การเปลี่ยนแปลงโครงสร้างทางกายวิภาคและเมแทบอลิซึมของผนังเซลล์ของเปลือกกล้วยหอมทอง
ที่ผ่านการจุ่มน้ำร้อน

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CHANGES IN ANATOMICAL STRUCTURE AND CELL WALL METABOLISM
OF 'HOM THONG' BANANA PEEL FOLLOWING HOT WATER TREATMENT

Ms. Nuankamol Amnuaysin

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By   Miss Nuankamol Amnuaysin
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กล้วยหอมทองที่ผ่านการจุ่มน้ำร้อน. (CHANGES IN ANATOMICAL STRUCTURE AND CELL WALL
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121 หน้า.

การนิ่มของผลเป็นการเปลี่ยนแปลงที่สำคัญอย่างหนึ่งในกระบวนการสุกของผลไม้ โดยมีผลต่อคุณภาพและอายุ
การเก็บรักษาของผล   เอนไซม์ที่ย่อยสลายผนังเซลล์นับเป็นปัจจัยหลักที่มีบทบาทสำคัญต่อการแยกของผนังเซลล์ระหว่าง
การสุก และการเปลี่ยนแปลงเนื้อสัมผัสของผล การศึกษาผลของการจุ่มน้ำร้อนที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 10
นาที ก่อนการเก็บรักษาที่อุณหภูมิ 25 องศาเซลเซียส ต่อการนิ่มของผลกล้วยหอมทอง (Musa acuminata, AAA group)
โดยวัดความแน่นเนื้อ องค์ประกอบของผนังเซลล์ แอกทิวิตีของเอนไซม์ที่ย่อยสลายผนังเซลล์ และการแสดงออกของยีนที่
เกี่ยวข้องในเปลือกกล้วย   พบว่าการลดลงของความแน่นเนื้อของผลกล้วยที่ผ่านการจุ่มน้ำร้อนจะเกิดช้ากว่าผลกล้วยหอม
ทองที่ไม่ผ่านการจุ่มน้ำร้อน เมื่อเก็บรักษาที่อุณหภูมิ 25 องศาเซลเซียส การจุ่มน้ำร้อนที่อุณหภูมิ 50 องศาเซลเซียส
สามารถชะลอการเพิ่มขึ้นของปริมาณเพกทินที่ละลายในน้ำซึ่งสอดคล้องกับการเปลี่ยนแปลงของความแน่นเนื้อระหว่าง
การเก็บรักษา นอกจากนี้พบว่าผลกล้วยหอมทองที่ผ่านการจุ่มน้ำร้อนมีปริมาณเพกทินที่ละลายใน HCl สูงกว่าผลกล้วย
หอมทองที่ไม่ผ่านการจุ่มน้ำร้อน แสดงว่ามีการลดการละลายของโพลิเมอร์ในผนังเซลล์ รวมทั้งการลดลงของปริมาณเฮมิ
เซลลูโลสเกิดช้าลงเมื่อผลกล้วยหอมทองผ่านการจุ่มน้ำร้อนก่อนการเก็บรักษา แต่การจุ่มน้ำร้อนก่อนการเก็บรักษาไม่มีผล
ต่อการเปลี่ยนแปลงของปริมาณเพกทินที่ละลายใน EDTA และปริมาณเซลลูโลส สำหรับผลของการจุ่มน้ำร้อนต่อการ
เปลี่ยนแปลงแอกทิวิตีของเอนไซม์   และการแสดงออกของยีน   พบว่าการจุ่มน้ำร้อนสามารถลดแอกทิวิตีของเอนไซม์
pectate lyase (PL) และเอนไซม์ β-galactosidase (β-Gal) รวมทั้งลดการแสดงออกของยีนของเอนไซม์ทั้งสองคือ MaPL1
และ MaGAL อีกด้วย นอกจากนี้การจุ่มน้ำร้อนยังยับยั้งแอกทิวิตีของเอนไซม์ polygalacturonase (PG) และชะลอการ
แสดงออกของยีน MaPG1 ในเปลือกกล้วยอีกด้วย อย่างไรก็ตาม การจุ่มน้ำร้อนมีผลกระทบต่อเอนไซม์ pectin
methylesterase (PME) และเอนไซม์ β-1,3 glucanase (Glu) เพียงเล็กน้อย โดยพบการลดลงของแอกทิวิตีของเอนไซม์
PME เมื่อผลกล้วยได้รับความร้อน แต่หลังจากนั้นแอกทิวิตีของเอนไซม์ PME จะกลับมามีค่าเท่ากับผลกล้วยที่ไม่ได้รับ
ความร้อน ซึ่งการเปลี่ยนแปลงของแอกทิวิตีของเอนไซม์ PME ไม่สอดคล้องกับการแสดงออกของยีน MaPME1 ส่วน
เอนไซม์ Glu ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของ   แอกทิวิตีของเอนไซม์ Glu และการแสดงออกของยีน
MaGLU ระหว่างผลกล้วยหอมทองที่ผ่านและไม่ผ่านการจุ่มน้ำร้อน การศึกษาผลของการจุ่มน้ำร้อนต่อการเปลี่ยนแปลง
โครงสร้างของผนังเซลล์ด้วยกล้องจุลทรรศน์   พบว่าโครงสร้างของเปลือกกล้วยหอมทองที่ผ่านการจุ่มน้ำร้อนมีการ
เปลี่ยนแปลงช้ากว่าเปลือกกล้วยที่ไม่ผ่านการจุ่มน้ำร้อน   และพบว่าการจุ่มน้ำร้อนสามารถชะลอการลดลงของความหนา
เปลือกและจำนวนชั้นของเซลล์ในเปลือกกล้วยหอมทองได้   รวมทั้งทำให้เกิดช่องว่างระหว่างเซลล์ในเนื้อเยื่อช้าลงด้วย
จากผลการทดลองแสดงให้เห็นว่า การจุ่มน้ำร้อนภายหลังการเก็บเกี่ยวมีประสิทธิภาพในการป้องกันการย่อยสลายของผนัง
เซลล์ในเปลือกกล้วยหอมทอง และสามารถลดแอกทิวิตีของเอนไซม์ที่ย่อยสลายผนังเซลล์ เช่น PL β-Gal และ PG รวมทั้ง
ลดการแสดงออกของยีนที่เกี่ยวข้องกับเอนไซม์ดังกล่าว ซึ่งส่งผลให้คงความแน่นเนื้อของผลกล้วยหอมทองไว้ได้

สาขาวิชา   วิทยาศาสตร์ชีวภาพ   ลายมือชื่อนิสิต
ปีการศึกษา   2554   ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก
                                           ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม

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LIST OF ABBREVIATIONS

AIR	alcohol insoluble residue
ACC	1-aminocyclopropane-1-carboxylic acid
As	air space
°C	degree Celsius
Ca^{2+}	calcium ions
cDNA	complementary deoxyribonucleic acid
CH_3COONa	sodium acetate
CRD	completely randomized design
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
EDTA	ethylenediaminetetraacetic acid
Ep	epidemis
ESP	EDTA-soluble pectin
EST	expressed sequence tag
FAA	formalin-aceto-alcohol
Fb	fiber
g	gram
β-Gal	β-galcturonase
Glu	β-1,3 glucanase
HCl	hydrochloric acid
H_2SO_4	sulfuric acid
HSP	HCl-soluble pectin
HWT	hot water treatment
h	hour
Ig	inner ground tissue
kb	kilo base pair
kg	kilogram
kPa	kilopascal

Lt	laticiferous
M	molar
mM	milimolar
mg	milligram
Mg^{2+}	magnesium
min	minute
ml	millilitre
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
nmol	nanomole
Og	outer ground tissue
PCR	polymerase chain reaction
PG	polygalacturonase
PGA	polygalacturonic acid
Ph	phloem
PL	pectate lyase
PME	pectin methylesterase
PMSF	phenylmethylsulfonyl fluoride
RG-I	rhamnogalacturonan I
RG-II	rhamnogalacturonan II
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SAM	S-adenosylmethionine
S.E.	standard error
Vb	vascular bundle
WSP	water-soluble pectin
Xy	xylem
μl	microlitre

CHAPTER I

INTRODUCTION

Banana fruit (*Musa acuminata*, AAA group, Gros Michel subgroup, cultivar 'Hom Thong') is one of the most economically important fruit of Thailand. The softening of banana fruit occurs rapidly when ripening is initiated which also becomes a major factor limiting shelf life, pathogen infection and physical damage during postharvest handling and storage (Goulao and Oliveira, 2008).

Fruit softening is associated with texture changes that are a consequence of polysaccharide component modifications that result in the disassembly of the middle lamella and primary cell wall structure (Brummell, 2006). Biochemical studies reveal that changes and rearrangements of the cell wall structure related to softening during ripening occur in pectin, hemicellulose and cellulose (Seymour et al., 1990). The solubilization and depolymerization of cell wall polysaccharides have been observed in several fruits including tomato (Chun and Huber, 1998), avocado (Wakabayashi et al., 2000), peach (Brummell et al., 2004) and strawberry (Rosli et al., 2004). Modification of cell wall polysaccharides involves the coordinate action of multiple cell wall degrading enzymes like polygalacturonase (PG) and pectin methylesterase (PME), pectate lyase (PL), β-galactosidase (β-Gal) and β-1,3 glucanase (Glu). Fruits contain the respective enzyme classes but the abundance and type of isoforms present and the timing of expression of the relevant genes or gene families during ripening may vary among species or even cultivars (Hadfield et al., 1998), and this may account for differences in the rate of fruit softening.

Heat treatments were originally used for insect disinfestation and disease control. Many researchers discovered that heat treatments could also maintain fruit quality during storage, induce fruit resistance to stresses and also delay fruit softening in several species (Lurie, 1998). Cell wall study in apple fruit demonstrated that treatment of apple with 38 $^{\circ}$C air for 4 days led to lower soluble pectin and higher insoluble pectin as compare to untreated fruit, indicating the inhibition of pectin degradation (Ben-Shalom et al., 1996). Firmness of strawberry fruit was maintained

by treating with hot air at 45 $^{\circ}$C for 3 h, and the treatment could delay pectin solubilization and hemicelluloses degradation (Vicente et al., 2002, 2005). Postharvest heat treatment could profoundly modify the activity of cell wall degrading enzymes and consequently affect the modification of cell wall component and structure. Application of temperature up to 45 $^{\circ}$C could disrupt cell wall degrading enzymes leading to a delay in softening (Paull and Chen, 2000). The dissolution of middle lamella and disruption of cell wall papaya were inhibited following heating (49 $^{\circ}$C, 120 min), and the activities of PG and xylanase were also reduced (Bacay-Roldan and Serrano, 2005). Heat treatment caused decreased activity of PG and β-Gal including enzymes related to hemicellulose metabolism; endoglucanase and β-xylosidase (Vicente et al., 2005). In addition, disruption of fruit softening affected by heat treatment is possibly attributable to suppression of mRNA for enzymes associated with cell wall modification.

In the case of banana, softening during ripening may be regulated by several cell wall degrading enzymes, and the effect of heat treatment on activities and gene expressions of the enzymes associated with banana fruit softening has not been examined. Therefore, in this research, we determined if postharvest hot water treatments reduced the activity and expression of cell wall degrading enzymes. Furthermore, the compositional and structural changes in the cell wall of banana fruit affected by hot water treatment were investigated.

Objectives :

1. To investigate the compositional changes in cell wall of 'Hom Thong' banana fruit following hot water treatment.

2. To investigate the changes in cell wall degrading enzyme activities of 'Hom Thong' banana fruit following hot water treatment.

3. To determine the effect of hot water on expression of gene encoding cell wall degrading enzyme of 'Hom Thong' banana fruit.

4. To identify the structural alterations in peel of 'Hom Thong' banana fruit affected by hot water treatment.

Expecting benefits:

This research will offer a better understanding of the effects of hot water treatment on alteration of cell wall structure and metabolism of banana fruit during postharvest storage that associated with softening retardation and can be applied to other fruits in order to delay fruit ripening and softening.

Content of the thesis:

1. Literature review

2. Hot water treatment and measurement of banana peel firmness

3. Determination of cell wall compositions

4. Determination of cell wall degrading enzyme activities

5. Determination of expression of gene encoding cell wall degrading enzymes

6. Determination of anatomical alteration in peel structure

7. Results and discussion

8. Conclusion

CHAPTER II

LITERATURE REVEIWS

1. Banana

Banana (*Musa* L.) which belongs to the family Musaceae is cultivated for many purposes, such as food, medicinal and cosmetic values. Banana is cultivated in more than 100 countries throughout tropical and subtropical areas. The annual world production of banana is around 98 million tonnes with a third of the total production being produced in the following countries, African, Latin American, Asia-Pacific and Caribbean regions (Frison and Sharrock, 1999). Most edible bananas originated from two species, *M. acuminata* Colla and *M. balbisiana* Colla. The cultivars are either hybrids among subspecies of *M. acuminata* or between *M. acuminata* and *M. balbisiana*. The edible cultivars are mainly triploids of the acuminata genome (AAA) with varying degrees of parthenocarpy and sterility (Cheesman, 1947; Simmonds, 1962).

In Thailand, at least 110 *Musa* species and cultivars have been found including 'Hom Thong' banana (Wongniam et al., 2010). 'Hom Thong' (*Musa acuminata*) is an edible triploid cultivar which belongs to the AAA genomic group and Gros Michel subgroup. Homogenomic AAA triploid derived from the hybridization within *Musa acuminata* (AA), a wild diploid species. The AAA genomic group is the main cultivar of international commerce (Fortescue and Turner, 2005). According to the Office of Agricultural Economic (2011), 'Hom Thong' banana is one of the most important export cultivars of Thailand.

