EFFECT OF PECTINMETHYLESTERASE AND CALCIUM INFUSION ON TEXTURE AND STRUCTURE OF FRUITS AND VEGETABLES

by

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(Under the Direction of Louise Wicker)

ABSTRACT

Processed fruits and vegetables are susceptible to textural quality changes and excessive softening. The overall objectives were to improve quality and to evaluate analytical methods to quantify the effect of pectinmethylesterase (PME) infusion. Strawberry, mango and eggplants were infused with plant or fungal PME with or without $CaCl_2$, using vacuum. In strawberry, a print technique was developed to visualize PME activity after infusion. Although PME activity in infused fruit was about twice that of control fruit, no significant effects on firmness were found. Firmness of PME infused fruit was about twice that of water infused controls. Pectin, as water soluble pectin (WSP), was about twice that of chelator soluble pectin (CSP) or alkaline soluble pectin (ASP). In strawberry, mango and eggplant, PME activity increased, and % degree of esterification was changed, but did not correlate with the amount of infused PME. A putative inhibitor of PME was identified. WSP and alkaline ASP were the major pectic fractions in strawberry and mango, respectively. In mango, temperature gradient infusion with Valencia orange PME and/or $CaCl_2$ increased gumminess and chewiness, but had no impact on hardness and adhesiveness. In eggplant, the firmness of fruits infused with a commercial fungal or Marsh grapefruit (MGF) PME was significantly increased compared to controls, immediately after

treatment and after storage for 7 days at 4 °C. Ion-exchange chromatography revealed that water soluble pectin from fungal and MGF PME and/or $CaCl_2$ infused eggplant had greater charge density than pectin from control eggplant. Cryo-scanning electron microscopy showed that treated samples with fungal PME/$CaCl_2$ had greater cell to cell integrity than water-infused control. JIM5 (antibody to low-esterified pectin) and JIM7 (antibody to high-esterified pectin) labeled cell walls of non-infused control tissues. JIM5 showed more binding than JIM7 with cell walls of eggplant tissues from fungal PME/$CaCl_2$ treatment. From the results of this study, factors that influence effectiveness of PME infusion and textural changes include source of PME, pH, endogenous inhibitors, porosity of commodity, type of vacuum process, cell wall composition of commodity, and variability in raw materials. In addition, choice of PME and process treatments must be optimized for each commodity.

INDEX WORDS: Vacuum infusion, Pectinmethylesterase, Pectin, Calcium chloride, Strawberry, Mango, Eggplant, Firmness, Enzyme activity, Degree of esterification, Cryo-SEM, JIM5 and JIM7 antibodies.

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DEDICATION

I gratefully dedicate this dissertation to my family and friends for their love, support and encouragement.

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CHAPTER 1

INTRODUCTION

Texture, one of the sensory quality attributes appreciated by the consumer, plays an important role in the quality of fruits and vegetables. One major problem in processing fruits is their susceptibility to textural change during harvesting and handling. The changes in some fleshy fruits appear as tissue softening and loss of cohesiveness, as well as a decrease in the extent of intermolecular bonding between cell wall polymers (Van Buren 1979). Textural changes of fruit during ripening and senescence can arise from a loss of turgor or degradation of starch. However, modification of the fruit cell wall is frequently attributed to the enzymatic degradation of cell wall materials (Tucker and Grierson 1987; Hulme 1970). Several studies have been done on textural change, such as the effects of ripening, storage and cooking on the alteration of cell walls, particularly directed to changes in pectic substances (Bartolome and Hoff 1972; Chang and others 1993).

Pectic substances consist of a group of closely associated polysaccharides from the primary cell wall and intercellular regions of higher plants (Van Buren 1979). Pectin is a linear chain of α- (1$\rightarrow$4)-linked D-galacturonic acid unit backbone in which varying proportions of acid groups are methyl esterified. This homogalacturonan backbone is occasionally interrupted by rhamnose-rich regions, which can be highly substituted with neutral sugar-rich side chains, usually referred to as 'hairy,' or ramified, regions. The homogalacturonan regions are often referred to as 'smooth' regions (Daas and others 2000). The firming effect of pectin in tissues involves two separate phenomena; in fresh tissue, the formation of free carboxyl groups

increases the potential for the strength of calcium binding between pectic polymers, and in heated tissue there is a combination of increased calcium binding and a decrease in the susceptibility of the pectin to depolymerization by β–elimination (Sajjaanantakul and others 1989)

Pectic enzymes are closely correlated with increases in softening in many ripening fruits and vegetables along with the concurrent increase in soluble pectins. Polygalacturonase (PG) and pectinmethylesterase (PME) are the principal enzymes participating in pectin degradation (Kays 1997). During ripening, PME removes methyl ester groups from the cell wall pectin constituents, which are accessible to depolymerisation by PG, reducing intercellular adhesiveness and tissue rigidity (Alonso and others 1997). The PG activity correlates with the tissue softening of tomato (Hobson 1964), pear (Ahmed and Labavitch 1980) and peach (Pressey and others 1971), with the exclusion of strawberry, which softens rapidly without apparent PG activity (Neal 1965; Barnes and Patchett 1976). Although PME has a detrimental effect in ripening fruits, PME is also postulated to increase firmness of fruits and vegetables via demethylation of endogenous pectin and subsequent chelation of divalent cations by ionized carboxyl groups on adjacent pectic acids (Saurel 2002). PME is widely distributed in plants and microorganisms (Rexova-Benkova and Markovic 1976). In general, plant and bacterial PMEs have pH optima between pH 6 and 8, whereas some fungal PMEs have optima between pH 4 and 6. In the mode of action, plant PMEs generally remove blocks of methyl groups on a single chain, while fungal PMEs attack methyl groups on the pectin randomly, resulting in a random distribution of the unmethylated galacturonic acid groups (Benen and others 2003a). Plant PME has been extracted and/or purified from differences sources, including tomatoes (Giovane and others 1994), oranges (Versteeg and others 1978; Hou and others 1997), apples (Macdonald and

Evans 1996; Denes and others 2000), and grapefruits (Seymour and others 1991). Although PME occurs naturally in many fruits and vegetables (edogenous enzymes), they are also added as processing aids (exogenous enzymes). Exogenous PMEs are mostly derived from food-grade fungi, such as, *Aspergillus niger, A. aculeatus, A oryzae* (Benen and others 2003b). To preserve the firmness of fresh or processed fruits and vegetables, it is sometimes necessary to pretreat to modify the product's structure (Suutarinen and others 2000). Several studies have been done on maintaining or increasing the firmness of fresh and processed fruits or vegetables involving pretreatment with calcium, various other firming agents, or PME.

Calcium plays an important role in maintaining the quality of fruits and vegetables (Poovaiah 1986). Calcium may have two opposite effects on texture. Calcium firms the tissue via complexed formation with pectic substances; in addition, it enhances tissue softening by β - elimination. However, the net result of calcium addition has invariably been to firm the tissue (Van Buren 1979). The optimum concentrations of calcium, between 5 and 25 mM, can activate PME activity, but higher calcium concentrations have an inhibitory effect (Rexova-Benkova and Markovic 1976). Morris and others (1985) found that the use of 0.5% calcium lactate dip treatments increases firmness in sliced strawberries. One and two percent calcium lactate improved the firmness of overripe strawberry fruit, as indicated by shear-press values after freezing and thermal processing (Main and others 1986). Morris and others (1991) also revealed that the greatest firming effect on frozen-then-thawed strawberries was achieved with 0.18% calcium and 0.3% low methoxyl pectin dip treatment. The effect was greater with sliced fruit than whole fruit.

Vacuum technique is considered to be a pretreatment for processed fruit or vegetables, leading to improvement in their quality by active incorporation of functional ingredients in the

product structure (Saurel 2002; Baker and Wicker. 1996.). Ferguson and Malick (1983) showed that infusion with microcrystalline cellulose (Avicel) increased firmness and reduced weight loss during canning of mushrooms. In a study of Ponappa and others (1993), vacuum infiltration with polyamines increased firmness of strawberry slices under various storage conditions. In the presence of calcium, the firming effect is proportional to the PME activity preceding the thermal treatment and can reinforce by vacuum- assisted infusion of exogenous PME (Saurel 2002). Javeri and others (1991) reported that infusion under vacuum with Marsh grapefruit PME (14-18 Unit/ml) and calcium chloride (0.01%, w/v) showed significantly increased firmness in blanched peach halves after thermal processing nearly four times when compared to non-infused control. According to Suutarinen and others (2002), jam made from strawberries treated with 1%$CaCl_2$ and fungal (*Aspergillus oryzae*) PME (100,000 nkat=6000 Unit/ml) under vacuum had the highest firmness as compared to the control or other pretreated jam strawberries. Recently, Degraeve and others (2003) also showed that the firmness of strawberries was maximal after impregnation in solution containing commercial *Aspergillus niger* PME (0.12% w/w) and/or calcium (0.5% w/w). However, fruits and vegetables with a relatively impenetrable skin, such as cherries, blueberries, peas, and corn, are more difficult to infuse, and results are variable. In particular, infusion with commercial pectinolytic and cellulytic enzymes in carrot dices did not improve either their dehydration or subsequent rehydration properties (Baker and Wicker 1996).

The microscopic structure and molecular architecture of the cell wall has an important bearing on its function (Suutarinen and others 1998). Several techniques have been developed to locate the distribution of pectic polymers and enzymes, such as PG and PME, in the cell wall of fruits and vegetables. Ruthenium red is a common staining method used to microscopically visualize pectic substances. However, the reliability of ruthenium red is minimal because it is

not specific for pectin, and, in the case of tissue fixation, results in inconsistent staining (Albersheim and others 1960). According to Reeve (1958), hydroxylamine-$FeCl_3$ staining of light microscope slides can localize esterified pectins microscopically because of its specificity for high methoxyl pectic substances. Delincee (1976) developed the print technique, which employed a paper impregnated with pectin at a suitable pH for detecting PME in the thin-layer isoelectric focusing. Several biochemical and immunological techniques, including tissue-printing (Tieman and Handa 1989) and immunocytochemistry at the ultrastructure level (Steele and others 1997), have been studied. Knox and others (1990) used monoclonal antibodies for un-esterified pectin (JIM5), with the degree of esterification (DE) above 35% and methyl-esterified (JIM7) with the range of 35 to 90% DE, to detect pectin in the root apex of carrot. The JIM5 and JIM7 antibodies were used in localization of pectin in kiwi (Sutherland and others 1999) and tomato fruit (Blumer and others 2000) at different stages of ripening. In a study of Parker and others (2001), JIM5 and JIM7 were also used to investigate the distribution of pectic polysaccharides at the potato surface of separated cells. To study the effects of blanching treatment in carrots, Lo and others (2002) used JIM7 antibody to locate the pectin distribution in the cell wall of blanched carrots.

Enzyme infusion is one new process development used to alter the textural features of fruit and vegetable products. Many aspects of this methodology, however, need clarification and enhancement, especially basic studies of the movement of enzymes with tissues (McArdle and Culver 1994).

The overall objectives of the following experiments were to improve quality of fruits and vegetables using PME and to evaluate analytical methods to quantify the effect of PME infusion. The objectives of the first part were to develop a rapid method to visualize the location of PME

in fruit after vacuum infusion and to study the effect on pectic substance and texture. The second one was to evaluate infusion of exogenous PME into mango, to determine PME activity in tissue, and to determine the effect on textural properties of mango. The last one were to compare the textural changes in eggplants using fungal and plant PME and use JIM5 and JIM7 antibodies to determine the change of pectin in cell wall of infused eggplants.

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CHAPTER 2

LITERATURE REVIEW

Fruit and Vegetable

Fruits and vegetables are derived from living organisms, which are composed of cells. The structure of these cells is one of the major contributors to the characteristic texture of food (Christiansen 1984). Most fruits and vegetables are composed of the edible parts of plants. The word 'fruit' has a different meaning to food technologists and botanists. To a food technologist, fruits are the fleshly tissues of plant origin that are eaten. However, to a botanist, fruits are usually regarded as the reproductive organs of plants, containing the seed-bearing structure of flowering plants (Bourne 1983). Vegetables are generally classified as the nonreproductive parts of plants, such as roots, leaves, or stems (Edwards 1999). Almost any part of a growing herbaceous plant may be used as a vegetable, including bulbs (onion), immature flowers (cauliflower), mature fruits (tomato), immature fruits (cucumber), leaves (celery, lettuce), roots (carrot), stem (asparagus), and tubers (potato). However, the distinction between a fruit and a vegetable is often unclear (Bourne 1983, Edwards 1999). For example, watermelon and cantaloupe are considered to be vegetable crops although they are customarily eaten for dessert and botanically would be classified as fruits (Bourne 1983).

Plant anatomy and cell structure

The edible parts of fruits and vegetables are composed predominantly of fleshy parenchyma cells (Figure 2.1). The individual parenchyma cells, bound by a semipermeable

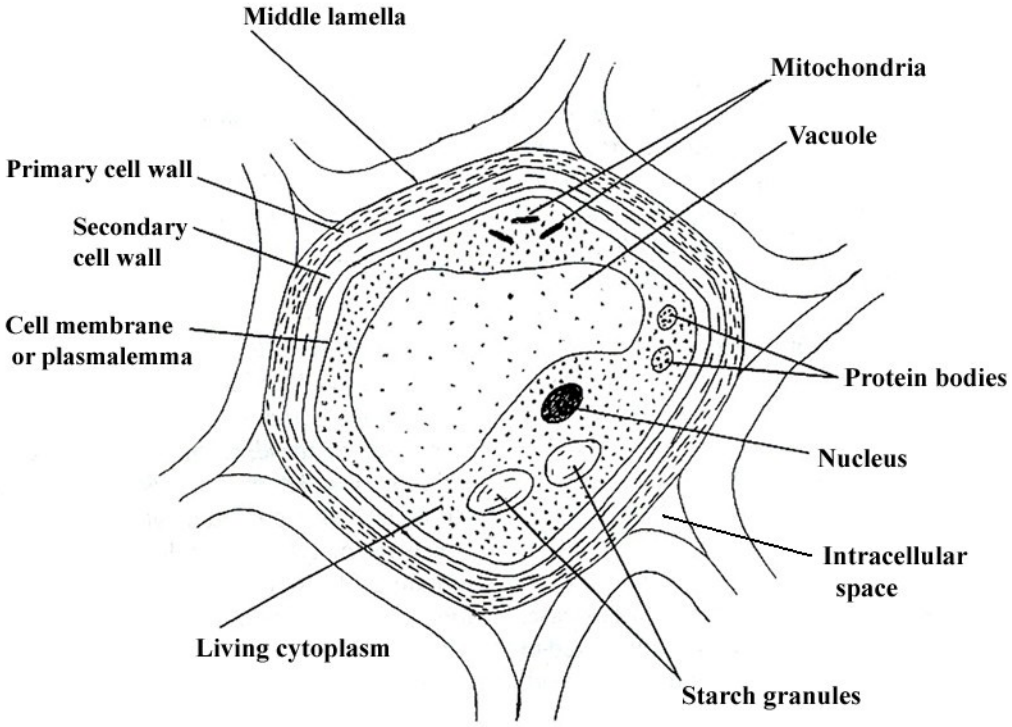

Figure 2.1. Diagram showing the main structure feature of a plant cell (Edwards 1999)

membrane (plasmalemma), are separated by a morphologically distinct region known as middle lamella or the interlamella layers (Edwards 1999). Parenchyma cells contain the cytoplasm of the cell, which in turn contains the cell nucleus, mitochondria, and other organelles, as well as the cell vacuole. The vacuole is surrounded by a membrane, and is filled with a watery solution of sugars, acids, and salts called the 'cell sap.' The physiological processes within the living parenchyma cell enables it to absorb water, generating hydrostatic pressure called turgor pressure. It causes the vacuoles to enlarge and press tightly against one another imparting turgidity, rigidity, and crispness to the plant tissues (Bourne 1983). When turgor pressure is lost, the structure collapses to a greater or lesser degree, as illustrated in a plant that wilts for lack of water (Edwards 1999).

Exterior to the plasmalemma are the primary cell wall and middle lamella, both of which contain polysaccharides as well as smaller amounts of glycoproteins and phenolic compounds (Jackman and Stanley 1995). Primary cell walls are composed of cellulose microfibrils embedded in a matrix of pectic substances and hemicelluloses (Bourne 1983). Most fruits and vegetables lack a secondary cell wall, but, if present, it lies interior to the primary wall. The secondary cell walls also consist of hemicellulose and lignin, which are associated with the development of 'woodiness,' such as is seen in asparagus (Jackman and Stanley 1995).

Middle lamella between adjacent cells is composes mainly of heat-labile pectic substances and constitutes the outer layer of the cell wall. The middle lamella acts like an adhesive and bears some of the compressive or tensile stress, but readily transmits shear. Much of the texture of plant foods can be attribute to the structural integrity of the primary cell wall and the middle lamella, as well as to the turgor generated within cells by osmosis (Jackman and Stanley 1995). The interlamella layer consists principally of the calcium salts of polymers of

galacturonic acid that have been partially esterified with methyl alcohol, and known as the pectic material. The degree of polymerization and esterification of the polygalacturonide chains and the amount of crosslinking of adjacent pectin molecules by salt bridge formation have profound effects on the physical properties of the middle lamella as well as the overall textural properties of fruits and vegetables (Bourne 1983). In green fruits the pectic material is principally in the form of partially esterified polygalacturonic acid of very high molecular weight called protopectin (water insoluble pectic substances) (Joslyn 1962). It imparts great strength to the tissue. As the fruit ripens, the chain length of the pectin polymer decreases, forming water soluble pectin, which is not as strong as the protopectin, and the structure becomes increasingly soft and, eventually, mushy.

Texture is modified by the presence of other cells. These include supporting cells (collenchyma cells) and conducting cells. The conducting cells are long tube-shaped structures that transport food (phloem cells) and water (xylem cells) throughout the tissues. Collenchyma cells are thick-walled cells that take the form of fibers. These supporting and conducting cells form an interconnected branching network throughout the fleshy parenchyma tissues. The texture of the conducting tissues stands out in fibrous celery, asparagus, and mango (Bourne 1983).

Fruits and vegetables are covered with one layer of epidermal cells, and often several layers of hypodermal cells, that constitute the skin and protect the underlying tissues. The epidermal layer may be very thin (lettuce, strawberry), moderately thick (cherry), or very thick (grapefruit, watermelon). The epidermal cells excrete a wax (cuticle wax) that helps protect the surface and retard water loss. For example, this wax may be thin and unnoticeable as in strawberry, or may be quite prominent as in apples and pears (Bourne 1983). The texture of

fruits and vegetables is affected by traits such as cellular organelles and biochemical constituents, water content or turgor, and cell wall composition (Sams 1999).

Methods for measuring texture

Puncture test

Puncture tests consist of measuring of the force required to push a punch or probe into a food. Many hand-operated devices are available for this test, such as Magness-Taylor, Effegi, Ballauf, and Chatillon (Bourne 1982). In origin, these devices are used by plant bleeders to evaluate fruit and vegetable maturity. Thereafter, they are used for a wide range of purposes such as assessment of the penetration force for potato tubers, skin development in tomatoes, resistance of cherries, blackberries, and raspberries to mechanical injury (Edwards 1999).

The process of driving a probe into fruit and vegetable tissues produces a combination of shear and compression stresses. The shear force is associated with the perimeter of the probe, while the compression force is associated with the cross-sectional area of the probe contact surface (Bourne 1975). Yang and Mohsenin (1974) studied the mechanics of puncturing fruits using cylindrical probes with slightly convex tips. The results indicate that probe size, shape, radius of curvature, and the mechanical properties of fruit influence the overall puncture force. Qurecky and Bourne (1968) used a star-shaped probe to determine the different tissue zones (skin toughness, flesh firmness, and core tissue resistance) of a whole strawberry. Puncture tests with small-diameter probes (3.15 mm diameter) can be applied in different tissue zones of slice cucumbers for firmness measurement (Thompson and others 1982). According to Bourne (1975), if the diameter of the probe is less than 2 mm, it will deviate from expected area-dependent and perimeter-dependent characteristics of large probes. In a study of strawberry fruit firmness by Hietaranta and Linna (1999), they recommended using of a large cylindrical probe

(6.4 mm dia) to measure firmness when the measured forces are very small, since the 6.4-mm probe increased the forces sufficiently for as compared to a small probe. Wu and Abbott (2002) measured firmness of outer pericarp and columellar tissue on 7-mm thick tomato slices. They indicated that a 4-mm flat-faced cylindrical probe provided more consistent firmness measurement than a 6.4-mm spherical probe.

Texture profile analysis

The texture profile analysis (TPA) was first developed by the General Foods Cooperation Technical Center using the General Foods (GF) Texturometer (Friedman and others 1963). TPA comprises the compressing a bite-size piece of food two times in a reciprocating motion that imitates the action of the jaw, then extracting from the resulting force-time curve a number of textural parameters that correlate well with sensory evaluation of those parameters (Friedman and others 1963; Szczesniak and others 1963). In 1968, Bourne was first to adapt the Instron machine to determine a modified TPA of pears. Bourne defines the individual parameter of Friedman and others (1963) fairly closely, except in the definition of cohesiveness. According to a typical TPA curve from a GF Texturometer (Figure 2.2), cohesiveness is obtained from measuring the total areas under the curve (A2+A2*/A1+A1*). In contrast, Bourne (1968) measured the area under the compression portion only (A2/A1) and excluded the area under the decompression portions (Figure 2.2). Consequently, the definition of seven textural parameters for TPA in this review, five measured and two calculated, is based on both Bourne (1978) and Szczesniak (1975) as follows (Figure 2.2):

(1). **Fracturability** (originally called brittleness) is defined as the force at the first significant break in the curve.

(2). **Hardness** is defined as the peak force during the first compression cycle (first bite).

(3). **Cohesiveness** is defined as the ratio of the positive force during the second compression portion to that during the first compression (A2/A1), excluding the area under the decompression portion in each cycle.

(4). **Adhesiveness** is defined as the negative force area for the first bite, representing the work necessary to pull the plunger away from the food sample.

(5). **Springiness** (originally called elasticity) is defined as the height that the food recovers during the time that elapses between the end of the first bite and the start of the second bite.

(6). **Gumminess** is defined as the product of hardness x cohesiveness.

(7). **Chewiness** is defined as the product of gumminess x springiness (which equals to hardness x cohesiveness x springiness).

Several authors have proposed several modifications to the names or definitions. For example, 'Stickiness' is defined as the negative peak obtained during probe retraction and is to measure the stickiness of starch gels (Singh and others 2002). 'Resilience' is a measurement of how the sample recovers from deformation both in the terms of speed and forces derived. This parameter was developed from by looking more closely at the elastic recovery of the sample. 'Stringiness' is the distance the product extends during decompression before separating from the compression probe. The two latter parameters are not from the original TPA, but from the Stable Micro System of TA-XT2 Texture Analyser (Stable Micro Systems, Surrey, UK). Presently, it is possible to perform TPA tests and directly obtains all TPA parameters by means of its software, without any previous selection of curve value for calculations such as the Stable Micro System of TA-XT2 Texture Analyser (Stable Micro Systems, Surrey, UK).). To obtain TPA parameters, there are many techniques.

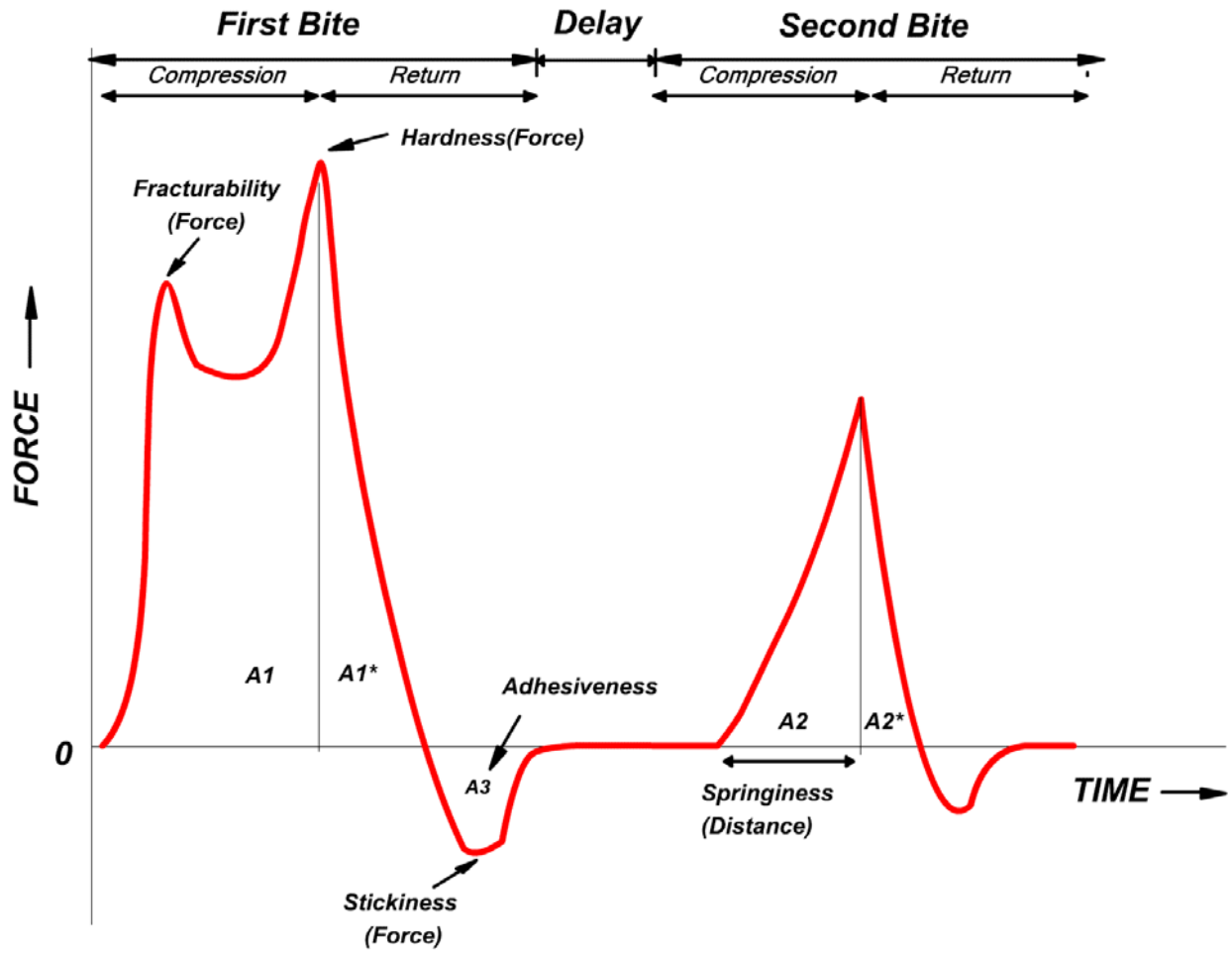

Figure 2.2. Aspect of a Texture Profile Analysis adapted from Friedman and others (1963) and Bourne (1968)

These differences may be due to experimental conditions such as sample size and shape, ratio of compressing probe size versus sample, extent of deformation, cross-head speed, number of bites, and replicates per mean value (Pons and Fiszman 1996).

The size of the compressing unit (sometimes called 'punch,' 'plunger' or 'probe') is very important, especially in relation to sample size. Most recent research done on TPA uses compression devices larger than the sample size, so that the forces registered in TPA tests are largely due to uniaxial compression forces (Pons and Fiszman 1996).

This technique has been used for a wide range of foods, including fruits and vegetables. Lee and others (1999) applied TPA to relate descriptive analyses of processed diced tomatoes. Processed tomatoes were separated according to the processing method by using firmness variables, such as hot-fill, and aseptic samples were grouped in the region of cohesiveness (TPA measurement), chewy (sensory), and metallic variables. Ratnayake and others (1999) studied the texture of buttercup squash using TPA. The textural parameters of hardness, fracturability, and gumminess tended to decrease during storage, whereas cohesiveness, chewiness, and springiness remained unchanged.

