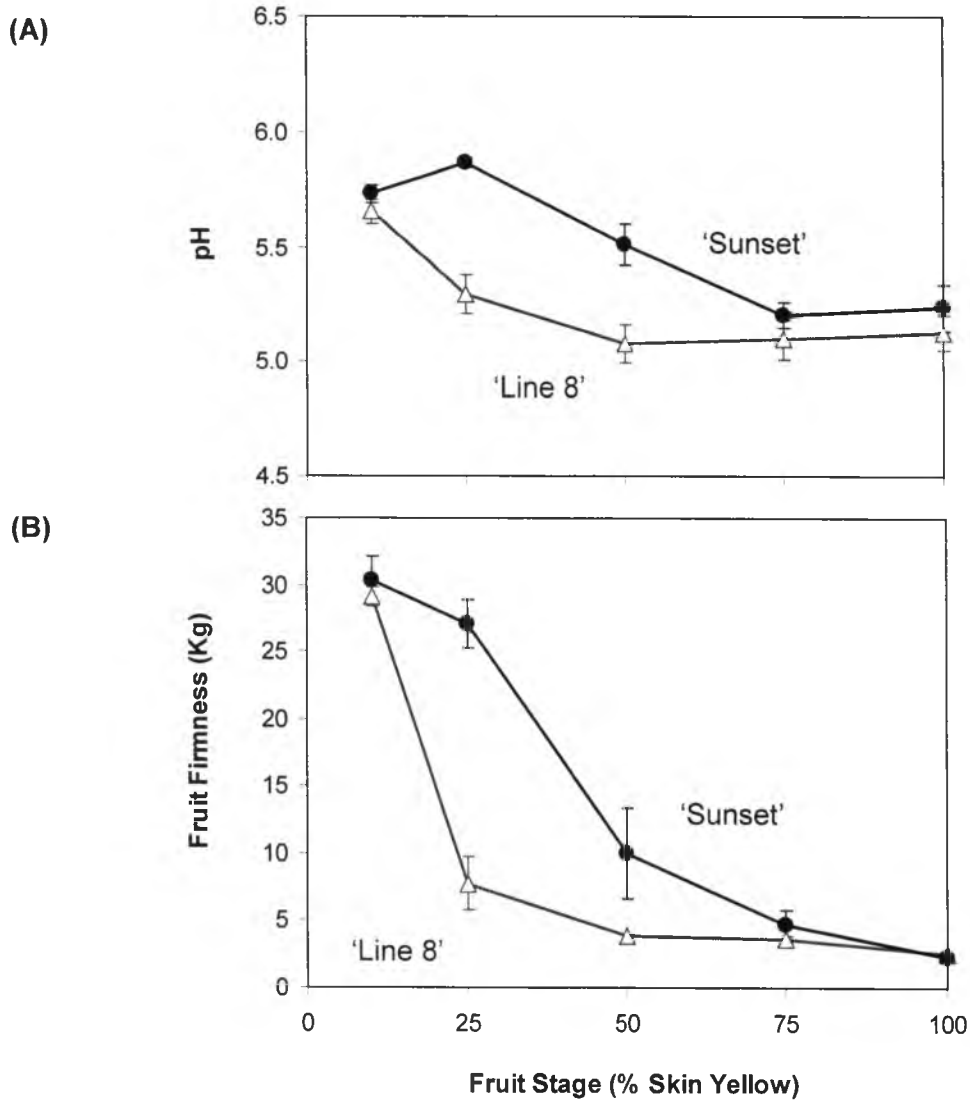
6.3 Results

6.3.1 Papaya Firmness and Acidity Changes during Ripening

Changes in acidity of mesocarp homogenates coincided with changes in fruit firmness. At the color break stage, 'Line 8' and 'Sunset' had similar mesocarp homogenate pH (Figure 6.1 A). Homogenate pH of both papaya lines at the color break