Banana is a monoecious plant having male flowers at the tip of the inflorescence and female flowers behind. The fruit of banana is a product of parthenocarpy and characterised as berry with a leathery outer peel that contains much collenchyma (Daniells et al., 2001). Pseudostem, the above ground trunk is formed by the leaf sheaths of the spirally arranged leaves. The underground corm, true stem,

grows up through the center and develops into terminal inflorescence. The initiation of flower begins when the vegetative axis in the corm is changed into the reproductive apex and flowers emerge in groups along the stem. The immature inflorescence is enclosed by purple bracts. The bracts shed sequentially from base to tip. Banana fruit develops from the inferior ovaries of female flowers. The outer protective layer of banana fruit is known as peel which derived from the ovary wall. The triploid sweet banana cultivars including 'Hom Thong' banana are parthenocarpic fruit, developing without fertilization. In principal, growth of the ovary in parthenocarpic fruit occurs in two ways; the first is the inward growth of the pericarp tissue and the second is the expansion of central floral axis, placenta and septa. Finally, the ovarian cavity is obliterated completely by filling with a soft and fleshy tissue but there is no development of ovules into seeds (Mohan Ram et al., 1962; Fortescue and Turner, 2005). The number of hands along a central stem in each bunch varies with cultivars and species. Each hand composes of two transverse rows of fruit called a finger (Simmonds, 1966).

Bananas can be divided into 8 stages of ripeness according to CSIRO standards (1972), as followed (Sukuntaros Tadakittisarn et al., 2009):

Stage 1: 100% green peel, hard fruit

Stage 2: 95% green peel, 5% yellow peel emerging

Stage 3: 70% green peel, 30% yellow peel emerging

Stage 4: 30% green peel, 70% yellow peel

Stage 5: 95% yellow peel, 5% green at the tip

Stage 6: 100% the whole fruit yellow

Stage 7: Yellow peel with brown spots appearing, characteristic aroma distinct

Stage 8: Yellow peel with more brown spots over ripe, pulp softens
        and pungent aroma

2. Fruit ripening and softening

Fruit ripening is an important physiological change that occurs during later stages of fruit growth and development and early stages of senescence (Asif and Nath, 2005). Ripening process is coordinated, genetically determined and an irreversible event involving a number of physiological and biochemical modifications which affect appearance, texture, aroma and flavour (Goulao and Oliveira, 2008). These changes which take place during ripening are initiated and proceed together under the correct circumstances leading to the development of edible and desirable quality attributes of fruit (Thompson, 2003). The activation of many metabolic pathways occurs during ripening, resulting in alteration in biochemical composition of fruits and the developmental stage of fruits indicates the biochemical composition and quality attributes.

Ethylene is an important phytohormone which is known to regulate numerous physiological and developmental processes in plants including fruit ripening. Ethylene biosynthesis occurs via common pathway using methionine as the precursor. Methionine is converted to S-adenosylmethionine (SAM) by methionine adenosyl transferase. Then, the conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) which is the intermediate precursor of ethylene biosynthesis is mediated by action of ACC synthase and ethylene is finally generated from ACC using the enzyme ACC oxidase (Figure 2.1).

Fruit ripening is initiated when cellular quantities of ethylene reach the threshold level. Ethylene's arrival to threshold level will occur naturally at fruit maturity. However, it can occur earlier if the fruit is subjected to stresses such as infection by pest and disease, water shortage during production or physical damages during handling and storage (Thompson, 2003). The application of exogenous ethylene to fruits can also trigger the initiation of ripening and endogenous production of ethylene. Ethylene is synthesized autocatalytically at a level as low as $0.01 \mu l L^{-1}$. Johnson et al. (1997) found that the level of ethylene at $0.05 \mu l L^{-1}$ can initiate ripening in banana fruit. Ethylene binds to its receptors and elicits a signal transduction resulting in the ripening process (Fluhr and Mattoo, 1996) (Figure 2.1).

The ethylene biosynthesis in the pulp is the major source of ethylene in banana fruits (Reviewed in Thompson, 2003). Increasing of ethylene synthesis is followed by modifications in fruit physiology, texture, composition and also climacteric rise in respiration.
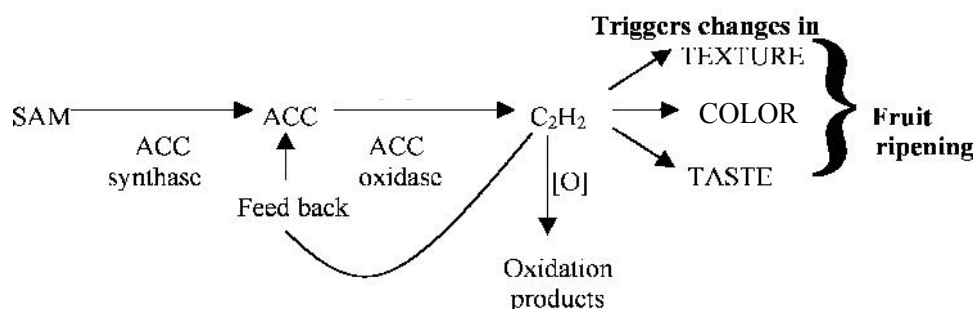
Figure 2.1 Pathway of ethylene biosynthesis and metabolism (Prasanna et al., 2007)

Color is considered to be a major parameter which attracts a consumer to fruit. The change of color during ripening process is a consequence of chlorophyll degradation which unmasks previously presented pigments. A change in peel color from green to yellow or degreening is the distinct external symptom during ripening of banana fruit, and it is important as an indicator to determine internal ripeness (Lizada et al., 1990 ; Li et al., 1997). The external color of banana fruit is associated with the relative amounts of pigments in the peel, chlorophylls and carotenoids (Meddlicott et al., 1992). The degreening during banana ripening is a result of chlorophyll breakdown. During fruit maturity and ripening, chlorophylls degrade to a colorless chlorophyll catabolic unmasking carotenoid pigments in plastids (Matile et al., 1999). The decrease in total carotenoid was detected in early stage of banana ripening before carotenoid biosynthesis in later stage (Pokorny et al., 2001). Compositional analysis documented that major types of carotenoid in ripening banana are lutein and α- and β-carotene. The degreening of Cavendish bananas is affected by temperature. The failure to degreen in peel was detected in Cavendish banana ripened at 27 ^{o}C even though the pulp had softened (Ding et al., 2007). Non-degreening of

banana peel could be due to the retention of thylakoid membranes in chloroplast which delayed chlorophyll degradation (Blackbourn et al. 1990).

Flavor is the principle component to the quality perception combining taste, smell and texture. An increase in soluble sugars, a decrease in organic acids and phenolic compounds and an increase in volatiles are observed during fruit ripening, producing the characteristic flavor (Pantastico, 1975). The development of sweetness is due to the hydrolysis of starch to simple sugars. Bananas accumulate sugars from a stored starch reserve. The starch content, which constitutes 20-25% of unripe banana fresh weight, is mostly converted to sugars with less than 5% lost during ripening (Hubbard et al., 1990). Sucrose is the predominant sugar in early stage of ripening while glucose and fructose predominate during later stage (Reviewed in Thompson, 2003). Starch reduction in bananas is mediated primarily by action of phosphorylase. The association of phosphorylase activity with the breakdown of starch to produce sugars was reported previously.

Although the conversion of starch to sugars is responsible for increasing sweetness of ripe fruit, organic acids present in fruit are also important in affecting fruit flavor. Decreased acidity was found during fruit ripening with types of organic acid varying among different fruits. Early studies reported that citric, malic and oxalic were the main acid in banana fruit (Palmer, 1971). Moreover, banana fruit, especially in the peel, contains high levels of phenolic compounds such as tannins. This compound gives astringency in fruit and is converted to insoluble compound as fruit ripen, leading to the reduction of astringent taste in ripen banana (Lizada et al., 1990). The reduction of astringency during ripening seems to be related to structural change of tannin rather than the decline in tannin level (Thompson, 2003).

The characteristic of ripe fruit aroma is derived from the production of a complex mixture of volatile compounds. More than 350 flavors and aroma compounds have been observed in ripe banana and have been generally synthesized in the late climacteric with approximately 70% acetate and butyrate of the total volatile compounds (Tressl and Drawert, 1973). Esters, alcohols, carbonyls and ketone have also shown to contribute to the banana flavor in 'Hom thong' banana, and their rates

of production increase as ripening proceed (Thaipanit and Anprung, 2010). The major compounds were 3-methyl-1-butyl acetate, 3-methylbutyl butanoate, 2-methylpropyl ethanoate and 3-methylbutyl 3-methylbutanoate.

The development of several organoleptic components like sweetness, sourness, astringency, and production of volatile compounds which give fruit its characteristic aroma during the ripening process is linked to changes in fruit texture (Lurie, 2008). These changes produce desirable quality attributes for consumer's perception, but some changes related to softening cause fruits to become unacceptable. The presence of off-flavors increases with excessive softening. Shelf life, pathogen infection, and physical injury during postharvest handling and storage were also affected by softening that increase proportionally with firmness loss (Goulao and Oliveira, 2008). Thus, textural quality is considered to have commercial importance in fleshy fruits including banana. Softening of banana fruit occurs rapidly when ripening is initiated (Duan et al., 2008) (Figure 2.2).
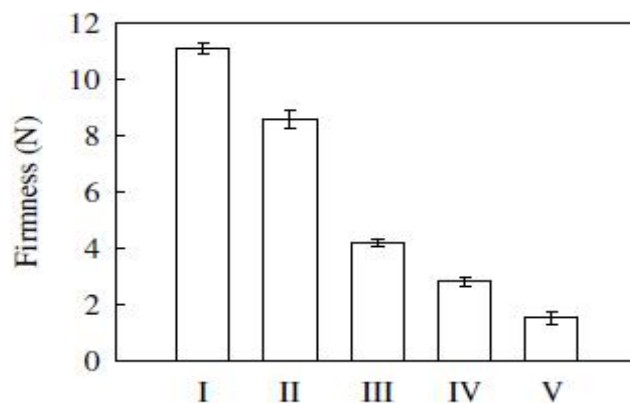
Figure 2.2 Firmness of banana (*Musa* spp., AAA group, cv. Brazil) at various stages of ripening. I: mature green; II: green, III: green>yellow; IV: yellow>green and V: yellow. (Duan et al., 2008)

The changes in fruit texture during ripening in association with softening appear to be correlated with various metabolic events including loss in turgidity, degradation, and physiological changes in membrane composition, breakdown of starch to form sugars, and modification in cell wall structure (Goulao and Oliveira, 2008). However, compositional modification in cell wall is believed to be a major factor responsible for the textural changes, especially cell wall mechanical strength and cell adhesion (Fischer and Bennett, 1991; Hadfield and Bennett, 1998).

3. Cell wall structure and composition

Ripening associated softening of fruit is usually represented by a reduction in firmness of tissues involving a coordinated series of polysaccharide component modifications of primary cell wall and middle lamella that result in weakening of the structure (Negi and Handa, 2008).

Cell wall is a complex network structure providing mechanical and structural support to the plant body. It is composed of various polysaccharides, such as pectin, hemicelluloses, and cellulose microfibrils (Prasanna et al., 2007). Besides polysaccharides, structural and enzymatic proteins, inorganic molecules, and hydrophobic compounds also exist in the cell wall. Generally, cellulose microfibrils that are synthesized by cellulose synthase complexes are enclosed by a layer of tightly bound hemicelluloses. Hemicellulose hydrogen bonds to microfibrils and cross-link between cellulose microfibrils. The microfibrils and associated hemicelluloses are embedded in a matrix phase consisting of pectic polysaccharides. The cell wall matrix, hemicellulose, pectin and glycoprotein are secreted through Golgi vesicles (Kohorn, 2001) (Figure 2.3).
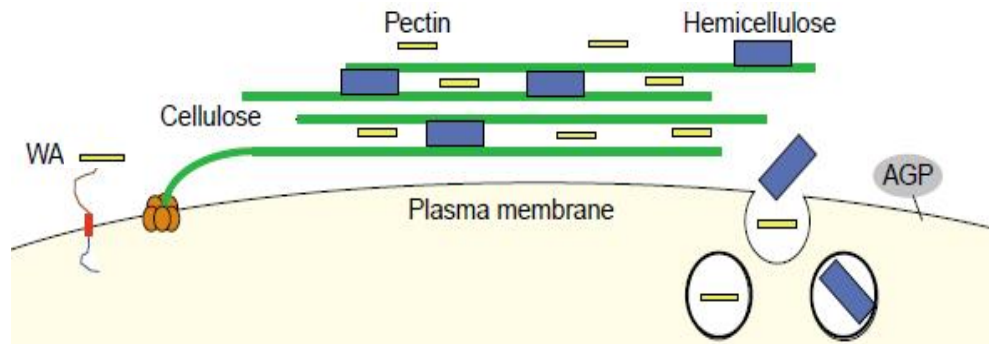
Figure 2.3   Cell wall structure where cellulose microfibrils are enclosed by hemicelluloses and embedded in a matrix of pectins (Kohorn, 2001).

Cellulose are composed of unbranched associated β-1,4-linked glucan chains which are held together via hydrogen bonds.   The abilily of β-1,4-linked glucan chains in forming intermolecular and intramolecular hydrogen bonds (Figure 2.4) affects the physical and chemical property of cellulose. The hydrogen bonds lead to lateral aggregation and crystallization of the β-1,4-linked glucan backbones into microfibrils structures (Albersheim et al., 2011).   The intermolecular and intramolecular hydrogen bonds provide a well-defined structure and the mechanical strength to cellulose microfibrils.   The β-1,4-linked glucan chains are approximately 30-50 chains self-associated by hydrogen bonding to form cellulose microfibril with a diameter of 3-5 nm.   Cellulose microfibrils consist of highly ordered crystalline regions which are interrupted by less ordered crystalline regions (amorphous) from the studies in their structure. It is proposed that the physical properties of cellulose microfibril in cell wall are affected by the distribution and the degree of crystalline and amorphous regions in cellulose microfibrils (Albersheim et al., 2011)
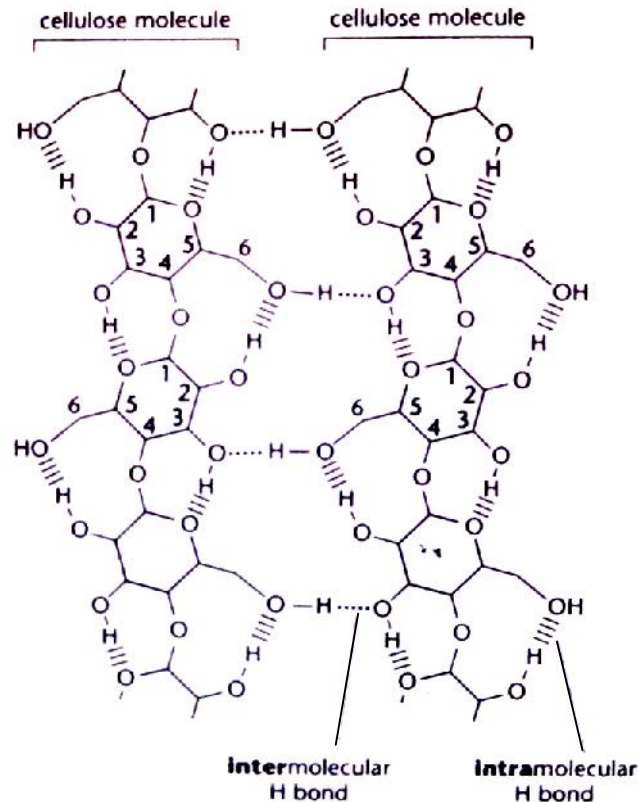
Figure 2.4 Intermolecular and intramolecular hydrogen bonds between two glucan chains within a microfibril (Albersheim et al., 2011).