Pectic substances

Pectic substances are a group of closely associated polysaccharides found in the cell wall of higher plants (Voragen and others 1995). They make up about one-third of the dry substances of the primary cell walls of fruits and vegetables and constitute a much greater proportion of the dry substances of the middle lamella (Van Buren 1979). Most plants contain pectin in the intercellular layer between the primary cell walls of adjoining cells. The highest concentration of pectins in the cell wall is in the middle lamella, with a gradual decrease in content from the primary cell wall toward the plasma membrane (Thakur and others 1997). The

pectic substances contribute to both the mechanical strength of the cell wall and to the adhesion between cells (Jarvis 1984). They are brought into solution more easily than other cell wall polymers and have a higher degree of chemical reactivity than do other polymeric wall compounds (Van Buren 1979).

For the nomenclature associated with pectic substances, Andersson and others (1994) used the terminology of pectic substance that conforms with the suggestion by Kertesz (1951). Protopectin stands for water insoluble pectic substances. Pectic acid is the polygalacturonic acid with low methoxyl content. Pectinic acid is galacturonic acid with a high methoxyl content. Pectin is water soluble pectinic acid of varying methylester content and degree of neutralization. In some cases, the term pectin is used instead of pectic substance.

Pectin structure

The main component of pectin is a linear chain of α–(1 →4) linkage D-galacturonic acid (GalA) units,on which varying proportions of the acid groups are present as methoxyl esters (Voragen and others 1995) (Figure 2.3). Neutral sugars, primarily L-rhamnose, are also inserted into the galacturonan backbone by joining to the reducing end of uronide with (1→2) linkages and the nonreducing end of the next uronide unit with (1→4) bonds. Rhamnose introduces a kink into the otherwise straight chain. Arabinan, galactan, or arabinogalactan side-chains are often linked (1→4) to the rhamnose (Van Buren 1991). Native pectins are considered to consist of rather pure homogalacturonan parts (poly of α–(1→4) –linked GalA residues), in addition to more complex subunits. Complex segments of pectic molecule include rhamnogalacturonan I and II (RG-I, RG-II). These highly branched parts of pectin are usually referred to as ‘hairy’, or ramified, regions. The homogalacturonan regions are often referred to as ‘smooth’ regions.

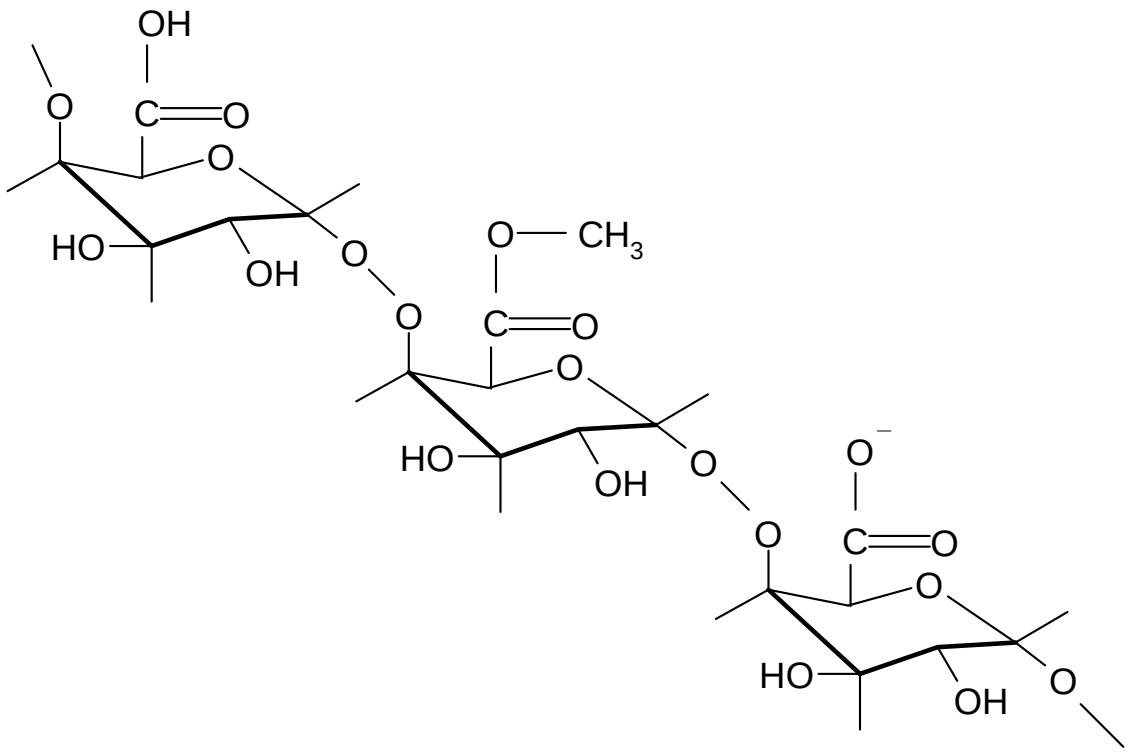

Figure 2.3. A repeating segment of the pectin molecule (Oakenfull 1991)

The proportion of ‘smooth’ to ‘hairy’ regions can vary greatly, depending on the type of tissue or its development stage (Dass and others 2000).

The esterification of galacturonic acid residues with methanol and/or acetic acid are very important structural characteristic of pectic substances. The degree of esterification (DE) is defined as the percentage of carboxyl groups esterified with methyl alcohol. Pectins with DE > 50% of the carboxyl groups in the methyl ester form (-$COOCH_3$) are called high-methoxyl (HM) pectins; those with DE < 50% are low-methoxyl (LM) pectins. When less than 10% of the carboxyl groups are methylated, it is called pectic acid (Voragen and others 1995). Generally, the %DE of pectin tissue ranges from 60% to 90 %, depending on species, tissue, and maturity (Van Buren 1991). Protopectins and water soluble pectins have slightly higher DE values than do chelator soluble pectins.

The degree of acetylation (DAc) is defined as the percentage of galacturonosyl residues esterified with an acetyl group. Degree of amidation (DA) indicates the number of amidated carboxylic groups per 100 galacturonic acid residues (Voragen and others 1995). The DE and DA values strongly influence the solubility, gel-forming ability, conditions required for gelling, gelling temperature, and gel properties of the preparation (Whistler and BeMiller 1997). Pectin content and % DE of pectin can differ among fruits and vegetables (Table 2.1 and 2.2). At present, apple pomace and citrus peels are the main sources of commercially acceptable pectin (Table 2.1). Other sources of pectins that have been considered are sugar beet and residues from the seed heads of sunflowers (Thakur and others 1997). Besides % DE, Table 2 also indicates that two procedures which are commonly used to measure degree of esterification and can give quite different results. However, regardless of the analytical procedure employed,

in no case has completely esterified or deesterified pectin been isolated from cell walls (McFeeters 1985).

Table 2.1. Pectin content of some fruits (Thakur and others 1997)

Fruit	%Pectin substances (wet weight)
Apple (*Malus spp*)	0.5-1.6
Apple pomace	1.5-2.5
Banana (*Musa acuminata* L.)	0.7-1.2
Beet pulp (Beta vulgaris)	1.0
Carambola (Averrhoa carambola)	0.66
Carrot (*Daucus carota*)	0.2-0.5
Giant granadilla (Passiflora quandrangularis L.)	0.4
Guava (Psidium quajava L.)	0.77-0.99
Lemon pulp (*Citrus limon*)	2.5-4.0
Lychee (Litchi chinesis S.)	0.42
Mango (Mangifera indica L.)	0.26-0.42
Orange peel (*C. sinesis*)	3.5-5.5
Papaya (*Carcia papaya*)	0.66-1.0
Passion fruit (*Passiflora edulis* S.)	0.5
Passion fruit rind	2.1-3.0
Peaches (*Prunus persica*)	0.1-0.9
Pineapple (*Ananas comosus* L.)	0.04-0.13
Strawberries (*Fragaria ananassa*)	0.6-0.7
Tamarind (Tamarindus indica L.)	1.71
Thimbleberry (Robus rosalfolius)	0.72
Tomato fruit (*Lycopersicon esculentum*)	0.2-0.6

Table 2.2. Degree of esterification of uronides estimated from methanol release and copper binding by the pectin fraction (McFeeters 1985)

	Methanol release	Copper binding
Apple	72[a]	81
Carrots	45	63
Cherries	36	44
Cucumber	51	73
Mango	78	79
Onions	50	78
Papaya	56	68
Pear	51	61
Pineapple	22	25
Raspberries	20	55
Strawberries	60	88

[a]percentage esterification

Pectic substances and textural change during fruit ripening

The textural changes during fruit ripening are related to alteration in cell structure (Huber 1983). These changes usually involve an increase in water soluble pectin, decrease in protopectin, and a loss of pectic natural sugar, such as arabinose and galactose, from one or more of the pectic fractions (Seymour and others 1990). The increase in water soluble pectin is attributed to enzymatic degradation involving polygalacturonase (PG), pectinmethylesterase (PME) or β-galactosidase (Muda and others 1995). In peaches, water soluble pectin increased whereas alkali soluble pectin fraction decreased during ripening (Shewfelt 1965; Pressey and others 1971). In strawberry, the total amount of galacturonic acid in the separated pectin fraction also decreases with ripening (Inari and Takeuchi 1997). In mango fruits, some studies showed different changes in pectin fractions from different cultivars during ripening. According to Tandon and Kalra (1984) water soluble (high methoxyl) and ammonium oxalate soluble (low methoxyl) pectin fractions increased, while the alkali soluble (protopectin) fraction decreased, during ripening of mango fruit (cv Dashehari). As the fruits ripened, they softened largely because of enzymatic deesterification and the depolymerization of cell-bound pectin, which yielded water and ammonium oxalate (pectinic acid) soluble pectin. Protopectin, upon hydrolysis, yields water soluble pectins (Mizuta and Subramanyam 1973). In addition, Mitcham and McDonald (1992) found that the ripening of ‘Keitt’ and ‘Tommy Atkins’ was associated with an increase in soluble pectin as well as a decline in arabinosyl, rhamnosyl,and galactosyl residues. Muda and others (1995) confirmed that the chelator soluble pectin fraction is progressively depolymerized and becomes more polydisperse during ripening in ‘Tommy Atkins’. However, in a study by Roe and Bruemmer (1981), softening in ‘Keitt’ mango was accompanied by a decline in the amount of both water and alkaline soluble pectin. In contrast,

oxalate soluble pectin increased during ripening. The decline in alkaline soluble pectin was most closely correlated with the loss of firmness of the mango fruit. Roe and Bruemmer (1981) mentioned that the conversion of protopectin (alkali soluble) to water soluble pectin in peach was accompanied by polymer degradation but not to the extent to be observed in 'Keitt' mango. Degradation of mango pectin apparently proceeded to the stage that the product molecules were small enough to be soluble in ethanol, and were not precipitated in alcohol insoluble solid (AIS). Robertson and Swinburne (1981) also reported a significant inverse relationship between the firmness of unpeeled kiwifruit and water soluble pectin content.

Sequential pectin extraction with water, chelating agents, acid and alkali

Several procedures for fractional extraction have been developed to study pectic changes during growth, ripening, storage, and processing as well as for conducting structural studies on pectins from plant cell walls. The majority begins with grinding the plant material in warm ethanol or acetone, followed by washing with ethanol to inactivate endogenous enzymes instantaneously and to remove alcohol soluble solids known as alcohol insoluble solids (AIS). Thereafter, pectin fractions are obtained by sequential extraction of the purified cell-wall material with cold and/or hot water or buffer solutions, cold and/or hot solutions of chelating agents [ammonium oxalate, sodium hexametaphosphate, ethylene diamine tetraacetate (EDTA), cyclohexane diamine tetraacetate (CDTA)], hot diluted acids, and, finally, cold dilute sodium hydroxide or cold and /or hot dilute sodium carbonate. Water extraction generally yields highly esterified pectins with neutral sugar content higher than that of the chelator soluble pectins. Pectin extracted with hot chelating agents originates from the middle lamella, where they are presumed to be present in the form of a calcium pectate gel (Voragen and others 1995).

Pectic enzyme

Pectic enzymes are important factors in the texture of fruits and vegetables during ripening, postharvest storage, and processing (Pilnik and Rombouts 1981, Voragen and others 1995). Pectic enzymes occur naturally in many fruits and vegetables (endogenous enzymes), but they are also added as processing aids (exogenous enzymes) (Benen and others 2003b). Pectic enzymes are composed of main-chain depolymerases and esterases active (e.g. pectinmethlyesterase) on methyl-and acetylesters of galacturonic acid residues in the galacturonan and rhamnogalacturonan structure. The depolymerizing enzymes comprise hydrolases (e.g. exo-and edo-polygalacturonase) as well as lyases (e.g. petin lysase, pectate lysase) (Benen and others 2003b). The points of attack of pectic enzymes are illustrated in Figure 2.4. Pectin esterases act on methyl-and acetylesters of galacturonosyl uronic acid in the galacturonan and rhamnogalacturonan structure. Pectin lyase depolymerizes highly esterified pectin by splitting glycosidic linkages next to methyl esterified groups through a β-elimination mechanism (Figure 2.5). Pectate lyase also attacks glycosidic linkages next to a free carboxyl group (Figure 2.5). Another depolymerization pathway is by the combined action of pectinmethylesterase (PME) and polygalacturonase (PG). In the presence of a water molecule, PME splits off methanol from highly esterified pectin, transforming it into low ester pectin, which is hydrolyzed by PG, attacking glycosidic linkages next to a free carboxyl group (Figure 2.4, 2.5) (Pilnik and Voragen 1989). Both classes of enzymes contain enzymes that act on the homogalacturonan backbone, or ‘smooth’ part, and enzymes that degrade the rhamnogalacturonan part, also known as ‘hairy regions’ (Benen and others 2003b). Protopectinases are those enzymes active on protopectin, the insoluble pectin that cannot be extracted from the plant tissue by chemical or enzymatic treatment without degradation.

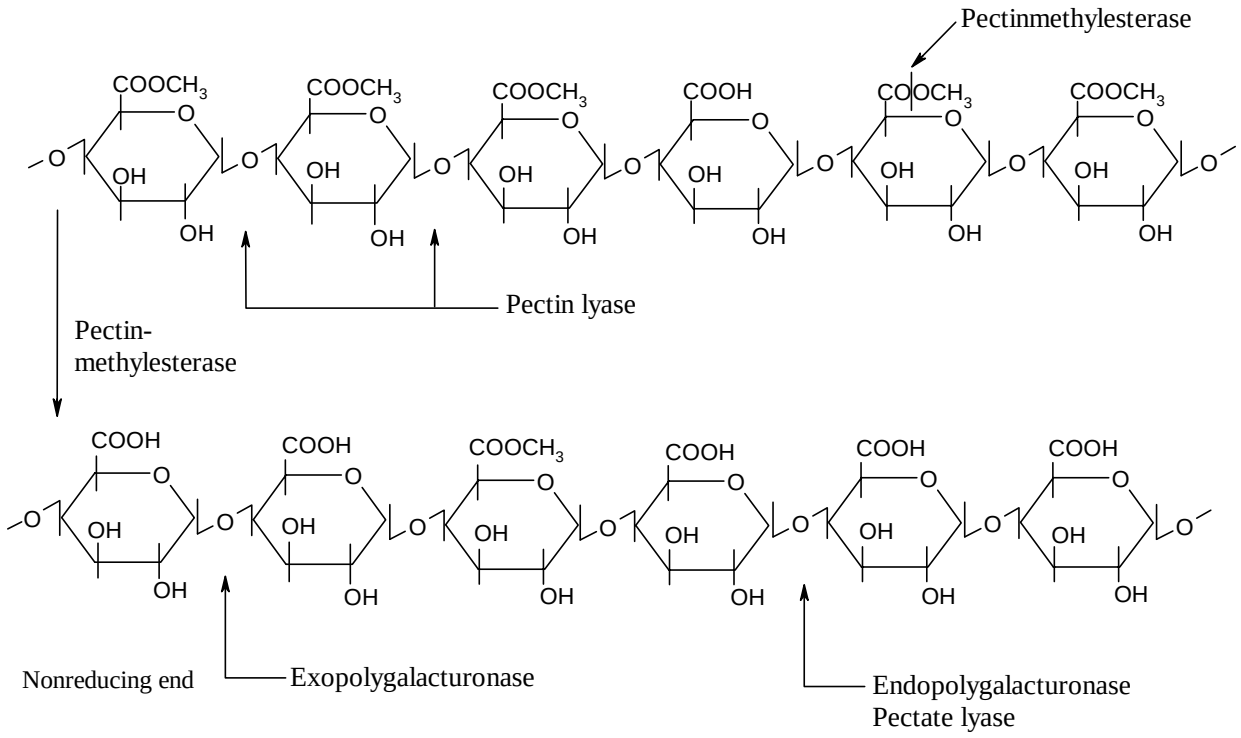

Figure 2.4. Fragment of a pectin molecule and points of attack by pectic enzymes (Pilnik and Voragen 1989)

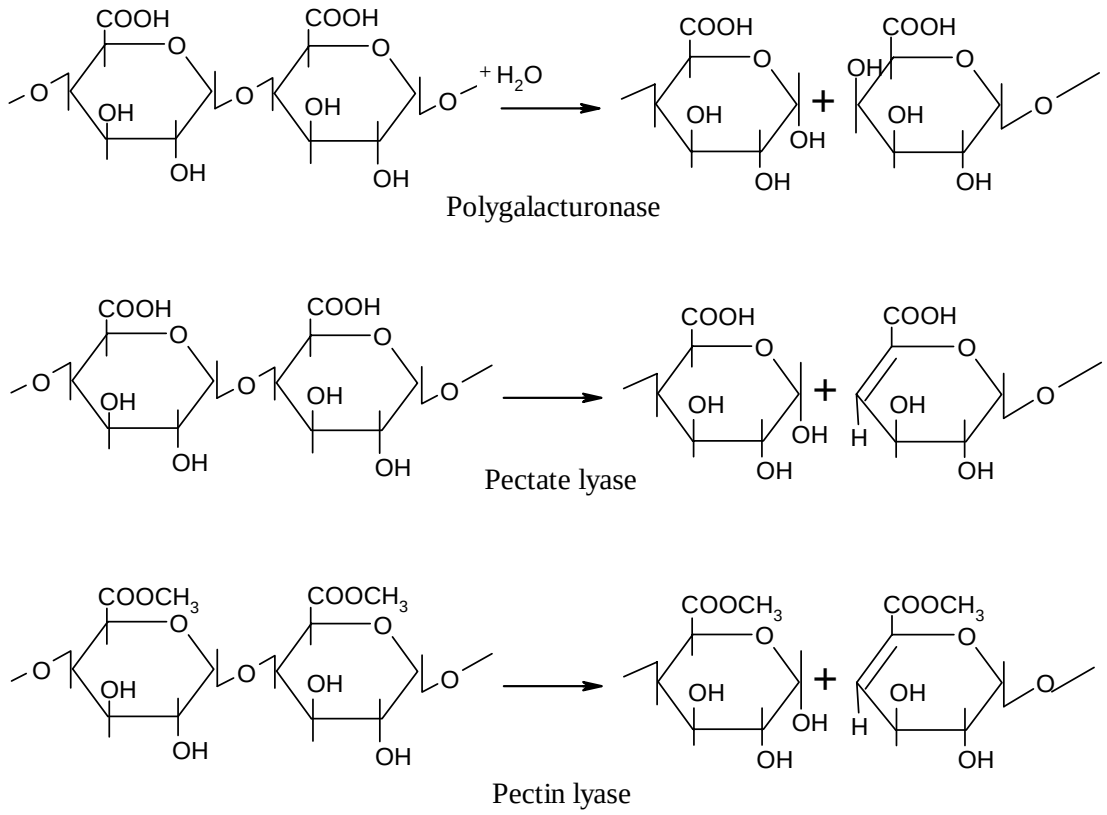

Figure 2.5. Splitting of glycosidic bonds in pectin by hydrolysis (polygalacturonase) and by β–elimination (pectate lyase and pectin lyase) (Pilnik and Voragen 1989).

The action of a protopectinase results in the release of soluble pectin or pectin constituents (Benen and others 2003b). However, it is not yet clear whether the protopectinase activity in plants is due to the combined action of PME and PG or to a true protopectinase (Whitaker 1996).

Pectinmethylesterase

Pectinmethylesterase (PME), pectinesterase (PE), pectate, or pectylhydrolase (EC 3.1.1.11) appear to hydrolyze only a methylester group if it is next to a free carboxyl group, thus proceeding linearly along the polyuronides (Sajjaanantakul and Pitifer 1991). Also, it does not completely deesterify pectin; the action stops at a certain methoxyl content (5%) (Kertesz 1951), possibly limited by the effect of small substrate polymer size (Sajjaanantakul and Pitifer 1991). PME is able to deesterify HM pectin, resulting in LM pectins, and releases methanol (Kertesz 1951). Both the appearance of free carboxyl groups and of free methanol can be used for activity measurement such as automatic titration, pH shift, or gas chromatographic methanol determination (Sajjaanantakul and Pitifer 1991).

PME has been detected in various plants as well as fungi, some bacteria, and yeast (Table 3). In higher plants, PME has been identified in apple, banana, berries, citrus (lime, orange, grapefruit, and mandarin), cherry, grape, mango, papaya, passion fruit, pear, plum, beans, carrot, cauliflower, cucumber, leek, onion, pea, potato, radish, and tomato (Benen and others 2003a). Plant PME deesterifies galacturonic chains linearly, creating blocks of free carboxyl groups. These blocks are extremely sensitive towards complex formation and precipitation with Ca^{2+} (Rexova-Benkova and Markovic 1976). Plant PME can also exhibit differing modes of action depending on the pH (Denes and others 2000). Two types of plant PME have been distinguished on the basis of their biochemical properties, including activation by cations (salt-independent or salt-dependent) and tissue expression patterns (Hotchkiss and others 2002). Savary and others

(2002) established that the major PME from Valencia orange peel was a salt-independent isozyme.

The action of microbial PME may be blockwise or random. *Trichoderma reesei* PME deesterified pectin in a blockwise manner (Markovic and Khon 1984) whereas PME from *A. foetidus, Aspergillus japonicus, Aspergillus niger, F. oxysporum, Penicillium chrysogenum, Sclerotinia archidis,* and *Sclerotinia libertiana* attack the methyl groups on the pectin randomly, resulting in a random distribution of the unmethylated galacturonic acid groups (Ishii and others 1979, 1980; Baron and others 1980; Kohn and others 1983). King and others (1986) observed that by increasing the pH from 3.5 to 8.5 to 10.0, at constant ionic strength of 0.3M NaCl, increased the solubilization efficiency of lime PME from 38% to 70% to 90%, respectively. PME is most effectively extracted by high ionic strength (0.1-1.0 M NaOH) alkaline pH buffers (pH 7-9) (MacDonnell and others 1945).

Factors affecting PME activity

The activity of PME is affected by pH, temperature as well as the presence of salts and various inhibitors (Rexova-Benkova and Markovic 1976). In general, the pH optima of plant PME differs from those of microbial PME, which, for fungal PMEs, have pH optima in the acid region (pH 4 and 6). All plant PMEs have their pH optima in the range of pH 7 to 9, whereas some of the microbial PME also have pH optima in the alkaline region (Table 2.3). Above pH 8.0, PME activity cannot be determined accurately without correction from alkali deesterification; alkaline deesterification of pectin begins to set in, providing a more suitable substrate for the action of PME. Therefore, the subtraction of values obtained by alkaline deesterification without PME may not provide the true value for the original substrate. There is

also the possibility of inactivation of some PME at pH values above 9 (Miller and Macmillan 1970).

PME can be activated in the presence of either monovalent or divalent cations, but not anions. The presence of salts of monovalent and divalent cations increases the activity of plant PME by several-fold, while the activity effect of salts on microbial PME is not as great displaying, an increase of 1.5 to 2.0 fold. Divalent cations were 5-20 times more effective than monovalent cations in the activation of PME (Rexova-Benkova and Markovic 1976). NaCl and KCl have been noted to stimulate the maximal activation of plant and microbial PME (Table 2.3). Vijayalakshmi and others (1979) suggested that cations might remove an anionic inhibitor from the terminal amino group in the protein of PME at the active site, thereby permitting the enzyme to be activated. In 1991, Nari and others explained the activation of PME by metal ions, noting that metal ions interact with the substrate (pectin) rather than with the enzyme. They also described the existence of blocks of carboxyl groups on pectin that may trap enzyme molecules, thus preventing the enzyme reaction from occurring. Metal ions may react with these negatively charged groups, allowing the enzyme to interact with the ester bonds to be cleaved. However, at high concentration, metal ions inhibit the enzyme reaction. This is explainable on the basis of the view that some carboxyl groups must be adjacent to the ester bound to be cleaved in order to allow the reaction to proceed. Thus, if these groups are blocked by metal ions, the enzyme reaction cannot occur. In contrast, Marcus and Schejter (1983) proposed that the main effect of the salt was direct competition for the cationic site in the enzyme, thus decreasing the enzyme-substrate affinity.

Besides the inhibitory effect of a high concentration of cations, PME activity is also inhibited by pectic acid, but is lessened by cations (Lineweaver and Ballou 1945).

Table 2.3. pH Optima of some plant and microbial pectinesterase and concentrations of Na and Ca causing maximum activation (Rexova-Benkova and Markovic 1976)

Origin	Purification	pH optimum	Optimal concentration	
			NaCl (mM)	$CaCl_2$ (mM)
Plant				
Alfalfa	Crude	7.0	130	20
Apple	Crude	7.0	n.d.[a]	n.d.[a]
Avena coleoptile	Crude	n.d.[a]	100	n.d.[a]
Banana	Pure	6.0-9.5	n.d.[a]	20
Bean hypocotyls	Crude	7.0	100	n.d.[a]
Snap beans	Crude	8.2	200	50
Citrus	Pure	8.0	n.d.[a]	n.d.[a]
Snap beans	Crude	8.2	200	50
Southern peas	Crude	7.5-8.0	250	n.d.[a]
Orange	Crude	7.5	100	5
Papaya	Crude	7.5	200	n.d.[a]
Tobacco	Crude	7.5	n.d.[a]	n.d.[a]
Tomato	Crude	7.0-7.8	100	n.d.[a]
	Pure	7.5-8.5	50	5
	Pure	8.0	100	5
Turnip	Crude	7.5-8.5	100	n.d.[a]
Microbial				
Acrocylindrium	Pure	7.0-7.5	100	10
Aspergillus niger	Pure	4.5	n.d.[a]	n.d.[a]
Cercosporella herpotrichoides	Crude	7.5	85	n.d.[a]
Coniothyrium diplodiella	Pure	4.5-5.0	100	25
Diplodia gossypina	Crude	6.5	n.d.[a]	3
Fusarium oxysporum	Crude	5.0	n.d.[a]	n.d.[a]
Monilia laxa	Pure	4.0	n.d.[a]	n.d.[a]
Rhizoctonia solani	Crude	6.0-7.0	No effect	n.d.[a]
Sclerotinia libertiana	Pure	4.8-5.2	150	5
Pectinol (commercial preparation)	Crude	4.3-5.0	100	n.d.[a]
Pellicularia filamentose	Crude	7.0	n.d.[a]	n.d.[a]
Pseudomonas prunicola	Crude	7.8	50	n.d.[a]
Clostridium multifermentans	Pure	9.0	50	n.d.[a]

[a] n.d.= not determined

Fungal PME is generally less influenced by cations than is plant PME (Macmillan and Sheiman 1974). In addition, many PMEs are also inhibited by polygalacturonic acid, the product of its action (Benen and others 2003a). All PMEs in fruits (papaya, banana, and apples) are inhibited by high sugar concentrations as indicated a noncompetitive inhibitor (Lee and Wiley 1970). Phenolic compounds, including catechins, tannic, gallic, shikimic, chlorogenic, and caffeic acids also inhibit some PMEs. Tannins are reversible inhibitors of tomato as indicated by Hall (1966). Iodine inhibition, a non-competitive and irreversible inhibitor, was found in tomato by Markovic and Patocka (1977). A glycoprotein inhibitor of PME from orange, tomato, apple, banana, and potato has also been found in kiwi fruit (Balestrieri and others, 1990). This inhibitor is effective in inhibiting PME in the pH range 3.5-7.5. The interaction between the PME and inhibitor appeared to be a 1:1 ratio. According to Giovane and others (1995) this inhibitor was present in the unripe fruit as an inactive precursor with a higher molecular mass (30 kDa), and was transformed into the active protein by proteinase action during the ripening process. Recently, a PME inhibitor has also been found in jelly fig (*Ficus awkeotsang* Makino) achenes as well as rubbery banana (*Musa sapientum* L.) (Jiang and others 2001;Wu and others 2002).