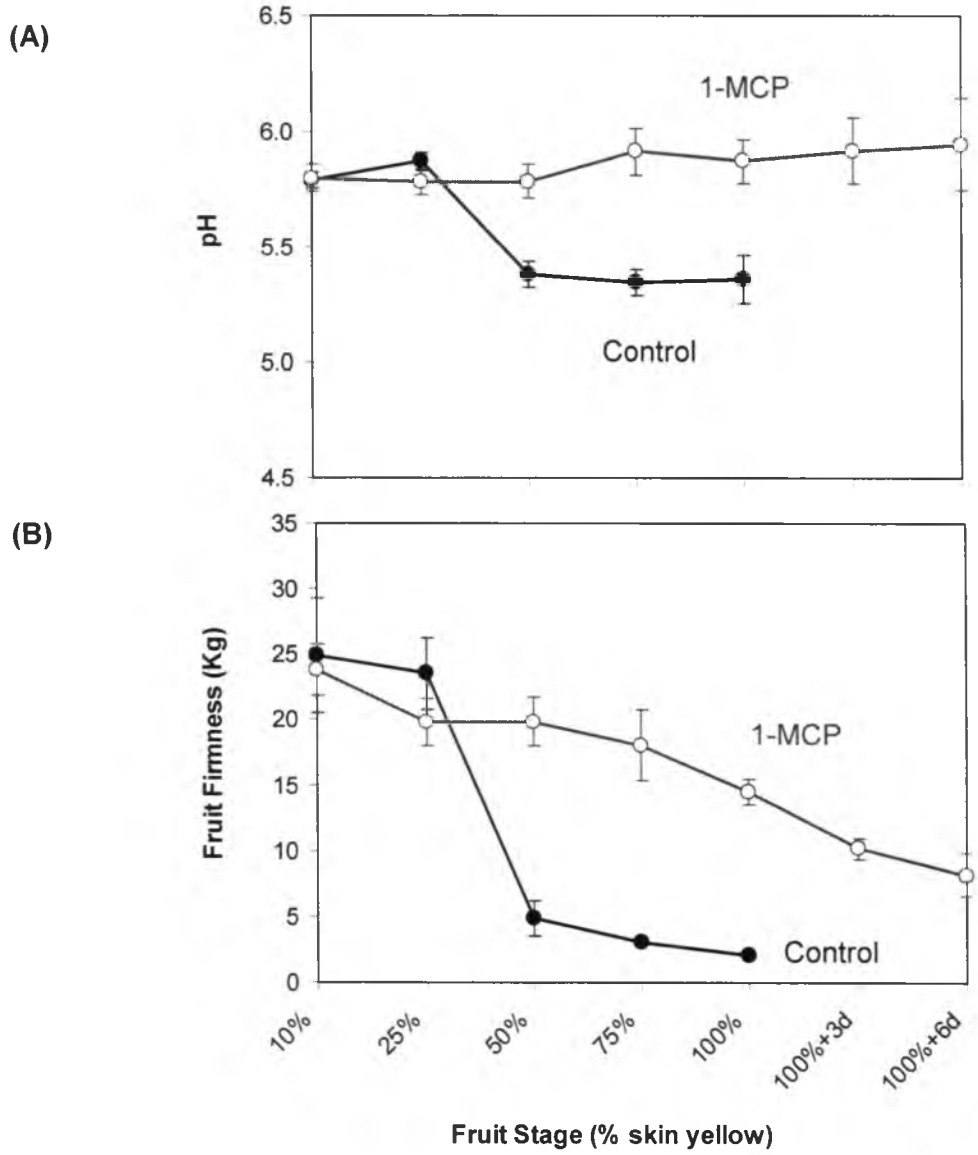
stage ranged from 5.50 to 5.80 while the original pH of diluted extraction solution (blank) was 5.28. The pH of 'Line 8' mesocarp significantly declined at the 25% skin yellow stage coincident with the significant decline in fruit firmness (Figure 6.1 B). Whereas, the pH of 'Sunset' mesocarp at 25% skin yellow was similar to that at harvest. The pH of 'Sunset' mesocarp decreased at the 50% skin yellow stage when fruit firmness began to decline. The homogenate pH of 'Sunset' papaya was significantly higher than that of 'Line 8' papaya at the 25 and 50% skin yellow stages. 'Line 8' fruit homogenate maintained pH after the 50% skin yellow stage while 'Sunset' declined in pH and had similar pH to that of 'Line 8' papaya at later stages of ripening. The lowest pH of 'Line 8' and 'Sunset' mesocarp homogenates were 5.1 and 5.2 at the 50% and 75% skin yellow, respectively (Figure 6.1 A).

Comparison of mesocarp pH between 1-MCP-treated and non-treated 'Sunset' fruit (Figure 6.2 A) showed that papaya after 1-MCP treatment had similar mesocarp pH as the control papaya at harvest. The control papaya showed a marked decline in mesocarp pH at 50% skin yellow stage as the fruit firmness declined (Figure 6.2 B). 1-MCP-treated papaya showed a slight increase in homogenate pH throughout ripening up to pH 6.2 (Figure 6.2 A) during modified softening and development of the "rubbery" texture. A significant difference in mesocarp pH and firmness began to occur in 1-MCP-treated papaya from the control papaya at 50% skin yellow stage to the end of ripening (Figure 6.2 A and B).



T-test of pH	ns	**	**	ns	ns
T-test of firmness	ns	**	ns	ns	ns

Figure 6.1 Mesocarp homogenate acidity (A) and fruit firmness (B) of 'Line 8' and 'Sunset' fruit during ripening. Markers were the mean ± SE of five fruit. T-test for equality of means was compared between two papaya lines: ns = not significant, and *and ** = significant at 0.05 and 0.01 levels, respectively.



T-test of pH	ns	ns	**	**	*
T-test of firmness	ns	ns	**	*	**

Figure 6.2 Mesocarp homogenate acidity (A) and fruit firmness (B) of non-treated and 1-MCP-treated 'Sunset' fruit during ripening. Markers were the mean ± SE of four fruit. T-test for equality of means was compared between treatments: ns = not significant, and *and ** = significant at 0.05 and 0.01 levels, respectively.

6.3.2 Mesocarp Firmness Responding to Exogenous pH

Mesocarp cubes from mature green 'Line 8' showed a significant difference in outer mesocarp firmness after submergence in various pH solutions at room temperature (22°C). Outer mesocarp cubes in distilled water (pH 5.5) had the greatest firmness at 24 hours after submergence, and the firmness was significantly greater than in citrate buffer at pH 5.0 and pH 6.0. However, cubes held in citrate buffer at pH 6.0 had the greatest firmness among all the cubes in other treatments 48 hours after submergence at room temperature. The firmness of cube held in citrate buffer at pH 6.0 differed significantly from the firmness of cube held in citrate buffer at pH 4.5 (Figure 6.3 A). Mesocarp cubes submerged in various pH solutions at 1°C showed a significant difference in firmness after a longer period of submergence compared to those were held at the room temperature. Outer mesocarp firmness started to show differences between treatments after 48 hours. At 72 hours of submergence at 1°C, mesocarp firmnesses of cubes held in citrate buffers at pH 5.5 and pH 6.0 did not differ from those of cubes held in distilled water. Mesocarp cubes in pH 4.5 and 5.0 buffers had lower firmness than mesocarp cubes held in distilled water and they tended to be less firm than mesocarp cubes held in pH 5.5 buffer (Figure 6.3 B).