The hemicelluloses (matrix glycans) are neutral or weakly acidic and consist of neutral sugars. This polysaccharide that is tightly bound in the cell wall has variation in structure and composition. Hemicelluloses have the same basic structure as that of celluloses but do not form microfibrils because of its branching and sugar modifications (Negi and Handa, 2008). Xyloglucan and arabinoxylan are predominant among several components of hemicellulose. As cellulose, xyloglucans are composed of a highly branched polymer and a β-1,4-linked glucan backbone, but approximately 75% of the glucose residues are substituted by xylose side chains (Albersheim et al., 2011). Xylose is occasionally extended with either *β*-galactosyl-*α*-fucose or *α*-arabinose in some species. The arabinoxylan has a *β*-1,4-xylan backbone with single unit arabinose and glucuronic acid side chains. Moreover, glucomannan is another component which may have similar roles to those of xyloglucan and

arabinoxylan. Its backbone is made of *β*-1,4-glucan and *β*-1,4-mannan in nearly equal amounts. Hemicellulose polysaccharides that present in ripening fruit also include galactomannans and mannans (backbone containing a β-1,4-mannose with or without galactose linked to mannose residues by an α-1,6 bond) and xylans (backbone contains β-1,4-xylose residues) (Sozzi, 2004).

Pectins are the most abundant class of polysaccharides within the cell wall matrix and in the middle lamella between the cells (Figure 2.5). They are highly hydrophilic and have the ability to form gels where the cellulose-hemicellulose network is embedded (Evert, 2006).
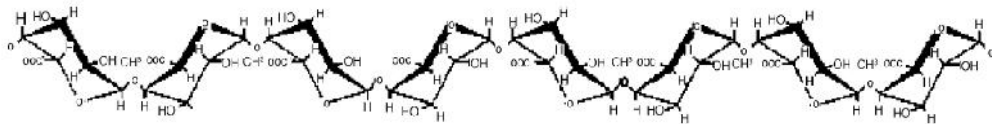
Figure 2.5 Primary structure of pectin (Jayani et al., 2005).

Homogalacturonan, rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) constitute pectin network of cell wall. Homogalacturonan is homopolymer formed with α-1,4-galacturonic acid. It is initially synthesized and secreted into cell wall in a highly methyl-esterified form (Carpita and McCann, 2000). The adjacent nonesterified homogalacturonan regions can form calcium cross-links between two chains (Figure 2.6). RG-I is a major branched, heterogenous and hydrated component of middle lamella which is also present in primary cell wall. The backbone of RG-I is consisted of the repeating unit of disaccharide *α*-1,4-galacturonic acid, *α*-1,2-rhamnose (Sozzi, 2004). RG-I has different side chains attached to C4 of rhamnose units. Arabinose and galactose predominate in side chain components and fucose, glucosyluronic acid, and 4-*O*-methyl glucosyluronic acid are also present in small amounts (Albersheim et al., 2011). RG-II has α-1,4-galacturonic acid backbone, similar to that of homogalacturonan, but with complex side chains of many types of neutral sugar. Although, it is a minor cell wall component, RG-II monomers can

dimerize together by single borate diester cross-link and may affect the wall porosity (Brummell and Harpster, 2001).
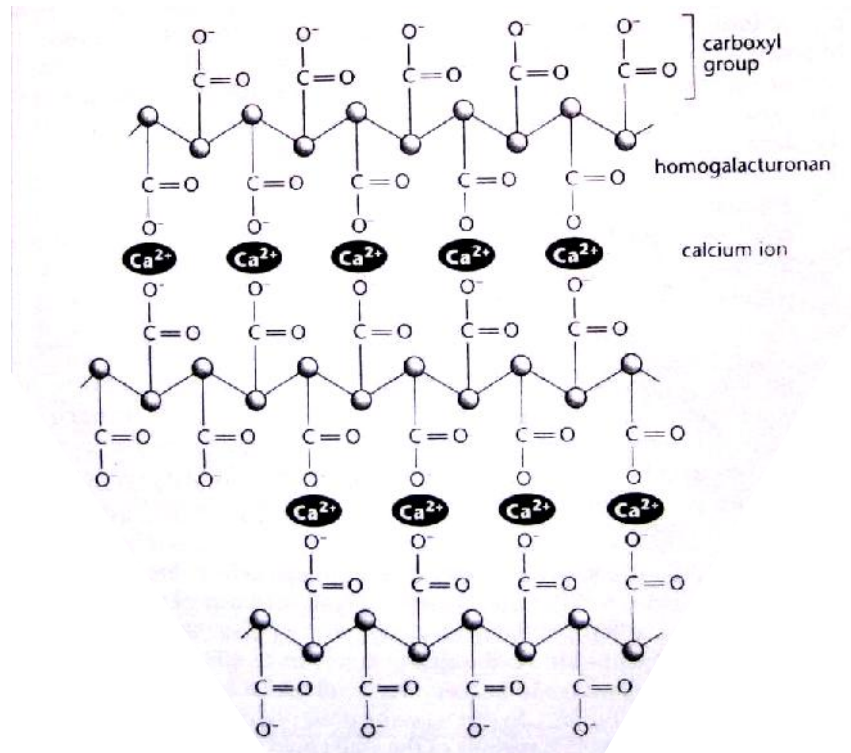
Figure 2.6 Calcium cross-linking bridges between nonesterified homogalacturonans (Albersheim et al., 2011).

In addition, cell wall matrix includes proteins with varying amount of linked carbohydrates, glycoproteins (Kohorn, 2001) that may account for 10% of the dry weight of primary cell wall. Hydroxyproline-rich proteins (HRGPs), proline-rich proteins (PRPs), and glycine-rich proteins (GRPs) are the major classes of structural proteins in cell wall (Evert, 2006). Arabinogalactan proteins (AGPs) are also found in cell wall but do not have structural function. AGPs have multiple roles in plant development including messengers in cell-cell interaction during differentiation. Moreover, expansins are another cell wall protein that mediate the loosening of the cell wall and promotes cell expansion (Cosgrove, 2000).

The cell wall-associated kinases (WAKs) span the plasma membrane and are linked directly to the cell wall. They have a cytoplasmic kinase domain. The association of WAKs with the wall is very strong (Kohorn, 2001). WAKs are covalently bound to pectin and some of WAKs that is linked to pectin is also phosphorylated (Figure 2.3). In addition to pectin, WAKs bind to an abundant cell wall protein, GRP. WAKs are somehow related to signaling from the pectin extracellular matrix in cooperation with GRPs (Anderson et al., 2001). It suggests that WAKs may play either adhesive or signaling roles.

Many enzymes like peroxidases, phosphatases, laccases, invertases, malate dehydrogenases, cellulases, pectinases, pectin methylesterases, β-1,3-glucanase and chitinases have also been identified in primary cell walls (Evert, 2006).

4. Cell wall changes during ripening associated softening

Fruit softening, an important aspect of the ripening process, is associated with extensive structural alterations within pectin matrix, pectin-rich middle lamella, and hemicellulose-cellulose network. Biochemical studies indicate that cell wall changes associated with softening during ripening occur in pectin, hemicelluloses, and cellulose polysaccharides (Seymour et al., 1990). The solubilization and depolymerization of cell wall polysaccharides have been observed in several fruits.

Pectin is the most abundant class of macromolecules in the cell wall matrix and the middle lamella which is found between the primary cell walls. The pectic matrix is a major intercellular adhesive materials and provides an environment for the deposition, slippage, and extension of the hemicellulose-cellulose network (Willats et al., 2001). Pectin typically undergoes solubilization and depolymerization during softening process, and it is thought to contribute to loosening and disintegration of cell wall leading to textural changes.

Numerous studies reported that water-insoluble pectin is converted to water-soluble form during fruit ripening (Reinders and Their, 1999; Majumder and Mazumdar, 2002; Manrique and Lajolo, 2004; Yashoda et al., 2005), and this change is important to fruit texture. The increase in water-soluble pectin contents during ripening was accompanied with the decline of water-insoluble pectin contents. This indicated pectin solubilization during ripening originated from polymer tightly integrated to cell wall or linked to hemicelluloses (Redgwell et al., 1997a). Similarly, banana fruit showed a remarkable increase of water-soluble pectin level, whereas acid-soluble pectin level decreased during ripening (Duan et al., 2008) and the amount of water-soluble pectin fraction in strawberry also increased from the small green to the white stage (Rosli et al., 2004). The increase of pectin solubilization and depolymerization is usually associated with the decline of fruit firmness (Redgwell et al., 1997b). The involvements of pectin degradation in the reduction of fruit firmness have been extensively studied.

The progressive depolymerization of polyuronides from unripe to mid ripe and full ripe was observed in tomato and avocado, but polyuronides in peach exhibited little change at mid ripe stage compared with unripe stage before showing dramatic depolymerization in full ripe fruit (Negi and Handa, 2008). Polyuronides depolymerization occurring in late stage of ripening is considered to be a contributor to the weakening of the middle lamella and reduction of adhesion between cells. The depolymerization occurs late in ripening is correlated with a decrease in tissue firmness. However, fruit softening does not seem to be involved in polyuronide depolymerization early in ripening. Duan et al. (2008) reported that softening of banana fruit occurred during ripening was a consequence of the changes in polysaccharide compositions and glycosyl linkage, decreased distribution of molecular mass and enhanced depolymerization of pectin.

In addition to pectin degradation, the modification of hemicelluloses polysaccharides is considered to be correlated to fruit softening. Hemicellulose degradation was observed in numerous fruits and was found to occur in both the xyloglucan component and total matrix glycans. Previous study on tomato showed a reduction of xyloglucan level at about 30% from mature green to red stage and a

downshift in the molecular mass of hemicellulose polysaccharide (Sakurai and Nevins, 1993). The molecular mass of xyloglucan also reduced in the early stage of tomato softening which was further accompanied by a decline of tissue firmness (Maclachlan and Brady, 1994). In avocado, the progressive downshift in xyloglucan molecular mass was observed at the early onset of fruit softening (O'Donoghue and Huber, 1992; Sakurai and Nevins, 1997). Besides, the depolymerization of hemicellulose polysaccharides was detected in avocado, kiwi fruit, and banana during fruit softening period (MacRae and Redgwell, 1992; Sakurai and Nevins, 1997; Cheng et al. 2009). The significant degradation of hemicellulose polysaccharide was found during banana fruit ripening after cell wall disassembly analysis (Kojima et al., 1997; Prabha and Bhagyalakshmi, 1998). The study of Cheng et al. (2009) suggested that the modification and depolymerization of hemicellulose in cell wall were responsible for fruit softening during banana ripening. Moreover, the disassembly of hemicelluloses in peach fruit was closely implicated to softening process (Brummell et al., 2004).

These finding revealed that the breakdown of hemicellulose was related to textural changes of fruit tissue. The hemicellulose degradation occurring during early stage of softening may partially disrupt the cellulose-hemicellulose network resulting in reduction of cell wall rigidity (Wakabayashi, 2000). Loosening of the cellulose-hemicellulose network might also be part of cell wall swelling that involved in solubilization of pectins (Negi and Handa, 2008).

The hemicellulose breakdown often occurs in parallel with the extensive degradation of pectin polysaccharides in ripening fruit. Rose et al. (1998) found in melon fruit the degradation of tightly bound hemicelluloses in the early stage of softening process, whereas the depolymerization of pectin polysaccharides was detected in the later stage. The breakdown of both hemicellose and pectin was cooperatively associated with fruit softening. The hemicellulose degradation may play a role in the initiation and the modification of pectin contributing to the excessive softening (Wakabayashi, 2000). It is anticipant that structural changes in cellulose may be related to softening of ripening fruits. Loss of hemicelluloses and cellulose has been reported in strawberry and the decline of cellulose amount was found in

Grape fruit (Deng et al, 2005). The observations in cell wall ultrastructure of apple, pear, and avocado demonstrated the dissolution of cell wall microfibril network during ripening (reviewed in Fischer and Bennett, 1991)

In the last decade, more attentions have been paid to the loss of neutral sugars as one of the major factors responsible for the development and ripening of fruits. Loss of neutral sugars, especially galactose and arabinose, seems to be a common change that accompanies ripening in many fruits (Fischer and Bennett, 1991). Galactose and arabinose play an important role in cell wall structure and function. They are present in primary cell wall as the galactan, arabinan and branched arabinogalactans side chains of RG-I and in the side chains of xyloglucan, glucuronoarabinoxylan, and RG-II (Carpita and Gibeaut, 1993). The loss of galactan and arabinan side chains of RG-I during fruit ripening may alter cell wall rigidity and intercellular adhesion, increase cell wall porosity and affect pectin solubilization (Negi and Handa, 2008). Generally, pectin polysaccharide which was solubilized during ripening process had low content of galactose and arabinose (Redgwell et al., 1997a; Brummell et al., 2004). This indicated that solubilization and neutral sugar loss had an effect on pectin molecules or loss of side chain led to pectin solubilization.