The temperature stability is moderate (40-70 °C), with slightly higher stability for the plant enzyme as compared to the microbial enzymes (Benen and others 2003a). Activation of PME in tissue, caused by relatively low temperature (50-70°C), was observed in canned green bean and carrot (Stanley and others 1995), jalapeno pepper (Howard and others 1997), and frozen cherry (Alonso and others, 1993; 1997a). A purified PME was completely inactivated by heating between 65 and 90 °C for 1 to 5 min (Sajjaanantakul and Pitifer 1991). A thermostable form of orange PME was inactivated by heating for 1 min at greater than 90 °C (Versteeg and others 1980).

Pectic enzyme in relation to softening of fruit ripening

The general concept of fruit softening involves a weakening of the cell wall and a decrease in adhesion between the cells. The modes of softening appear to involve enzyme degradation and solubilization of pectin (Van Buren 1991). During ripening, PME removes methyl groups from the cell wall pectin constituents, which can then be depolymerized by polygalacturonase, reducing intercellular adhesiveness and tissue rigidity (Alonso and others 1997b). PG hydrolyzes the glycosidic bond between two adjacent α-1, 4-linked D-galacturonic acid residues. There are two general types of PG: endopolygalacturonase (endo-PG, EC 3.2.1.15) and exopolygalacturonase (exo-PG, EC 3.2.2.67) (Van Buren 1991). Endo-PGs cleave the α–1,4-glycosidic linkages randomly along the pectin chain, and effectively reduce its molecular size, while exo-PGs remove galacturonic acid subunits from the nonreducing end of the pectin chains, with a minimal effect on the macromolecule (Figure 2.4) (Pressey and Avants 1978).

A number of fruits contain both endo-and exo- PG, including freestone peach, pear, cucumber, and papaya (Pressey and Avants 1973, 1975, 1976; McFeeters and others 1980; Chan and Tam 1982). In apple and clingstone peach, only exo-PG activity has been detected (Knee and Bartley 1981). The presence of the endo-PG in freestone peaches is associated with a higher level of water soluble pectins than in clingstone peaches (Postlmayr and others 1966). Endo-PG activity has been identified in a number of ripening fruits and is correlated with an increase in soluble pectins as well as the softening that accompanies ripening (Fischer and Bennett 1991). It has been suggested that PG is primarily responsible for ripening-associated pectin degradation and fruit softening (Brady 1987; Huber 1983). A correlation between the increase in PG activity with increases in soluble pectin and softening has been observed in various fruits, such as mango. However, there is evidence that this correlation does not always hold. Abu-Sarra and

Abu-Goukh (1992) observed that 'Abu-Samaka' which shows a slow rate of softening, had higher levels of PG than the 'Kitchner' mango which has a faster softening rate. In mango, it seems that only exo-PG is presents (Roe and Bruemmer 1981; Brinson and others 1988; Abu-Sarra and Abu-Goukh 1992)

PG seems to be more active in degrading demethylated pectin than methylated pectin (Seymour and others 1987). Therefore, PME may have an important role in determining the extent to which pectin is accessible to degradation by PG (Fisher and Bennett 1991). Unlike PG, PME is usually present in abundance in fruit tissue before softening takes place (Van Buren 1991). Reports on the changes in PME activity during ripening are often contradictory since the changes do not appear to have a common pattern (Gomez-Lim 1997). PME has been shown to increase during ripening of banana (Hultin and Levine 1965), tomato (Hobson 1963), orange (Tahir and others 1975), and strawberry (Barnes and Patchett 1976), to remain constant in banana (Brady 1976), tomato (Sawamura and others 1978), and mango (Ashraf and others 1981), or to decrease in tomatoes (Pressey and Avants 1972), avocado (Rouse and Barmore 1974) and mango (Roe and Bruemmer 1981). In mango fruit, the changes in PME activity during ripening vary depending upon the varieties. PME activity shows a decrease in 'Keitt' mango (Roe and Bruemmer 1981), remains constant in some Pakistani mango varieties (Ashraf and others 1981), or increases in African mango (Aina and Oladunjoye 1993). According to Tucker and others (1982) several PME isozymes in tomatoes have been identified, and the ratio of some of these isozymes may change during ripening. It is speculated that there are also several isozymes in mango which may fulfil different roles during ripening (Gomez-Lim 1997). Strawberry also appears to be different from other fruits, as solubilization of pectin increases in the apparent absence of PG (Neal 1965, Barnes and Patchett 1976, Huber 1984). Only PME has been found

in strawberry and increased from the green to early ripe stages but decreased during the senescent (over ripe) stage (Barnes and Patchett 1976). The presence of PG during the ripening strawberries remains controversial and PG has been found by Gizis (1964), but not by others (Neal 1965; Barnes and Patchett 1976). Recently, reports on ripening strawberry fruit have implicated endo-glucanase in the depolymerisation of strawberry xyloglucans (Llop-Tous and others 1999; Woolley and others 2001). According to Koh and Melton (2002), pectin solubilization during strawberry ripening could be associated with disentanglement of pectin in the cell walls due to the gradual degradation of arabinan side chain. The authors suggested that the presence of α-L-arabinofuranosidase and rhamnogalacturonase which could affect an increase in the pectin solubilization should be investigated on the strawberry cell walls.

Calcium and quality of fruits and vegetables

Calcium has been shown to have considerable effects in delaying senescence and controlling physiological disorders in fruits and vegetables (Poovaiah 1986). Calcium is essential for the synthesis of enzymes as well as the functional macromolecular structure of cell membranes, microtubules, and microfilaments (Pooviah 1985). The association of calcium with cellular membranes by linking of phospholipids with the membrane proteins is required to maintain membrane integrity and control membrane-associated functions (Hanson 1984). Calcium also serves as an intermolecular binding agent that stabilizes pectin-protein complexes of the middle lamella (Dey and Brinson 1984). Rossignol and others (1977) have estimated that at least 60% of the total calcium in plants is associated with the cell wall fraction. A large portion of calcium is located in the cell wall and plasma membrane, where it plays a major role in senescence and ripening (Chardonnet and others 2003).

The firming effect of calcium in the structure and function of cell walls and membrane of fruit and vegetables can be explained as follows. First, calcium plays a special role in maintaining the cell wall structure in fruit and other storage organs by interacting with the pectic substances in the inner part of the cell wall to form calcium pectate (Demarty and others 1984). This is demonstrated by the fact that most of the pectic substances which cannot be extracted from the wall by hot water are released into solution with the presence of a chelating agent of calcium, such as EDTA or oxalate (Demarty and others 1984).

To understand calcium effects on the structure and texture of fruits and vegetables, an 'egg box' model has been proposed by Grant and others (1973). This model was first used to explain the aggregation and gelation of alginate and polypectate molecules in dilute solutions of neutral pH when calcium or other divalent cations are added. In this model, calcium ions fit between two or more chains of nonesterified galactosyluronic residues in such a way that the calcium ions chelate to the oxygen atoms of 4 galactosyluronate residue present between 2 galacturonan chains, thus packing the ions like eggs within a box composed of galacturonans (Figure2.6). However, this model is inadequate to explain the observed textural effects of calcium and multivalent metal ions on brined cucumber tissue (McFeeters and Fleming 1989). In this case, the textural effects of calcium are a result of calcium binding at sites other than pectin carboxyl groups. In addition, a direct involvement of calcium ion in maintaining fruit firmness by modifying chemical changes in cell wall composition may not always explain the observed effect of exogenously applied calcium on flesh firmness (Ferguson 1984; Siddiqui and Bangerth, 1995a, b). In 1996, Siddiqui and Bangerth studied the effects of calcium infiltration on structural changes in the cell walls of stored apples. They revealed that the effect of calcium ions in maintaining an intact middle lamella might not be due to its direct ionic effect.

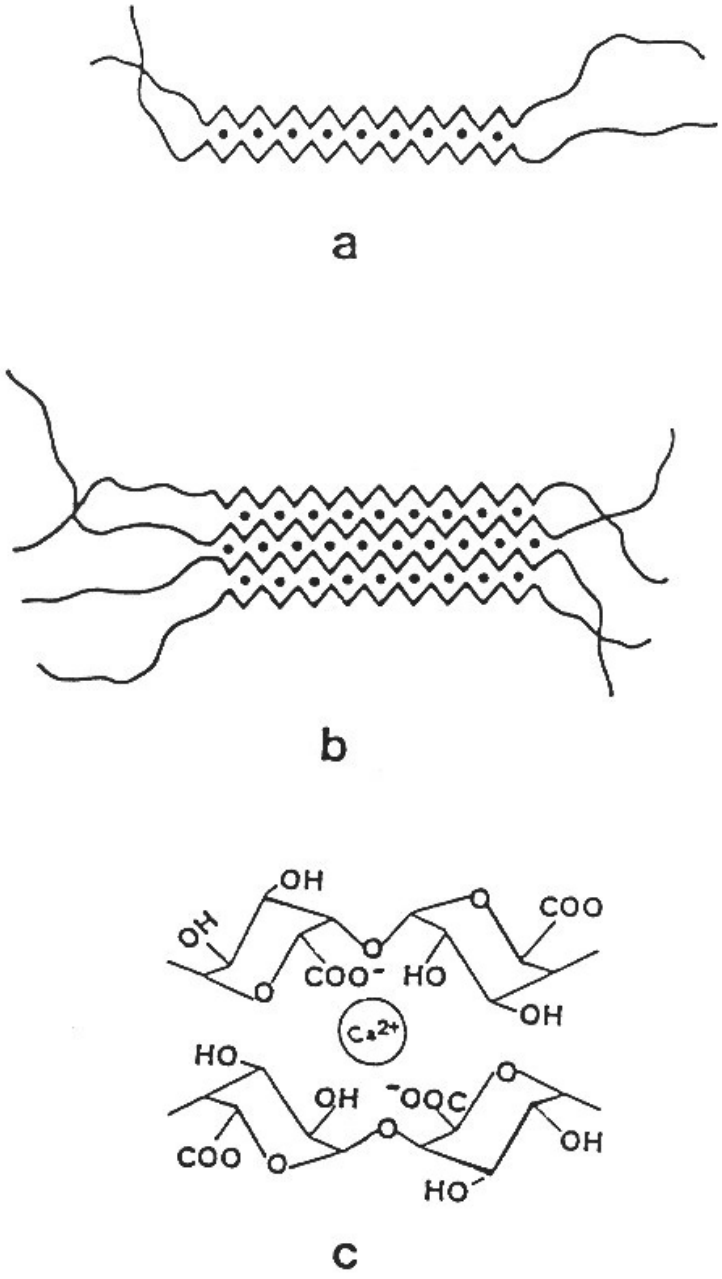

Figure 2.6. Schematic representation of calcium binding to polygalacturonate sequence: (a) ‘egg- box’ dimmer; (b) aggregation of dimmers; (c) an ‘egg-box’ cavity

The authors suggested an indirect role of calcium in maintaining intact middle lamella. It has been proposed that calcium cross-linking with pectic polymers may restrict the access of hydrolytic enzymes to wall components, particularly hemicelluloses. This may prevent the liberation of pectins covalently attached to hemicelluloses as well as retain the attachment of the middle lamella with the primary wall, thereby maintaining cell cohesion and fruit firmness.

Secondly, calcium interacts with cellular membranes by modifying their structure and exerting a regulating role on the permeability of this membrane and the transport of some substances involved in product ripening and senescence (Saurel 2002). The works of Picchinoni and others (1995) demonstrated that $CaCl_2$ infiltration increased the fruit firmness by affecting the membrane organization and function of apple fruit tissues. Moreover, the effect of calcium ions on fruit firmness may be turgor-dependent, mediated through the stabilizing properties of calcium ion on membranes (Shackel and others 1991). Mignani and others (1995) also found that tissue softening in ripening tomato pericarp was related to cellular turgor decrease resulting from alterations in the pattern of pectin solubility due to calcium ions.

Thirdly, calcium may directly affect the activity of cell wall-degrading enzymes (Poovaiah and others 1988). The calcium concentration in the cell wall is estimated to be in the millimolar range (Poovaiah 1985). These millimolar concentrations inhibit the activity of both exo-PG and endo-PG in vitro, whereas this concentration range may stimulate PME activity. Rexova-Benkova and Markovic (1976) reported that the concentrations of calcium between 5 and 25 mM cause the maximal activation of pectinesterases of plant and microbial origin, whereas higher calcium concentrations have an inhibitory effect. Furthermore, calcium treated apples also displayed a reduction in activity of β-D-galactosidase (Siddiqui and Bangerth 1995a). Ferguson (1984) observed that relatively high Ca^{2+} concentrations in fruit tissue were required

for delaying fruit softening. The author suggested the possibility that Ca^{2+} binding to the outer surface of plasmalemma was influencing or regulating glycoside secretion. Poovaiah (1986), in a study of a calcium-dependent protein phosphorylation in mature and ripening tomatoes, found that phosphorylation of many soluble polypeptides was promoted by calcium in immature fruits, whereas calcium-dependent phosphorylation decreased dramatically as soon as the fruits started to ripen. This suggests the presence of stage-specific changes in calcium-regulated protein phosphorylation in fruits.

Calcium chloride is commonly used industrially as a firming agent for canned tomatoes and cucumber pickles (Luna-Guzmán and Barrett 2000). According to FDA (2002) regulations, calcium chloride as a firming agent in processed fruits and vegetables can be used at a maximum level of 0.2% and 0.4%, respectively. Several products used calcium chloride to improve or maintain texture in different concentration. Many techniques have also been developed to increase calcium content in the cell walls of fruit tissue after harvest or during processing. Dipping treatment is usually used for fresh cut products, such as in strawberry, 1% calcium chloride dip treatment increased firmness of whole strawberry (Rosen and Kader 1989; Garcia and others 1996); in pear, a 1.0% $CaCl_2$ dip was effective in reducing browning and loss of firmness (Rosen and Kader 1989). In diced tomato, relatively low calcium concentrations (0.43%) and longer contact time (≈3.5 min) resulted in diced tomatoes with low Ca^{2+} concentration and high firmness (Floros and others 1992). Vacuum infiltration with calcium chloride is used both postharvest and pre-treatment in processing of fruits and vegetables. For example, vacuum infiltration with 2%-4% $CaCl_2$ to increase firmness and delay senescence of apple (Poovaiah 1986) or 4%-8% (w/v) $CaCl_2$ to delay mango ripening (Tirmazi and Wills, 1981). Conway and Sams (1983) found that vacuum infiltration of a 12% $CaCl_2$ solution

increased the Ca content of fruit two-fold as compared to a dip treatment in the same solution and resulted in 30% less decay. More calcium applications in vacuum infusion with others products are listed in Table 2.4. Pressure infiltration (68.95 kPa) of 8% $CaCl_2$ decreased the decay of apples after harvest (Conway and Sams 1985). $CaCl_2$ (0.14 –0.34 M) is used as surfactants or coating agents to maintain the quality and prevented fruit-surface injury in apples (Saftner and others 1998).

Despite the benefits of calcium as related to product texture, the use of calcium chloride may impact bitterness or cause flavor differences. In apricot, no taste differences were noted between control and sliced apricot dipped with 3% calcium chloride solution (200 ppm in tissue), but in a 5% solution, a flavor difference was observed (Bolin and Huxsoll 1989). Calcium chloride greater than 0.30% minimized the softening of processed apple, but the typical crispy structure of the fresh apple was not restored (Monsalve-Gonzalez and others 1993). In a study by Luna-Guzman and Barrett (2000), 2.5% solutions of either calcium chloride or calcium citrate maintained the firmness of fresh-cut cantaloupes throughout cold storage, but only calcium chloride imparted undesirable bitterness to the fruit piece. Calcium lactate represents an alternative calcium source. Calcium lactate (0.5%-2.0%) has been used as a firming agent for processed strawberries (Morris and others 1985; Main and others 1986), and 0.1%-0.5% calcium lactate increased the firmness of grapefruit sections in juice or syrup (Baker 1993) without reported flavor differences.

Vacuum infusion technology

Principle

Vacuum technology is considered to be a pretreatment for processed fruit or vegetables leading to improvement in quality by active incorporation of functional ingredients in the

product structure (Baker and Wicker 1996; McArdle and Culver 1994; Saurel 2002). In passive impregnation, the penetration of preservatives or humectants of agents by soaking can be required in dried products. However, the processing times of this treatment are long, extending from several hours to several days, and mass transportation phenomena are mainly governed by molecular diffusion of the compounds present in aqueous solution (Saurel 2002). In contrast, vacuum infusion yields faster water loss in short-time treatments, as compared to the time-consuming, atmospheric processed, due to the occurrence of a specific mass transfer phenomenon as called the hydrodynamic mechanism (HDM) (Alzamora and others 2000). This allows the occluded air initially contained in the fruit or vegetable pores to be replaced by the impregnation solution; this replacement is due to the positive pressure differential that results when atmospheric conditions are restored.

The vacuum operation is carried out in two steps after the product immersion in the tank containing the liquid phase. In the first step, vacuum pressure is applied in the tank, thus promoting the expansion and outflow of the product internal gas. The release of the gas takes the product pore native liquid with it. In the second step, the atmospheric pressure is restored in the tank, and compression leads to a great volume reduction of the remaining gas in the pores and, thus, the subsequent inflow of the external liquid in the porous structure (Fito and Chiralt 2000). In HDM phenomena (Fito and Pastor 1994; Fito 1994), intercellular spaces in plant products are described by elementary cylindrical pores occupied by an ideal gas undergoing isothermal compression. The penetration of solution into the ideal rigid pores occurs in two stages. Initially, the pores fill by capillary action in the first part of the treatment, corresponding to atmospheric immersion and vacuum application. Secondly, when restoring normal pressure, the

resulting driving force induces liquid flow in the pores. The quantity of external liquid transferred can be almost as great as the available void space in the food structure.

A proper formulation of the impregnation solution allows expeditious compositional modifications of the solid matrix that may result in quality and stability enhancement of final products (Salvatori and others 1998). During vacuum impregnation of porous fruits, important modifications in structure and composition occur as a consequence of external pressure changes. The final products may exhibit structural, physical, and chemical properties very differently from those of atmospheric infused fruit (Alzamora and others 2000).

Factors affecting vacuum process

The magnitude of applied vacuum and infusion times will be different for each fruit and vegetable, depending on the cellular nature and geometry of that particular piece of produce. In strawberry, whole strawberries displayed more drip loss and were not as firm as the infused strawberry halves. The greater surface area to volume ratio and the more permeable surface down the length cut in the strawberry halves can be attributed to their better performance (Margaret and others 1988). In a study of Mujica-Paz and others (2003), vacuum infusion times of 3 and 45 min and vacuum pressures between 14-76 kPa were applied to fruits (mango, apple, papaya, banana, peach and melon) with isotonic solution. Vacuum pressure had a significant effect on the volume of isotonic solution impregnation in fruit slices. The infusion also depends significantly on the vacuum time, except as applied to apple. Gras and others (2002) studied the response of some vegetables to vacuum impregnation at vacuum pressure 5 kPa, with a range of process times between 5 and 15 min. Mushroom reached the maximum impregnation level, followed by oyster mushroom and eggplant. Diced zucchini showed vacuum infusion responses different to sliced samples, due to a high ratio of empty spaces of diced shape. Carrot tissue

showed almost no intercellular spaces, and no significant action of hydrodynamic mechanisms will be expected in carrot parenchyma. According to Ferguson and Malick (1983), pulsed vacuum infusion of AVICEL solution can be used to reduce weight loss of mushrooms during canning. They observed that, after the first vacuum application (100 kPa for 5 min) and released, approximately 20% of the mushrooms remained floating. Secondly, the vacuum was released for 1 min and reapplied for 5 min. After releasing the vacuum the second time, virtually 100% of the mushrooms sank to the bottom of the liquid, indicating complete saturation.

The characteristics of the raw materials influence how easily and quickly they can be infused. Not all fruits and vegetables can be successfully infused. Some, such as raspberries, are too delicate, and tend to disintegrate under the treatment and form a puree rather than infused pieces (Baker and Wicker 1996). Products with a skin or tough surface, such as peas, corn, cherries, and blueberries, resist infusion unless the skin is broken or scarified to facilitate the movement of infused solution (Kuntz, 1995). Plant tissue contains intercellular spaces that may contain a gas or liquid phase and that are susceptible to impregnation with external solution. In a study by Fito and Chiralt (2000), apple tissue showed large voids among cells, whereas strawberry cells were densely packed, showing very small pores. Pineapple and potato showed a great part of intercellular spaces filled with native liquid, which is revealed by their dentritic aspect. Thus, vacuum technique seems to adapt well in porous fruit or vegetable products.

Several factors affecting the rate and degree of the infusion process include time, temperature, pressure, characteristics of the raw materials, type of infused solutions, and solids gradient. The higher the temperature and the greater the pressure, the faster the transfer occurs. However, raising the temperature too high creates problems such as volatiles flashing off, as well

as color and flavor degradation (Kuntz, 1995). Based on these factors, experimentation with each fruit or vegetable is necessary to develop the required treatment.

Applications

The use of vacuum technology was proposed as a pretreatment in many processing and product applications such as postharvest storage, thermal processing (blanching, canning, drying, and pasteurization), fruit peeling, and others. The major role of vacuum technology is the modification of food structure in order to improve the strength and firmness of products after a physical treatment for preservation and /or during storage (Saurel 2002). Different applications of vacuum infusion have been developed to meet several objectives in the fruit and vegetable products listed in Table 2.4 (Saurel 2002). Browning inhibitors has been applied to cut apple and potato by vacuum infiltration (Sapers and others 1990). Vacuum infusion with polyamines increased the firmness of strawberry slices (Ponappa and others 1993). Vacuum infusion with cryoprotectants/cryostablilizers, such as pectin, improves frozen product stability (Martinez-Monzo and others 1998). Enzyme infusion under vacuum has also been developed for several objectives. In peach, the infusion of plant pectinmethylesterase (Marsh grapefruit PME) with calcium increased firmness in peach halves after thermal processing (Javeri and others 1991). Fungal PME infusion is also used to increase strawberry firmness after making jam (Suutarinen and others 2002) and pasteurization (Degraeve and others 2003). In addition, vacuum infusion of pectinase is used to soften the peel of citrus fruit for removal (Rouhana and Mannheim 1994; Soffer and Mannheim 1994; Pretel and others 1997; Prakash and others 2001). In terms of health benefits, vacuum infusion is applied to produce dried fruit products enriched with probiotics (Betoret and others 2003) as well as in the calcium fortification of vegetables (Gras and others 2003).

Table 2.4. The applications of vacuum infusion methods in fruits and vegetable processing (adapted from Saurel 2002)

Process application	Raw material	Solution composition	Vacuum treatment	Quality improvement	Reference
Blanching	Green bean & Green bell pepper	$CaCl_2$ $2H_2O$ (0.24%)	60 kPa, 4 h	Firmness & color retention	Seow and Lee (1997)
	Turnip	Water	101 kPa, 15 min	Firmness	Moreira and others (1994)
Canning	Apricot	$CaCl_2$, MgCl, KCl, potassium citrate or malate	< 75 kPa, 1 h.	Firmness	French and Malick (1989)
	Button mushroom	Water	0.3 kPa, 5 min	Yield	McArdle and others (1974)
	Mushroom	Xanthan gum (0.5% or 0.1%) with 0.25% Sodium metabisulfite	75 kPa, 30 min	Yield, lower shrinkage	Gormley and Walshe (1986)
	Mushroom	Mycrocrystalline cellulose (Avicel) (4%)	Two steps: 1.100 kPa, 5 min and released for 1 min 2.Vacuum is reapplied for 5 min	Reduction of weight loss, tender texture	Ferguson and Malick (1983)
	Strawberry	Calcium lactate (1%)	17 kPa, 8 min	Firmness	Main and others (1986)
	Peach	Pectinmethylesterase (PME) from marsh grapefruit (14-18 Unit/ml) with $CaCl_2$ (0.01%)	85 kPa, 0.5-2 h	Firmness	Javeri and others (1991)
Jam process	Strawberry	Fungal PME (12,000 Unit/kg fresh fruit) with $CaCl_2$ (1%)	17 kPa, 10 min	Firmness	Suutarinen and others (2002)

*Vacuum absolute pressure values are converted in kPa unit: times correspond to the maintaining times under vacuum

Table 2.4. Continued

Process application	Raw material	Solution composition	Vacuum treatment*	Quality improvement	Reference
Pasteurization	Apple, Strawberry and Raspberry	Aspergilluse niger PME (Rapidase® FP Super) (3%) with $CaCl_2.2H_2O$ (6%)	7 kPa, 2 min	Firmness increased only in strawberry	Degraeve and others (2003)
Freezing	Apple	High methoxyl pectin (3%)	5kPa, 30 min followed by 25 min at atmospheric pressure	Texture	Martinez-Monzo and others (1998)
	Kiwi and Strawberry	Sodium alginate (0.5%-6.0%), calcium salt (0.1%-3.0%), Tetra sodium pyrophosphate (0.1%-3.0%)	40 kPa, < 30 min	Texture, drip-loss	Margaret and others (1988)
	Strawberry	Calcium lactate (1%)	17 kPa, 8 min	Firmness	Main and others (1986)
	Strawberry	Fungal PME (252 Unit/kg fresh fruit), $CaCl_2$ (1%), sucrose (15%)	17 kPa, 10 min	Firmness	Suutarinen and others (2000b)
Air drying	Apple	Different microorganisms (*Saccharomyces cerevisiae and Lactobacillus caseil*)	5 kPa, 10 min	Enrichment of dehydrated fruit product with probiotic	Betoret and others. (2003)
Ohmic heating	Potato	NaCl (3%)	40-45 kPa, 5 min	Electrical conductivity	Wang and Sastry (1993)

*Vacuum absolute pressure values are converted in kPa unit: times correspond to the maintaining times under vacuum

Table 2.4. Continued

Process application	Raw material	Solution composition	Vacuum treatment*	Quality improvement	Reference
Postharvest storage	Apple	$CaCl_2$ (4%-8%)	15-87 kPa, 1-2 min	Reduction of 'bitter pit' and internal breakdown	Scott and Wills (1977, 1979)
	Apple	Different flavonoid and phenolic acid compounds	5.1 kPa, 2.5 min	Firmness	Lidster and others. (1986)
	Avocado	$CaCl_2$ (1M)	33-50 kPa	Ripening delaying	Wills and Sirivatanapa (1988)
	Lemon	Putrescine or $CaCl_2$ (1mM), gibberellin (100 ppm)	27 kPa, 8 min	Firmness, color change delaying during ripening	Valero and others (1998a, 1998b)
	Mango	$CaCl_2$ (4%-8%)	33 kPa, 4.5 min	Ripening delaying	Tirmazi and Wills (1981)
	Strawberry	$CaCl_2$ (100 mM) and different polyamines (10-100 mM)	17 kPa, 8 min	Firmness	Ponappa and others (1993).
	Passion fruit	$CaCl_2$ (1%-2%)	25 kPa, 1 min	Firmness	da Silva and Vieites (2000)
	Tomato	$CaCl_2$ (4%-8%), other divalent metal ions: Mn, Co, Mg	60-86 kPa, 4.5 min	Retardation of ripening	Wills and Tirmazi (1979)
Citrus fruit peeling	Grapefruit	Pectinases and cellulases	10kPa 95 kPa, 2- 10 min	Appearance Appearance, firmness	Baker and Bruemmer (1989 Rouhana and Mannheim (1994)
	Indian grapefruit	Commercial enzyme (1 ml/L)	101 kPa,0.5-4 min	Easy peeling	Prakash and others (2001)
	Valencia orange and pomelo	Pectinases and cellulases	95 kPa,3 min (orange), 12 min (pomelo)	Appearance, firmness	Soffer and Mannheim (1994)
	Orange	Pectinases and cellulases	93 kPa, 10 min	Peeling of whole oranges with excellent quality	Pretel and others (1997)

*Vacuum absolute pressure values are converted in kPa unit: times correspond to the maintaining times under vacuum

Table 2.4. Continued

Process application	Raw material	Solution composition	Vacuum treatment*	Quality improvement	Reference
Osmotic dehydration	Apple	$CaCl_2$ (2%)	9 kPa, 4 min	Texture	Del Valle and others (1998)
	Kiwi	Glocose monohydrate (Cerelose, 59%)	8 kPa, 10 min	Firmness	Muntada and others (1998)
	Melon	Glucose monohydrate (550 g/kg), calcium lactate (10 g/kg) and potassium sorbate (2 g/kg)	21 kPa, 10 min	Texture	Mastrangelo and others (2000)
	Mango	Sucrose (65 °Brix)	21 kPa, 30 min	Prevention of membrane integrity loss	Tovar and others. (2001)
	Strawberry	Sucrose (65 °Brix)	5 kPa, 5 min	Volatile profile of fresh fruit	Escriche and others (2000)
Fresh cut product/ refrigeration	Eggplant and Orange	Different calcium salt, Iron salts	5 kPa, 15 min	Formulation of functional fresh or vegetable products	Fito and others (2001)
	Eggplant, carrot and oyster mushroom	Sucrose(Suc) and calcium lactate(Lca) mixture (33 g Suc/20 g Lca)	5 kPa, 10 min	Calcium fortification	Gras and others (2003)
	Potato and apple	Na ascorbate, Ca ascorbate, $CaCl_2$	17-98 kPa, 0.5-2 min	Browning inhibition	Sappers and others (1990)

*Vacuum absolute pressure values are converted in kPa unit: times correspond to the maintaining times under vacuum

Application of vacuum infusion has the capability to modify composition and structure for use in industrial development. In addition, vacuum infusion can bring about improvements in the color and flavor of dried products.