The pH of the citrate buffer and distilled water changed during submergence of mature green papaya mesocarp cubes (Figure 6.3 C and D). Buffers and distilled water had less change in pH during submergence at 1°C than during submergence at the room temperature. The buffer at pH 4.5 showed an increase in pH while the buffer at pH 5.0 and 5.5 appeared to maintain pH during the first 24 hours of submergence. Buffer at pH 6.0 declined in pH after submergence. Distilled water showed a dramatic increase in pH at the start of submergence then declined. During early submergence, within each

buffers and distilled water some small variation in pH was seen among experimental replicates (SE values in Figure 6.3 C and D). However, following submergence for 24 hours at the room temperature, each buffers and distilled water had higher pH variation among replicates (Figure 6.3 C).

The firmness of mature green 'Line 8' papaya cubes responded to exogenous pH differently between submergence at the room temperature and at 1°C (Table 6.1). Mesocarp cubes submerged in buffer at pH 4.5 and pH 5.0, and distilled water at the room temperature lost their firmness faster than mesocarp cubes submerged in the same solutions at 1°C. At 48 hours of submergence, firmnesses of mesocarp in these solutions at the room temperature were lower than firmnesses of mesocarp in the same solution at 1°C. Whereas in buffer pH 5.5 and pH 6.0 for 48 hours no significant differences in mesocarp cube firmness was found between submergence at the room temperature or at 1°C (Table 6.1).

The mesocarp of color break 'Line 8' papaya showed high variation in firmness. Submergence with various pH buffers did not cause a significant difference in mesocarp firmness at both temperatures (Figure 6.4 A and B). However, submergence of color break papaya cubes showed a similar trend in solution pH changes (Figure 6.4 C and D) when compared to submergence of mature green papaya cubes (Figure 6.3 C and D). Firmnesses of color break papaya cubes submerged in each buffer and distilled water were not different between those held at room temperature and at 1°C (Table 6.2).

The firmness of 'Line 8' outer mesocarp cubes at the mature green stage was similar to that at color break stage (Table 6.3). However, following submergence at 1°C

in various pH buffers, the outer mesocarp of papaya at the color break stage softened faster than that of the mature green papaya cubes.

The outer and inner mesocarp of color break 'Line 8' pieces submerged in buffer at pH 6.0 tended to have a higher average firmness than those held in buffer at pH 4.5 after 120 hours at 1°C, although the difference was not significant (Figure 6.5 A and B). Comparison of the response between the outer and inner sides of the mesocarp to the same exogenous pH (Figure 6.5 D, E, and F) showed that the outer mesocarp seemed to decline in firmness more slowly than the inner mesocarp though it was not significant (Table 6.4).

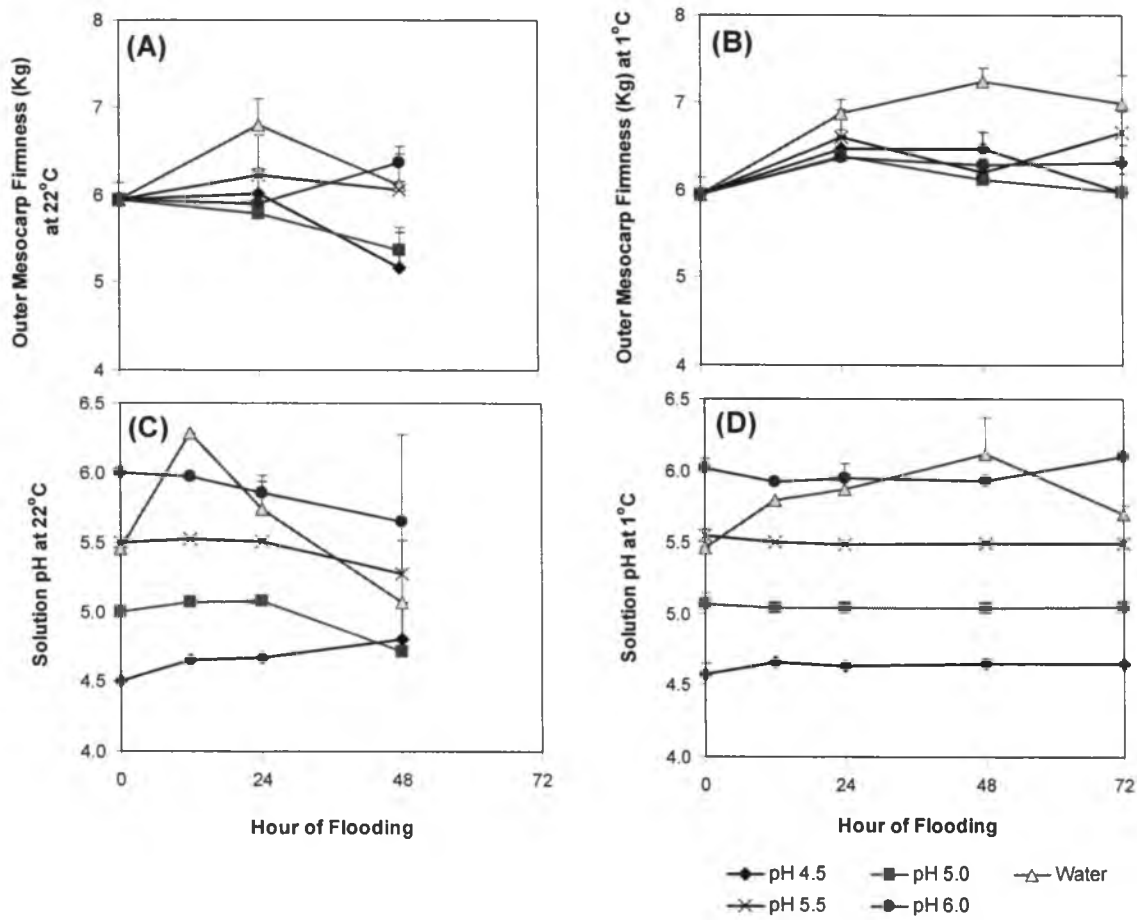


Figure 6.3 Outer mesocarp firmness of mature green 'Line 8' cubes flooded with different pH of citrate buffers and distilled water at 22°C (A) and 1°C (B), and acidity changes (C) (D) of the solutions during flooding. Markers on (A) and (B) were the mean + SE of six replicates (fruit). Markers on (C) and (D) were the mean + SE of two experiments.