Variation in cell wall component and significant differences in cell wall polysaccharide modifications during ripening among species and even among different cultivars contribute to the difference of fruit softening rates.

5. Cell wall degrading enzymes

The changes in cell wall structure and composition, which accompany fruit softening during ripening, are involved in the concerted actions of a range of cell wall degrading enzymes secreted from the symplast into cell wall space (Brummell and Harpster, 2001). These enzymes mostly exist in low level and are constitutive throughout fruit development but generally increase during ripening (Prasanna et al, 2007). The expression of many genes encoding the cell wall degrading enzymes increases during ripening process and the product of these genes may affect the

modification of cell wall polysaccharides. Therefore, the relationship between fruit softening and cell wall enzymes has been demonstrated at both molecular and biochemical level.

Among cell wall modifying enzymes, pectin degrading enzymes are mainly associated with fruit softening. Polygacturonase (PG) is a pectin degrading enzyme that catalyses the hydrolysis of the glycosidic bonds in galacturonans (Figure 2.7). PG can be classified into exo-PG and endo-PG. The exo type cleaves the glycosidic bonds between galacturonic acid from the reducing-end, releasing single galacturonic acid. The action of exo-PG causes the formation of reducing groups in a large amount. Conversely, the endo type randomly depolymerizes pectin polysaccharide, which results in a decrease in viscosity (Prasanna et al., 2007).

It is accepted that the integrity of the middle lamella, which controls intercellular connections, is affected by PG and thereby influences fruit texture (Wei et al., 2010). The appearance of PG has shown a clear correlation with the initiation of dissolution of middle lamella and primary cell wall during fruit ripening (Crookes and Grierson, 1983). The PG activity has been extensively studied in many ripening fruits and is consistent with soluble pectin increase and fruit softening. PG has been suggested to be primarily responsible for pectin degradation and softening during fruit ripening (Fischer and Bennett, 1991). The PG activity in tomato showed direct correlation to ethylene induction and responded to ethylene treatment in concentration dependent manner (Sitrit and Bennett, 1998). Additionally, an increase in activity of PG with a peak at climacteric stage was reported in capsicum (Priyasethu et al., 1996), mango (Prabha et al., 2000) and banana (Prabha and Bhagyalakshmi, 1998). The study of Asif and Nath (2005) found an increase in PG activity during banana ripening to further suggest PG's role in softening process.

Belonging to a multi-gene family, PG shows the expression patterns in differential tissues at different times and responds to different induction (Hadfield et al., 1998). PG, present in banana, is a multi-gene family like many other fruits. Four cDNAs encoding PG were cloned from banana during ripening and their expression was also studied (Asif and Nath, 2005). This finding indicated that softening during

banana ripening was due to the coordinated action of at least four PG genes which were expressed in fruit with different patterns. Tomato fruit suppressed in PG activity had decreased level of water soluble pectin which coincided with the increased level of sodium carbonate soluble fraction indicating that covalently bound pectin was depolymerized by PG action and then solubilized into water soluble pectin (Carrington et al., 1993).

Polygalacturonans are synthesized and secreted into the cell wall with a high degree of methylesterification which are deesterified during fruit development. PME catalyzes the hydrolysis of methyl esters from the C6 position of galacturonic acid residues resulting in the deesterification of pectin (Sozzi, 2004) (Figure 2.7). PME was suggested to be related to pectin degradation by lowering the degree of pectin methoxylation, making polygalacturonan available for degradation by PG (Koch and Nevins, 1989). The degree of methylesterification of pectin dropped significantly during tomato ripening which correlated with PME activity (Koch and Nevins, 1989).

The activity of PME had been involved in various processes like cell wall maturation, abscission and plant pathogen infection (Blumer et al., 2000), and was also observed during ripening in several fruits like avocado, mango, papaya, banana, pear, and strawberry (Goulao and Oliveira, 2008). In tomato, PME activity and protein was found throughout fruit development and ripening, increasing from the early stage of green fruit through different stages to the ripening stage and peaking early in ripening and decreasing slightly thereafter (Harriman et al., 1991; Tieman et al., 1992). The pattern of PME mRNA accumulation was different in that it increased to reach its maximum in the mature green stage and then reduced rapidly when fruit further ripen (Harriman et al., 1991).

Tomato fruit PME was present as a small gene family composing of at least four genes (Brummell and Harpster, 2001). Three isoforms of PME were isolated and cloned from banana fruit that were differentially expressed during fruit ripening (Mbeguie-A-Mbeguie et al., 2009). Transgenic antisense PME tomato had higher degree of pectin methylesterification by 15–40% (Tieman et al., 1992; Hall et al., 1993) and also showed a decreased pectin depolymerization and reduced content of

chelator soluble pectin during ripening (Tieman et al., 1992). Diminished PME activity had no effect on ripening associated with fruit softening but resulted in an almost complete loss of tissue integrity during overripe stage (Tieman and Handa, 1994). This was related to a reduction of bound divalent cations, Ca^{2+} and Mg^{2+}.

Pectate lyase (PL) also plays a role in pectin degradation. Pectate which is the de-esterified product of pectin polysaccharides is the principle component maintaining the cell wall integrity in higher plants (Carpita and Gibeaut, 1993). This polymer is depolymerized by action of PL which cleaves α-1,4-galacturonoside linkages by β-elimination generating an unsaturated bond at the non-reducing ends of pectin polymer reaction product (Fischer and Bennett, 1991) (Figure 2.7).

The EST programme displayed a high expression of PL like gene in ripe tomato (Marin-Rodriguez et al., 2002). Clones with homology to PL from banana, strawberry and grape berry were also reported (Dominguez-Puigjaner et al., 1997; Pua et al., 2001; Medina-Escobar et al., 1997; Nunan et al., 2001).

The result from an early study in strawberry fruit demonstrated that PL was associated with strawberry ripening (Medina-Escobar et al., 1997). High level of PL transcript accumulated in ripe fruit but not in other organs like flower, root, leaf, stem including unripe fruit. The transcript accumulation of PL in Dwarf Cavendish banana was consistent with ethylene level during ripening that suggested the regulation of PL at transcriptional level during ripening (Dominguez-Puigjaner et al., 1997). Two distinct cDNA clones with homology to PL were isolated from banana (Pua et al., 2001). The two PL showed differential expression in fruit during ripening and their expression were affected by ethylene treatment. The investigation of Payasi and Sanwal (2003) to correlate PL expression of banana during ripening observed previously with enzyme activity of PL revealed that PL activity increased steadily from early climacteric and showed maximum level at climacteric peak. This supported the finding of Dominguez-Puigjaner et al. (1997) on the expression of cDNA clone from banana fruit. The consistency of maximum activity of PL with climacteric peak indicated the role of PL in banana ripening (Payasi and Sanwal, 2003). In addition, transgenic strawberry fruit with suppressed PL mRNA showed

lower degree of in vitro cell wall swelling, lower content of ionically bound pectins, and significant higher firmness during ripening and storage as compared to control fruit (Jiménez-Bermúdez et al., 2002).

Loss of cell wall galactosyl residue had been reported as one of the cell wall compositional changes during ripening. β-Galactosidase (β-Gal) is the only enzyme identified in higher plants that catalyzes the cleavage of β-galactosidic residues (Figure 2.7). The enzyme partially degrades pectin and hemicellulose polysaccharides of cell wall. The role of β-Gal in fruit development and ripening had been extensively studied in many fruits. In mango, the increase in β-Gal activity was reported during the development stage of fruit (Rahman et al., 2000). Three isoforms of β-Gal were purified and characterized from tomato fruit (Pressey, 1983) and β-Gal II isoform was found to be capable of catalyzing the cleavage of β-1,4-galactan isolated from cell wall of tomato. The temporal correlation of softening with the increased activity of β-Gal II was showed during tomato ripening (Pressey, 1983; Carey et al., 1995). Moreover, seven cDNAs encoding β-Gal (TBG1-TBG7) were isolated from ripening tomato (Carey et al., 1995; Smith et al., 1998; Smith and Gross, 2000) and TBG4 was likely to contribute to the loss of cell wall galactosyl residue occurring during ripening (Smith et al., 1998; Carey et al., 2001). Its expression first appeared at the beaker stage and displayed maximum level at the turning stage. Three strawberry β-Gal cDNAs (Faβgal1-Faβgal3) were also identified (Trainotti et al., 2001).

The coincidence of increased β-Gal expression with increased activity, free galactose release and fruit softening was reported during tomato ripening (Smith et al. 1998, 2002; Lazan et al. 2004). Similarly, softening of Japanese pear was consistent with increasing enzyme activity and gene expression of β-Gal (Tateishi et al., 2001). The role of β-Gal in banana fruit softening was also studied (Zhuang et al., 2006). The results indicated that increased β-Gal gene expression corresponded with enhanced levels of enzyme activity and the decline in fruit firmness during ripening suggesting the possible role of β-Gal in banana fruit ripening and softening.

β-1,3-glucanase (Glu) is a widely distributed enzyme found in all higher plants (Stone and Clarke, 1992). Glu catalyzes the hydrolysis of β-1,3-glucosidic linkages in β-1,3-glucans (Figure 2.7). It is known that Glu plays a role in the major biological functions in plant reproductive biology and plant defense mechanisms against pathogen infection. In recent years, significant evidences have been obtained that Glu is associated with various processes including cell division, seed germination, fertilization and embryogenesis, pollen germination, wound, and abiotic stress responses (Leubner-Metzger and Meins 1999). Furthermore, the possible function of Glu in fruit ripening has been proposed (Hinton and Pressey, 1980).

The transcript abundance of gene encoding Glu was found to increase during banana ripening after differential screening of cDNA library in banana fruit (Clendennen and May 1997) suggesting that Glu is ripening upregulated gene in banana. The enzymatically active form of Glu from banana fruit had been purified and characterized and probable physiological function in softening during banana ripening had also been predicted (Peumans et al. 2000). Early study in strawberry fruit reported that two Glu genes were induced during ripening (Shi et al. 2006).

The study of the expression pattern and enzyme activity of Glu gene along with fruit firmness changes during banana ripening which indicate a possible function of Glu in fruit softening (Choudhury et al., 2009, 2010). Glu was induced by treatment with ethylene at both mRNA and protein level which was accompanied by an increased activity of Glu and decreased firmness (Kesari et al 2007; Choudhury et al., 2010).

The Glu is present as multiple isoforms with the differences in primary structure, size, cellular localization, and regulation pattern (Leubner-Metzger and Meins, 1999). Glu is classified into three classes (class I-III) in tobacco. Class I Glu is usually basic protein in nature restricted in the cell vacuole. Class II and III Glu are acidic proteins and secreted in the extracellular space (Meins et al. 1992). In banana, Glu belongs to class I, and the moderated expression of Glu was detected in several parts of banana flower that interpreted the function of Glu in flower formation. Glu showed a weak expression in root, leaf, and stem which indicate that Glu had a

negligible role in banana vegetative tissues, but in ripe peel and pulp tissue, the enhanced expression of Glu was observed which suggest that Glu was ripening regulated (Choudhury et al., 2010).
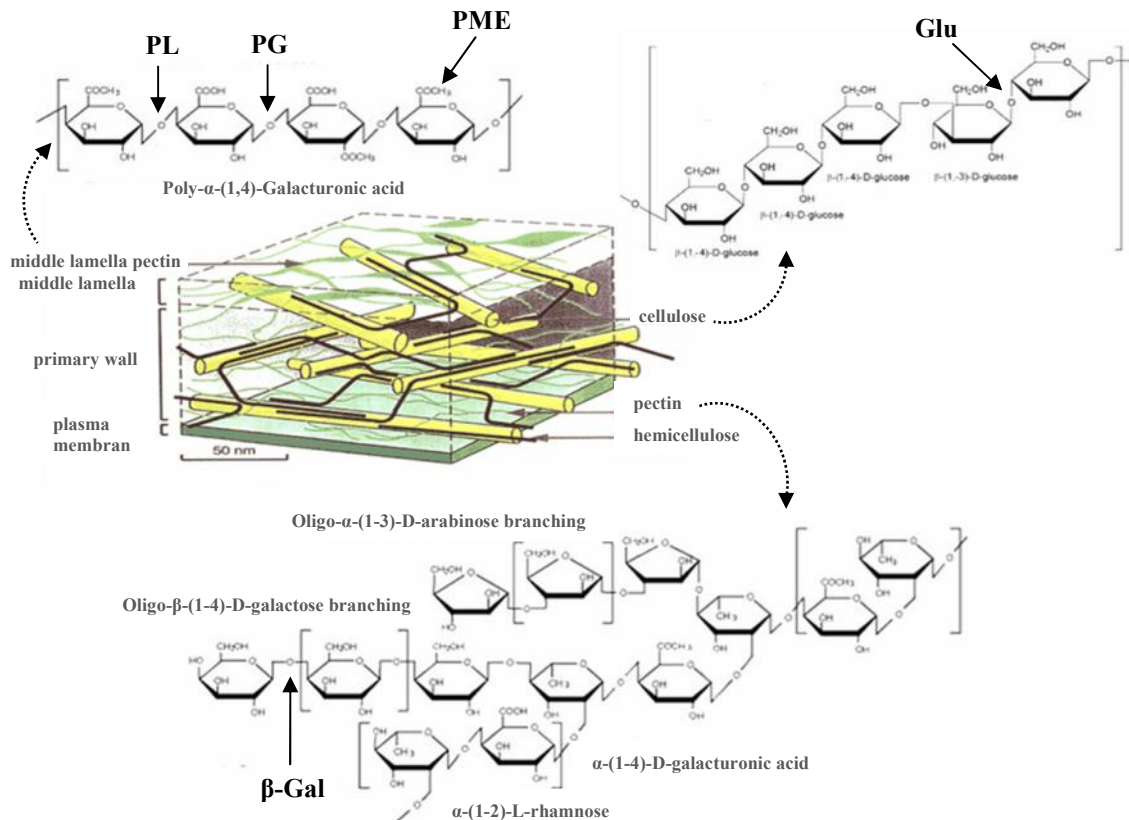
Figure 2.7   Mode of action of cell wall degrading enzymes reacts with cell wall components.