Microscopy and localization techniques

Light microscopy

Microscopy techniques (e.g. optical or light, electron and atomic microscopy) vary in method of image production, resolution, and type of signal detected, and give a particular type of structural information that is unique to the technique used (Kalab and others 1995). Bright-field, polarizing, and fluorescence microscopy techniques are used most frequently. In conventional bright-field microscopy, illumination is transmitted sequentially through a condenser, the specimen, and the objective, producing a real image that is upside down and reversed as well as magnified within the microscope tube. The real image is then magnified within by the ocular lens, which produces either a virtual image that appears to be ~25 cm from the eye, or a real image on photographic film placed above the microscope tube. If the specimen is not highly colored, contrast must be introduced to make it visible. This is commonly achieved by the use of dyes or stains of known specificity for different components of the specimen.

Advances in instrumentation have been made in light microscopy, most notably in the development of confocal laser scanning microscopy (CLSM). This method not only provides an image with better resolution than conventional light microscopy or fluorescence microscopy, but also provides an opportunity to observe a 3-dimensional image without creating the need to physically section and observe the same sample in the z-direction (Vodovotz and others 1996). In CLSM, a laser source is focused by the objective lens to illuminate a single, precisely defined point in the specimen (the focal point). A scanning device deflects the beam in the X/Y, X/Z, or

Y/Z dimension, thereby scanning the focused spot on the specimen to create an image of the X/Y, X/Z, or Y/Z focal plane. Reflected and fluorescent light returns via the illumination path, and is then focused by the optics of the microscope at the confocal point at the center of a pinhole. Since the spot on the pinhole and the spot on the specimen are both located in the focal plane of the imaging lens, they are said to be confocal. The CLSM is most advantageous in its ability to provide extraordinarily thin, in focus, high-resolution optical sections through a thick specimen (Aguilera and others 2000).

CLSM has been used to examine the microstructure of pretreated frozen strawberries as compared to fresh strawberries (Suutarinen and others 2000a), as well as to visualized the fracture properties of stored fresh apple tissue (Alvarez and others 200). In addition, the use of antibody JIM5 in conjunction with CLSM has indicated patterns of pectin deposition in relation to pit fields at the plasma-membrance-face of tomato pericarp cell walls (Casero and Knox 1995).

Scanning electron microscopy (SEM)

SEM is used to examine surfaces. The sample is either dry (conventional SEM) or below –80 °C (Cryo-SEM). A 5-20 nm thick metal (gold) coating provides electrical conductivity. The sample is scanned by a focused electron beam, and secondary or back scattered electrons are processed to form an enlarged image. The absence of water in dried samples exposes their solid structures for examination. SEM images have a great depth of focus, and are relatively easy to understand (Kalab and others 1995). However, the drawback of conventional SEM is dimensional changes and shrinkage of soft biological specimen (Aguilera and Stanley 1999). Cryogenic scanning electron microscopy (Cryo-SEM) is very useful for observing high-fat samples and other food samples that are difficult to stabilize using conventional preparation

methods (Allan-Wojtas and Yang 1987). In this technique, hydrated samples are cryofixed by immersion in slush nitrogen to retain water and convert it into vitreous ice without crystal growth, cryofractured, then directly observed after surface etching (sublimation of a thin layer of the ice) and coating with gold (Fito and Chiralt 2000). Cryo- SEM has been widely used to study the relationship of microstructure and mechanical properties in fruit and vegetable tissues subjected to vacuum infusion. Microstructural changes of some vegetables (beetroot, carrot, eggplant, zucchini, mushroom and oyster mushroom), induced by vacuum infusion were shown by Cryo-SEM (Gras and others 2002). In a study by Gras and others (2003), calcium interaction with the cell matrix of carrot, eggplant and oyster mushroom by vacuum impregnation was observed by Cryo-SEM technique. Their results showed that calcium impregnation occurred in the intercellular spaces of eggplant and oyster mushroom and in the xylem of carrot.

Transmission electron microscopy (TEM)

TEM visualizes the internal structure of food samples. Thin (15-90 nm) sections of samples embedded in epoxy resin or platinum-carbon replicas of the sample are placed in the path of the electron beam and the enlarged image is observed on a fluorescent screen or photographed on film. The electrons are transmitted through the sample with varying degrees of energy loss. Variations in the electron density of structures stained in the resin sections with heavy-metal salts (e.g. of uranium or lead) or differences in the thickness of the metal replica due to differences in the angles at which the metal is deposited on the fractured sample result in the formation of the image (Kalab and others 1995). One of the most significant drawbacks of TEM is that sample preparation is difficult and invariably causes structural artifacts, often as a result of the drying or sectioning steps (Aguilera and Stanley 1999).

Localization techniques for pectic substance in plant tissues

The goal of food microscopy is to examine the structural composition of materials and observe how the various structural elements interact; in addition, determining the spatial distribution of specific structures (e.g. particular macromolecules or elements). Localization probes are introduced during the sample preparation procedure. These probes range in specificity from the long-used stains and fluorescent dyes employed in light microscopy, stains and dyes that can identify classes of compounds, such as proteins, lipids, and carbohydrates, to molecule-specific labels (Aguilera and Stanley 1999). Ruthenium red and hydroxylamine-ferric chloride ($FeCl_3$) are stains that commonly used for the localization of pectic substances (Dashek 2000). Ruthenium red selectively binds to the intramolecular spaces of carboxyl groups of pectin. However, this stain is recognized to be non-specific for pectin (Albersheim and others 1960). Hydroxylamine-$FeCl_3$ has the ability to localize microscopically esterified pectins (Reeve 1958). The reaction of the carbomethoxy groups of pectin with alkaline hydroxylamine produces pectin hydroxamic acids which react with ferric ion to form insoluble red complexes. In 1959, Gee and others developed the colored spots of the ferric-pectin hydroxamic complex on filter paper strips to measure the degree of esterification of pectic substances in fruit tissue sections. Delincee (1976) developed print techniques in thin-layer isoelectric focusing by using a paper impregnated with pectin at suitable pH to detect the forms of tomato pectinesterase.

To localize interest molecules within the tissues, immunocytochemical techniques, which offer potent probes for clarifying the location, metabolism and function, as well as the structure of cell wall polymer have been widely used (Nielsen 2000). Antibodies, lectins, and enzymes could be used for this purpose but antibodies are the most suitable due to more precision than the other candidates (Hoson 1991). These may be employed in both light and electron microscopy

modes. Immunolocalization techniques start with the preparation of antibodies having a strong binding affinity to the target molecule (Aguilera and Stanley 1999). Visualization of the antibodies after their binding to the target can be accomplished by labeling the primary, or, more often, the secondary antibodies with fluorescent dyes, enzymes, or colloidal gold particles (Nielsen 2000). For light microscopy, the usual approach involves fluorescent labeling using a fluorescence compound such as fluorescein isothiocyanate, rhodamine, tetramethylrhodamine isothiocyanate and cyanine (Vodovotz and others 1996). For electron microscopy, colloidal-gold probes attached to a secondary antibody can be used to label the target component (Aguilera and Stanley 1999). Fluorescent dyes are used on secondary antibodies giving more intense labeling and conserving labor and primary antibodies (Nielsen 2000).

Pectic polysaccharides have been localized using different types of antibodies (Hoson 1991). Three antipectin monoclonal antibodies are now used extensively, predominantly in immunofluorescence and immunogold localization studies: JIM5, JIM7 and 2F4 (Knox 1997). The JIM5 antibody binds to pectin with up to 50% DE, and JIM 7 with a range of 35 to 90%DE. The JIM5 and JIM 7 epitopes can occur on the same pectin molecule (Knox and others 1990). The 2F4 antibody recognizes homogalacturonan with up to 30% random and 40% blockwise degree of esterification (Liners and others 1992). The major observation made with these antibodies has been the heterogeneous distribution of pectin epitopes, indicating spatial variation in pectin and particularly its esterification within cell walls (Knox 1997). In 1999, Sutherland and others studied the localization of pectin in kiwifruit ripening using JIM5 and JIM7. They found that a labeling pattern for both JIM5 and 7 was not consistent with the ripening-associated chemical changes to the cell wall polysaccharides. JIM7 displayed only low-density binding throughout the wall of both unripe and ripe fruit. From chemical analyses, the degree of

esterification of cell wall material was noted to decrease during ripening (Redgewell and others 1992). A possible reason for the poor JIM7 labeling was that most epitopes were inaccessible to the antibody in unripe kiwi fruit. However, the high level of labeling of JIM5 was found in unripe fruit. Possible explanations include cell wall pectins becoming less esterified in kiwifruit ripening while the %DE remained greater than 35%. Thus, it is possible that there is a specific epitope of a low-esterified pectin available in unripe kiwifruit which either was lost during ripening or became masked as the cell wall swelled. At the surface of potato parenchyma cells, antibodies JIM5 and JIM7 were used to locate weakly esterified and highly esterified pectin via silver-enhanced immunogold SEM (Parker and others 2001). The edge of face structures labeled strongly with JIM5 but not JIM7, indicating that they contained polygalacturonic acid of low ester content. Recently, Lo and others (2002) studied the blanching effects on the chemical composition and the cellular distribution of pectins in carrots using JIM5 and JIM7 antibodies with immunogold labeling TEM. They reported that the differences in methyl-esterified pectins between high-temperature short-time (HTST) and a long time at low temperature (LTLT) blanching, as determined by JIM7 antibodies, was not consistent with the change in DE determined by GC analysis. LTLT-blanched carrots showed higher levels of JIM7-labeled pectin than HTST-treated carrots, which had a higher DE than that of LTLT carrots. The lack of strong binding of methyl-esterified pectin with JIM7 in HTST carrots could be due to the fact that the epitopes of JIM7 were partly masked by hydration of the cell wall in blanched cell walls. Another possible reason was that JIM7 bound to methyl-esterified pectin in the range of DE from 35 to 90%. Although a lower %DE was produced after LTLT treatment as compared to HTST blanching, it was still higher than 35%.

The binding of an antipectin antibody, or lack of it, does not directly correlate with the presence or absence of pectin, but of the pectin epitope, which may be absent or modified by metabolic reactions in the cell wall (Knox 1997).

The overall objectives of the following experiments were to improve the quality of fruits and vegetables using PME and to evaluate analytical methods to quantify the effect of PME infusion. The objectives of the first part were to develop a rapid method to visualize the location of PME in fruit after vacuum infusion and to study the effect on pectic substance and texture. The second one was to evaluate infusion of exogenous PME into mango, to determine PME activity in tissue, and to determine the effect on textural properties of mango. The last one was to compare the textural changes in eggplants using fungal and plant PME and use JIM5 and JIM7 antibodies to determine the change of pectin in cell wall of infused eggplants.

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CHAPTER 3

DETECTION OF VACUUM INFUSION OF PECTINMETHYLESTERASE (PME) IN STRAWBERRY BY ACTIVITY STAINING[1]

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ABSTRACT

Strawberry halves were infused with Valencia orange pectinmethylesterase (PME) under vacuum for 15 min at room temperature. Fruits were blotted onto pectin paper and stained for activity. Activity of PME infused fruit was about twice (0.36 Unit/mL or 0.86 Unit/mg protein) that of non-infused control (0.19 Unit/mL) or water infused control (0.16 Unit/mL). Instron firmness values were not significantly different ($p \leq 0.05$) between non-infused and PME infused fruit. Firmness of PME infused fruit was about twice that of water infused control. Water soluble pectin (WSP), chelator soluble pectin (CSP), alkaline soluble pectin (ASP) and total pectin (TP) ranged from 8.24-8.70, 3.24-4.56, 3.77-5.39 and 17.86-26.16 mg/100mg Alcohol Insoluble Solid (AIS), respectively for all treatments.

Keywords: strawberry, pectinmethylesterase, pectin, vacuum infusion, firmness

Introduction

Textural properties play an important role in the quality of fruits and vegetables. A major problem in the processing of fruits is the susceptibility to textural loss during harvesting and handling. The changes in some fleshy fruits appear as tissue softening and loss of cohesiveness as well as a decrease in the extent of intermolecular bonding between cell wall polymers (van Buren 1979). Pectic substances are a group of closely associated polysaccharides from the primary cell wall and intercellular region of higher plant, which contribute to the firmness and structure of plant tissues. The softening of fruit during ripening and senescence is frequently attributed to the enzymatic degradation of cell wall materials (Hulme 1970).

Softening during ripening fruits due to pectic enzymes, such as polygalacturonases, is influenced by pectinmethylesterase (PME; EC 3.1.1.11). PME is widely distributed in all higher plants, plant-pathogenic bacteria and fungi and PME function is related to cell wall extension during cell growth and fruit ripening by de-esterification of pectin to low ester pectin (McMillian and Perombelon 1995). PME can increase firmness in fruits and vegetables by deesterification of pectin and chelation of calcium as crosslinks between carboxyl groups of adjacent chains (van Buren 1979). PME is activated at low-temperature thermal treatment (54-77 °C) resulting in firmness enhancement in potato (60-70°C) (Bartolome and Hoff 1972), carrot (54-77 °C) (Lee and others 1979), and frozen sweet cherry (70 °C) (Alonso and others 1993).

The activity of desirable enzymes, such as PME, may be enhanced by pre-processing treatments, such as vacuum infusion. Vacuum infusion facilitates the incorporation of sugar (Barton 1951; Main and others 1986), firming agents (Ponappa and others 1993) and PME (Suutarinen and others 2000) in strawberries. Vacuum infiltration of peaches with PME and $CaCl_2$ significantly increased firmness of canned peaches (Javeri and others 1991). Infusion

under vacuum of commercial fungal PME under optimized pre-processing conditions of pH and calcium level resulted in improvement in the texture of strawberry halves (Suutarinen and others 2000). Jams made from strawberries treated with $CaCl_2$ and crystallized sucrose were about twice as firm as reference samples (Suutarinen and others 2000). Firm pieces of fruit are desirable for industrial applications that use sliced or diced fruit in ingredients for dairy products, ice-cream, jam, confectionary, or cakes (Degraeve and others 2003).

Not all fruits and vegetables are amenable to infusion of exogenous ingredients (Baker and Wicker 1996) and infusion of large molecules, like enzyme, may not be uniformly distributed. Many aspects of the infusion process would benefit from development of analytical techniques to quantify translocation of enzymes within intact tissue (McArdle and Culver 1994). An indirect method for visualizing enzyme infusion into apple tissue was developed using a fluorescently labeled enzyme (Culver and others 2000). A direct technique to visually identify localization of enzyme infusion would be beneficial. The objective of this study is to develop a rapid method to visualize the location of PME in fruit after vacuum infusion and to study the effect on pectic substances and texture.

Materials and Methods

Fruit preparation and vacuum infusion

Fresh strawberries were purchased from local vendor and kept for 2-3 days at refrigerated temperature before experimentation. The fruits were halved, submerged in Valencia orange PME with an activity of 14-18 Unit/mL and infused under vacuum of 85 kPa for 15 min at room temperature. Non-infused and water infused strawberries were used as controls.

Pectinmethylesterase extraction

Valencia orange pulp was donated by Tropicana Products, Inc. (Bradenton, FL). Valencia PME extraction was modified from the method of Javeri and others (1991). PME was extracted from frozen pulp using deionized water at a ratio of water to pulp of 3 to 1. The Proscientific homogenizer (Pro 300A, Proscientific Inc., Monroe, CT) was used to homogenize (speed no 2 for 10 min) at 4 °C and the pH of the homogenate was adjusted to pH 8.0 by a few drops of 10 M NaOH to bring the pH close to 7.5 and 0.1 M NaOH to bring pH to 8.0 and to maintain at pH 8.0. The homogenate was filtered through Miracloth (CalBiochem, La Jolla, CA) and the filtrate was used for infusion.

Pectinmethylesterase activity

PME was extracted from a 5g aliquot of infused strawberries in 20 mL of buffer solution (0.1 M NaCl, 0.25 M Tris, pH 8.0) and that was homogenized in a Sorvall Omni Mixer (Du Pont, Newtown, CA) for 20 s at 4 °C. The homogenate was filtered through Miracloth. The PME activity of the filtrate was determined at pH 7.5, 30 °C by a pH stat titrator (Brinkmann, Westbury, NY) in 20 ml of 1% high methoxyl pectin and 0.1 M NaCl. The reaction was maintained at pH 7.5 and. A unit of PME activity was defined as the microequivalent of ester hydrolyzed/min at 30 °C.

PME activity print

PME activity in intact strawberry fruit was detected by modification of the hydroxylamine, ferric chloride staining procedure for PME isozymes (Delincee 1976). The pectin paper was prepared with blotting paper (S&S Filter paper, Item No: 10318311,Schleicher and Schuell Inc., NH) that was impregnated with 1% pectin buffered (0.1 M NaCl and 0.3 M Na_2HPO_4) at pH 7.5 and dried at room temperature for 1 h. The impregnating and drying

processes were repeated. The paper was dried overnight, wrapped in plastic wrap and stored in a Ziploc® bag at 4 °C for several weeks until use. The fruits were quartered to facilitate handling and the surface exposed to PME infusion was immediately blotted onto pectin paper for 10 min at room temperature and hot air dried. The print paper was immersed for 7 min in 60 mL of the alkaline hydroxylamine reagent, prepared by mixing equal volume of solution A (2.0 M hydroxylamine chloride ($NH_2OH \bullet HCl$) in 60% ethanol) and B (3.5 M NaOH in 60% ethanol). Then, the solution was acidified with 60 mL of C [25% HCl/ 95% ethanol (1:2, v/v)] and mixed on Red Rotor (Model PR70, Hoeffer Scientific Instruments, San Francisco, CA, USA) for 10 min. The above solution was discarded and the print paper was stained in 20 mL of solution D (0.15 M $FeCl_3$ in 0.1 N HCl, 60% ethanol) for 2 min. The paper was dried at room temperature.

Determination of firmness

Firmness of strawberries was determined with an Instron Universal Testing Machine Model 1122 (Instron Corporation, Canton, MA). The compression force was measured with a 5 Kg capacity load cell. A plunger (round shaped probe, 5.5 mm) penetrated to a depth of 7 mm at a plunger speed of 90 mm/min. The strawberry was placed cut surface up and the firmness was expressed as maximum force in Newtons. The firmness of both sides of cut fruit was determined. The mean of maximum force from two measurements per berry for 10 berries was used to determine average firmness of each treatment.

PME inhibitor

PME inhibitor (PMEI) was extracted by a modified method of Giovane and others (1995) and Wu and others (2002). Fresh strawberries were halved, homogenized in water (1:1 wt/vol), and centrifuged at 15,000 x g for 30 min at 4 °C. The extract was filtered through Miracloth and divided into two parts. The first part was adjusted to pH 6.0 by 1.0 M and 0.1 M NaOH (PMEI-

6). The second part was not adjusted to pH 6.0 (PMEI-N). Endogenous PME was extracted from strawberries by homogenization in 0.1 N NaCl, pH 7.5 (1:4 wt/vol), centrifugation at 15,000 x g for 30 min at 4 °C and filtration through Miracloth. A Valencia orange PME and PMEI-N or PMEI-6 was mixed at different ratios (1:3, 1:4, or 1:5). The mixtures were vortexed, incubated at 30 °C for 30 min, and assayed for the residual activity by titration. Deionized water was used as control.

Alcohol insoluble solids (AIS)

AIS was prepared as described by Huber and Lee (1986). A 200 g of strawberry sample was homogenized for 20 s in a Waring blender at room temperature at a ratio of one part of sample to four part of 95% ethanol. The homogenate was heated to boiling for 5 min and cooled to room temperature. The residue was filtered through a medium pore, sintered glass funnel (Fisher Scientific, Atlanta, GA). Based on the initial fresh weight, the residue was successively washed in four volumes of 95% ethanol and six volume of acetone. AIS was dried under the hood overnight, weighed, ground with a mortar and pestle and stored at –20 °C.

Extraction of pectin from AIS

The extraction of pectin from AIS was performed according to a modified method of de Vries and others (1981). A 1 g aliquot of AIS was extracted with 90 mL of 0.05 M sodium acetate buffer, pH 5.2 for 90 min at 60 °C. The water-soluble pectin (WSP) was collected by centrifugation at 8,000 x g, 20 min at 4 °C and filtered through Miracloth. The pellet was collected and resuspended in 90 mL of 0.05 M EDTA, 0.05 M ammonium oxalate, and 0.05 M sodium acetate buffer, pH 5.2 at 60 °C for 90 min. The extract was centrifuged at 8,000 x g, 20 min at 4 °C and filtered through Miracloth. The supernatant, chelator soluble pectin (CSP), was

collected. The pellet was resuspended with 90 mL of 0.05 M NaOH for 90 min at 60 °C, centrifuged and filtered as above. This supernatant was referred to alkaline soluble pectin (ASP).

Determination of degree of esterification (DE)

The degree of esterification (DE) was determined using a modified (Banjongsinsiri and others 2003) titration method (Anon. 1997). An aliquot of 1 g of AIS was extracted with 90 mL of deionized water for 90 min at 60 °C. The extract was centrifuged at 8,000 x g, 20 min at 4 °C and filtered through Miracloth. The supernatant was collected and analyzed for %DE. A volume of 20 mL of sample were titrated with 0.05 N NaOH, saponified 60 min at room temperature with 20 mL of 0.05 N NaOH, and neutralized with an equivalent amount of 0.05 N HCl. The total carboxylic acids groups were estimated by titration with 0.05 N NaOH. The endpoint of titration was estimated in the presence of phenolphthalein, but was quantified to an endpoint pH between 8.0 and 8.5. The %DE was estimated as the mole ratio of free to total carboxylic acids.

Total pectin

Total pectin (TP) of AIS was extracted by the method of Ahmed and Labavitch (1977). An aliquot of dried AIS (150 mg) was weighed into a 10 mL screw-capped tube. A total volume of 10 mL of concentrated sulfuric acid was incrementally added to the tube, vortexed for 2 s and cooled on ice after each addition. The sample was transferred to a 50 mL volumetric flask and made to volume with deionized water. This solution was filtered through glass wool and analyzed for the total pectin by using the m-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973).

Statistical analysis

Analysis of variance (ANOVA) was performed by SAS (SAS Institute 2000). The least significant difference (LSD) was used for comparison between means at significant level $p \leq 0.05$. All treatments were carried out in duplicate.

Results and Discussion

The infusion process affected the overall appearance of strawberry halves (Figure 3.1). Non-infused control strawberry halves had the greatest integrity (Figure 3.1A). Strawberry halves that were vacuum infused with water (control) exhibited the greatest surface damage with a soft and water soaked appearance (Figure 3.1B). Strawberry halves that were infused with PME had an intact appearance and were firm to the touch (Figure 3.1C). Baker and Wicker (1996) reported that gases were removed from fruit by vacuum and infusion of enzyme occurred when the vacuum was released. The advantage of this method is that enzyme solution remained in the fruit tissues after infusion. Sapers and others (1990) studied the application of browning inhibitors to cut apple and potato by vacuum infiltration. They reported that vacuum infused apple plugs, (17-98 kPa) had extensive water-logged appearance and browning discoloration while pressure infusion (34 kPa) was less detrimental. Sapers and others (1990) suggested that gaseous voids in apple tissue were removed by vacuum and replaced by treatment solution. Using pressure infiltration the gaseous voids were compressed, resulting in less absorption of treatment solution and less water-logging. Ponappa and others (1993) also reported that strawberry slices that were vacuum infiltrated with water caused a soft, water-soaked appearance, and polyamine-infiltrated slices exhibited less surface damage.

Preliminary work with whole strawberry showed that PME was infused from the bundle zone and not skin (epidermis). Previously, Baron-Epel and others (1988) showed that cell wall

pectins moderated the sieving effect of the cell wall. Strawberries were cut in half prior to vacuum infusion to facilitate infusion and detection of PME into fruit tissues.

PME visualization using print technique

The reaction of alkaline hydroxylamine with methyl ester of pectin produces pectin hydroxamic acids which react with ferric ion to form insoluble red complexes and is used as an estimation of the degree of esterification of pectic substances in fruit (Gee and others 1959). In addition, Delincee (1976) also developed the print technique, which was adapted in thin-layer isoelectric focusing to detect the activity of tomato pectinesterases. De-esterified pectin and high ester pectin react with alkaline hydroxylamine and stain with ferric chloride differently. De-esterified pectin was detected as yellow-white bands on a red-brown background of high ester pectin zone.

The PME activity from a spot of Valencia PME extract was seen as a yellow zone on a red-brown background paper (Figure 3.2A). Strawberries that were not treated (Figure 3.2B) or water infused (Figure 3.2C) had evidences of some native PME, but most of the blotted area remained brown, indicating low PME activity. The more yellow zone in the water infused fruit is probably due to cellular damage and release of endogenous PME. Fruit pieces after Valencia PME infusion showed the greatest yellow zone on the strawberry halves (Figure 3.2D). PME activity appears uniformly distributed across the exposed surface of the strawberry.

Effect of PME infusion on activity and firmness

The firmness of strawberry halves that were infused with PME was compared to non-infused and water control (Table 3.1). Water infused fruits were significantly less firm than non-infused control and PME infused fruit. There was no significant difference ($p \leq 0.05$) in firmness between non-infused control and PME treatment. According to Ponappa and others

(1993), there was no significant difference in firmness of strawberry slices immediately after vacuum infiltration with polyamines or calcium compared to control (water treatment) and untreated slices, but firmness increased after storage for 9 days at 1 °C. In this study, firmness was significantly greater than water infused control at the same day after infusion. Firmness measurement had high variation between and within replications. The firming effect involves the formation of free carboxyl groups, which increases the possibility and strength of binding of divalent cations, such as calcium, between pectin polymers (Sajjaanantakul and others 1989).

PME activity in non-infused (0.19 Unit/mL) and water infused control (0.16 Unit/mL) were significantly lower than that of PME treatment (0.36 Unit/mL) (Table 3.1). According to Javeri and others (1991), specific activity of PME in peach halves increased more than 20 fold after infusion. The PME activity of Valencia PME used for infusion was 14-18 Unit/mL and 0.36 Unit/mL was detected in strawberry after infusion, an increase of nearly 2 fold.