Table 6.1 Outer mesocarp firmness of mature green 'Line 8' cubes responding to exogenous pH at different temperatures

pH	Hour of Flooding	Mean[x] ± SE of Outer Mesocarp Firmness (kg)		T-test for Equality of Means	F-test for Equality of Variances
		22°C	1°C		
	Initial	5.94 ± 0.20	5.94 ± 0.20	-	-
Buffer pH 4.5	24	6.01 ± 0.39	6.46 ± 0.14	ns	**
	48	5.17 ± 0.45	6.47 ± 0.19	*	ns
Buffer pH 5.0	24	5.78 ± 0.26	6.38 ± 0.31	ns	ns
	48	5.37 ± 0.22	6.11 ± 0.08	*	*
Water	24	6.80 ± 0.36	6.86 ± 0.16	ns	ns
	48	6.11 ± 0.37	7.25 ± 0.15	*	ns
Buffer pH 5.5	24	6.23 ± 0.09	6.59 ± 0.32	ns	**
	48	6.05 ± 0.20	6.19 ± 0.14	ns	ns
Buffer pH 6.0	24	5.90 ± 0.18	6.37 ± 0.25	ns	ns
	48	6.37 ± 0.39	6.28 ± 0.08	ns	ns

[x] = Mean and stand error (SE) were calculated from six replicates

* = Means or variances at the same row were significant different at 0.05 level.

** = Means or variances at the same row were significant different at 0.01 level.

ns = Means or variances at the same row were not significant different at 0.05 level.

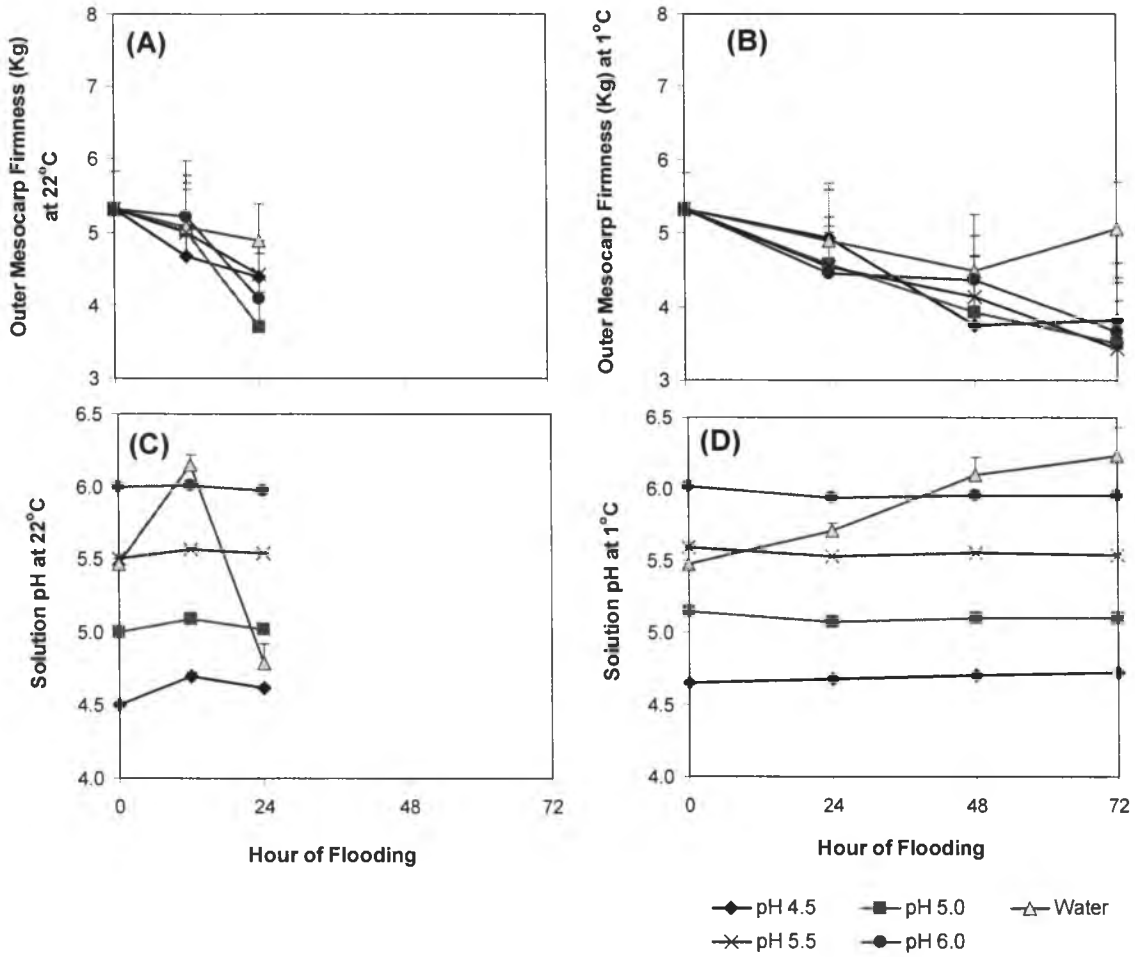


Figure 6.4 Outer mesocarp firmness of color break 'Line 8' cubes flooded with different pH of citrate buffers and distilled water at the room temperature (A) and 1°C (B), and acidity changes (C) (D) of the solutions during flooding. Markers were the mean + SE of five replicates (fruit).