6. Postharvest technology

Fruits are one of the economically important horticultural products. The postharvest deterioration in fruits easily occurs during handling and storage and thus appropriate postharvest techniques are required to extend shelf life whereas to maintain quality. Many postharvest handling techniques have been developed to control or delay the metabolic processes to provide optimal fruit quality for consumption and marketing.

Controlled atmosphere has been extensively used to prolong storage life of various fruits. The basic objective of these techniques is the introduction of adequate levels of gases, low oxygen (O_2) and/or high carbon dioxide (CO_2) to the produces for maintaining their quality and delaying senescence (Sozzi, 2004). The storage life of 'Kensington Pride' mango was prolonged under controlled atmosphere storage and high concentration of volatile compounds responsible for ripe mango aroma was also maintained (Lalel and Singh, 2004; Lalel et al., 2005). Extended shelf life of mangoes by properly selected atmospheres was the result of slowing the ripening processes. Controlled atmosphere storage (10 kPa of CO_2 and 11 kPa of O_2) lengthened strawberry fruit shelf life by maintaining the quality attributes without alteration of consumer acceptance (Almenar et al., 2006). The sensory evaluation revealed that, litchi was rated good throughout 56 days of storage in controlled atmosphere (Mahajan and Goswami, 2004). A previous experiment showed that postharvest controlled atmosphere storage was effective in delaying firmness changes during tomato ripening, 20% CO_2 or 3% O_2 decreased the rate of ethylene production and fruit softening and also postponed the increase of galactosidase activities (Sozzi et al., 1999).

Fruit surface coating has shown to be beneficial in improving appearance and delaying quality changes. The component of the coating material is a water-soluble polymer (i.e., polysaccharides) and a hydrophobic substance from a group composing of hydrophobic solids and hydrophobic and nonvolatile liquids (i.e., natural waxes) (Thompson, 2003). Banana coated with material consisting of sucrose esters of fatty acids and carboxymethylcellulose demonstrated the retardation of ripening (Bank,

1984). It was the result of the restriction of gas exchange between fruit and atmosphere causing higher level of CO_2 and lower level of O_2. This was similar to the beneficial effect of controlled atmosphere (Thompson, 2003). Peach treated with chitosan was fimer than control fruit and also had higher titratable acidity and ascorbic acid content (Li and Yu 2001). The study of Jiang and Li (2001) suggested that chitasan coating prolonged longan storage life and maintain quality. Moreover, a sucrose fatty acid ester called Biofresh was applied to apples (1% Biofresh for 20 sec and cold storage for 3 month). The results revealed that the treatment could delay firmness and weight loss, retain titratable acidity and decrease ethylene production and respiration rate (Xuan and Streif, 2000).

A variety of chemicals is applied to fruits after harvest to prevent ethylene action. 1-Methylcyclopropene (1-MCP) is a competitive inhibitor of ethylene responses by blocking the receptor (Sisler and Serek, 1997). There were many reports in its efficacy to extend shelf life and retard fruit softening. The shelf life and softening of banana fruit was delayed after treatment with 1-MCP (Jiang et al., 1999). Peel color change, respiration, and ethylene production were also affected by this treatment. The application of 1-MCP inhibited the increase of PG activity in avocado for up to 12 days (Jeong et al., 2002). 1-MCP had shown the ability to reduce the activities of fruit softening enzymes (PME, cellulase exo-PG and endo-PG) during ripening of plum (Khan and Singh, 2007). In addition, diazocyclopentadiene and 2,5 norbornadiene were found to control the ripening and softening of apple fruits (Blankenship and Sisler, 1993). Cellulase which was abundant during peach ripening, was suppressed after 2,5 norbornadiene application (Bonghi et al., 1998). However, these compounds are commercially unacceptable because of toxicity.

Irradiation may affect the postharvest life of various fruits. Tomato treated with 3.7 kJ m^{-2} UV-C (beneficial doses) was firmer than non-treated fruit and showed decreasing in PG, β-Gal, PME and xylanase activity (Barka et al., 2000). The softening rate of papaya fruit was slowed down by treatment with γ-irradiation (500 Gy) (D'Innocenzo and Lajolo, 2001). However, the abnormal textural changes could developed in fruits after irradiation treatment depending on the species, dose applied, and exposure time (Sozzi, 2004).

7. Heat treatment

Heat treatments were originally used for insect disinfestation and disease control however there has been growing interest in the use of heat treatments to modify fruit responses to stresses and maintain fruit quality instead of chemical treatments which are potentially harmful to humans and environments. Heat treatments, non-damaging physical treatments were also found to have impact on ripening and softening in numerous fruits (Paull and Chen, 2000).

Heat treatments can be applied to produces in several ways including hot water dips, hot water brushing, vapor heat, and hot air (Lurie, 1998). Due to the difference in produces' sensitivity to high temperature among species and cultivars, the suitable method, temperature, and length of exposure time were needed to achieve desired effect without causing damages.

The response of fruits to heat treatment depends on several factors including species and cultivars, fruit size and morphological characteristics, physiological ages and field-induced thermotolerance (Paull and Chen, 2000).

7.1 Heat treatment and fruit ripening

The heat induced inhibition of fruit ripening may be a consequence of its effect on ethylene, ripening phytohormone (Lurie, 1998). Yu et al. (1980) stated that the conversion of ACC to ethylene is highly susceptible to temperature over 30 $^{\circ}$C (Paull and Chen, 2000). The activity of ACC oxidase was rapidly lost, 90 %, in apple fruit treated in 38 $^{\circ}$C air which is consistent with the ethylene inhibition by heat treatment (Klein, 1989). The accumulation of ACC in heated apple was higher than that in unheated fruit indicating that ACC oxidase was inhibited by heating at 38 $^{\circ}$C more than ACC synthase. ACC synthase was also affect by heat but still, less sensitive to heat than ACC oxidase (Lurie, 1998). Loss of ACC synthase activity was slower compared to ACC oxidase in apple and kiwi fruit (Roh et al., 1995; Antunes and Sfakiotakis, 2000). The disruption of ethylene synthesis produced by heat was

mediated by direct inhibition of enzyme activity and reduction of new enzyme synthesis (Lurie, 2008). The production of endogenous ethylene was not only inhibited during heat treatment but response to exogenous ethylene was also affected. Pear, tomato, and banana showed no response to exogenous ethylene during heating period (Maxie et al., 1974; Seymour et al., 1987; Yang et al., 1990) suggesting that loss of sensitivity was correlated with inactivation of ethylene receptors or inability of signal transfer (Lurie, 1998).

It had been found that heat treatment inhibited degreening in banana (Blackbourn et al., 1989). The inhibition of degreening was due to the absence of chlorophyll oxidase leading to the retention of chlorophyll in banana peel. The study in tomato found the inhibition of lycopene synthesis during hot air treatment at 38 $^{\circ}$C or higher (Cheng et al., 1988). This appeared to be due to the inhibited transcription of mRNA for lycopene synthase and the inhibition recovered after transferring to ambient temperature (Lurie et al., 1996). An accelerated rate of degreening was observed in apple, plantain, and tomato following treatment at 35-40 $^{\circ}$C for 4 days (Seymour et al., 1987; Lurie and Klein, 1990, 1991) while the rate was delayed in tomato heated at 40 $^{\circ}$C for 2 days (Sozzi et al., 1996). The response to heat treatment in tomato was different suggesting that threshold duration had been exceeded. Besides, the temperature above 30 $^{\circ}$C could inhibit the synthesis of carotenoid (Paull and Chen, 2000).

Heat treatment had effect on flavor characteristics of fruits. Heating in 38 $^{\circ}$C for 4 days caused a reduced titratable acidity in apple fruit but had no effect on soluble solids content (Klein and Lurie, 1990) leading to a higher sugar to acid ratio. The finding of Lay-Yee and Rose (1994) showed that the titratable acidity declined in nectarines heated at 41-46 $^{\circ}$C for 24-48 hours. Similar result was reported in heated strawberry (Garcia et al., 1995). Loss of titratable acidity and soluble solid concentration was delayed in mei fruit subjected to heat treatment (47, 50 and 53 $^{\circ}$C for 3 min) (Luo, 2006). Volatile production was also affected by exposure to high temperature. The production of apple volatile was immediately inhibited after hot air treatment at 38 $^{\circ}$C and recovered slowly thereafter (Fallik et al., 1997). Volatile flavor level and profile in tomato fruit was changed, with two components increasing

and five decreasing, out of the 15 analyzed by heat treatments ranging from 39-48 ^{o}C for 1 hour (McDonald et al., 1996, 1999).

7.2 Heat treatment and fruit softening

The softening in many fruits can be delayed by a chronic exposure to temperature ranging from 30-46 ^{o}C for some days or an acute exposure to higher temperature up to 50 ^{o}C for short times (minutes or hours) (Sozzi, 2004). The firmness of cherry fruit was maintained after exposure to 70 ^{o}C before freezing (Alonso et al., 1993). Firmness of 'Golden Delicious' apple was maintained during storage after exposure to 38 ^{o}C for 4 days (Sam et al., 1993), and cell wall studies found that heated apple had less soluble pectin and more insoluble pectin as compare to untreated control, indicating the disruption of pectin modification (Ben-Shalom et al., 1996). Tomato heated for 96 hours at 40 ^{o}C had lower soluble pectin content (Mitcham and McDonald, 1992) and loss of galactose and arabinose residues from tomato cell wall was also affected by this treatment. Strawberry fruit treated with hot air at 45 ^{o}C for 3 hours remained firmer than unheated fruit, and the treatment delayed pectin solubilization and hemicelluloses degradation (Vicente et al., 2002, 2005).

It is hypothesized that heat treatment could prevent cell wall modification by inhibiting cell wall degrading enzymes. The application of heat treatment up to 45 ^{o}C reduced activities of cell wall degrading enzymes and consequently, delayed fruit softening (Paull and Chen, 2000). The inhibition of middle lamella dissolution and cell wall disruption of papaya was observed after treating with hot water at 49 ^{o}C for 120 min, and the activities of PG and xylanase were also reduced (Bacay-Roldan and Serrano, 2005). Tomato held at 33 ^{o}C showed the suppressed activity of PME (Ogura et al., 1975). Nevertheless, little difference in the degree of pectin esterification was reported in apple fruit after exposure to 38 ^{o}C for 4 days (Klein et al., 1995). Loss of endo-mannase and galactosidase activity was found in tomato fruit heated at 40°C for 2 days (Sozzi et al., 1996). The PG activity extracted from heated mango (38 ^{o}C for 3 days) rose 2-3 fold during storage at 25 ^{o}C

for 8 days whereas unheated fruit showed increased activity by almost 8 fold (Ketsa et al., 1998). In the same study, the disruption of PME activity was detected following heat treatment and also, the partial inhibition of β-Gal activity was immediately observed after treatment but the activity rose rapidly when heated fruit was returned to 25°C (Ketsa et al., 1998). The decreased activity of enzymes related to pectin degradation (PG and β-Gal) and hemicellulose metabolism (endoglucanase and β-xylosidase) was detected following heating at 45 $^{\circ}$C for 3 hours, while PME activity increased (Vicente et al., 2005). According to Luo (2006) regarding study in mei fruit (*Prunus mume*), exposure to hot water for 3 min in the range 47-53 $^{\circ}$C slowed down the increase in PME and PG activity. In addition, heat treatments led to a modification of gene expression involved in cell wall disassembly. Hot air treatment at 45 $^{\circ}$C for 3 h caused the reduction in the expression of genes encoding PG, endoglucanse and β-xylosidase in strawberry fruits during the first hour after treatment, which limited their enzyme activities and delayed fruit softening (Martinez and Civello, 2008).

The disruption of softening could be ascribed to reduction of cell wall degrading enzymes. Even though inhibition of cell wall modification by heat had been considered to be the cause of fruit softening delaying, the exact enzyme playing an important role in softening process had not been determined (Lashbrook et al., 1998; Rose et al., 1998). Effect of heat treatment in preventing fruit softening may occur from the combination of various causes including disruption of the enzymes associated in ethylene production, inactivation or loss of ethylene receptors, inhibition of signal transfer that lead to cell wall enzyme synthesis, inhibition of the synthesis and stability of mRNA or the synthesis and degradation of protein and disruption of enzyme export to the apoplast (Paull and Chen, 2000; Sozzi, 2004).

However, application of heat treatment can cause damage on fruits both external and internal. External damages include peel blackening and browning, skin scald and increasing in decay development. Internal damages produced by heat treatment result in abnormal softening and color development, formation of internal cavities, failure of starch degradation and flesh darkening (Lurie, 1998).

CHAPTER III

MATERIALS AND METHODS

I. MATERIALS

1. Plant materials

Hands of banana fruits (*Musa acuminata*, AAA group, Gros Michel subgroup, cultivar 'Hom Thong') were obtained from a local farm in Pathumtani province at the mature green stage and transferred immediately to the laboratory within 2 h, where fruits with uniform size and color and lack of noticeable defects were selected.