The level of detectable PME activity in infused strawberry may be related to several natural characteristics of the fruit beyond the concentration of PME. Balestrieri and others (1990) reported the finding of a glycoprotein inhibitor of PME in ripe kiwi fruit that also inhibited effectively PME from orange, tomato, apple, banana and potato at a 1:1 molar ratio of PME and PME inhibitor. Based on this information, we tested for PME inhibition in strawberry (Table 3.2). Inhibition by extracts of strawberry was tested on Valencia PME (12.7 Unit/mL). No PME activity was detected in PMEI-6 or PMEI-N. At ratios of Valencia PME to PMEI-6 or PMEI-N or 1:3 or 1:4, no inhibition was observed. At 1:5 ratio of PME to PMEI-6 or PMEI-N, an inhibition of 30% and 50% were observed, respectively. No inhibition was observed at 1:4 or 1:5 ratio of Valencia PME and water control. Thus, based on *in vitro* experiments there is some evidence of an inhibition of Valencia PME in strawberry. In addition to a possible PME

inhibitor, pH affects activity of Valencia PME. The optimum pH of orange PME activity is in the range of 7.6-8.0 (Pilnik and Rombouts, 1981), but the pH of strawberry is about 3.6. According to Versteeg and others (1978), the PME activity of pH 5.0 was decreased to 10% of the activity observed at pH 7.5. In addition, three forms of orange PME (Type I, II, and high mol. wt.) was inactivated at 70 ºC, 60ºC, and 90ºC, pH 4.0 (Versteeg and others 1980). There is a possibility that the inactivation or inhibition of exogenous Valencia PME can be attributed to the low pH of strawberry fruit. Finally, the pK_a of pectin carboxyl groups is about 3.6 (Cesaro and others 1982). The ionized carboxyl groups of pectin would be negatively charged at pH above 3.6. Metal ions such as calcium ion may competitively interact with these negatively charged groups (Nari and others 1991). Recently, Degraeve and others (2003) studied vacuum impregnation with fungal PME to improve firmness of pasteurized fruits. The authors observed that the final firmness of strawberry samples was not affected by significant variations in final PME activity or calcium content.

Pectin fractions and %DE

The major contribution to intercellular adhesion comes from CSP and the protopectin and softening during ripening or heating is accompanied by a loss of protopectin and increases in WSP (Massey and others 1964; Gross 1984). The WSP, CSP, ASP and TP content of strawberries are shown in Table 3.3. WSP accounted for the greatest proportion of pectic substances for the control and PME infused fruits. The TP on a dry weight basis decreased by infusion treatment. There were no significant differences ($p \leq 0.05$) in WSP, CSP, ASP, TSP and TP among the three treatments. The percentage of solubilized pectin varied between treatments. In non infused control and water infused control, 62% and 82% of the total pectin was solubilized as WSP, CSP and ASP. In PME infused strawberry, 80% of the total pectin was

solubilized. Hence, nearly 40% and 20% of the pectic substances in non infused and water infused controls are not considered in the analysis. The CSP represents 21%, 20% and 26% of the soluble pectin in non infused, water infused control and PME infused fruit, respectively. Yu and others (1996) found the positive correlation between the oxalate soluble pectin (chelator soluble pectin) content and firmness of irradiated strawberry.

The %DE of WSP three treatments showed no significant differences among them and were in the range from 69.3% to 70.8% (Table 3.3). The %DE values in this study are close to the 63% DE value reported by Carle and others (2001). They reported a decrease in %DE from 63% to 44% which was correlated with an increase of strawberry firmness during cold storage. In contrast, Wade (1964) revealed that the %DE of strawberry fruit is low and constant during ripening.

Conclusions

The PME print technique has been successfully adapted as a rapid method to detect localization of PME after infusion. Using this technique, it was shown that PME was uniformly infused into strawberry halves. PME assay confirmed an increase of PME activity in fruit. The localization of the enzyme in specific area of the strawberry fruit may impact on any overall firmness of strawberries. The distribution of pectic substances was not affected by PME infusion, which might due to an inhibitor or inactivation of Valencia PME in strawberry fruit. Firmness of non-infused and PME infused strawberries were greater than water infused strawberries. Infusion of PME with the vacuum technique may be a potential process aid to improve quality, particularly of strawberry pieces or purees.

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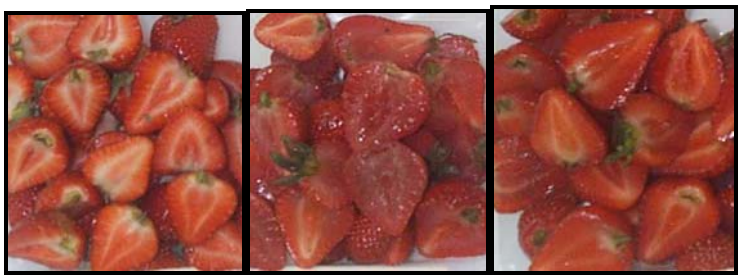

(A) (B) (C)

Figure 3.1. Strawberry after vacuum infusion (A) Non –infused control (B) Water infused control (C) PME infusion.

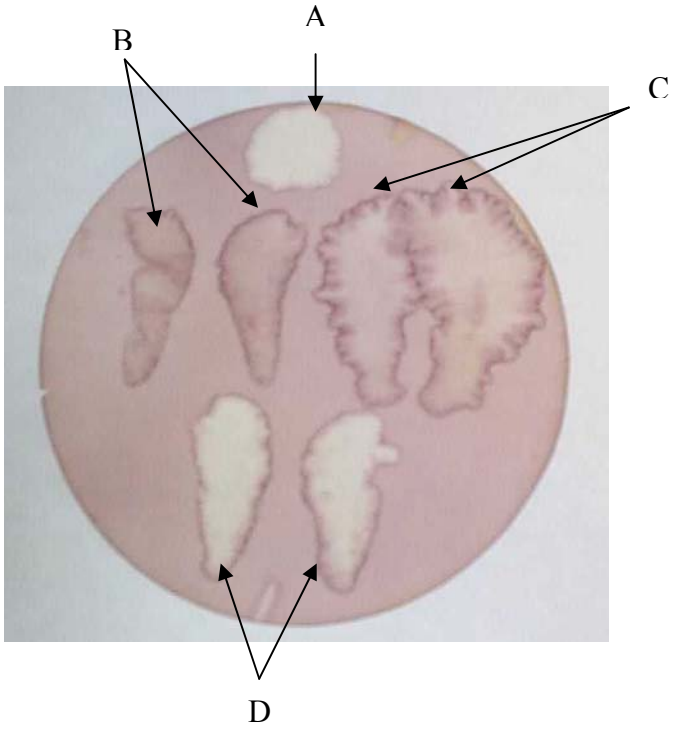

Figure 3.2. The PME print technique of strawberry (A) Valencia orange PME extract (B) Non-infused control (C) Water infused control (D) PME infusion.

Table 3.1. Firmness and PME activity of strawberry after vacuum infusion

Treatment	Firmness (N)	*PME activity in strawberry	
		Unit/mL	Unit/mg protein
Non-infused control	2.97 [a]	0.19 [b]	0.49
Water infused control	1.49 [b]	0.16 [b]	0.42
PME infusion	2.86 [a]	0.36 [a]	0.86

[a-b] Means with the same letter in each column are not significantly different at $p \leq 0.05$ as determined by LSD. *PME from non-infused control, water infused control and PME infused strawberry was extracted using 0.1 M NaCl, 0.25 M Tris at pH 8.0.

Table 3.2. PMEI activity of strawberry extract mixed with Valencia pulp extract.

Sample	Ratio	Enzyme activity (Unit/mL)
Valencia PME		12.7
Strawberry PME[a]		0.04
Strawberry PMEI-6[b]		No activity
Strawberry PMEI-N[c]		0.07
Val PME and PMEI-6	1:3	11.8
	1:4	12.1
	1:5	9.1
Val PME and PMEI-N	1:3	12.7
	1:4	14.5
	1:5	6.1
Val PME and deionized water	1:4	12.7
	1:5	13.3

[a]Strawberry PME was extracted by 0.1 NaCl. [b]Strawberry PME inhibitor extract was adjusted to pH 6.0. [c] Strawberry PME inhibitor extract was not adjusted pH to 6.0.

Table 3.3. Characterization of pectic substances of strawberry after vacuum infusion

Treatment	Pectin content (mg galacturonic acid /100mg dry wt.)					%DE
	WSP	CSP	ASP	TSP	TP	(WSP)
Non-infused control	2.13	0.83	0.96	3.92	6.31	69.3
Water infused control	2.29	0.96	1.50	4.74	5.78	69.5
PME infusion	2.41	1.25	1.14	4.79	6.00	70.8

TP =Total pectin, TSP= Total soluble pectin, WSP = Water soluble pectin, CSP = Chelator soluble pectin, ASP = Alkaline soluble pectin. TSP = WSP+CSP+ASP

CHAPTER 4

TEXTURE AND DISTRIBUTION OF PECTIC SUBSTANCES OF MANGO AS AFFECTED BY INFUSION OF PECTINMETHYLESTERASE AND CALCIUM[1]

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ABSTRACT

Fresh cut mangos were infused with pectinesterase (PME) and calcium chloride, and the effect on textural properties, distribution of pectic substance, and degree of esterification was determined. Temperature gradient infusion with PME and/or calcium chloride increased gumminess and chewiness, but had no impact on hardness and adhesiveness. The distribution of pectic substances, as protopectin or alkaline soluble pectin, was approximately twice that of water or chelator soluble pectin. The degree of esterification of water and chelator soluble pectic substances was near 50%-60%, and less than 10%, respectively. Heat sensitive PME inhibitor in mango was detected. The initial hardness of Kent mango was variable, and differences in distribution of pectic substances were observed. Texture of Kent mango is most likely moderated by changes in the solubility of insoluble pectin or by non-pectin components in the cell wall.

Keywords: mango, pectinmethylesterase, calcium chloride, texture profile analysis, pectin

INTRODUCTION

Softening and loss of texture is a problem in fresh cut and minimally processed fruits and vegetables. Mango (*Mangifera indica, L.*) is one of the most popular tropical fruits worldwide.[1] This fruit softens very rapidly in the mesocarp and, as the fruit approaches the fully ripened stage, becomes undesirable for consumption.[2] The softening is presumably related to the depolymerization and solubilization of pectic substances in the middle lamella of the cell wall, and is believed to involve cell wall hydrolases.[3] The activities of polygalacturonase (PG) and cellulase progressively increase during ripening, and are likely to be responsible for the extensive softening of Nam Dokmai mango.[4] β-galactosidase also increases during the ripening of Harumanis mango, and is associated with increased solubility and depolymerization of cell wall pectin.[5] In contrast, pectinmethylesterase (PME) and hemicellulase activities decrease in Tommy Atkins, Nam Dokmai, Harumanis, and Keitt mango during ripening.[1,4-6]

To minimize softening of fruits and vegetables, numerous process treatments are described in the literature. Low temperature blanching[7-9] and stepwise blanching[10] increase firmness. Low temperature blanching increases firmness in fruits and vegetables by stimulation of PME activity, which is activated between 50°C and 70°C. PME catalyzes the hydrolysis of methyl ester along the pectin chain to produce free carboxyl groups, which bind with cations, such as calcium, to form salt-bridges between adjacent pectin molecules.[11] Calcium in combination with low temperature blanching, improves the firmness in canned green beans, carrots[9] and frozen sweet cherries.[12]

However, low temperature blanching also activates detrimental enzymes, and may result in quality loss. Infusion of desirable, exogenous enzymes to improve quality or to inhibit deterioration is a useful technology and minimizes some of the deleterious reactions of low

temperature blanching.[13] Infusion of exogenous PME increases firmness of peaches[14] as well as strawberry pieces in jam.[15,16] Although infusion of exogenous PME into blanched fruits improves firmness of some fruits, it is not effective with others.[17] In particular, fruits and vegetables with a skin or tough surface, such as peas, corn, cherries and blueberries, resist infusion. Furthermore, fruits that are too soft, too hard, or without structural porosity or air voids, are not amenable to enzyme infusion.[17] Additionally, endogenous enzyme inhibitors may be present. For example, an inhibitor of PME in kiwi fruit (PMEI) inhibits PME from orange, tomato, apple, banana, and potato.[18] PMEI in unripe fruits is present as an inactive precursor, and is transformed into the active protein by proteinase action during ripening.[19] A PMEI is also found in jelly fig (*Ficus awkeotsang Makino*) and rubbery banana (*Musa sapientum L.*).[20,21] Our objectives were to evaluate infusion of exogenous PME into mango, to determine PME activity in tissue, and to determine the effect on textural properties of mango.

MATERIALS AND METHODS

PME and inhibitor extraction

For infusion, PME was extracted from Valencia orange pulp (Tropicana Products, Inc., Bradenton, FL) without sodium chloride using deionized water at a 3:1 ratio of water to pulp, homogenized using a Proscientific homogenizer (Pro300A, Proscientific Inc., Monroe, CT) at 4°C, and adjusted to pH 8.0 with 0.1 M and 10 M NaOH. The filtrate was collected through Miracloth (CalBiochem, La Jolla, CA) and used for infusion. The PME activity of the filtrate was analyzed by titration using a pH stat titrator (Brinkmann, Westbury, NY). The assay was conducted in 20 ml of 1.0% high methoxyl citrus pectin in 0.1 M NaCl, at pH 7.5 and 30°C. One unit of PME activity was defined as the amount of enzyme that released 1 μmol of carboxylic acid group per minute.

To determine the PME activity of mango, a 5 g aliquot of infused mango was homogenized (Proscientific homogenizer) in 20 ml of buffer solution (0.1 M NaCl, 0.25 M Tris, pH 8.0) for 20 s at 4°C. The homogenate was filtered through Miracloth. The PME activity of mango was determined using the titration method described above.

To test for mango PME inhibitor levels, mango (cv. Kent) was peeled, cut in 1.5 cm^3 cubes, and blanched in boiling water (100°C) at discrete time intervals between 60 and 150 s. Blanched or fresh mango cubes were homogenized and extracted (0.1 M NaCl, 0.25 M Tris, pH 8.0). The homogenate was filtered through Miracloth, and used as mango PME inhibitor. Valencia orange PME extracts were mixed with fresh or heated mango extracts at equal ratios.[20]

Raw material and process treatment

Mango fruits were purchased from a local supermarket. Mangos were initially sliced around 1-3 mm in depth to remove the skin using a meat slicer (Hobart Model 1612E, Hobart Corp., Troy, OH) and slices of 1.5 cm were obtained in a second cut. Finally, 1.5 cm^3 cubes were cut using a grid. Mango cubes without traces of peel were randomized for each process treatment.

In preliminary experiments to establish an infusion process, mangos (cv. Tommy Atkins) were infused with PME and calcium chloride by three processes. In the first process, unblanched mango cubes were infused with Valencia PME (13-16 Unit ml-1) under vacuum (85 kPa) for 600 s at room temperature. The second process consisted of pulsing infusion, in which the vacuum was pulled for 300 s, released, and repeated for another 300 s. In the third process, a temperature gradient infusion method was evaluated. Mango cubes were blanched for 150 s at 100°C, and immediately submerged in Valencia PME (13-16 Unit ml-1) with and without $CaCl_2 \cdot 2H_2O$ (100 or 1000 ppm) at 4°C for 2 h. In all process treatments, water infused mangoes and non–infused

mangoes were used as controls. The process treatments were replicated three times. Temperature gradient was selected for use in this study. In subsequent experiments, to test the effects of PME and calcium chloride on textural properties, mangoes (cv. Kent) were purchased from a local supermarket and stored at room temperature until used for analysis.

Microscopy

The preparation of sections for microscopy was modified based on a previously published method.[22] A subsection (1.5 x 1.5 x 0.5 cm^3) was excised from the original sample using a razor blade. This subsection was further reduced to 1 mm^3 cubes. The cubes were placed in glass vials, and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 21°C for 2 h. The fixative was decanted from the vials; the cubes were washed three times with distilled water, then placed in fresh fixative for overnight fixation. Fixative was again decanted from vials, and the cubes were washed three times with distilled water. Cubes were post-fixed with OsO_4 for 1 h. Prior to embedding in low viscosity Spurr resin, the cubes were dehydrated in a graded ethanol series (900 s per step).

Sections (60 μm) of the sample were obtained using a RMC Products MTX ultramicrotome (Research Manufacturing Company, Tucson, AZ). Sections were mounted on clean 200 mesh grids, and stained using uranyl acetate/lead citrate. Stained sections were visualized with a JOEL 100 CX II (Tokyo, Japan) transmission electron microscope (TEM).

Textural analysis

Samples of mango cubes were equilibrated to room temperature before textural analysis. Texture Profile Analysis (TPA) was used to evaluate mango texture using a Texture Analyzer (TA-XT2i, Texture Technologies Corp, Scarsdale, NY). TPA was performed using a cylindrical probe (35 mm diameter) with a 25 kg load cell. Samples were compressed in two consecutive

cycles to 25% deformation from the initial sample height of 1.5 cm, at a compression speed of 2 mm s-1. The textural parameters determined were hardness, stickiness, cohesiveness, springiness, gumminess, and chewiness[23,24]. Hardness, measured in Newtons (N), is defined as the first maximum force necessary to compress the sample. Stickiness (N) is defined as the negative force to pull the probe from the sample after the first compression. Cohesiveness is assessed using the ratio of the area of work during the second compression divided by the area of work during the first compression. Adhesiveness (N*S) consists of the negative force area for the first compression, and represents the work required to overcome the attractive forces between the surface of a food and the surface of other materials. Springiness (mm) is defined as the distance the sample was compressed during the second compression to the peak force. Gumminess (N) is the product of hardness and cohesiveness, and chewiness (N.mm) is the product of gumminess and springiness. At least fifteen samples were analyzed for each treatment.

Alcohol insoluble solid preparation

Alcohol insoluble solids (AIS) were prepared using a modified method.[25] Fruit tissue (50-100 g) was homogenized for 20 s with a Proscientific homogenizer in a ratio of one part of sample to four parts of 95% ethanol. The homogenate was boiled at 100°C for 300 s and cooled to room temperature. The residue was filtered through a sintered glass funnel (Fisher Scientific, Atlanta, GA) under vacuum. Based on the initial fresh weight, the residue was successively washed in four volumes of 95% ethanol and six volumes of acetone. Finally, the residue was dried under the hood overnight, weighed, ground with a mortar and pestle, and stored at –20°C.

Extraction of pectin fraction from AIS

The pectin from AIS was fractionated based on solubility into water, chelator, or alkali soluble pectins according to a modified method.[26] An aliquot of 0.5 g of AIS was extracted for

1.5 h at 60°C with 90 ml of 0.05 M sodium acetate buffer at pH 5.2. The water-soluble pectin (WSP) was collected by centrifugation at 8,000 x g for 20 min at 4°C, and filtered through Miracloth. The pellet was collected and resuspended for 1.5 h at 60°C in 90 ml of 0.05 M EDTA, 0.05 M ammonium oxalate, and 0.05 M sodium acetate buffer at pH 5.2. The extract was centrifuged at 8,000 x g for 20 min at 4°C, and filtered through Miracloth. The supernatant, consisting of chelator soluble pectin (CSP), was collected. The pellet was resuspended with 90 ml of 0.05 M NaOH for 1.5 h at 60°C, and centrifuged and filtered as described above. The resulting supernatant was collected as alkaline soluble pectin (ASP). Pectin (galacturonic acid) content of each fraction was analyzed using an m-hydroxydiphenyl method.[27]

Total pectin determination

Total pectin (TP) was extracted from AIS.[28] A dried AIS aliquot (10 mg) was weighed into a 10 ml screw-capped tube. Concentrated sulfuric acid was incrementally added to the tube, vortexed for 2 s, and cooled in an ice bath, until a total of 10 ml of sulfuric acid was added. The dissolved sample was transferred into a volumetric flask, and deionized water was added to make a 50 ml volume of solution. This solution was then filtered through glass wool, and analyzed for TP.[27]

Determination of degree of esterification (DE)

The percent degree of esterification (%DE) was determined using a modification of the titration method.[29] A volume of 15 ml of WSP and CSP fractions, with an estimated concentration between 0.1 to 0.4 mg/ml, was used. The free carboxylic acid groups were titrated with 0.01 M NaOH. Samples were saponified with 10 ml of 0.05 M NaOH for 30 min at room temperature and neutralized with an equivalent amount of 0.05 M HCl. The total carboxylic acids groups were estimated by a second titration with 0.01 M NaOH. The endpoint of titration

was estimated in the presence of phenolphthalein, but was quantified by titration to an endpoint pH near 8.0. The %DE was estimated as the ratio of free to total carboxylic acid groups.

Statistical analysis

The randomized complete block design was conducted to evaluate the influence of treatments (water, PME, calcium chloride, and PME plus calcium chloride). The analysis of variance was performed using Proc GLM with mixed effect model and SAS statistical package V8.2 (SAS Institution Inc., Cary, NC). Means and standard deviations were reported and differences between means were compared using Fisher's least significant difference (LSD) at $p \leq 0.1$.

RESULTS AND DISCUSSION

Vacuum infusion, pulsed vacuum infusion, and temperature gradient infusion influenced the appearance of mango cubes. The firmness of unblanched, PME, and calcium chloride vacuum infused mango cubes was not significantly different from the control (no treatment) and control (water infused mango cubes). Pulsed infusion of PME and calcium chloride also did not increase firmness (data not shown). Vacuum and pulsed infusion resulted in a swollen, water-logged appearance of mango cubes. Other studies have reported that vacuum infusion altered the appearance of produce. For example, vacuum infiltration of preservative solutions in apple slices resulted in a water-logged and translucent appearance.[30] In addition, apple plugs infused with browning inhibitors under vacuum (68-98 kPa) resulted in extensive water-logging and development of dark color.[31] Of the infusion methods evaluated, temperature gradient infusion gave the greatest effect on texture with minimal change of integrity of mango cubes. Temperature gradient infusion with Valencia PME and/or calcium chloride influenced firmness of mango with variable results. Mangoes have variable fiber content [32, 33] which may influence

infusion efficacy and measurement of textural properties. Quantity and orientation of fiber may act as a barrier to infusion of exogenous compounds as well as obscure textural measurements. Tommy Atkins cv has moderate fiber and Kent cv has little fiber content.[32, 33] In subsequent experiments, Kent cv and temperature gradient infusion was used.

PME inhibitor

When Kent mango was tested for the presence of a PME inhibitor, no PME activity was found in unblanched or blanched (60-150 s) mango cubes (Figure 4.3B). The PME activity values of mixtures of equal volumes of heated mango extract and Valencia extract were in the range of 13.1 to 13.7 Units ml-1 (Figures 4.1D-4.1G), which are similar to that found in the control Valencia PME (11.6 ± 1.6 Units ml-1) (Figures 4.1A and 4.1H). However, the PME activity in the mixture of unheated mango and Valencia PME decreased (Figure 4.1C). Thus, it is possible that a heat sensitive inhibitor of Valencia PME is present in the mango extract. A PME inhibitor has been identified in kiwi fruit that inhibits PME from orange, tomato, apple, banana, and potato.[18] Recently, a PME inhibitor was also found in jelly fig (*Ficus awkeotsang Makino*)[20] and rubbery banana (*Musa sapientum* L.).[21] Based on these results, a PME inhibitor is likely to be present in fresh mango (cv. Kent) as well.

Microstructural studies

To further evaluate the impact of infusion on the texture, the ultrastructure of temperature gradient infused Kent mango was determined. The ultrastructure appeared intact in the untreated sample (Figure 4.2A). The ultrastructure of the samples treated with water (Figure 4.2B) appeared damaged compared to untreated controls. Further, greater loss of structural integrity was observed in samples treated with PME plus calcium chloride (Figure 4.2C). The cell wall of the samples treated with a combination of PME and calcium chloride had a more undulating

appearance than the untreated control or the water infused control. The untreated control was the only sample in which the cytoplasm was closely associated with the cell wall (Figure 4.3A). The internal structures of cell wall from water (Figure 4.3B) and PME plus calcium chloride (Figure 4.3C) infused samples were not as clearly defined as the internal structure of the untreated samples. The cellular structures within the tissue of the PME plus calcium chloride treated samples appeared ruptured. The cellular structures within the tissue of the water treated samples appeared to be beginning the rupturing process as evidenced by the bleb (Figure 4.3B, arrow) protruding through the membrane of the organelle.

Texture profile analysis (TPA)

The hardness of the non-infused control samples was significantly different between replications (Table 4.1). The hardness of non-infused control samples measured in replication 1 (70 N) was greatest when compared to replications 2 (33 N) and 3 (50 N). Thus, results for each replication of analysis are presented separately. Significant differences ($p \leq 0.1$) in adhesiveness, gumminess, and chewiness were observed depending on treatment. For example, the gumminess and chewiness of water treated mangos were significantly less than those values measured in other treatment groups (Replication 1). Mango treated with calcium chloride was more adhesive and sticky than the water infused control and PME with or without calcium chloride samples (Replication 2). No significant differences ($p \leq 0.1$) in treatment effects were seen for hardness, cohesiveness, and springiness within replications.

Pectic substances and DE

The measured total pectin, solubilized from AIS with sulfuric acid, ranged from 0.027–0.062 g kg^{-1} dry weight. In all cases, infusion significantly increased the amount of total pectin. The range of % total soluble pectin (TSP) from non-infused control, water infused control, PME,

calcium chloride, and calcium chloride plus PME temperature gradient infusion (3 replications) was 70%-125% (Table 4.2). Values of %TSP greater than 100% and an increase in total pectin on a dry weight basis with infusion may partly be due to an artifact of measurement. Although the m-hydroxydiphenyl method improved the specificity for uronic acid in the presence of neutral sugars, nonspecific color development in the first heating overestimated uronic acid.[34] Since infusion ruptured the cell wall (Figures 4.2, 4.3), neutral sugars may be released that result in overestimation of uronic acid content.

Of the AIS that fractionated according to solubility, the amount of alkali soluble pectin was the highest compared to water or chelator soluble pectin, regardless of infusion treatment. Of the solubilized pectin, the levels of WSP (17%-38%) and CSP (12%-28%) were lower than the content of ASP (47%-60%) (Table 4.2). The values are in contrast to a previous report[6] that the amount of water soluble (29%-36% of AIS) was greater than alkali-soluble pectins (4%-10% of AIS) and declined, while oxalate soluble pectin (3%-4.5% of AIS) increased during mango ripening. The authors suggested that pectin depolymerization increased the amount of alcohol soluble material in mangos and influenced the extraction and distribution of pectin fractions.[6] Others reported that ASP is the major pectic substance in Keitt and Tommy Atkins mangos, regardless of the ripening stage.[1] In this study, ASP also represented the major pectic substance in Kent. After infusion treatment, alkali soluble pectin remained about twice water or chelator soluble pectin. Water and chelator soluble pectin were extracted in similar amounts. Infusion is probably not only influencing the relative distribution of pectin fractions in water, chelator, or alkali, but also influencing the extent of hydrolysis in sulfuric acid (g kg^{-1} total pectin) and relative amounts of total soluble pectin to total pectin. Most values of %TSP were close to 100%.

The %DE of water soluble pectin in all treatments ranged from 38%-61%. The range of %DE for water soluble pectin extracted from the water infused control fruit (52%-61%), PME infused fruit (52%-53%), calcium chloride infused fruit (54%-57%), and PME plus calcium chloride infused fruit (38%-50%) was generally lower than typical water soluble pectin. Water soluble pectin from PME treated mango tended to have lower %DE values than controls or calcium infused fruit. The range of %DE for pectin soluble in EDTA/oxalate buffer was low, 4-8 %DE, regardless of treatment. Previously reported values for average %DE of mango pectin range from 68%[35] to 80%-90%DE[36]. In addition, %DE values between 70% and 80% were typical in water soluble apple and citrus pectins and %DE values greater than 75-80% were not naturally present.[37,38] The lower %DE of water soluble pectin in PME treated fruit in this experiment was interesting. First, de-esterification of pectin occurred, in the absence of detectable PME in mango extracts as well as in the presence of a PME inhibitor in mango. Second, water soluble, low %DE pectin may occur if low molecular weight pectins were present. To measure MW of pectin fractions, WSP was precipitated with ethanol, to concentrate the samples for gel permeation chromatography. However, no solids were recovered (data not shown), which suggested depolymerization of pectin and increased alcohol soluble material, as previously reported.[6]

PME and calcium infusion had some influence on mango texture and properties of pectic substances. Previous research reported that calcium chloride has a minimal effect on the texture of canned mango fruit, if the stage of ripeness was past that suitable for canning.[2] In addition, softening of different mango cultivars during ripening was related to the cell wall degrading enzymes PME, PG, cellulase, and β-galactosidase.[5,39] The PME activity decreased continuously during ripening of Kitchner and Dr. Knight mango. However, in Abu-Samaka, PME activity

increased and subsequently decreased. In contrast, PG and cellulase activity progressively increased during ripening of all three cultivars and was highly correlated with the loss of fruit firmness.[5] In Pakistani mangoes, no correlation between ripening and changes in PME activity was observed due to the inconsistent pattern of the PME during ripening.[40]

CONCLUSIONS

Mango cubes infused with Valencia orange PME and calcium chloride had greater gumminess and chewiness, but displayed no difference in firmness compared to mango cubes without treatment. AIS extracted from treated and untreated mangos was high in insoluble pectin. Water soluble pectins had degree of esterification values near 38%-61%. The incongruity in pectin distribution and presence of a PME inhibitor, suggests altered solubility of pectin by infusion treatment. Kent mango texture is most likely moderated by the change in solubility of large molecular weight, insoluble pectin. Other components in the cell wall, such as cellulose and hemicellulose, may contribute to softening or inhibition of firming by infusion of PME and calcium chloride.