Table 6.2 Outer mesocarp firmness of color break 'Line 8' cubes responding to exogenous pH at different temperatures

pH	Hour of Flooding	Mean[x] ± SE of Outer Mesocarp Firmness (kg)		T-test for Equality of Means	F-test for Equality of Variances
		22°C	1°C		
	Initial	5.32 ± 0.51	5.32 ± 0.51	-	-
Buffer pH 4.5	24	4.39 ± 0.70	4.93 ± 0.67	ns	ns
Buffer pH 5.0	24	3.69 ± 0.48	4.57 ± 0.43	ns	ns
Water	24	4.89 ± 0.49	4.90 ± 0.78	ns	ns
Buffer pH 5.5	24	4.43 ± 0.45	4.54 ± 0.68	ns	ns
Buffer pH 6.0	24	4.08 ± 0.64	4.45 ± 0.64	ns	ns

[x] = Mean and stand error (SE) were calculated from five replicates

* = Means or variances at the same row were significant different at 0.05 level.

** = Means or variances at the same row were significant different at 0.01 level.

ns = Means or variances at the same row were not significant different at 0.05 level.

Table 6.3 Outer mesocarp firmness of 'Line 8' cubes at different stages after submergence in different exogenous pH at 1°C

pH	Hour of Flooding	Mean[x] ± SE of Outer Mesocarp Firmness (kg)		T-test for Equality of Means	F-test for Equality of Variances
		Mature Green	Color Break		
	Initial	5.94 ± 0.20	5.32 ± 0.51	ns	ns
Buffer pH 4.5	24	6.46 ± 0.14	4.93 ± 0.67	*	ns
	48	6.47 ± 0.19	3.74 ± 0.69	**	ns
	72	5.97 ± 0.21	3.82 ± 0.77	*	**
Buffer pH 5.0	24	6.38 ± 0.31	4.57 ± 0.43	**	ns
	48	6.11 ± 0.08	3.92 ± 0.78	*	**
	72	5.96 ± 0.22	3.50 ± 0.58	**	ns
Water	24	6.86 ± 0.16	4.90 ± 0.78	ns	**
	48	7.25 ± 0.15	4.49 ± 0.77	*	*
	72	6.99 ± 0.32	5.06 ± 0.63	*	ns
Buffer pH 5.5	24	6.59 ± 0.32	4.54 ± 0.68	*	ns
	48	6.19 ± 0.14	4.14 ± 0.55	*	**
	72	6.65 ± 0.24	3.43 ± 0.89	*	**
Buffer pH 6.0	24	6.37 ± 0.25	4.45 ± 0.64	*	ns
	48	6.28 ± 0.08	4.36 ± 0.60	*	**
	72	6.32 ± 0.20	3.65 ± 0.76	*	**

[x] = Mean and stand error (SE) were calculated from six replicates for mature green stage and from five replicates for color break stage

* = Means or variances at the same row were significant different at 0.05 level.

** = Means or variances at the same row were significant different at 0.01 level.

ns = Means or variances at the same row were not significant different at 0.05 level.