2. Instruments

2.1 Equipments for hot water treatment and storage

- Digital water bath (DAIHAN LABTECH CO., LTD)

- Thermometer

- Cardboard corrugated box

- Phytotron room

2.2 Equipments for sample collecting

- Knife and cutting board

- Aluminium foil

- Balance

- Liquid nitrogen

- Deep freezer -80 °C

2.3 Equipment for firmness measurement

- Penetrometer (Hardness tester FHM-1, Takemura, Japan)

2.4 Equipments for cell wall extraction and analysis

- Homogenizer

- Glass funnel and filter paper

- Digital water bath (DAIHAN LABTECH CO., LTD)

- Incubator (memmert, BE600)

- Spectrophotometer (G1103A, Agilent technologies, Germany)

- Hotplate stirrer

- Vortex mixer

- Glass pipette and micropipette

- Shaker

- Test tube

2.5 Equipments for enzyme extraction and activity assay

- Homogenizer

- Centrifuge (Universal 32R Hettich, Germany)

- Digital water bath (DAIHAN LABTECH CO., LTD)

- Hot plate

- Test tube

- Spatula

- Glass cylinder

- Micropipette

- Vortex mixer

2.6 Equipments for RNA extraction

- Mortars and pestles

- Liquid nitrogen

- Spatula

- Digital water bath (DAIHAN LABTECH CO., LTD)

- Centrifuge (Universal 32R Hettich, Germany)

- Eppendorf tubes

- Spectrophotometer (NanoDrop Technologies, Wilmington, DE)

- Micropipette

- Vortex mixture

2.7 Equipments for quantitative PCR

- iQ5 Thermocycler (Bio-Rad, Hercules, CA)

- 96-well PCR plate

- Multichannel pipette

2.8 Equipments for anatomy observation

- Blade and microtome blade

- Multipurpose Microscope (Olympus BX-51)

- Vacuum pump

- Rotary microtome (MICROM, HM 340E)

- Microscope slide and cover glass

- Nail polish

- Forcep, paint brush and needle

- Paraffin oven

- Aluminium foil

3. Chemicals and reagents

3.1 Chemicals for cell wall extraction and analysis

- Absolute ethanol

- Distilled water

- 0.05 M Sodium acetate

- 0.04 M Ethylenediaminetetraacetic acid (EDTA)

- 0.05 M Hydrochloric acid

- 4 M Sodium hydroxide

- 72% (w/w) Sulfuric acid

- *m*-Hydroxydiphenyl

- Galacturonic acid

- Anthrone reagent

- Glucose

3.2 Chemicals for enzyme extraction

- 0.02 M Sodium phosphate buffer

- 0.02 M Ethylenediaminetetraacetic acid (EDTA)

- 1% Triton x-100

- 0.02 M Cysteine-HCl

- 1mM Phenylmethylsulfonyl fluoride (PMSF)

3.3 Chemicals for PG activity assay

- 1% Polygalacturonic acid

- 50 mM Sodium acetate

- 0.2 M Sodium acetate buffer

- DNS reagent (see in Appendix A)

- Galacturonic acid

3.4 Chemicals for PL activity assay

- 0.3 % Polygalacturonic acid

- 20 mM Sodium acetate

- DNS reagent (see in Appendix A)

- Galacturonic acid

- Distilled water

3.5 Chemicals for β-Gal activity assay

- 13 mM 2-Nitrophenyl-β-D-galactopyranoside

- 0.1 % (w/v) Bovine serum albumin

- 0.1 M Sodium citrate

- 0.2 M Sodium carbonate

- 2-Nitrophenol

3.6 Chemicals for PME activity assay

- 0.5 % (w/v) Citrus pectin

- 0.01% (w/v) Bromothymol blue

- 0.003 M Potassium phosphate buffer

- Distilled water

3.7 Chemicals for Glu activity assay

- 2% (w/v) Laminarin

- 50 mM Potassium acetate buffer

- DNS reagent (see in Appendix A)

- Distilled water

- Glucose

3.8 Chemicals for RNA extraction

- Liquid nitrogen

- 1M Tris-HCl pH 8.2

- NaCl

- 0.5 M EDTA

- CTAB

- 2-Mercaptoethanol

- Saturated phenol pH 4.3 (Fisher Co., USA)

- Chloroform: Isoamyl alcohol (24:1) (v/v)

- Absolute ethanol

- 10 M LiCl

- Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich Co., USA)

- 3M Sodium acetate pH 5.2

3.9 Chemicals for anatomy observation

- Formalin-aceto-alcohol (FAA)

- Absolute ethanol

- 95% Ethanol

- Paraffin

- Distilled water

- n-Butyl alcohol

- Eosin

- Paraffin oil

- Xylene

- Petroleum ether

- Safranin O

- Fast green

II. METHODS

1. Hot water treatment

Hands of mature banana fruits (*Musa acuminata*, AAA group, Gros Michel subgroup, cultivar 'Hom Thong') were cut into fingers and classified into two groups. The control group was dipped in water at room temperature for 10 min, and the treated group was dipped in hot water at 50 ^{o}C for 10 min. After treatment, all fruits were air-dried and kept in corrugated boxes. Thereafter, the fruits were allowed to ripen at 25±1 ^{o}C. Peel tissue samples were taken from control and treated fruit every 2 days, frozen in liquid nitrogen and stored at -80 ^{o}C until used for enzyme and RNA extraction.

2. Banana firmness determination

Peel firmness was determined using penetrometer (Hardness tester FHM-1, Takemura, Japan). Each fruit was measured at three different regions of peel including the blossom end, middle and stem end. Firmness readings were recorded as kilogram-force and were converted to Newtons (N). The average of the three readings was taken as a measure of firmness for individual fruit.

3. Cell wall analysis

3.1 Isolation of cell wall polysaccharide

Cell wall polysaccharides were isolated according to Rosli et al. (2004) with some modifications. Approximately 10 g of frozen peel was homogenized with 40 ml of absolute ethanol and then boiled for 30 min with shaking to inactivate cell wall degrading enzymes. The homogenate was filtered and the residue was washed three times with 15 ml of absolute ethanol. Then, the residue was incubated overnight at 37 $^{\circ}$C to evaporate solvent and weighed. The dried residue or alcohol insoluble residue (AIR) was used to extract cell wall fractions.

3.2. Extraction and quantification of cell wall fractions

AIR was extracted by sequential chemical that allow its separation to obtain several cell wall fractions. Extraction of cell wall fractions was carried out according to the method described by Rosli et al. (2004) and Figueroa et al. (2010) with slight modifications. A 100 mg aliquot of AIR was suspended in 100 ml of water and shaken overnight at 20 °C. The homogenate was filtered and washed three times with 10 ml of water. The filtrates were pooled and collected as water-soluble pectin fraction (WSP). Then, water-insoluble residue was shaken in 100 ml of 0.05 M sodium acetate (pH 4.5) containing 0.04 M EDTA for 4 h at 20 °C. The homogenate was filtered and washed three times with 10 ml of same buffer. The filtrates were pooled and collected as EDTA-soluble pectin fraction (ESP). The residue was resuspended in 100 ml of 0.05M HCl and heated at 100 °C with stirring for 1 h. The homogenate was filtered after cooling to room temperature and then washed three times with 7 ml of 0.05M HCl. The filtrates were pooled and collected as HCl-soluble pectin fraction (HSP). The remaining residue from pectin extraction was extracted in 100 ml of 4M NaOH and shaken for 8 h at 20 °C. The homogenate was filtered and washed three times with 5 ml of 4M NaOH. The filtrates were pooled and collected as hemicellulose. Finally, the last residue was hydrolyzed in 12 ml of 72% (w/w) H_2SO_4 and shaken for 1 h at 30 °C and then diluted to 4% (w/w) H_2SO_4 (d'Amour et al., 1993).

Uronic acid concentration in WSP, ESP and HSP were quantified by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) using galacturonic acid as standard. Hemicellulose and cellulose concentration were quantified by the anthrone method (Nigam and Ayyagari, 2007) using glucose as standard.

4. Enzyme extraction and activity assay

Banana peel was homogenized in 0.02 M sodium phosphate buffer pH 7.0, 0.02 M EDTA, 1% triton X-100, 0.02 M cysteine-HCl and 1 mM phenylmethylsulfonyl fluoride (PMSF). Then, homogenate was centrifuged at 15,000 x g for 30 min at 4 ^{o}C. Clear supernatant was used for enzyme activity analysis (Lohani et al., 2004).

4.1 Polygalacturonase (PG)

PG activity was determined according to the modified method of Pathak and Sanwall (1998). Five hundred μl of enzyme extract was mixed with 300 μl of 1% polygalacturonic acid (PGA, 50 mM sodium acetate pH 4.5) and 200 μl of 0.2 M sodium acetate buffer pH 4.5 in a total volume of 1 ml and then incubated at 37 ^{o}C for 1 h. To measure the amount of reducing sugar released, 0.5 ml of dinitrosalicylic acid (DNS) was added to reaction mixture (Miller, 1959). After the reaction mixture was boiled for 5 min and cooled to room temperature, absorbance was measured at wavelength of 520 nm (G1103A Spectrophotometer, Agilent technologies, Germany). One unit of PG activity was expressed as nmol of galacturonic acid per min per mg protein.

4.2 Pectate lyase (PL)

PL activity was assayed by measuring the increase of reducing sugar released from PGA (Miller, 1959). The activity was analyzed in a reaction mixture containing 600 μl of 0.3 % PGA (20 mM sodium acetate pH 4.5), 200 μl of 20 mM sodium acetate pH 4.5 and 100 μl of enzyme extract, brought up to a volume of 1 ml

with distilled water. Following incubation at 37 ^{o}C for 1 h, 0.5 ml of DNS was added and the mixture was heated in boiling water for 5 min to stop the reaction. The absorbance was measured at 520 nm. One unit of specific activity was calculated as nmol of galacturonic acid released per min per mg protein.

4.3 β-Galactosidase (β-Gal)

β-Gal activity was determined using a modified version of the method described by Ali et al., 1995. One hundred μl of enzyme extract was added to 400 μl of 13 mM 2-nitrophenyl-β-D-galactopyranoside, 400 μl of 0.1 % (w/v) bovine serum albumin and 500 μl of 0.1 M of sodium citrate pH 4.1 and mixed thoroughly. After incubation for 15 min at 37 ^{o}C, the reaction was stopped by adding 2 ml of 0.2 M sodium carbonate. The amount of released 2-nitrophenol was measured at a wavelength of 415 nm. One unit of β-Gal activity was expressed as nmol of 2-nitrophenol released per min per mg protein.

4.4 Pectin methylesterase (PME)

The activity of PME was analyzed following the spectrophotometric method of Hangerman and Austin (1986). The enzyme assay mixture consisted of 2 ml of 0.5 % (w/v) citrus pectin, 200 μl of 0.01% (w/v) of bromothymol blue (0.003 M potassium phosphate buffer pH 7.5), 300 μl of water and 500 μl of enzyme extract. The reaction was initiated by adding enzyme. The absorbance was measured immediately at a wavelength of 620 nm. One unit of enzyme was expressed as nmol of methyl ester hydrolyzed per min per mg protein.

4.5 Glucanase (Glu)

The assay of Glu activity was performed based on the methods of Choudhury et al. (2009). The assay was carried out in a mixture containing 100 μl of 2% (w/v) laminarin, 800 μl of 50 mM potassium acetate buffer (pH 4.8) and 100 μl of enzyme extract. The reaction mixture was incubated at 50 ^{o}C for 1 h. DNS was added to the mixture, and subsequently, the reaction was stopped by boiling for 5 min. The reducing groups released from laminarin were spectophotometrically measured at

520 nm. One unit of Glu activity was defined as nmol of glucose per min per mg protein.

In all assays, denatured enzyme was used as control and prepared by boiling for 10 min. The concentration of protein was estimated by the Lowry method (1951) using bovine serum albumin as a standard.

5. RNA isolation and gene expression

Musa acuminata genes related to cell wall modification were identified using key word searches of the GenBank™ database. Genes encoding polygalacturonase (*MaPG*; GenBank #EU269469), pectate lyase (*MaPL*; GenBank #AF206319), pectin methylesterase (*MaPME*; GenBank #FJ264505), beta-1,3-glucanase (*MaGLU*; GenBank #AF001523), and beta-galactosidase (*MaGAL*; GenBank #AJ585757) were identified for gene expression analysis. Transcript abundance was quantified using RT-qPCR analysis. Total RNA was isolated from hot water treated and control banana peels according to the procedure described by Asif et al. (2000). RNA was quantified by a spectrophotometer (NanoDrop Technologies, Wilmington, DE) and quality was assessed on a 1% agarose gel.

After RQ1 RNase-free DNase treatment (Promega, Madison, WI) to remove contaminating genomic DNA, the first strand of cDNA was synthesized from 2 µg of total RNA using the Omniscript Reverse Transcriptase kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Quantitative PCR was carried out on the iQ5 Thermocycler (Bio-Rad, Hercules, CA) with iQ SYBR Green Master Mix (Bio-Rad, Hercules, CA). One µl of cDNA was used as templates in a 20 µl reaction volume. Reactions were performed in triplicate. The oligonucleotide primers were designed from coding sequences of the banana genes using IDT Primer Quest (Table 1). A *Musa acuminata* actin gene was also identified from GenBank (*MaACT*; GenBank #AF246288). RT-qPCR confirmed that *MaACT* was constitutively expressed in banana peel from all treatments. Melt curves were generated to check amplification specificity of the primers, and relative target gene expression was normalized to

MaACT expression for each cDNA sample. Amplicons were sequenced to confirm the identity of the products.

Table 1 Sequences of specific primer for real-time RT-PCR

Gene	Name	Primer sequence
PG	*MaPG-F*	5'-CGGATGAGCAATGTTTCCAACCCA-3'
	MaPG-R	5'-ACATGGAGAACTGTCGCTGCAAGA-3'
PL	*MaPL-F*	5'-AAGACCTGGTTCAGAGGATGCCAA-3'
	MaPL-R	5'-TGGCTGTTTATAGTGGGAGCAGCA-3'
β-Gal	*MaGAL-F*	5'-TGTGCAAGCAAGATGATGCACCTG-3'
	MaGAL-R	5'-CTGATAACCTGCACGGCAAAGCAA-3'
PME	*MaPME-F*	5'-TGTCCAATGTGTCAAAGCCAGTGC-3'
	MaPME-R	5'-TGGAATGCAAATCCGGAATGGTGG-3'
Glu	*MaGLU-F*	5'-TTGCCTTGCTGGTTTCAGTCCTTG-3'
	MaGLU-R	5'-TCTCATCCTCGCGATGTTGTTGGA-3'
Actin	*MaACT-F*	5'-TGTAGGTGATGAGGCCCAATCCAA-3'
	MaACT-R	5'-ATCTTCTCCCTGTTCGCTTTGGGA-3'

6. Peel anatomy observation

Small pieces (5x5 mm) of banana peel were cut from the mid zone and fixed in formalin-aceto-alcohol (FAA). Then, the fixed tissue was dehydrated in ethanol dehydration series and embedded in paraffin wax according to the method of Ruzin (1999). The paraffin embedded tissues were sectioned to a thickness of approximately 10 μm using microtome and attached to microscope slides. The sections were stained with safranin O and fast green and then, the structural changes of banana peels of hot water and control treatment were observed under light microscope.