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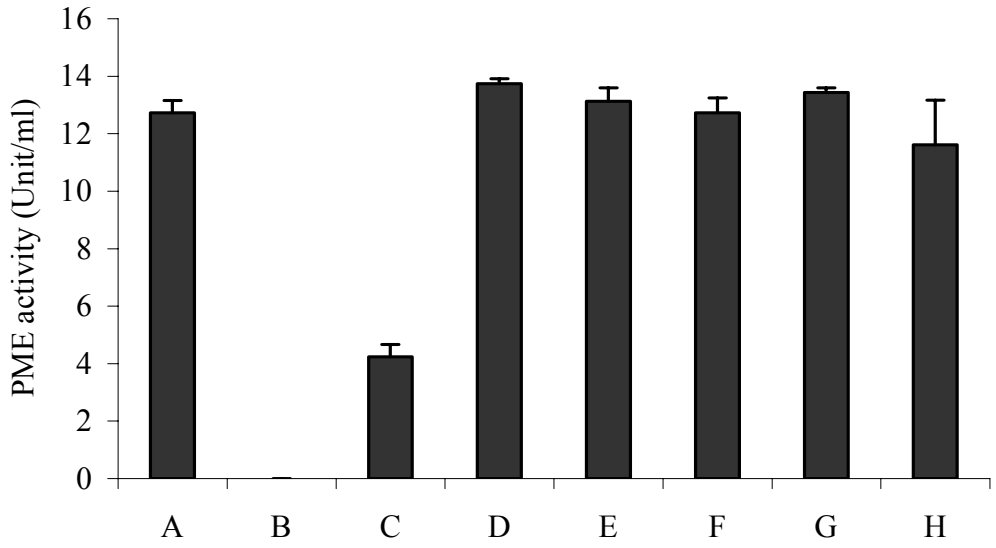

Figure 4.1. PME activity of Kent mango extract, Valencia orange pulp extract, and Kent mango extract mixed with Valencia extract. A: PME activity extracted from Valencia pulp. B: PME activity from blanched mango cubes (60-150 s). Unheated mango extract also displayed no PME activity (data not shown). C: The mixture of equal ratio of A and B (extract from fresh raw material). D: The mixture of equal ratio of A and B (mango blanched for 60 s). E: Mixture of equal ratio of A and B (mango blanched for 90 s). F: The mixture of equal ratios of A and B mango blanched for 120 s). G: The mixture of equal ratios of A and B (mango blanched for 150 s). H: The mixture of equal ratios of A and deionized water (control).

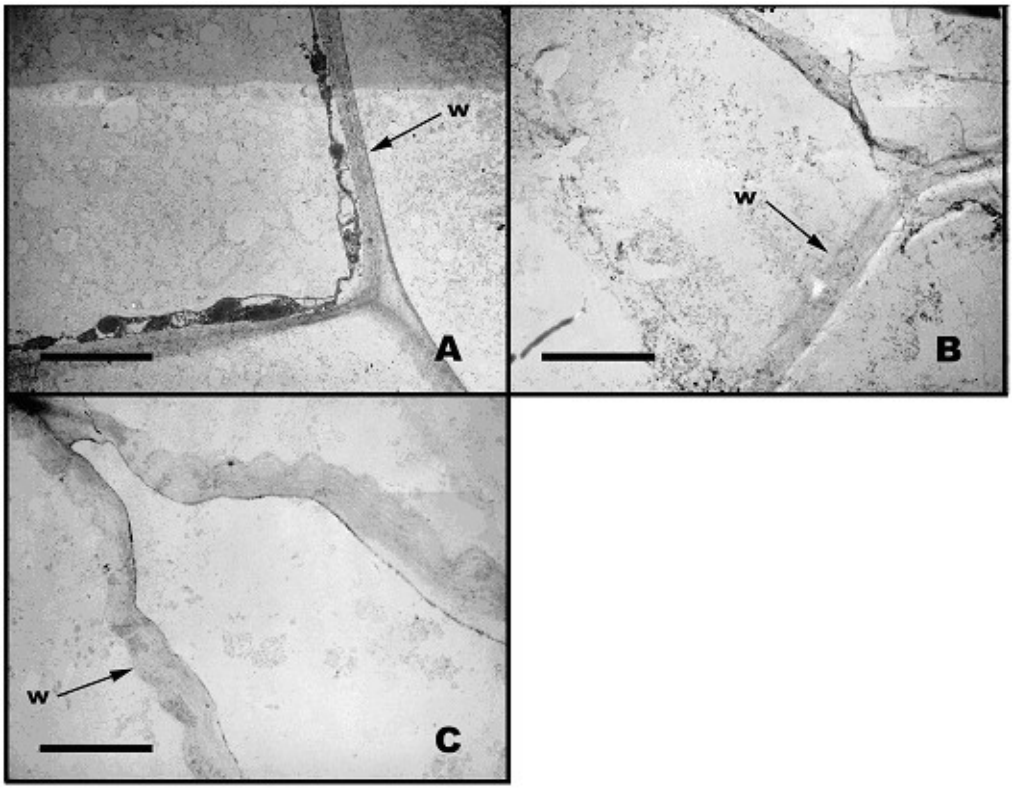

Figure 4.2. Transmission electron micrographs of temperature gradient infusion Kent mango tissue. (A) Untreated mango, intact cell wall (w) structure, (B) mango treated with water, change in cell wall, (C) mango treated with PME and calcium, change in cell wall. Bar = 500µm

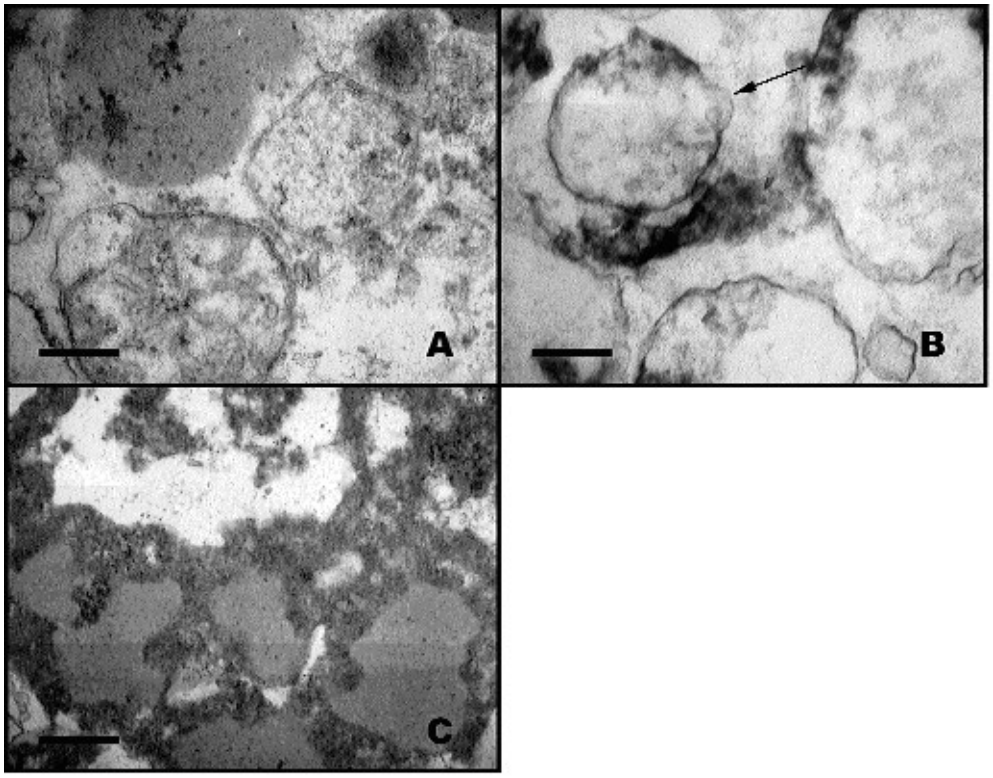

Figure 4.3. Transmission electron micrographs of temperature gradient infusion Kent (A) untreated mango, no visible change in internal cellular structure, (B) mango treated with water, bleb visible protruding through membrane of organelle (arrow), (C) mango treated with PME and calcium, internal structure of organelle is disrupted. Bar = 25µm

Table 4.1. Texture Profile Analysis of Kent mango after temperature gradient infusion

Test ID	Hardness (N)	Stickiness (N)	Cohesiveness (unitless)	Adhesiveness (N.s)	Springiness (mm)	Gumminess (N)	Chewiness (N.mm)
Replication 1							
RW	70.07^{x}	-2.03^{x}	0.42^{x}	-8.18^{x}	2.13^{x}	30.2^{x}	65.26^{x}
Water	29.71^{a}	-0.71^{a}	0.31^{a}	-3.44^{a}	2.05^{a}	8.86^{b}	18.19^{b}
PME	31.09^{a}	-0.66^{a}	0.33^{a}	-2.97^{a}	2.12^{a}	9.94^{a}	21.02^{a}
$CaCl_2$	30.22^{a}	-0.52^{a}	0.34^{a}	-1.79^{a}	2.27^{a}	10.05^{a}	23.05^{a}
PME + $CaCl_2$	32.50^{a}	-0.61^{a}	0.33^{a}	-2.59^{a}	2.11^{a}	10.59^{a}	22.52^{a}
Replication 2							
RW	33.34^{y}	-2.37^{y}	0.37^{y}	-10.15^{y}	1.98^{y}	12.32^{y}	24.46^{y}
Water	19.31^{a}	-0.53^{b}	0.36^{a}	-2.46^{a}	2.07^{a}	6.98^{a}	14.35^{a}
PME	19.91^{a}	-0.72^{b}	0.37^{a}	-3.54^{a}	2.04^{a}	7.16^{a}	14.55^{a}
$CaCl_2$	20.31^{a}	-0.84^{a}	0.35^{a}	-4.43^{b}	1.83^{a}	6.88^{a}	12.62^{a}
PME + $CaCl_2$	20.14^{a}	-0.70^{b}	0.37^{a}	-3.50^{a}	1.98^{a}	7.30^{a}	14.35^{a}
Replication 3							
RW	48.90^{z}	-3.04^{z}	0.39^{z}	-7.05^{z}	2.10^{y}	19.46^{z}	40.23^{z}
Water	21.44^{a}	-0.54^{a}	0.35^{a}	-2.39^{a}	2.14^{a}	7.42^{a}	15.98^{a}
PME	22.97^{a}	-0.52^{a}	0.36^{a}	-2.35^{a}	2.21^{a}	8.09^{a}	18.00^{a}
$CaCl_2$	21.38^{a}	-0.50^{a}	0.36^{a}	-2.13^{a}	2.18^{a}	7.54^{a}	16.39^{a}
PME + $CaCl_2$	23.18^{a}	-0.58^{a}	0.35^{a}	-2.69^{a}	2.12^{a}	7.94^{a}	16.91^{a}

RW=Unblanched non-infused control, Water =water infused control, PME (13-16 Unit ml-1), $CaCl_2$ =$CaCl_2{\cdot}2H_2O$ (1000ppm), and PME (13-16 Unit ml-1 + $CaCl_2{\cdot}2H_2O$ (1000ppm)).

$^{a-b}$Columns with the same letter within each replication are not significantly different. ^{x,y,z} Different letters (x,y,z) in the same column of RW indicate significant differences ($p \leq 0.1$) among replications.

Table 4.2. Distribution of pectic substance of Kent mango after temperature gradient infusion

Treatment	%TP (mg 100mg-1 dry wt.)	Pectin fraction %WSP	%CSP	%ASP	%TSP (mg 100mg-1 dry wt)
Replication 1					
RW	3.71^{d}	20.44^{c}	24.64^{a}	54.93^{b}	85.63^{c}
Water	6.15^{a}	24.97^{ab}	22.42^{ab}	52.62^{c}	98.40^{ab}
PME	4.25^{c}	27.61^{a}	20.16^{b}	52.23^{c}	90.71^{bc}
$CaCl_2$	5.44^{b}	18.59^{c}	21.31^{b}	60.11^{a}	90.90^{bc}
PME+$CaCl_2$	4.40^{c}	22.06^{bc}	21.05^{b}	56.89^{b}	103.68^{a}
Replication 2					
RW	2.66^{d}	25.16^{b}	27.70^{a}	47.15^{b}	91.36^{c}
Water	3.73^{b}	26.99^{ab}	21.15^{c}	51.87^{b}	101.99^{b}
PME	3.74^{b}	28.41^{a}	21.20^{c}	50.41^{b}	105.07^{b}
$CaCl_2$	4.00^{a}	20.18^{c}	21.00^{c}	58.83^{a}	97.93^{bc}
PME+$CaCl_2$	3.07^{c}	25.43^{ab}	24.43^{b}	50.15^{b}	125.38^{a}
Replication 3					
RW	3.39^{c}	25.18^{c}	19.67^{a}	55.15^{b}	70.01^{b}
Water	5.69^{a}	30.08^{b}	14.33^{dc}	55.60^{b}	67.20^{b}
PME	4.05^{b}	37.29^{a}	15.50^{bc}	47.22^{c}	97.30^{a}
$CaCl_2$	5.37^{a}	23.29^{c}	17.74^{ba}	58.98^{a}	76.08^{b}
PME+$CaCl_2$	4.22^{b}	28.42^{b}	12.49^{d}	59.09^{a}	75.37^{b}

RW=Unblanched non-infused control, Water=Water infused control, PME (13-16 Unit ml-1), $CaCl_2$=$CaCl_2 \cdot 2H_2O$ (1000ppm), and PME (13-16 Unit ml-1 + $CaCl_2 \cdot 2H_2O$ (1000ppm)).

Three replications of extraction are presented separately. Data presented within one replication is the average of three assays.

TP=Total pectin, TSP=Total soluble pectin, WSP=Water soluble pectin, CSP=Chelator soluble pectin, ASP=Alkaline soluble pectin.

TSP=WSP+CSP+ASP %WSP=(WSP/TSP)*100, %CSP=(CSP/TSP)*100, %ASP=(ASP/TSP)*100, %TSP=(TSP/TP)*100,

%TP=Uronic acid/ dry wt. $^{a\text{-}b}$Columns with the same letter within each replication are not significantly different ($p \leq 0.1$).

CHAPTER 5

VACUUM INFUSION OF PLANT, FUNGAL PECTINMETHYLESTERASE AND CALCIUM AFFECTS TEXTURE AND STRUCTURE EGGPLANT[1]

[1]Banjongsinsiri P, Shields J, Wicker L. To be submitted to *Journal of Food Science*.

ABSTRACT

The effect of vacuum infusion on eggplant quality of a commercial fungal (*Aspergillus niger*) and Marsh grapefruit (MGF) pectinmethylesterase (PME) with $CaCl_2$ (4000 ppm) was investigated after processing and during storage. Firmness of infused eggplants using both PMEs were significantly increased compared to control (fresh non-infused and water-infused control) after processing and during a storage time of 7 days at 4 °C. Activity of fungal PME infused eggplant was increased almost 32 times, while activity of eggplant infused with MGF PME increased 2 folds. Degree of esterification of infused eggplants with both PMEs decreased slightly. Cryo-SEM that showed that treated samples with fungal PME/ $CaCl_2$ displayed more integrity among cells as compared with water-infused control. The change of pectin in cell wall was visualized using monoclonal antibodies JIM5 (low esterified pectin) and JIM7 (high esterified pectin). Both antibodies labeled the cell wall of non-infused control. JIM5 showed more binding than JIM7 with the cell walls of eggplant tissues from fungal PME/ $CaCl_2$ treatment.

Keywords: eggplant, pectinmethylesterase, degree of esterification, vacuum infusion, firmness

Introduction

Pectinmethylesterase (PME, EC 3.1.1.11) is widely distributed in plant and microorganisms, and catalyzes the hydrolysis of methoxyl ester groups of polygalacturonic acids (Rexova-Benkova and Markovic 1976). PME plays an important role in the textural changes of fruits and vegetables. In fruit softening during ripening, PME removes methyl groups from the cell wall pectin, which enhances polygalacturonase activity and results in lower intercellular adhesiveness and tissue rigidity (Alonso and others 1997b). However, firmness and quality of fruit and vegetable products can be enhanced by PME and formation of calcium pectate (Alonso and others 1997a; Alvarez and others 1999; Fuchigami and others 1995).

Differences between plant and fungal PMEs have been reported in mode of action, pH optima, and salt dependency (Savary and others 2002; Cameron and others 2003). According to Denes and others (2000), the action patterns for apple PME at pH 7.5 consisted of a blockwise distribution by a single chain mechanism, while the action at pH 4.5 was also a blockwise distribution with shorter blocks by a multiple chain. PMEs from *A. niger* act by a multiple chain mechanism, removing the methoxyl groups at random (Massiot and others 1997). Optimal pH of plant PMEs was found in the range between 6.0-9.5, while optimal *A. niger* PME activity is in pH 4.5 (Rexova-Benkova and Markovic 1976). Several studies reported that calcium gel strength of low methoxyl pectin produced by blockwise acting PMEs was weaker than that observed for randomly deesterified pectins (Hills and others 1949; Speiser and Eddy 1946; Powell and others 1982). In addition, low methoxyl pectin deesterified with a randomly acting *Aspergillus* PME produced relatively strong calcium gels (Ishii and others 1979).

Vacuum infusion technology has been used as a pretreatment in many different processing applications to improve the quality of product structure by active incorporation of

functional ingredients (Baker and Wicker 1996; McArdle and Culver 1994; Saurel 2002). Vacuum infusion with plant and fungal PME with/or without calcium chloride as a pretreatment increased firmness of several fruit products. In canned peaches, vacuum infusion of Marsh grapefruit PME and/$CaCl_2$ increased firmness (Javeri and others 1991). The pretreatment of strawberries with commercial *Aspergillus* PMEs and $CaCl_2$ in a vacuum also increased firmness of strawberry jam (Suutarinen and others 2002). Recently, vacuum infusion pretreatment with plant or fungal PME and $CaCl_2$ improved the firmness of strawberries (Degraeve and others 2003; Banjongsinsiri and others 2003a) and mango (Banjongsinsiri and Wicker 2003b). The response of different kinds of vegetables to vacuum infusion treatment in terms of sample volume deformation and infusion levels was studied by Gras and others (2002). Eggplants had a porosity of 60% compared to carrot (0.3%), mushroom (37%), and zucchini (18%), and wider pores of the eggplant enhanced infusion. Based on this information, eggplants were selected to determine the effect of two types of plant PME as well as commercially derivable fungal PME on physical and chemical properties. Microstructural features were also determined using Cryo-SEM. Antibodies specific to low ester pectin (JIM5) and high ester pectin (JIM7) were used to investigate changes in pectin of the cell wall of eggplant tissue after treatment.

Materials and Methods

Raw material and sample preparation

Fresh eggplants (*Solanum melongena* L. cv. Classic) were obtained from a local grocery and kept at refrigerated temperature until use. Samples were sliced (1.0 cm thick) with a meat slicer (Hobart Model 1612E, Hobart Corp, Troy, OH). To avoid seeds, cylindrical samples (8 mm in diameter) were cut with a borer (No 6) (Boekel Brass-plated cork borer, Fisher Scientific,

Atlanta, GA) at the parenchyma cells close to the epidermis. To prevent browning reaction of samples, ascorbic acid (200 ppm) was mixed with test solution before vacuum infusion.

Microbial pectinmethylesterase

A microbial pectinmethylesterase derived from *Aspergillus niger* (ROHAPECT® MPE, Rohm Enzyme Gmbh, Darmstadt, Germany) was donated. The optimal pH of this enzyme is 4.5.

Plant pectinmethylesterase extraction

Valencia orange pulp and Marsh grapefruit pulp were donated by Citrus World (Lake Wales, FL). PME extraction was modified from the method of Ackerley and others (2002). Crude extract was extracted from frozen pulp with 0.1 M NaCl, 0.25 M Tris buffer, pH 8.0 at a ratio of buffer to pulp of 3 to 1. The extract was homogenized (Pro 300A, Proscientific Inc., Monroe, CT) at 4 °C. The pH of the homogenate was adjusted to pH 8.0 with a few drops of 10 M NaOH to bring the pH close to 7.5, and 0.1 M NaOH to bring the pH to 8.0 and to maintain at pH 8.0. The homogenate was filtered through Miracloth (CalBiochem, La Jolla, CA). Ammonium sulfate cut was used to minimize the viscosity of crude PME pulps. The filtrate was concentrated by 30% ammonium sulfate precipitation overnight at 4 °C, and centrifuged (Sorvall RC-5B centrifuge, Dupont Instruments, Doraville, GA) at 8000 × g at 4 °C for 20 min. The supernatant was dialyzed overnight against deionized water at 4 °C and used for infusion. The dialysis tubing (Spectra/Por, MWCO 12-14,000, Fisher Scientific, Atlanta, GA) was boiled in 10% acetic acid for 5 min, and rinsed with deionized water.

Pectinmethylesterase activity

PME was extracted from a 5g aliquot of infused eggplant with plant PME in 20 mL of buffer solution (0.1 M NaCl, 0.25 M Tris, pH 8.0), and homogenized in a Sorvall Omni Mixer

(Du Pont, Newtown, CA) for 20 s at 4 °C. The homogenate was filtered through Miracloth. The PME activity of the filtrate was determined by a pH stat titrator (Brinkmann, Westbury, NY) at 30 °C in 1% high methoxyl pectin (Citrus pectin type CC104, Citrus Colloid Ltd., Hereford, U.K.) and 0.1 M NaCl. A set point pH of 7.5 and 4.5 was used to assay plant and fungal PME, respectively. A unit of PME activity was defined as the microequivalent of ester hydrolyzed/min at 30 °C. The method to extract PME from infused eggplant with microbial PME was similar to plant PME, except utilized 0.25 M acetate in 0.1 M NaCl at pH 4.5.

Vacuum infusion procedures and process pretreatments

In the preliminary experiments, three infusion procedures were evaluated. The treatments were (A) vacuum infusion at 68 kPa for 15 min at 30 °C, (B) pulsed vacuum (85 kPa for 5 min, release vacuum to atmospheric pressure for 1 min, reapply vacuum for 5 min, and release again for 5 min at 30 °C), and (C) temperature gradient without vacuum from 30 °C to 4 °C and stored at 4 °C for 26 hr.

To determine the effects of PME and calcium, twelve tests were performed using the three infusion pretreatments (A, B and C). Test 1(RW): non-treated eggplants were used as non-treated control. Test 2 (Water): eggplant cylinders were infused in deionized water as water-infused control. Test 3 (Ca): eggplant cylinders were infused with 4000 ppm $CaCl_2 \cdot 2H_2O$. Test 4 (ValPME): Valencia orange PME (25 Unit/ml). Test 5 (FungalPME): eggplant cylinders were infused with fungal PME (10 Unit/ml). Test 6 (MGFPME): eggplant cylinders were infused with Marsh grapefruit PME (10 Unit/ml). In tests 7, 8 and 9, eggplant cylinders were infused with 4000 ppm $CaCl_2 \cdot 2H2O$ with ValPME (ValPME+Ca), FungalPME (FungalPME+Ca) and MGFPME (MGFPME+Ca). Test 10: eggplant cylinders were infused in 4000 ppm $CaCl_2 \cdot 2H_2O$ under vacuum of 68 kPa for 5 min at 30 °C, followed by treatment A. Test 11: eggplant

cylinders were infused in 4000 ppm $CaCl_2 \cdot 2H_2O$ under vacuum of 85 kPa for 5 min at 30 °C, followed by pretreatment B. Test 12: eggplant cylinders were soaked in 4000 ppm $CaCl_2 \cdot 2H_2O$ for 1 hr at 4 °C and followed by treatment C.

Experimental procedure

Pulsed vacuum infusion was selected to use in further studies with 7 test pretreatments: Test 1 (RW), 2 (Water), 3 (Ca), 5 (FungalPME), 6 (MGFPME), 7 (FungalPME+Ca) and 8 (MGFPME+Ca). In the experimental procedure, eggplant cylinders were infused with test solutions under pulse vacuum procedure. For storage study, samples were drained for 3 min, put in a Ziploc® bag, and stored at –34 °C and 4 °C for one week. Two replicates of the experiments were carried out.

Yield and weight loss (or drip loss)

Eggplant cylinders were weighed before and after each treatment. Samples were drained on screener for 3 min. In stored samples, eggplants were taken from storage room, kept at 4 °C for about 3 hr and taken after 1 hr equilibration at 20±1 °C. Yield and weight (wt.) loss were calculated as follows:

$$\text{Yield (\%)} = (\text{Wt. after process} / \text{initial wt.}) \times 100$$

$$\text{Wt. loss (\%)} = \frac{(\text{Wt. after process} - \text{wt. after storage})}{\text{Wt. after process}} \times 100$$

Textural analysis

Eggplants were equilibrated to room temperature before textural analysis. Texture Profile Analysis (TPA) was used to evaluate eggplant texture using a Texture Analyzer (TA-XT2i, Texture Technologies Corp, Scarsdale, NY). TPA was determined using a modified method of Banjongsinsiri and others (2003b). TPA was performed using a cylindrical probe (35 mm diameter) with a 25 kg load cell. Samples were compressed in two consecutive cycles to

60% deformation from the initial sample height of 1.0 cm, at a compression speed of 2 mm/s. The textural parameters determined were hardness, cohesiveness, springiness, gumminess, and chewiness (Bourne 1978). Hardness, measured in Newtons (N), is defined as the first maximum force necessary to compress the sample. Cohesiveness is assessed using the ratio of the area of work during the second compression divided by the area of work during the first compression. Adhesiveness (N*mm) consists of the negative force area for the first compression, and represents the work required to overcome the attractive forces between the surface of a food and the surface of other materials. Springiness (mm) is defined as the distance the sample was compressed during the second compression to the peak force. Gumminess (N) is the product of hardness and cohesiveness, and chewiness (N*mm) is the product of gumminess and springiness. At least seven samples were analyzed for each treatment.

Alcohol insoluble solids (AIS)

AIS was prepared using a modified (Banjongsinsiri and others 2003b) method initially described by Huber and Lee (1986). A 20-50 g sample of eggplant sample was homogenized for 20 s in a Proscientific homogenizer, at a ratio of one part sample to four parts 95% ethanol. The homogenate was heated to boiling for 5 min, then cooled to room temperature. The residue was filtered through a medium pore, sintered glass funnel (Fisher Scientific, Atlanta, GA). Based on the initial fresh weight, the residue was successively washed in four volumes of 95% ethanol and six volume of acetone. AIS was dried under the hood overnight, weighed, and stored at –20 °C.

Degree of esterification (DE)

The degree of esterification (DE) was determined using a modified (Banjongsinsiri and others 2003b) titration method (Anon. 1997). An aliquot of 1 g of AIS was extracted with 90 mL of deionized water for 90 min at 60 °C. The extract was centrifuged at 8,000 × g, for 20 min at 4

°C, and filtered through Miracloth. The supernatant was collected and analyzed for %DE. A volume of 20 mL of sample were titrated with 0.05 N NaOH, saponified with 20 mL of 0.05 N NaOH, and neutralized with an equivalent amount of 0.05 N HCl. The total carboxylic acids groups were estimated by titration with 0.05 N NaOH. The endpoint of titration was estimated in the presence of phenolphthalein, but was quantified to an endpoint pH between 8.0 and 9.6. The % DE was estimated as the mole ratio of free to total carboxylic acids.