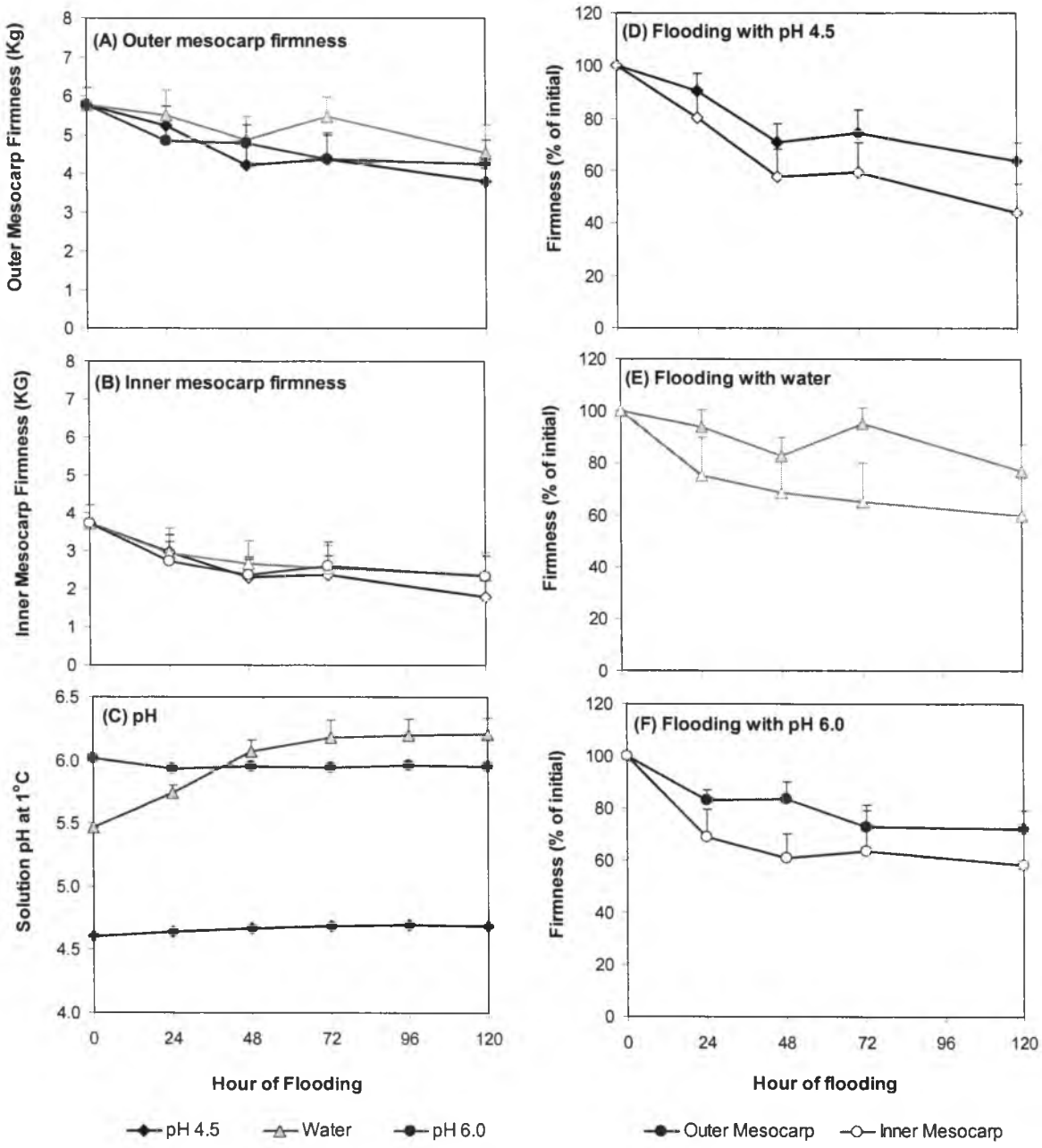


Figure 6.5 Mesocarp firmness of color break 'Line 8' cubes flooded with different pH of citrate buffers and distilled water at 1°C, and acidity changes of the solutions during flooding. Markers were the mean + SE of seven replicates (fruit).

Table 6.4 Outer and inner mesocarp firmness of color break 'Line 8' cubes responding to exogenous pH at 1°C

pH	Hour of Flooding	Mean[x] ± SE of Mesocarp Firmness (% of initial)		T-test for Equality of Means	F-test for Equality of Variances
		Outer	Inner		
	Initial (Kg)	5.75 ± 0.45	3.72 ± 0.49	-	-
	Initial (%)	100	100	-	-
Buffer pH 4.5	24	91 ± 6	80 ± 10	ns	ns
	48	71 ± 7	58 ± 11	ns	ns
	72	75 ± 9	59 ± 12	ns	ns
	120	64 ± 7	44 ± 11	ns	ns
Water	24	94 ± 7	75 ± 14	ns	*
	48	83 ± 7	68 ± 15	ns	ns
	72	95 ± 6	65 ± 15	ns (p=0.095)	*
	120	77 ± 10	60 ± 15	ns	ns
Buffer pH 6.0	24	83 ± 4	69 ± 11	ns	*
	48	83 ± 7	61 ± 9	ns (p=0.076)	ns
	72	73 ± 9	64 ± 15	ns	ns
	120	72 ± 7	58 ± 12	ns	ns

[x] = Mean and stand error (SE) were calculated from seven replicates.

* = Means or variances at the same row were significant different at 0.05 level.

** = Means or variances at the same row were significant different at 0.01 level.

ns = Means or variances at the same row were not significant different at 0.05 level.

6.4 Discussion

'Line 8' and 'Sunset' fruit had similar mesocarp pH at the color break stage (pH 5.6-5.7) and showed a marked reduction in pH that coincided with fruit ripening and softening. The patterns of mesocarp pH change in this experiment agree with that of the juice pH changes in papaya cv. 'Exp.15' (Proulx *et al.*, 2005), and consistent with apoplastic pH change in tomato (Almeida and Huber, 1999). 'Sunset' papaya following 1-MCP treatment did not show a reduction in mesocarp pH during ripening. Ethylene has been shown to promote fast net H^+ extrusion, causing apoplastic acidification in petiole of a semi-aquatic dicot (Vreeburg *et al.*, 2005). The acidification following submergence of this plant was inhibited by pre-treatment of 1-MCP (Vreeburg *et al.*, 2005). The stability of mesocarp pH in 1-MCP-treated papaya may reflect the effect of the suppression in H^+ extrusion to the cell wall.

To determine whether lowering the mesocarp pH causes a major effect on softening, papaya mesocarp cubes were submerged in citrate buffer at various pH. Buffer solutions and the distilled water control did change their pH to track the pH of the papaya mesocarp and the responses were similar in every experiment and consistent with the result in 'Sunset' papaya (data not shown) and other plants (Ugalde *et al.*, 1988; Ballarrin-Denti and Antoniotti, 1991). This pH tracking may explain the result of re-equilibration of the system pH by the fruit tissue (Almeida and Huber, 1999). The activity of ATPase which transport H^+ ions to intercellular space, has also been reported to increase during fruit ripening (Ben-Arie and Faust, 1980; Lurie and Ben-Arie, 1983) and could influence cellular pH. The evidence presented here supports a conclusion that fruit mesocarp pH of is highly regulated and could possibly involve ATPase.

Firmness of mesocarp cubes increased during early submergence, especially the mesocarp cubes in distilled water. The mesocarp firmness change during submergence may represent the combined effect of the applied pH and the water potential differential between mesocarp cells and the applied solution. Incubating papaya mesocarp cubes in distilled water showed the greatest increase in mesocarp due possibly to the water has nearly zero water potential and the buffers and mesocarp cells have water potential less than 0. If the water potential of the applied solution is higher than the water potential of mesocarp cells, water will flow from the solution into the cells and the cell turgor will increase. Buffer solutions used in this study had similar concentration and chemicals therefore would be expected to have similar water potential. Difference in firmness of mesocarp cubes in the buffer at different pH may be considered as the result of pH effect rather than the result of water potential differential. However, since citrate was a chelator, interaction with Ca^{2+} ions on the Ca^{2+} ion-bridge of pectin structure may facilitate mesocarp softening at all pHs tested. This chelation by citrate buffer may explain the small difference in mesocarp firmness responding to different pH buffers.