The tissue sections were chosen randomly for evaluation. In selected sections, epidermal cell, parenchyma cell, fiber and vascular tissues were evaluated as being collapsed cell or normal cell. A collapsed cell was defined as a cell with collapsed cell walls and large intercellular space. In addition, peel thickness, number of layer and percentages of air space per view (2x2mm tissues of section) were averaged to obtain the mean values for each tissue section.

7. Experimental design and statistical analysis

The experiment was performed by means of Completely Randomized Design (CRD) with 4 replicates. Data was subjected to statistical analysis using the independent sample *t-test* at a significant level of 0.05 using SPSS software version 14. The Pearson correlation test was conducted between firmness and cell wall degrading enzyme activities.

CHAPTER IV

RESULTS AND DISCUSSION

1. Changes in peel firmness of 'Hom Thong' banana fruit affected by hot water treatment.

During ripening, firmness of banana fruits declined slowly and continuously, resulting in softening. The firmness of 'Hom Thong' banana peel decreased throughout fruit development in both heated and non-heated fruit. Firmness decreased slowly during the first 4 days, with larger decreases observed at the later ripening stages (days 6 to 10). Firmness changes of hot water treated fruit followed the same trend as control fruit, but peel firmness of the hot water treated fruit was maintained at a higher level throughout the evaluation (0 to 10 days post treatment). Significant difference in firmness between heated and non-heated fruit was observed after 6 days of storage at 25 ^{o}C (Figure 4.1). The application of hot water was effective at preventing softening of 'Hom Thong' banana as reported previously in mei (*Prunus mume* 'Daqinghe') fruit, where immersion in water at 47-53 ^{o}C for 3 min caused enhanced firmness (Luo, 2006). In strawberry, the effect of heat treatment on fruit softening exhibited cultivar-related difference. Firmness of 'Chandler' strawberry treated with hot air was similar in untreated fruit (Yoshikawa et al., 1992), while hot air treated 'Selva' strawberry and hot water treated 'Tudla' strawberry exhibited delayed softening in comparison with untreated fruit (Garcia et al., 1995; Civello et al., 1997; Vicente et al., 2002).
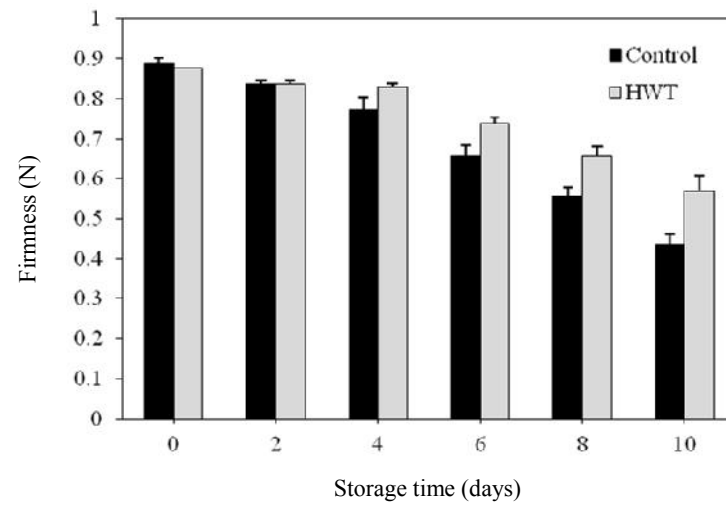
Figure 4.1 Changes in peel firmness of control and hot water treated (50 ^{o}C, 10 min) banana fruits during the storage of fruits at 25 ^{o}C.   Data represents the mean ± standard error.

Firmness change that occured in banana peel was coincident with visual appearance. Regarding peel color change, the ripening of 'Hom Thong' banana fruit started on day 4 of storage at 25 $^{\circ}$C (Figure 4.2). This was consistent with peel firmness that decreased rapidly on day 4. Treatment with hot water could delay peel color change from green to yellow of banana fruit. After 4 days of storage, treated fruit stayed at stage 4 of ripening (30% green peel, 70% yellow peel) while ripening stage of control fruit more proceeded (Figure 4.2). This result was supported by the early study in 'Hom Thong' banana that change in peel color of hot water treated fruit at 50 $^{\circ}$C was slower as compared to control fruit after the measurement of L value and hue value (Naunkamol Amnuaysin, 2007).

The inhibition of fruit ripening including softening in response to heat treatment is due to a combination of different causes. One of them is the disruption of ripening hormone, ethylene. Previous observation demonstrated that exposure 'Hom Thong' banana to 50 $^{\circ}$C hot water resulted in a decrease rate of ethylene production as well as a delay in appearance of ethylene climacteric peak (Naunkamol Amnuaysin, 2007). This indicated that heat-induced softening inhibition found in this study might be mediated by its effect on ethylene production.

Figure 4.2 Changes in control and hot water treated (HWT) banana fruits during the storage of fruits at 25 ^{o}C for 10 days. Hot water treatment at 50 ^{o}C for 10 min was applied to the treated group.

2. Changes in cell wall composition of 'Hom Thong' banana fruit following hot water treatment.

It is assumed that changes in fruit texture are consequences of modification in cell wall compositions which lead to disassembly of the middle lamella and primary cell wall structure. Since delay of banana softening was observed in response to 50 ^{o}C hot water, alteration in cell wall components of banana fruit was focused as a result of heat application.

Cell wall materials were sequentially extracted with water, EDTA, and HCl to obtain loosely both ionically and covalently-bound pectin fraction, respectively whereas NaOH and H_2SO_4 were used to solubilize hemicellulose and cellulose fraction, respectively. WSP content increased throughout the ripening period of banana. The content of WSP increased gradually at early stage of ripening, followed by marked increase. After heat treatment, the content of water-soluble fraction in control did not differ from that in hot water treated fruits, but treated fruits showed a significantly lower WSP level than control fruits thereafter (Figure 4.3), indicating that HWT led to a decrease in pectin solubilization. This observation was supported by Woolf et al. (1997) finding in persimmon where heat treatment resulted in delayed pectin solubilization and in a study where hot water treated strawberry also resulted in a similar decrease in soluble pectin and an increase in insoluble pectin (Lara et al., 2006).

ESP, attached to wall by ionic calcium bounds, is thought to be partially homogalacturonan from the middle lamella (Brummell et al., 2004). Moderated changes in ESP level were observed during fruit development (Figure 4.4). ESP remained at low level. HWT did not affect change of ESP content in 'Hom Thong' banana, and thus contrasting with previous study in strawberry fruit that found higher level of chelating soluble fraction after heat treatment (Lara et al., 2006).

HCl removed covalently bound pectin rich in branched rhamnogalacturonan I. HSP gradually increased and showed a maximum level on day 6 in control fruits and on day 8 in treated fruits, and after that, the fraction decreased (Figure 4.5). HSP content taken from hot water treated fruits showed higher value compare to control fruits. HWT could retain a high amount of HSP in 'Hom Thong' banana. Previously, heat treatment (45 ^{o}C, 3 h) was observed to decrease the reduction of HSP level in strawberry fruit (Vicente et al., 2005). The decline of covalently bound pectin level was accompanied by a rapid increase of WSP. This could indicate that the increased content of WSP during ripening is derived from covalently bound pectin that is released and becomes soluble (Figueroa et al., 2010).

For hemicelluloses change, hemicellulose amount increased initially and then decreased as ripening progress in both control and treated fruits (Figure 4.6). This is consistent with a early study on 'Brazil' banana where hemicelluloses yield decreased during fruit ripening (Cheng et al., 2009). However, loss of hemicellulose in banana fruit heated by hot water seemed to be slower than unheated fruit, but no significant difference was observed except day 8. In strawberry, application of 45 ^{o}C air for 3 h delayed hemicellulose solubilization; and heat treated fruits maintained a higher level of hemicelluloses (Vicente et al., 2005).

Cellulose is a major component that forms the primary and secondary cell walls of plants. In banana fruit, the cellulose level declined gradually throughout storage at 25 ^{o}C (Figure 4.7). The reduction of cellulose started on day 2. The decreased cellulose content may be due to an increase in cellulase activity. Prabha and Bhagyalashmi (1998) reported that cellulose level decreased during ripening of banana fruit which was accompanied by an increase in cellulase activity. However, change in cellulose amount was slight due to the crystalline structure of cellulose which is difficult to degrade by enzymes or chemicals. The chemical analysis of cellulose in ripening tomato and pear showed that cellulose content remained constant or even slightly increased. This did not support the dissolution of cell wall microfibril network that was suggested to result from cellulase activity. It could be explained by incomplete solubilization in cell wall cellulose by cellulase activity or modification of noncellulosic matrix leading to loss of microfibril organization. In this experiment,

no difference in cellulose level was observed between control and hot water treated fruits after treatment. Thereafter, cellulose content in hot water treated fruits showed a higher level compare to untreated fruits, but there was no significant difference except day 10.

These results indicate that exposure to 50 ^{o}C hot water delayed solubilization of some polysaccharide components which may be due to disrupting activity of enzymes related to cell wall degradation. Vicente et al. (2005) found that the activities of polygalacturonase, β-galactosidase, β-xylosidase, and glucanase in strawberry fruit were reduced after heat treatment and consequently affected the solubilization of pectin and hemicelluloses.

Figure 4.3 Changes in water-soluble pectin content of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.

Figure 4.4 Changes in EDTA-soluble pectin content of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.

Figure 4.5 Changes in HCl-soluble pectin content of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.
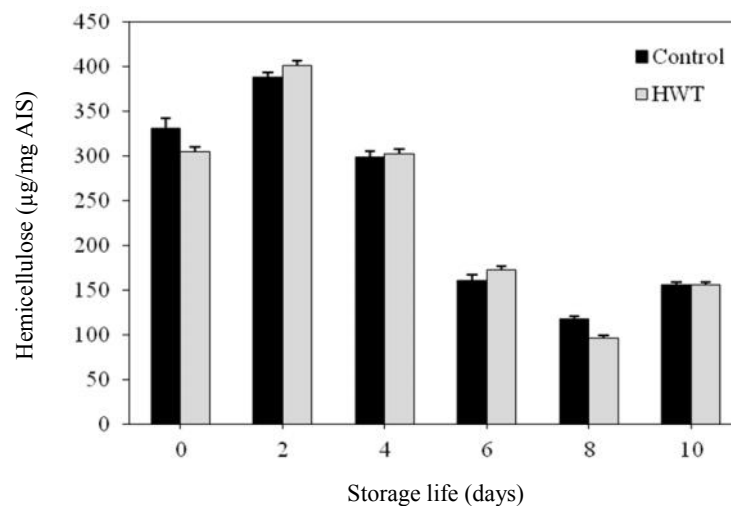
Figure 4.6 Changes in hemicellulose content of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.

Figure 4.7 Changes in cellulose content of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.

3. Changes in activities and gene expression of enzymes associated with cell wall modification in 'Hom Thong' banana fruit following hot water treatment.

Cell wall hydrolases are the main factors contributing to cell wall disassembly by decreasing cell-to-cell adhesion and cell dispersion at various stages of fruit development (Brummell, 2006). These processes, combined with the loss of pectic side chains, increase wall porosity, which later in ripening may allow increased access of degradative enzymes to their substrates.

3.1 Polygalacturonase (PG)

PG changes during fruit ripening and softening have been studied in many fruits. PG is a pectin degrading enzyme that catalyzes the hydrolysis of the glycosidic bonds of polygalacturonic acid. The integrity of the middle lamella, which controls intercellular connections, is affected by PG activity and thereby influences fruit texture (Wei et al., 2010). PG activity in banana peel increased continuously throughout ripening, slightly during the first 4 days and rapidly thereafter (Figure 4.8). This increase demonstrated significant negative correlation with firmness (r = -0.963**). A similar change in PG activity was observed after exposure to hot water. PG activity was not consistently lower in HWT peels, but was lower at 2 and 8 days of storage. The expression of *MaPG1* showed a general decline in both control and HWT peels from 6 to 10 days of storage (Figure 4.9). Similar to the trend observed with PG activity, relative expression of *MaPG1* was lower in HWT peels at both 2 and 8 days compared to control peels. The peaks in *MaPG1* mRNA in both control and treated fruits corresponded with the initial loss of firmness.

In this study, the increase in PG activity was not reflected in the expression patterns of the *MaPG1* gene. Four isoforms of PG have been described in banana fruit (Asif and Nath, 2005). Mbeguie-A-Mbeguie et al. (2009) reported that these four genes (*MaPG1 to MaPG4*) were differentially regulated during banana ripening. *MaPG1* and *MaPG2* are 98% identical at the nucleotide level, and the primers that we designed will amplify both of these genes (both the forward and reverse primers are 100% identical to both *MaPG1* and *MaPG2*). The increase in

mRNA abundance mid stage, with a decrease at the later stages of ripening, is similar to the gene expression patterns of *MaPG1* and *MaPG2* previously described in banana peels during fruit ripening (Mbeguie-A-Mbeguie et al., 2009). *MaPG3* and *MaPG4* share 90% nucleotide identity. *MaPG4* is the most highly expressed of the four genes in the peel, and both *MaPG3* and *MaPG4* show a steady increase in mRNA abundance during ripening.