Ion-exchange chromatography

An aliquot of dried AIS (50-175 mg) was suspended in 5 mL of 0.05 M sodium acetate buffer and 0.05 M EDTA (pH 5.0), and stirred for 12 hr at room temperature. The extract was filtered through 0.45 µm filtered paper (Whatman, Clifton, NJ). The filtrate (0.5-1.5 mg/ ml of uronic acid) was applied to a 12 × 53 mm anion exchange column of UNO^{TM} Q-6 (Bio-Rad Labs, Richmond, CA). The column was equilibrated with 0.05 M acetate /1.3 M acetate buffer (pH 5.0), and operated at room temperature at flow rate of 2.0 ml/min. After loading, pectin was eluted with a linear gradient from 0.05 M to 1.3 M acetate buffer. Fractions of 2.5 ml were collected and assayed for uronic acids using the m-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973).

Microstructural studies

Cryo-scanning electron microscopy (Cryo-SEM)

Eggplant structure from fresh and treatment samples were observed via Cryo-SEM technique using a method modified from Gras and others (2003). Sample was cryo-fixed by plunging into nitrogen slush (-200 ºC). The frozen sample was quickly transferred to an Alto 2500 cryo-preparation unit (Gatan Inc., Warrendale, PA) attached to a LEO 982 field emission (FE) SEM (LEO Electron Microscopy Inc., Thornwood, NY), fractured, and allowed to

sublimate at a vacuum of 10^{-4} Pa and -80 ºC for 15 min to remove frost on the surface. The sample was brought back down to -100 ºC before coating with gold palladium to a thickness of 30 nm. Once coated, the sample was transferred to a -100 ºC cold stage in the FE-SEM for viewing.

Immunofluorescence microscopy

Tissue fixation and microtomy was performed by a modified method of Knox and others (1989). Pieces of eggplant (8 mm in diameter, 1 cm in thickness) were excised with a razor blade, and immersed in freshly prepared 4% (w/v) formaldehyde in fixative buffer containing 100 mM PIPES (piperazine-*N*,*N'*-bis [2-ethanesulfonic acid]), 4.0 mM $MgCl_2$ and 4.0 mM EGTA (ethylene glycol bis [β-aminoethylether]-*N*,*N*,*N', N'* –tetraacetic acid) at pH 6.9, overnight. Pieces of tissue were washed in buffer for 15 min (three washes), embedded with Cryomatrix compound (Shandon, Pittsburgh, PA), and frozen at -20 ºC. Samples of 60 µm thickness were cut at -20 ºC using a Cryostat 2800 (Leica Microsystem Inc., Bannockburn, IL). Sections were collected on subbing slides coated with gelatin and Chromic potassium sulfate, and kept at 4 ºC before further processing.

The slides were washed with deionized water to remove the Cryomatrix compounds. The sections were treated at room temperature for 1 hr with a block solution of 3% w/v bovine serum albumin (BSA) (FisherScientific, Fair Lawn, NJ) in phosphate buffer saline (PBS) at pH 7.35-7.45 to avoid non specific binding. The samples were incubated in a dilution (1:5) of primary antibodies [rat monoclonal antibody against JIM5 or JIM7 was provided from Dr. Paul Knox (LS2 9JT, U.K.)] in 1% (w/v) BSA/PBS overnight, at 4 ºC. Control samples were incubated in 1% (w/v) BSA /PBS solution without primary antibodies. After overnight incubation, samples were washed in 1% (w/v) BSA/PBS for 15 min (three washes), and incubated in a dilution (1:50)

of goat anti-rat IgG conjugated to Alexa Flour® 633 (Molecular Probes, Inc., Eugene, OR) with 1% (w/v) BSA/PBS for 4 hr at 4 ºC. Samples were washed with 1% (w/v) BSA in PBS solution for 15 min (three washes), then mounted in a 1% (w/v) BSA in PBS solution containing a Citiflour anti-fade mountant pH 9.2 (Ted Pella, Inc., Redding, CA). Sections were observed with a confocal laser scanning microscopy (TCS NT SP2 Leica Confocal microscope, Leica Microsystems Heidelberg Gmblt, Germany). A Helium Neon laser excitation 633 nm and emission was set for 610-650 nm. Collected images were processed using Adobe Photoshop version 6.5 (Adobe Systems Incorporated, US)

Statistical analysis

A randomized complete block design was conducted. The analysis of variance was performed using Proc ANOVA and SAS statistical package V8.2, 2000 (SAS Institution Inc., Cary, NC). The least significant difference (LSD) was used for comparison between means at significant level $p \leq 0.05$. All treatments were carried out in duplicate.

Results and Discussion

Preliminary experiment

Vacuum infusion, pulsed vacuum infusion, and temperature gradient infusion were used to evaluate the effect of PME and calcium (12 tests) on eggplant quality. Table 5.1 shows the hardness and yield of eggplants from the 12 PME/Ca tests as compared to the 3 infusion methods. Selections of the test and vacuum processes were based on the hardness parameters from TPA. The initial raw material used in the experiment was consistent with respect to initial hardness since no significant differences ($p \leq 0.05$) were observed in the non treatment control (RW) between the infusion processes.

An increase in weight during infusion was observed in the preliminary experiment (Table 5.1). After comparison of yield among 3 infusion methods, infused eggplants indicated that pulsed vacuum method (B) had a higher yield than vacuum infusion (A) and temperature gradient method (C), except for the Ca_FungalPME test, vacuum infusion gave the greatest yield of infused products. Among the 3 infusion techniques, yield from the FungalPME test was high and similar to the water test. In tests 4-6, infused eggplants from FungalPME (200%) had the highest yield as compared to ValPME (127%) and MGFPME (157%), for all infusion processes. Among 3 infusion methods, the yield of infused eggplants from FungalPME+Ca test increased 1.4 to 1.7 times when compared to ValPME+Ca, and 1.4 to 1.5 times compared to the MGFPME+Ca test. Similarly, eggplants from the Ca_FungalPME test had a higher yield than in Ca_ValPME (1.0 –1.3 times) and Ca_MGFPME (1.0-1.2 times)

With regard to hardness value, infused eggplant with ValPME, ValPME +Ca, FungalPME +Ca, and MGFPME+Ca using pulsed vacuum had a higher firmness than those using vacuum infusion and temperature gradient (Table 5.1). In all infusion techniques, the hardness of infused eggplant between ValPME and MGFPME, ValPME + Ca and MGFPME +Ca, as well as Ca_ValPME and Ca_MGFPME did not display significant differences. In addition, no significant differences in hardness of infused eggplant between ValPME +Ca and Ca_ValPME, FungalPME +Ca and Ca_FungalPME, or MGFPME +Ca and Ca_MGFPME were found for the vacuum infusion and pulsed vacuum methods.

Based on firmness, the pulsed vacuum technique yielded the greatest firmness values when significant differences were observed. Likewise, pulsed vacuum resulted in higher compatible yield values for all tests. This finding agrees with the results of Ferguson and Malick (1983), who studied a rapid vacuum impregnation with microcrystalline cellulose into mushroom

to prevent weight loss during the canning or cooking of mushroom. They observed that, after a single vacuum application and release, approximately 20% of mushrooms remained floating. After drawing and releasing the vacuum the second time, 100% of the mushrooms sank to the bottom of the liquid, indicating complete saturation. In addition, Adam and Kirk (1991) used a pulsed pressure infusion to facilitate pectinase into fresh citrus fruits, which usually was blocked by collapsed tissues due to excessive pressure.

Using pulsed vacuum technique, higher eggplant yields of FungalPME, FungalPME+Ca or Ca_FungalPME were observed. Yet, the firmness of infused eggplant from FungalPME tests were lower than that of ValPME and MGFPME. The molecular size of FungalPME might be smaller than that of PME from Valencia orange and Marsh grapefruit, based on the weight gain after infusion. Although adding Ca in the FungalPME solution resulted in a decrease of infused eggplant yield compared to these without added Ca, the yield of these samples was higher than those with ValPME or MGFPME with Ca. It suggests that FungalPME can enter eggplant tissue better than plant PME, due to the smaller size. With greater potential of PME activity, it was expected that firmness of infused eggplant from FungalPME would be higher than plant PME. In contrast, infused eggplants from both Val and MGF PME had a higher firmness than FungalPME. This might be due to a different mechanism of de-esterification between plant PME and microbial PME. Plant PME deesterified pectin linearly, creating blocks of free carboxyl groups, whereas the action of microbial PME from *Aspergillus niger* creates a random de-esterification (Massiot and others 1997). According to Thibault and Rinaudo (1990), dependence of the binding of the counterions on the degree of esterification (DE) and the pattern (random and blockwise) has been investigated. They found that the concentration of calcium salts has a profound influence on the calcium activity coefficient for the highest charged

polymers or for plant enzyme-deesterified pectins. The degree of binding of counterions with alkaline-deesterified pectins (random distribution of free carboxyl groups) increased with increasing charge density, while the degree of binding of counterions was roughly independent of the DE for enzyme-deesterified pectin (blockwise distribution of free carboxyl groups). Thus, there is the possibility that blockwise de-esterification yields much stronger bonds with calcium ions than that of carboxyl groups created randomly. Another reason might be that infused eggplant from MGFPME+Ca (562 Unit/ g dried wt.) had a higher enzyme activity than that of FungalPME +Ca (257 Unit/ g dried wt.) (Table 5.4). Based on the pattern and enzyme activity, the texture of plant PME infused eggplants is likely to be firmer than that of microbial PME.

Effect of PME infusion on texture

Pulsed vacuum infusion with FungalPME, MGFPME, FungalPME+Ca and MGFPME+Ca tests were selected for further study. Ponappa and others (1993) found no difference in firmness of strawberry slices vacuum-infiltrated with polyamines or calcium after vacuum infiltration. Their results showed that storage condition (9 days at 1 °C) had a substantial effect on the firmness of all treatments. According to this research, infused eggplant texture was measured using TPA after processing and at a storage time of 7 days (4 and –34 °C).

The TPA change varied significantly ($p \leq 0.05$) by treatments at each storage temperature (4 and -34 °C) for 7 days, as shown in Table 5.2. The infused eggplants from FungalPME+Ca and MGFPME + Ca treatments had the greatest hardness values at day 0 and day 7 at 4 °C. Infused eggplants from FungalPME+Ca also had higher springiness, gumminess, and chewiness than non-infused control at day 0 and day 7 (4 °C). In contrast, thawed eggplants from all treatments had lower hardness, gumminess, and chewiness than non-infused control after storage of 7 days at –34 °C. Springiness values of thawed eggplants from all treatments were higher

than that of non-infused control at the same storage condition. The trend of cohesiveness and adhesiveness values in all treatments was found not to be consistent after processing and at each storage time. Thus, hardness value was an appropriate parameter to indicate the change of infused eggplant texture in this study.

Effect of vacuum infusion on weight loss during storage

The effect of treatments on yield of eggplants was significant at storage temperatures -34 °C and 4 °C of storage time 7 days (Table 5.3). Regardless of treatments, yield (1 and 2) increased and retention of water-soaked occurred if calcium, fungal, and MGF PME were present. Infused eggplants from MGFPME+Ca treatment had a relatively lower yield at -34 °C and 4 °C. No significant differences were found on the weight loss of infused eggplant for all treatments at 4 °C. At -34 °C, MGFPME+Ca (17%) treatment had a significant lower weight loss compared to the others, but no significant difference when compared to the weight loss of FungalPME treatment. From this result, there is the possibility that FungalPME +Ca and MGFPME+Ca pretreatment enhanced yield of eggplant after treatment, and also prevented weight loss during storage.

Enzyme activity and degree of esterification (%DE)

Eggplants from each treatment were assayed for PME activity, as shown in Table 5.4. Due to the different optimal pH from PME sources, pH 7.5 was set to assay PME activity of plant PME (MGFPME and MGFPME +Ca) including Ca, while optimal pH 4.5 was set for FungalPME and FungalPME+Ca treatment. The activity of control treatments (RW and Water) were assayed at pH 4.5 and 7.5. At pH 4.5, the activity of fresh non-infused (8.0 Unit/g dried wt.) and water infused control (27 Unit/ g dried wt.) was lower than that of pH 7.5 (247 and 654 Unit/ g dried wt.). This finding supports the fact that most plant PME activities have an optimal

pH of 7.5 to 8.0, while optimal fungal PME activity is in the pH range 4.0-5.2 (Rexova-Benkova and Markovic 1976). After vacuum infusion, PME activity of infused eggplants from MGFPME (814 Unit/ g dried wt.) increased significantly (3 fold) compared to RW (247 Unit/ g dried wt.), and 1.2 fold to Water control (654 Unit/ g dried wt.) at pH 7.5. However, the PME activity of MGFPME significantly decreased after adding Ca (4000 ppm) in MGFPME+Ca treatment (562 Unit/ g dried wt.). In contrast, there were not significant differences between FungalPME and FungalPME+Ca treatment in PME activity. The activity of infused eggplant from FungalPME and FungalPME+Ca was increased 42 and 32 folds, respectively, when compared to RW at pH 4.5. In comparison to the activity with Water control, FungalPME and FungalPME+Ca had 12 and 10-fold activities, respectively, higher than Water control at pH 4.5. In Ca treatment, PME activity of eggplant (469 Unit/g dried wt.) was relatively higher than that of RW (pH 7.5), FungalPME (328 Unit/ml) and FungalPME+Ca (257 Unit/g dried wt.) but lower than that of Water control. The presence of 50-250 mM NaCl or 5-20 mM $CaCl_2$ increases plant PME activity several-fold. In contrast, the activating effect of salts on several microbial PME was increased only 1.5 to 2-fold (Rexova-Benkova and Markovic 1976). The mechanism of the enzyme activation by salts was proposed by Nari and others (1991). Without salt ions, enzyme may be trapped by carboxyl groups on pectin. At the optimal salt concentration, salt ions may interact with negatively charged groups, allowing the enzyme to interact with the ester bonds to be cleaved. However, at higher concentrations, the enzyme reaction is inhibited since some carboxyl groups adjacent to the ester bond to be cleaved, allowing the reaction to proceed, are blocked by salt ions.

The %DE values of infused eggplants from each treatment are depicted in Table 5.4. The %DE value is high, and did not decrease considerably with increased PME activity. There was

no significant difference in %DE of fresh non-infused (RW), water infused control, and FungalPME treatment. The %DE of Ca (74%), MGFPME (65%) and MGFPME+Ca (71%) was relatively low when compared to control (RW and Water), FungalPME (94%), and FungalPME+Ca (80%). In general, %DE of tissue pectins is in the range of 60 to 90%, depending on species, tissue, and plant maturity (Van Buren 1991). The DE has a bearing on the firmness and cohesion of plant tissue. Reductions in DE result in greater cohesion. Since formation of free carboxyl groups increases the possibility and strength of calcium binding between pectin polymers. PME, present in most plant tissue, can decrease % DE by demethylation of pectin.

Ion- exchange chromatography (IEC)

The basic principles of IEC depend on separation achieved according to the charges of pectin molecules which depend on the number of dissociated carboxyl groups present on the individual molecules (Kravtchenko and others 1992). In the case of different patterns, blockwise distribution of free carboxyl or highly de-esterified pectin may account for firmness difference; therefore, pectin should be eluted later in IEC. The pectin extracts were analyzed for separation charge by ion-exchange chromatography (Figures 5.1A-C). Multiple and incompatible peaks of solution indicate that pectin from untreated and PME treated were heterogeneous. RW, Water (Figure 5.1A), MGFPME, and FungalPME (Figure 5.1B) showed two components after fraction number 9, the first one (fraction no 10-25) and at the second one (fraction no 25-33) eluting at high concentration of acetate buffer. Ca (Figure 5.1A), FungalPME +Ca, and MGFPME +Ca (Figure 5.1C) had many peaks, with most of them eluting at high ionic strength. According to Schols and others (1989), pectins de-esterified by acid or alkaline appear to be homogeneous on IEC, whereas pectins de-esterified by plant pectinesterase elute in large fractions of various

degree of methoxylation. The recoveries of ion-exchange fractions of all treatments were around 25 to 34%. This result was similar to Renard and others (1990), who characterized apple protopectin extracted by chemical means. They found that apple pectin extracted by alkaline (NaOH, hot and cold Na_2CO_3) solutions had a low % recovery for uronic acid (24, 27 and 51%), but a high % recovery for neutral sugars (108, 98 and 89%), which indicated that uronic acids mostly devoid of neutral sugars were retained on the column. In addition, some low methoxylated pectin molecules can be precipitated inside the column if calcium ions are present. Nonionic adsorption via phenolic compounds and/or precipitation with multivalent cations may be responsible for incomplete recovery of galacturonic acid (Kravtchenko and others 1992). With regard to fraction no 2 or 3 (Figures 5.1A-C), samples already showed pink color after boiling (before uronic acid assay). These sample fractions might be free neutral sugars, since they were eluted at considerably low ionic strength. Neutral sugars, phenolic and proteinaceous compounds were found to coelute with pectin molecules. Particularly free neutral polysaccharides are not retained in the column at low ionic strength (Kravtchenko and others 1992).

Microstructural studies

Cryo-SEM

Non-infused eggplant (RW) displays parts of intercellular spaces between parenchyma cells and vascular bundle tissues (xylem and phloem tissues), as presented in Figures 5.2A-C. According to Bomben and King (1892), the regions in the micrographs are termed ‘solute-water glass’ of the cell sap, cell membranes, and cell walls. Solute-water glass appears as a dentritic zone or sheets due to ice microcrystal sublimation, and can be observed in the intracellular zone and in some intercellular spaces containing native liquid. Alternatively, intercellular spaces may

appear completely empty (Fito and Chiralt 2000). In the Water treatment (Figure 5.2D), eggplant samples showed a deformation of cell structure and reticulated material from ice formation between cells, as compared to RW (Figure 5.2C). Eggplant treated with FungalPME +Ca (Figure 5.2E) also had deformed cells compared to RW, as well as larger sheets ('is' in Figure 5.2E) defined between cells, which differed from the material noted in Water treatment. In a study by Moreno and others (2000), differences in ultrastructural features between atmospheric pressure (OD) and pulsed vacuum treatments (PVOD) of treated strawberry fruits were observed. The authors found that the intercellular spaces of PVOD samples contained more compact dentritic structures compared to OD samples. Thus, a different aspect of liquid phase of infused eggplant could be present in the intercellular spaces between Water and FungalPME+Ca treatments. This liquid phase could be partially solubilized pectic substances of the middle lamella in the intercellular space, which could occur to a greater degree in FungalPME + Ca treatment (Moreno and others 2000). The differences seen with Cryo-SEM among the treatments were consistent with firmness measurements. The texture of FungalPME+Ca infused eggplants were more firm than that of RW and Water treatments.

Immunofluorescence labeling of eggplant tissues

JIM5 and JIM7 immunofluorescence of eggplant tissue cross-sections are shown in Figure 5.3A-F. According to Knox and others (1990), JIM5 monoclonal antibody detects low-esterified pectin in a % DE range of 0 to 50%, and JIM7 detects high-methyl-esterified epitope of pectin (35-90% DE) in carrot root apex. Although JIM5 and JIM7 are widely used to localize defined pectin within a single cell wall, the epitopes of JIM5 and JIM7 are not fully defined with respect to size or degree and patterns of methyl esterification. In a recent study (Willats and others 2000), the structure of epitopes recognized by JIM5 and JIM7 antibodies was investigated.

In their study, JIM 5 binds weakly to completely deesterified pectins but its binding greatly increases with the presence of methyl-esterified pectin up to 40%DE, then decreases when %DE is greater than 40%. JIM7 binding is strong in the range of 15 to 80% DE. In RW and Water control, both JIM5 and JIM7 bound to the cell walls of eggplant tissues (Figures 5.3A-D), although %DE value from our assay was in the range of 87 to 92%. This finding is similar to the pectin distribution in the root apex of carrot (Knox and others 1990) and the inner surface of potato parenchyma cells (Parker and others 2001). In Water and FungalPME + Ca treatment, binding of JIM5 was greater in the cell walls (Figures 5.3C, 5.3E), while JIM7 binding was less (Figures 5.3D, 5.3F). As noted earlier, (Knox and others 1990), JIM5 should have lower binding affinity, while JIM7 would have a stronger signal with greater than 80% DE pectin from FungalPME +Ca treatment. However, inconsistent binding of JIM5 and JIM7 with pectin epitopes were also found in kiwi (Sutherland and others 1999) and blanched carrots (Lo and others 2002). These researchers suggested that poor JIM7 binding with high methyl-esterified pectin is caused by inaccessible pectin epitopes. Strong binding of JIM5 to pectin with high %DE might be from cell wall swelling (after vacuum infusion), which involves modification of pectic polymers and enhances accessibility of binding sites.

Conclusions

In fused eggplant with Marsh grapefruit or fungal PME with calcium chloride, increased firmness and altered TPA quality factors were noted. The vacuum technique also made a difference in firmness and weight increase of infused eggplant ,with pulsed vacuum being optimal for eggplant. Pretreatment of eggplant with plant PME or fungal PME and calcium chloride improved texture and reduced weight loss during storage at 4 ºC. Changes in firmness were related to changes in pectin charge distribution, but not the total charge (%DE) in tissues.

Thus, choices of PME and infusion conditions are the main factors, which will influence almost any infusion process. The information obtained from pectin antibodies (JIM5 and JIM7) did not provide quantitative verification of %DE, but did correlate with our pectin charge distribution studies.

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Table 5.1. Firmness and % yield of eggplant compared to 3 process methods among different treatments

Test ID	Vacuum infusion (A)		Pulsed vacuum (B)		Temperature gradient (C)	
	Hardness (N)	Yield (%)	Hardness (N)	Yield (%)	Hardness (N)	Yield (%)
1. RW	$^{x}13.14^{bcd}$	-	$^{x}13.84^{cde}$	-	$^{x}13.68^{abc}$	-
2. Water	$^{x}10.94^{d}$	180	$^{x}10.44^{e}$	214	$^{x}9.71^{c}$	168
3. Ca	$^{x}14.87^{abc}$	179	$^{x}15.96^{bcd}$	183	$^{x}13.73^{abc}$	155
4. ValPME	$^{y}12.32^{bcd}$	104	$^{x}20.67^{a}$	127	$^{y}12.39^{abc}$	100
5. FungalPME	$^{x}11.58^{dc}$	180	$^{x}13.37^{de}$	200	$^{x}11.99^{abc}$	150
6. MGFPME	$^{x}15.62^{ab}$	124	$^{x}18.17^{abc}$	157	$^{y}9.72^{c}$	129
7. ValPME+Ca	$^{y}14.94^{abc}$	104	$^{x}20.61^{a}$	114	$^{y}14.58^{ab}$	95
8. FungalPME+Ca	$^{y}12.79^{bcd}$	165	$^{x}17.41^{abcd}$	190	$^{xy}15.53^{a}$	133
9. MGFPME+Ca	$^{xy}14.51^{abcd}$	109	$^{x}19.37^{ab}$	124	$^{y}11.52^{abc}$	95
10.Ca_ValPME	$^{x}14.72^{ab}$	150	$^{x}15.13^{bcd}$	170	$^{x}12.16^{abc}$	111
11.Ca_FungalPME	$^{xy}12.81^{bcd}$	191	$^{x}15.60^{bcd}$	183	$^{y}10.60^{bc}$	145
12.Ca_MGFPME	$^{xy}16.87^{a}$	163	$^{x}18.56^{ab}$	177	$^{y}11.82^{abc}$	120

RW=non-infused control, Water=water infused control, Ca= $CaCl_2 \cdot 2H_2O$ (4000ppm),ValPME=Valencial orange PME (25 Unit/ml), FungalPME (10 Unit/ml), MGFPME=Marsh grapefruit PME(10 Unit/ml), ValPME (25 Unit/ml) +Ca (4000ppm), FungalPME (10 Unit/ml)+ Ca (4000ppm), and MGFPME (10 Unit/ml) + Ca (4000ppm), Ca_ ValPME=Ca (4000ppm) infusion followed by ValPME (25 Unit/ml) infusion, Ca_ FungalPME= Ca (4000ppm) infusion followed by FungalPME (10 Unit/ml) infusion, Ca,_ MGFPME=Ca (4000ppm) infusion followed by Marsh grape fruit PME (10 Unit/ml) infusion.

Yield (%)=(Wt. after infusion/initial wt.) × 100

[a-d]Column with the same letter within each process method are not significantly different ($p \leq 0.05$). [x-y] Row with the same letter within each treatment are not significantly different ($p \leq 0.05$)

Table 5.2. Texture Profile Analysis of eggplant (Day0, Day7 at 4°C and -34 °C)

Test ID	Hardness (N)	Cohesiveness (unitless)	Adhesiveness (N.mm)	Springiness (mm)	Gumminess (N)	Chewiness (N.mm)
Day 0						
RW	16.46^{b}	0.25^{d}	-0.58^{abc}	2.90^{d}	4.17^{d}	12.48^{c}
Water	12.43^{c}	0.26^{d}	-2.30^{d}	3.45^{cd}	3.16^{d}	11.90^{c}
Ca	20.32^{a}	0.39^{ab}	-1.86^{dc}	4.13^{ab}	8.01^{ab}	36.90^{a}
FungalPME	16.70^{b}	0.36^{bc}	-1.91^{dc}	4.12^{ab}	6.01^{c}	26.14^{b}
MGFPME	17.47^{b}	0.34^{bc}	-0.45^{ab}	4.03^{bc}	6.00^{c}	24.87^{b}
FungalPME + Ca	20.89^{a}	0.44^{a}	-1.63^{bcd}	4.68^{a}	9.03^{a}	42.40^{a}
MGFPME + Ca	20.96^{a}	0.33^{c}	-0.118^{a}	3.43^{cd}	6.91^{bc}	24.00^{b}
Day7, 4 °C						
RW	13.89^{b}	0.28^{bc}	-0.56^{ab}	3.57^{b}	3.87^{b}	13.69^{b}
Water	10.56^{c}	0.42^{a}	-0.14^{a}	4.86^{a}	4.83^{b}	24.89^{a}
Ca	5.12^{d}	0.21^{cd}	-0.49^{ab}	2.82^{c}	1.29^{c}	5.04^{c}
FungalPME	2.41^{d}	0.33^{ab}	-0.42^{ab}	3.29^{bc}	0.81^{c}	3.23^{c}
MGFPME	4.62^{d}	0.18^{d}	-0.10^{a}	3.30^{bc}	0.86^{c}	2.85^{c}
FungalPME + Ca	17.48^{a}	0.36^{ab}	-1.11^{b}	4.48^{a}	6.29^{a}	28.81^{a}
MGFPME + Ca	17.57^{a}	0.28^{bc}	-0.10^{a}	3.06^{bc}	4.91^{ab}	15.12^{b}
Day7, -34 °C						
RW	8.22^{a}	0.36^{ab}	-0.070^{c}	2.31^{b}	3.12^{a}	7.18^{a}
Water	2.13^{b}	0.28^{c}	-0.035^{ab}	3.29^{a}	0.58^{b}	1.85^{b}
Ca	2.06^{b}	0.34^{bc}	-0.032^{ab}	3.53^{a}	0.65^{b}	2.26^{b}
FungalPME	1.96^{b}	0.30^{c}	-0.025^{a}	3.55^{a}	0.56^{b}	2.00^{b}
MGFPME	3.26^{b}	0.29^{c}	-0.031^{ab}	3.44^{a}	0.92^{b}	3.01^{b}
FungalPME + Ca	1.63^{b}	0.35^{b}	-0.059^{bc}	3.47^{a}	0.51^{b}	1.74^{b}
MGFPME + Ca	1.86^{b}	0.41^{a}	-0.046^{abc}	3.51^{a}	0.75^{b}	2.60^{b}

RW=non-infused control, Water=water infused control, Ca= $CaCl_2{\cdot}2H_2O$ (4000ppm), FungalPME (10 Unit/ml), MGFPME=Marsh grapefruit PME (10 Unit/ml), FungalPME (10 Unit/ml) + Ca (4000ppm), and MGFPME (10 Unit/ml) + Ca (4000ppm).

$^{a-d}$Column with the same letter within each day are not significantly different ($p \leq 0.05$).