The effect of pH on mesocarp firmness varied depending on the fruit stage at harvest and tissue used. During incubation, the inner mesocarp of color break papaya tended to respond to pH faster than outer mesocarp, and color break papaya softened faster than mature green papaya. Mature green papaya showed significant difference in their response to different exogenous pH buffers but the color break papaya softened very fast and did not show significant differences between the exogenous pH buffers. Softening of color break papaya cubes during submergence was possibly due to various mesocarp factors, and these factors had an effect on softening greater than the effect of applied pH. In mature green papaya, buffers at pH 4.5 and pH 5.0 caused the mesocarp

cubes to soften faster at the room temperature than at 1°C, while buffers at pH 5.5 and pH 6.0 did not show a significant difference in mesocarp firmness between the two incubation temperatures. Mesocarp cubes submerged in buffers at pH 4.5 and pH 5.0 had a lower firmness than those in buffer at pH 6.0. The lower pH may facilitate softening-associated enzymes in the fruit that react faster at the high temperature than at the low temperature, and showed optimum activity at the lower pH (4.5-5.0) rather than at the higher pH (6.0). Cell wall enzymes and proteins whose optimum activity occurs at the low pH include endoxylanase (Labavitch and Greve, 1983), exo-β-galactanase (Carey *et al.*, 1995), polygalacturonase (Chun and Huber, 1998), β-(1,3)-glucanase (Peumans *et al.*, 2000) and expansin (McQueen-Mason *et al.*, 1992).

Papaya mesocarp pH was highly regulated and the decline in pH during papaya ripening was correlated with fruit softening. The decline in pH during ripening may facilitate cell wall enzymes involving in papaya softening, rather than solely cause fruit softening.

CHAPTER 7

SUMMARY

Papaya cell wall modification is involved in softening during ripening. 'Line 8' and 'Sunset' fruit lost greater than one-half of the cell wall mass during ripening. This significant reduction of mesocarp cell wall mass occurred in the CDTA-soluble fractions, Na_2CO_3-soluble fractions, and the cellulose fraction. Beside uronic acids, galactosyl and xylosyl components of mesocarp cell wall showed significant decline from the mesocarp cell wall. The modifications of cell wall polysaccharides containing xylosyl and galactose seemed to play a significant role in normal softening of both papaya lines. Treatment with 1-MCP altered the papaya softening pattern and caused incomplete softening. The 1-MCP-treated papaya showed that although the major changes occurred in galactosyl components of the mesocarp cell wall comparable to that in untreated control papaya; the 1-MCP-treated papaya did not soften completely. The changes in the 1-MCP-trearted papaya suggested that modification of the xylosyl containing components of the cell wall was involved in papaya mesocarp softening. The changes in the fractions containing xylosyl residue in 1-MCP treatment included less solubilization of xylosyl residues, and a higher association of xylosyl residues to pectic polysaccharides of the middle lamella and to loosely bound matrix polysaccharides.

The activities of PG, PME, β-galctosidase, endoglucanase, endoxylanase, and xylosidase were detected during ripening. Hydrolases activity, except for PG, was correlated with normal softening. When softening was modified by 1-MCP treatment, the papaya showed a delayed rises in some hydrolases' activities. However, only endoxylanase activity was completely suppressed throughout ripening of 1-MCP-treated

papaya. The failure of 1-MCP-treated papaya to soften completely was possibly associated with a selective suppression of endoxylanase activity. Papaya softening, like other fruit, is a complex event that involves many cell wall hydrolases, such as endoxylanase, xylosidase, β-galactosidase and endoglucanase. These hydrolases may play their roles in concert, to provide the unique texture of a particular fruit.

During softening, changes in mesocarp pH are implicated. Mesocarp pH of 'Line 8' and 'Sunset' fruit declined from about 6 at the color break stage to about 5 when fruit started to soften. When treated with 1-MCP a similar reduction in mesocarp pH did not occurred during ripening. The reduction in pH during ripening was correlated with softening in the two papaya lines. Buffer with various pH were applied to papaya mesocarp cubes and showed that mesocarp pH was highly regulated. Exogenous pH at 4.5 tended to cause papaya mesocarp cubes to soften faster than exogenous pH at 6.0 and 5.5. Effect of the low pH on mesocarp firmness varied with the papaya stages at harvest and the tissues used. The pH reduction during papaya ripening may facilitate enzymes in papaya softening, rather than solely affect fruit softening.

Endoxylanase activity was highly correlated with normal softening of papaya and highly suppressed when softening was restrained by 1-MCP. The cell walls of papaya mesocarp showed a marked change in xylosyl components during fruit softening and showed a marked alteration of this change when softening was modified by 1-MCP treatment. The results emphasize the conclusion that endoxylanase plays a major role in papaya softening.

Further research needs to be conducted to confirm that endoxylanse is the major cause in papaya fruit softening. Purified papaya endoxylanase enzyme affects on

mesocarp firmness, *in vitro*, needs to be conducted. *In vivo* suppression of papaya endoxylanase activity by antisense endoxylanase gene or silencing of endoxylanase RNA will provide a clear picture of its role in fruit softening. Information on apoplastic pH of papaya mesocarp during ripening is necessary to obtain the actual activity of hydrolases at the cell walls.

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APPENDIX I

Extraction of Cell Wall Polysaccharides

Cell Wall Material (CWM) 0.25 g

CWM was added in 25 ml of de-ionized water and shaken at 150 rpm for 1 hr at room temperature. Mixture was centrifuged at 14,300 rpm for 10 min and supernatant filtered through a glass paper (Whatman GF/C) under aspiration. Residue was re-extracted.