Table 5.3. % Yield and % weight loss of eggplant after process and storage time at 4 ºC and -34 ºC for 7days

Treatment	% Yield (1)	%Weight loss	%Yield (2)
Day 7, 4 ºC			
RW	100^{d}	4.15^{a}	95.92^{d}
Water	226.00^{a}	4.43^{a}	216.00^{a}
Ca	213.05^{ab}	8.66^{a}	194.23^{ab}
FungalPME	218.00^{ab}	9.13^{a}	198.00^{ab}
MGFPME	197.26^{bc}	9.18^{a}	179.31^{bc}
FungalPME+Ca	200.32^{b}	3.00^{a}	194.23^{ab}
MGFPME+ Ca	172.12^{c}	3.51^{a}	166.03^{c}
Day 7, -34 ºC			
RW	100^{e}	12.27^{d}	87.71^{d}
Water	231.11^{a}	27.48^{a}	167.48^{a}
Ca	198.08^{c}	24.77^{ab}	149.00^{b}
FungalPME	211.85^{b}	28.71^{a}	151.00^{b}
MGFPME	194.16^{c}	17.19^{dc}	160.77^{a}
FungalPME+Ca	196.53^{c}	22.09^{bc}	152.78^{b}
MGFPME+ Ca	176.13^{d}	23.85^{ab}	134.14^{c}

RW=non-infused control, Water=water infused control, Ca= $CaCl_2 \cdot 2H_2O$ (4000ppm), FungalPME (10 Unit/ml), MGFPME=Marsh grapefruit PME (10 Unit/ml), FungalPME (10 Unit/ml) + Ca (4000ppm), and MGFPME (10 Unit/ml) + Ca (4000ppm).

%Yield (1) = (Weight (wt.) after infusion/ initial wt) ×100

$$\%\text{Weight loss} = \frac{\text{(Wt. after infusion-wt. after storage)}}{\text{(Wt. after infusion)}} \times 100$$

%Yield (2) = (Wt. after storage time/ initial wt.) ×100

[a-e]Column with the same letter within each storage condition is not significantly different ($p \leq 0.05$).

Table 5.4. Enzyme activity and %DE of eggplant after infusion

Treatment	Optimal pH	Enzyme activity (Unit/g dried wt.)	%DE
RW	4.5	7.80^{e}	87^{ab}
	7.5	246.67^{d}	
Water	4.5	26.76^{e}	92^{a}
	7.5	654.41^{b}	
Ca	7.5	468.54^{c}	74^{cd}
FungalPME	4.5	327.96^{d}	94^{a}
MGFPME	7.5	813.95^{a}	65^{d}
FungalPME+Ca	4.5	256.77^{d}	80^{bc}
MGFPME+ Ca	7.5	562.42^{bc}	71^{d}

RW=non-infused control, Water=water infused control, Ca= $CaCl_2 \cdot 2H_2O$ (4000ppm), FungalPME (10 Unit/ml), MGFPME=Marsh grapefruit PME (10 Unit/ml), FungalPME (10 Unit/ml) + Ca (4000ppm), and MGFPME (10 Unit/ml) + Ca (4000ppm).

$^{a-e}$Column with the same letter is not significantly different ($p \leq 0.05$).

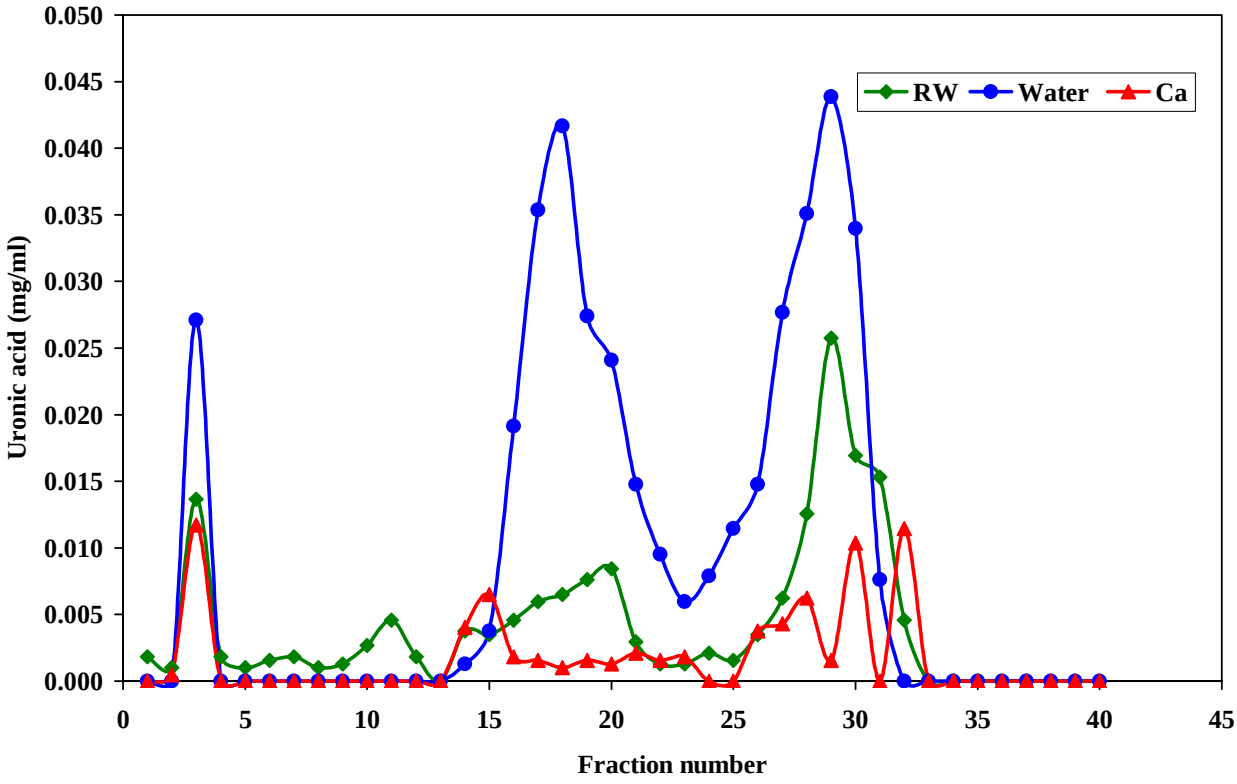


Figure 5.1A. Elution of the pectic substances extracted from eggplants (RW, Water, and Ca treatments)

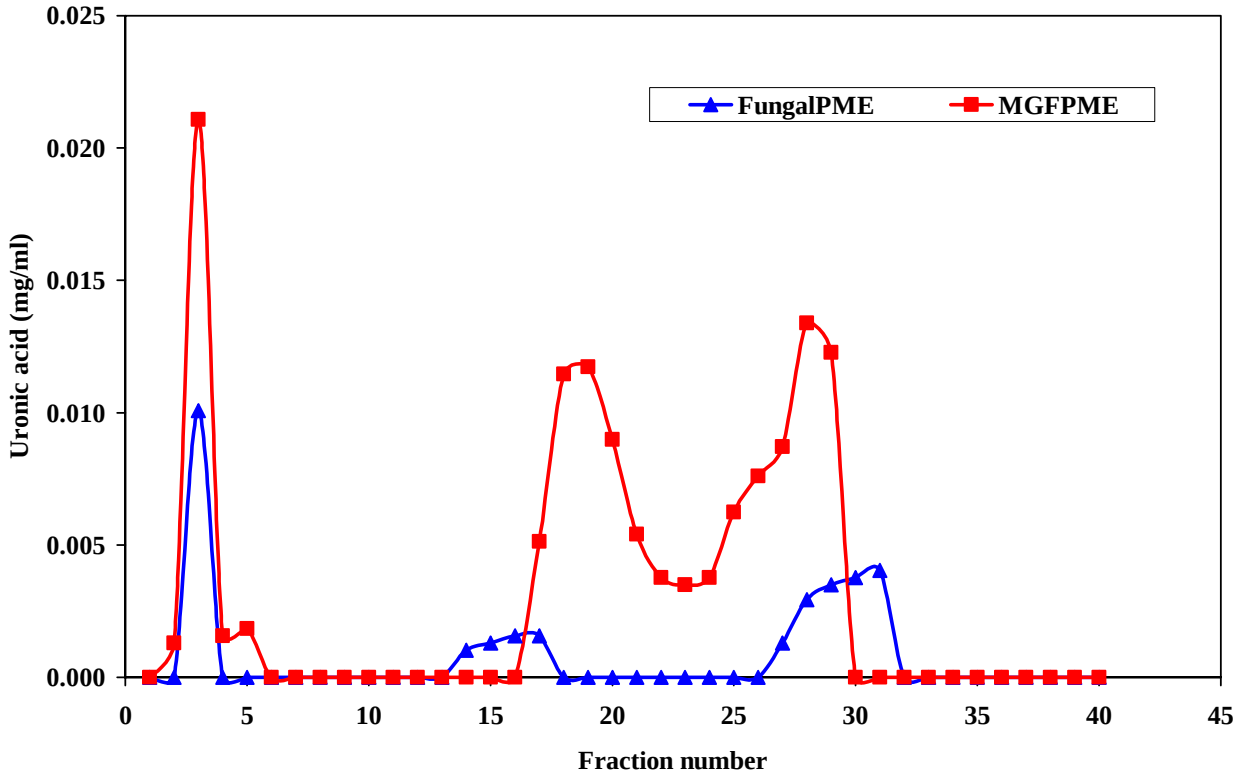


Figure 5.1B. Elution of the pectic substances extracted from eggplants (FungalPME and MGFPME treatments).

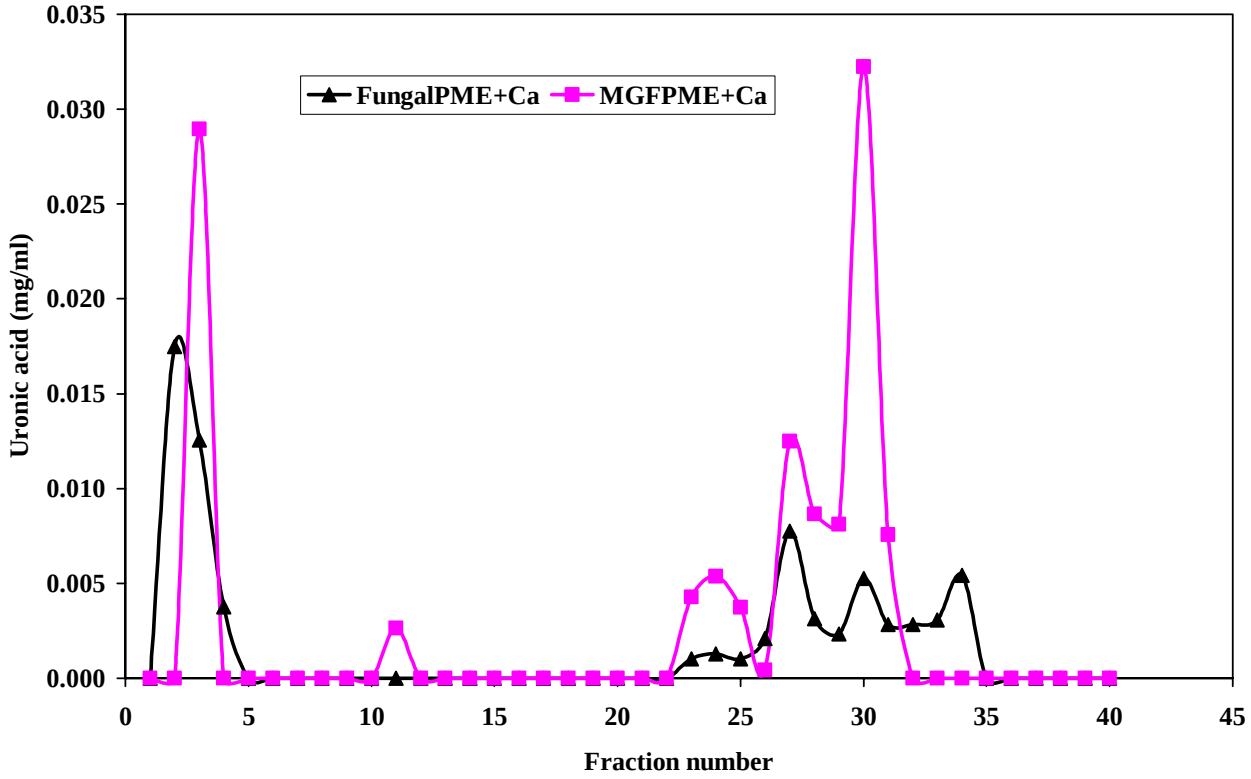
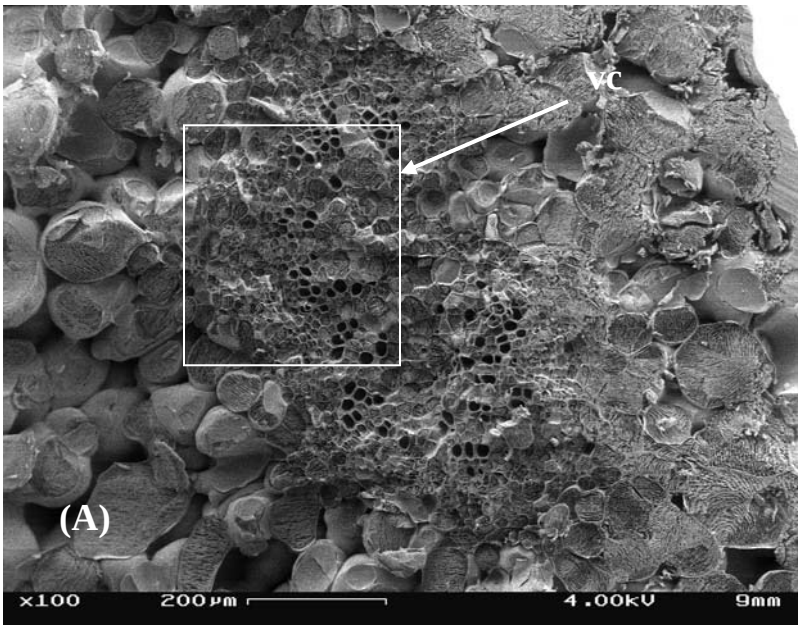
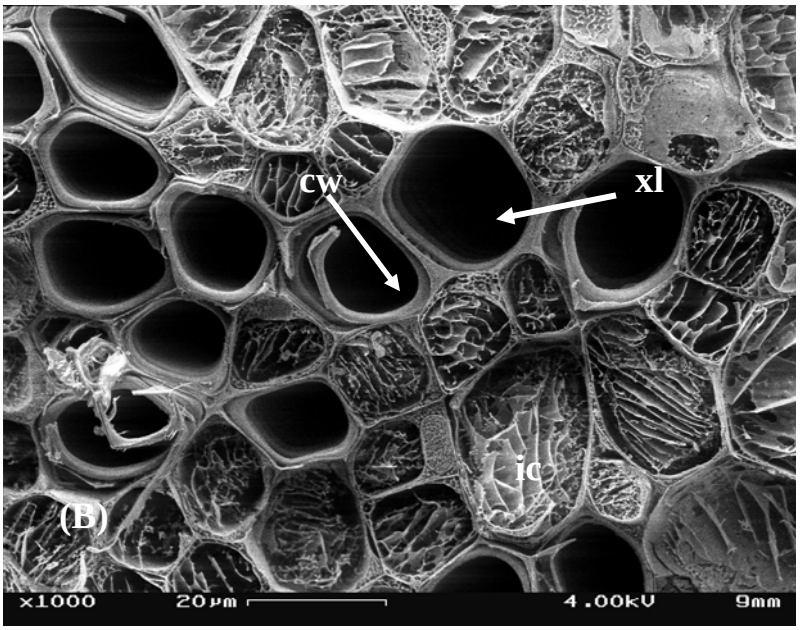
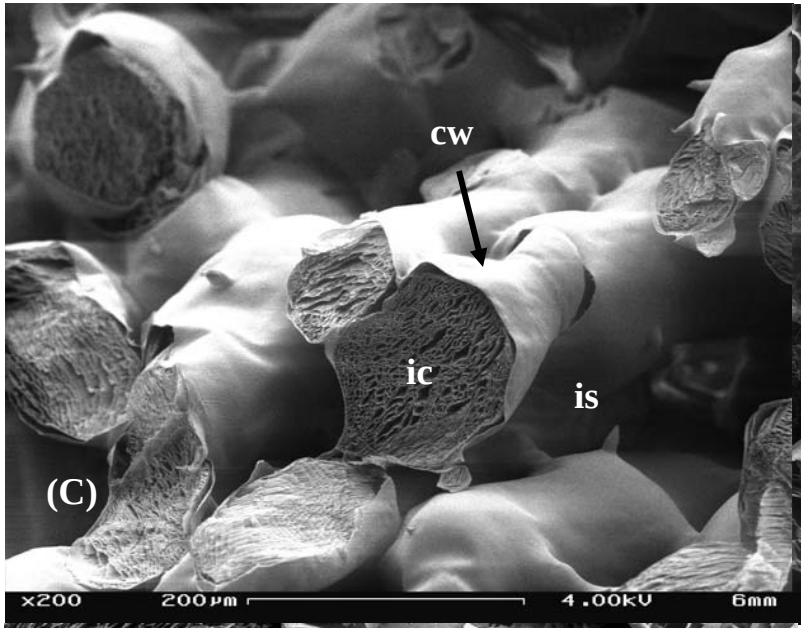
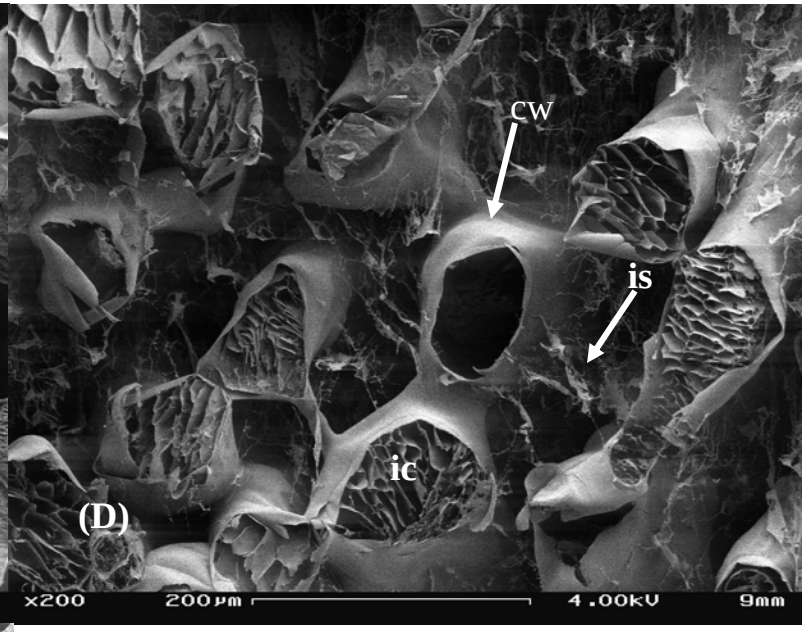


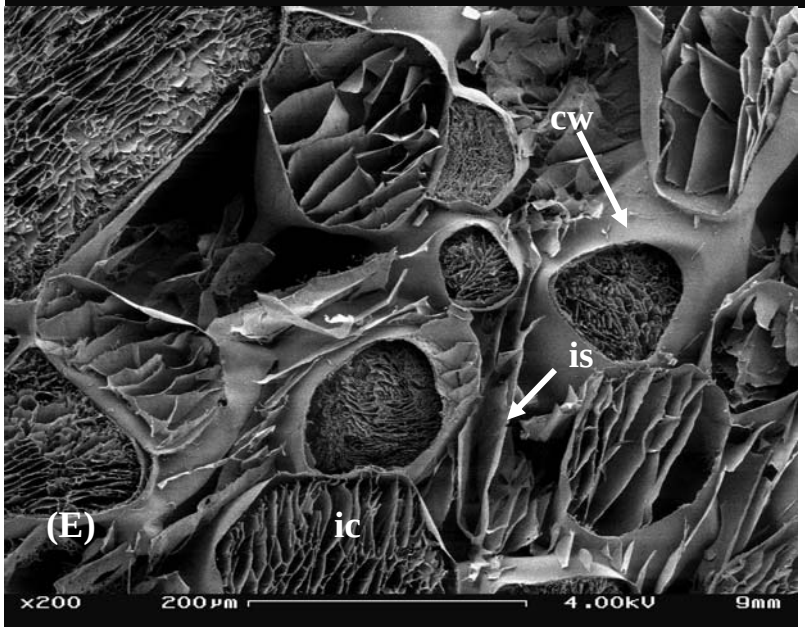
Figure 5.1C. Elution of the pectic substances extracted from eggplants (FungalPME+Ca and MGFPME+Ca treatments)


vc
(A)
x100
200µm
4.00kV
9mm


cw
xl
ic
(B)
x1000
20µm
4.00kV
9mm


cw
ic
is
(C)
x200 200µm 4.00kV 6mm


cw
is
ic
(D)
x200 200µm 4.00kV 9mm


cw
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(E)
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x200 200µm 4.00kV 9mm

Figure 5.2A-E. Cryo-SEM micrographs of eggplant: A-C, RW (non-infused control): D, Water infused- control: E, FungalPME +Ca treatment. is = intercellular space in RW(C) is space between cells located within the plant material, is with arrow (D and E) = defined as sheets of material which left from ice crystal formation, cw = cell wall, ic = intracellular content, vc = vascular bundle, xl = xylem.

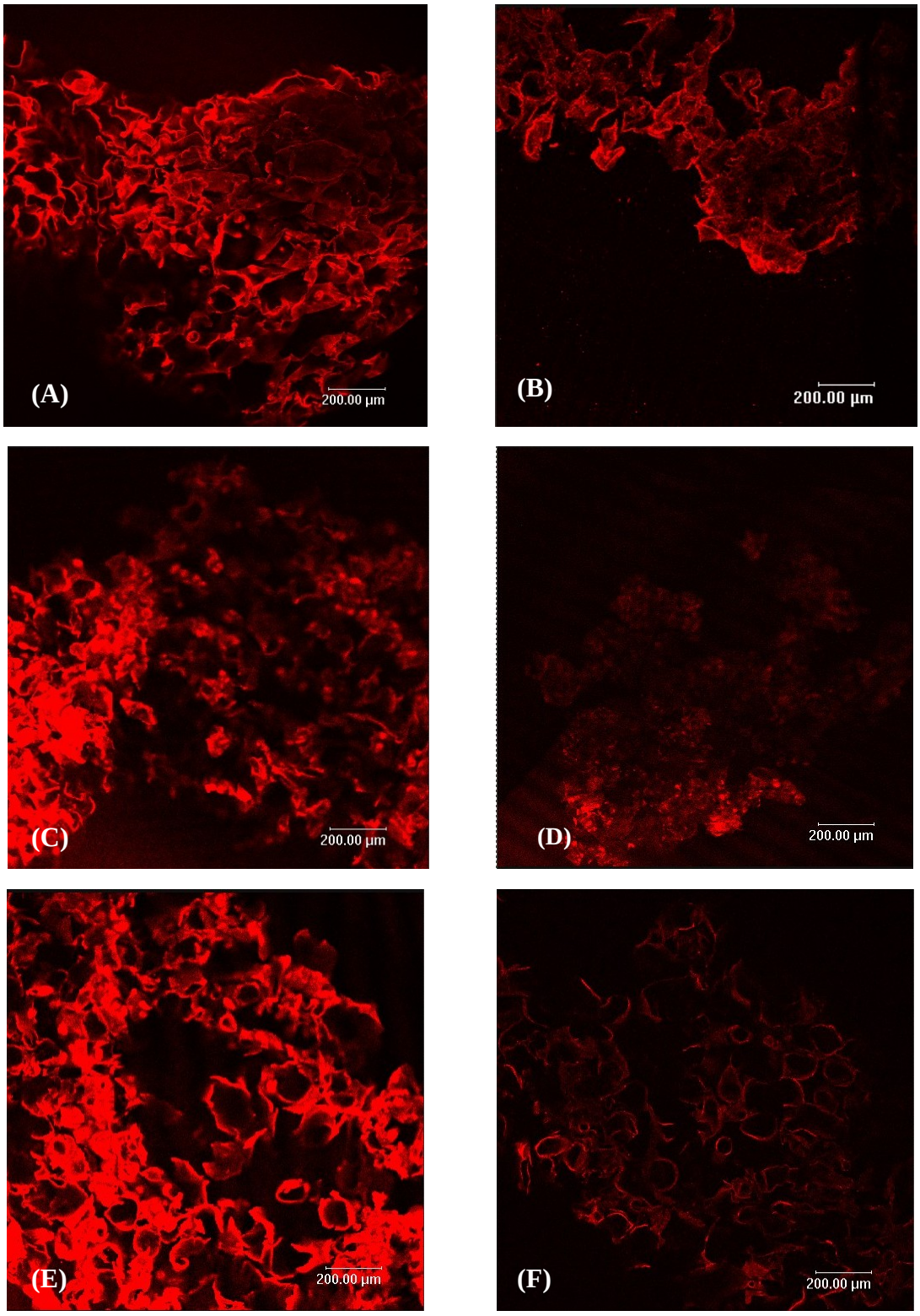
(A)
200.00 µm
(B)
200.00 µm
(C)
200.00 µm
(D)
200.00 µm
(E)
200.00 µm
(F)
200.00 µm

Figure 5.3A-F. Immunofluorescence labeled JIM5 (low esterified pectin, A, C, and E) and JIM7 (high esterfied pectin, B, D, and F) binding to a cross-section of eggplant tissues. A-B, RW (non-infused control): C-D, Water infuse- control: E-F, FungalPME +Ca treatment.

CHAPTER 6

CONCLUSIONS

The first part of the study involved development of a rapid method to visualize the location of PME in fruit after vacuum including the effect of PME infusion on pectic substances of fruits. According to the results, PME print technique has been successfully adjusted as a rapid method to detect location of PME after infusion. PME infusion did not affect the distribution of pectin fractions of infused fruits. This may be from an inhibitor or inactivation of Valencia PME in strawberry fruits. Firmness of infused PME fruit was greater than that of water infused fruit.

In the second part, infusion of exogenous PME was applied to mango fruits. The results showed that mango cubes infused with Valencia orange PME and $CaCl_2$ had greater gumminess and Chewiness, however, there were no differences in firmness compared to mango cubes without treatment. Water soluble pectins had degree of esterification values between 38% and 60%. The inconsistency of pectin distribution and presence of a PME inhibitor might be factors altering the solubility of pectin by infusion treatment. In addition, other components such as cellulose and hemicellulose may contribute to softening or inhibition of firming by PME and $CaCl_2$ infusion.

Finally, the effect of vacuum infusion on texture and structure changes of eggplant infused with a commercial fungal (*Aspergillus niger*) and Marsh grapefruit PME was investigated by physical measurement related to chemical changes and microstructure studies. The vacuum technique made a difference in firmness and weight increase with pulsed vacuum being optimal for eggplant. Pretreatment of eggplant with a fungal or Marsh grapefruit PME and

$CaCl_2$ improved texture and reduced weight loss during storage at 4 ºC. Changes in firmness were related to changes in pectin charge distribution, but not to total charge (%DE) in tissues. Thus, choices of PME and infusion conditions influence almost any infusion process. The information obtained from pectin antibodies (JIM5 and JIM7) did not provide quantitative verification of %DE, but did correlate with our pectin charge distribution studies.

Based on this study, the incorporation of PME enzyme with vacuum technique is possible to modify structure of fruits or vegetables by improving the texture of products for further processing (blanched, canned, or osmo-dehydrated products) and/or during storage. However, the condition of vacuum processes such as infusion time, magnitude of vacuum applied, and the concentration of infused solution, should be investigated for each produce due to differences of a cellular nature as well as the specific geometry of different fruits and vegetables. Sensory evaluation should be used to investigate the texture of finished products in order to correlate the mechanical measurement. Besides PME activity, calcium content of fruits or vegetables should be analyzed before and after infusion. 2F4 antibody was found to recognize a calcium-dependent conformation of homogalacturonan that is thought to be a dimer requiring at least nine contiguous galacturonic acid residues for formation (Liners and others 1989, 1992). In addition, 2F4 antibody is also specific for pectin with up to 30% random and 40% blockwise degree of esterification. Therefore, it is possible to use this antibody to locate low esterified pectin, which is associated through Ca^{2+} to form pectate gel.

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