Combine the two filtrates * = Water-soluble fraction

Residue was mixed in 25 ml of 0.05 M CDTA (pH 6.5) and shaken at 150 rpm for 6 hr at room temperature. Mixture was centrifuged at 14,300 rpm for 20 min and supernatant filtered through a glass paper under aspiration.

Filtrate 1

Residue was washed with 20 ml of distilled water and centrifuged at 14,300 rpm for 20 min. Supernatant was filtered through a glass paper under aspiration.

Filtrate 2 Combine Filtrate 1 and 2 * = CDTA_1-soluble fraction

Residue was mixed in 25 ml of 0.05 M CDTA (pH 6.5) and shaken at 150 rpm for 2 hr at room temperature. Mixture was centrifuged at 14,300 rpm for 20 min and supernatant filtered through a glass paper under aspiration.

Filtrate 3

Residue was washed with 20 ml distilled water and centrifuged at 14,300 rpm for 20 min. Supernatant was filtered through a glass paper under aspiration.

Filtate 4 Combine Filtrate 3 and 4 * = CDTA_2-soluble fraction

Residue was mixed in 25 ml of 0.05 M Na_2CO_3 + 20 mM $NaBH_4$ solution and shaken at 30% rotation for 16-19 hr at 1 °C. Mixture was centrifuged at 14,300 rpm for 20 min and supernatant filtered through a glass paper under aspiration.

Filtrate 5

Residue was washed with 20 ml distilled water and centrifuged at 14,300 rpm for 20 min. Supernatant was filtered through a glass paper under aspiration.

Filtrate 6 Combine Filtrate 5 and 6 [p] * = $NaCO_3$_1-soluble fraction

Residue was mixed in 25 ml of 0.05 M Na_2CO_3 + 20 mM $NaBH_4$ solution and shaken at 150 rpm for 3 hr at room temperature. Mixture was centrifuged at 14,300 rpm for 20 min and supernatant filtered through a glass paper under aspiration.

Supernatant 7

Residue was washed with 20 ml of distilled water and centrifuged at 14,300 rpm for 20 min. Supernatant was filtered through a glass paper under aspiration.

Filtrate 8 Combine Filtrate 7 and 8 [p] * = $NaCO_3$_rm-soluble fraction

(To be continued)

Residue was mixed in 25 ml of 1 M KOH + 10 mM $NaBH_4$ solution[n] and shaken at 30% rotation for 2 hr at 1 °C under N_2. Mixture was filtered through a glass paper under aspiration.

Filtrate 9 [p] * = 1M KOH_1 fraction

Residue was mixed in 25 ml of 1 M KOH + 10 mM $NaBH_4$ solution[n] and shaken at 150 rpm for 2 hr at room temperature under N_2. Mixture was filtered through a glass paper under aspiration.

Filtrate 10 [p] * = 1M KOH_2-soluble fraction

Residue was mixed in 25 ml of 4 M KOH + 10 mM $NaBH_4$ solution[n] and shaken at 150 rpm for 2 hr at room temperature under N_2. Mixture was filtered through a glass paper under aspiration.

Filtrate 11 [p] * = 4M KOH_1-soluble fraction

Residue was mixed in 25 ml of 4 M KOH + 10 mM $NaBH_4$ + 4% Boric acid solution[n] and shaken at 150 rpm for 2 hr at room temperature under N_2. Mixture was filtered through a glass paper under aspiration.

Filtrate 12 [p] * = 4M KOH_2-soluble fraction

Residue was washed with 2 liters of distilled water under aspiration.

Residue = Cellulose Fraction

Note:

[p] = Filtrate was adjusted to pH 5.0 by glacial acetic acid on ice.

* = Filtrate was recorded for final volume and collected 2 ml for starch, total sugar, and uronic acid analysis. The rest of filtrate was dialyzed in 3500 MW cut off dialysis tubing with at least 5 changes of distilled water, and lyophilized.

[n] = KOH solutions were prepared with distilled water that had been boiled for at least 10 min, flushed with N_2, and cooled under N_2. The solutions were prepared within a day before use and kept in a seal bottle under N_2.

APPENDIX II

Table of Critical Values for Pearson Product-Moment Correlation Coefficient

df = N-2 (N = number of pairs of data)	Level of significance for two-tailed test			
	.10	.05	.02	.01
1	.988	.997	.9995	.9999
2	.900	.950	.980	.990
3	.805	.878	.934	.959
4	.729	.811	.882	.917
5	.669	.754	.833	.874
6	.622	.707	.789	.834
7	.582	.666	.750	.798
8	.549	.632	.716	.765
9	.521	.602	.685	.735
10	.497	.576	.658	.708
11	.476	.553	.634	.684
12	.458	.532	.612	.661
13	.441	.514	.592	.641
14	.426	.497	.574	.628
15	.412	.482	.558	.606
16	.400	.468	.542	.590
17	.389	.456	.528	.575
18	.378	.444	.516	.561
19	.369	.433	.503	.549
20	.360	.423	.492	.537
21	.352	.413	.482	.526
22	.344	.404	.472	.515
23	.337	.396	.462	.505
24	.330	.388	.453	.495
25	.323	.381	.445	.487
26	.317	.374	.437	.479
27	.311	.367	.430	.471
28	.306	.361	.423	.463
29	.301	.355	.416	.456
30	.296	.349	.409	.449
35	.275	.325	.381	.418
40	.257	.304	.358	.393
45	.243	.288	.338	.372
50	.231	.273	.322	.354
60	.211	.250	.295	.325
70	.195	.232	.274	.302
80	.183	.217	.256	.284
90	.173	.205	.242	.267
100	.164	.195	.230	.254

Source: http://physics.mercer.edu/Younce/pearson.html