It has been reported that PG activity is responsible for solubilization and depolymerization of pectins during ripening. The content of water-soluble pectins decreased in fruit that had suppressed PG activity, paralleled to increased content of sodium carbonate-soluble pectins, indicating that PG depolymerizes covalently bound pectins and converts them into the water soluble form (Carrington et al., 1993). In this study the reduced PG activity in response to HWT might cause lower pectin solubilization and depolymerization and contribute to delayed fruit softening during storage.
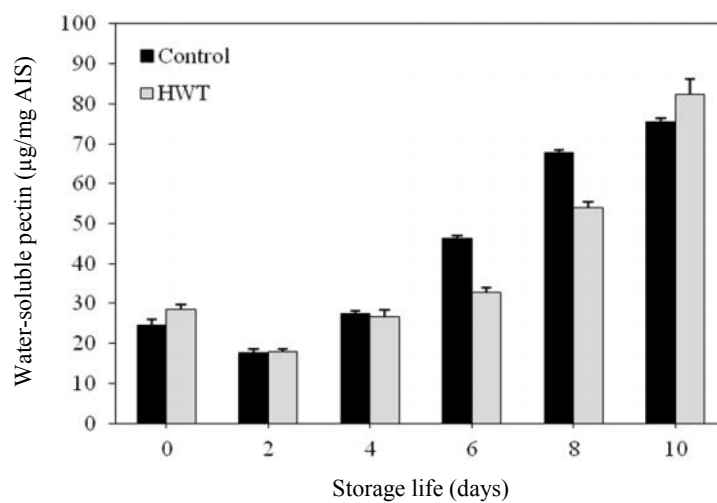
Figure 4.8 Changes in polygalacturonase activity of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 $^{\circ}$C. Data represents the mean ± standard error.
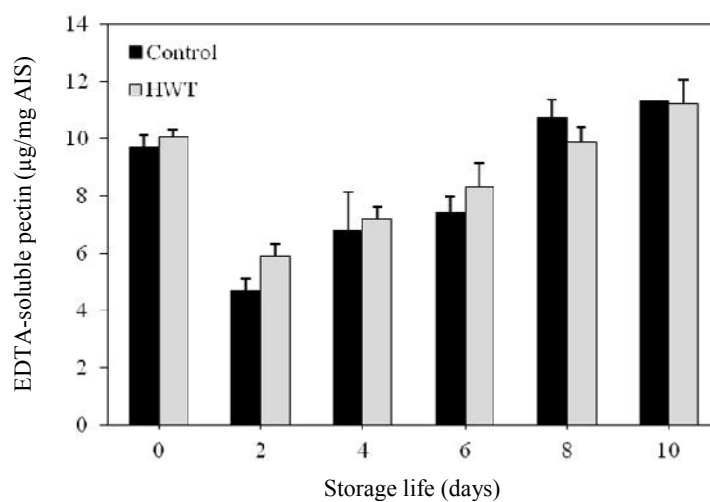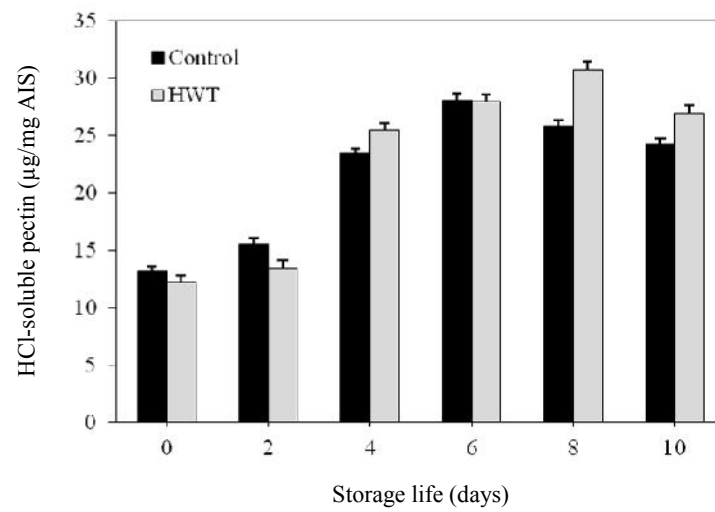

Figure 4.9 Changes in relative *MaPG* expression of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 $^{\circ}$C. Data represents the mean ± standard error.

3.2 Pectate lyase (PL)

Pectin degradation is also catalyzed by PL, which cleaves α-1,4 linkages between galacturonosyl residues via the β-elimination reaction. An increase in PL activity was detected in both control and hot water treated banana during the ripening period (Figure 4.10). The activity increased steadily until the overripe stage as has been previously reported (Lohani et al., 2004). With a correlation coefficient of -0.985**, the activity showed significant negative correlation with firmness, indicating that this increase was closely related to fruit softening. Treatment with hot water before storage at 25 $^{\circ}$C did not immediately reduce PL activity, but reduced activity was detected after 2 days of storage. At the late ripening stage, PL activity in HWT treated peels was the same as or higher than control peels. The expression of *MaPL1* followed the same trend as PL activity, increasing throughout the ripening period (Figure 4.11). Transcript levels were very low at the early stage of ripening and increasing when fruit softening began. This is similar to the expression pattern of *MWPL1* (same as *MaPL1*) and *MWPL2* observed in peels of ripening 'Williams' bananas (Pua et al., 2001). Except for day 4, *MaPL1* mRNA abundance was greater in non-heated fruit.

Fruit ripening corresponded with increased PL activity and gene expression. Low levels of PL activity and gene expression at the early stage and subsequent increases at the later stage suggested that PL could be associated with pectin degradation in the peel during ripening. This finding was consistent with previous studies in peach (Downs et al., 1992), avocado (Huber and O'Donoghue, 1993) and strawberry fruits (Martinez and Civello, 2008). Moreover, transgenic strawberry fruits with suppressed PL gene expression exhibited significant increases in their firmness (Jiménez-Bermúdez et al., 2002). PL activity appears to have an important role in fruit softening, and HWT could increase the storage quality of bananas by reducing the gene expression and activity of pectate lyase.

Figure 4.10 Changes in pectate lyase activity of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.
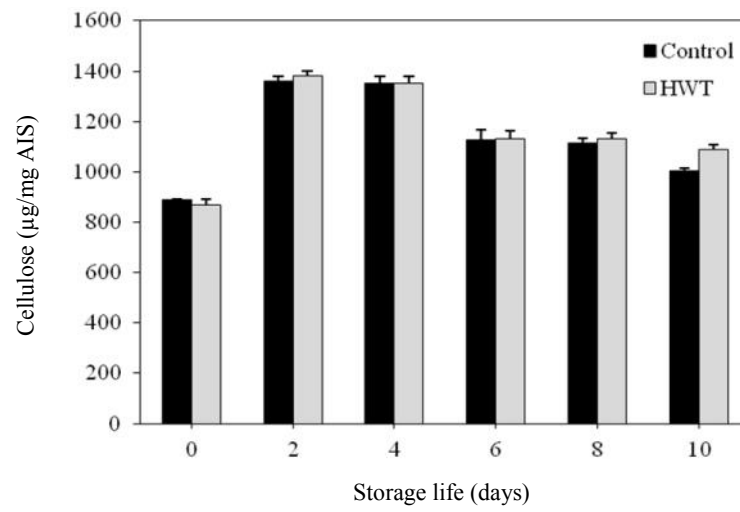

Figure 4.11 Changes in relative *MaPL* expression of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.

3.3 β-Galactosidase (β-Gal)

One of the important changes that occurs during fruit ripening is the loss of cell wall galactose that is present in side chains of rhamnogalacturonan I (RG I) attached to rhamnose residues. This is catalyzed by β-Gal, which hydrolyzes β-1,4 galactan bonds, and removes galactosyl residues from pectin side chains. β-Gal has been studied during ripening in several fruits. In 'Hom Thong' banana, β-Gal activity changed slightly during the early stage of ripening and increased during the later stages of ripening (Figure 4.12). The change in enzyme level correlated both significantly and negatively with firmness (r = -0.896*). HWT caused a reduction in β-Gal activity for up to 4 days, after which time the activity for both treatments was the same. Gene expression of *MaGAL* increased throughout the ripening process (Figure 4.13). Increases in *MaGAL* transcript abundance slightly preceded the increase in enzyme activity, with maximum expression detected at 8 and 10 days in both control and hot water treated peels. HWT did not affect the accumulation of *MaGAL* mRNA in early ripening, but difference in *MaGAL* mRNA abundance between control and HWT was detected after 4 days of storage.

The increase in activity and mRNA accumulation of β-Gal during ripening coincided with loss of firmness. The similar result was previously reported by Zhuang et al. (2006). Exposure to hot water delayed the softening of banana fruit and inhibited β-Gal activity and the expression of the β-gal gene, *MaGAL*. Activity and gene expression of β-Gal in strawberry fruit were similarly inhibited by heat treatment (Martinez and Civello, 2008). It has been proposed that de-galactosidation from the action of β-Gal is a key process in cell wall modification during fruit ripening (Smith and Gross, 2000). The loss of galactose in side chains of rhamnogalacturonans could diminish the interactions between polysaccharide chains and lead to increased pectin solubility (Smith et al., 1998). β-Gal activity increased 3-7 fold in both the pulp and the peel during banana fruit ripening and the largest levels of activity were associated with decreased fruit firmness (Zhuang et al., 2006). Decreased levels of β-Gal in banana peel following HWT of the whole banana could result in delayed softening via pectin modification.
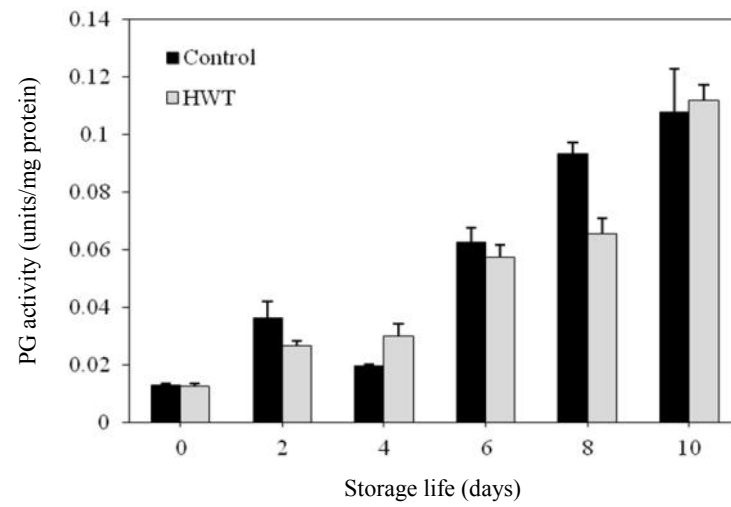
Figure 4.12 Changes in β-galactosidase activity of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.
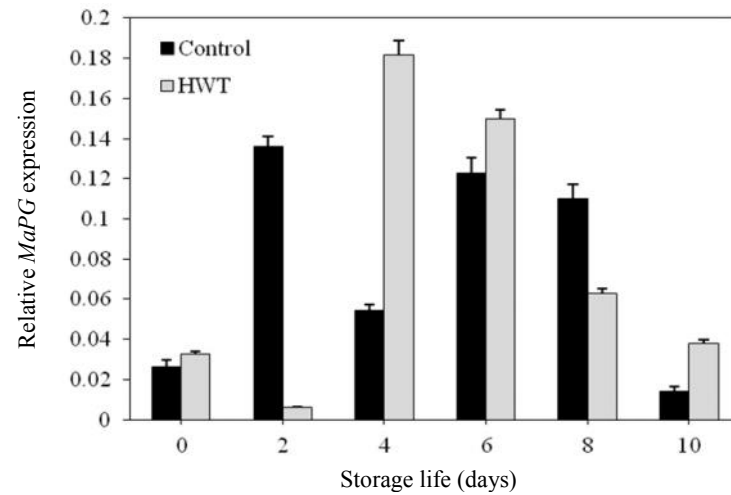

Figure 4.13 Changes in relative *MaGAL* expression of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.

3.4 Pectin methylesterase (PME)

PME catalyzes the hydrolysis of methylester groups from galacturonosyl residues. Demethylation of pectin to free carboxyl groups affects the changes of pH and charge in the cell wall that leads to aggregation of pectin into gel structure linked by calcium, providing a suitable substrate for PG-mediated hydrolysis (Brummell and Harpster, 2001).

PME activity remained constant until day 6, and then diminished slightly in late storage (Figure 4.14). This change was significant and positively correlated with firmness (r = 0.831*). Prestorage heat treatment reduced PME activity immediately after application. Subsequently, PME activity in HWT peels increased and remained at the same level as control fruit for the remaining storage period except on day 10. This result agrees with previous observation in apples that found no difference in PME activity between control and heat treated fruits (Klein et al., 1995). The expression of *MaPME1* decreased slightly during the beginning of storage, with an increase detected at 8 days (Figure 4.15). *MaPME1* transcript abundance was higher in HWT peels compared to controls at 2 and 6 days.

Mbeguie-A-Mbeguie et al. (2009) reported a gene expression pattern for *MaPME1* that was very similar to the pattern of enzyme activity presented in this experiment. While there was some expression in the peel at harvest time, gene expression was low and constant during ripening. This group also reported the cloning of two additional PME genes in banana (*MaPME2* and *MaPME3*) that were differentially expressed in peel during ripening. Although, increases in PME enzyme activity have been reported during ripening of several fruits, suppression of PME activity in transgenic tomato did not affect fruit firmness despite changes in pectin metabolism (Tieman et al., 1992). The present result revealed that banana softening was also not correlated well with PME activity.
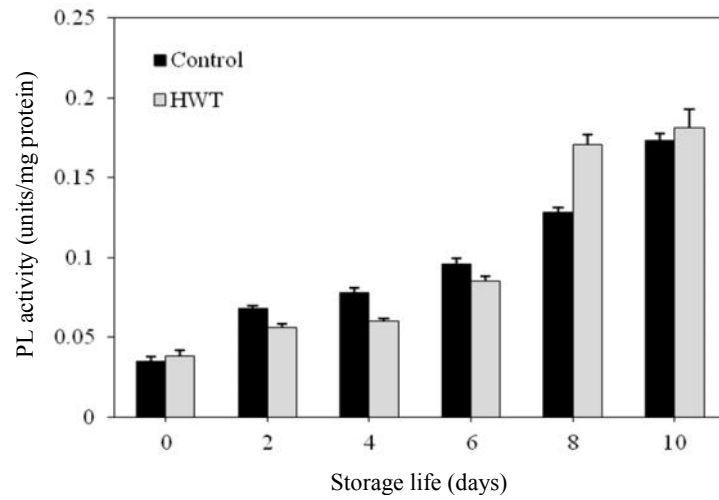
Figure 4.14 Changes in pectin methylesterase activity of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.
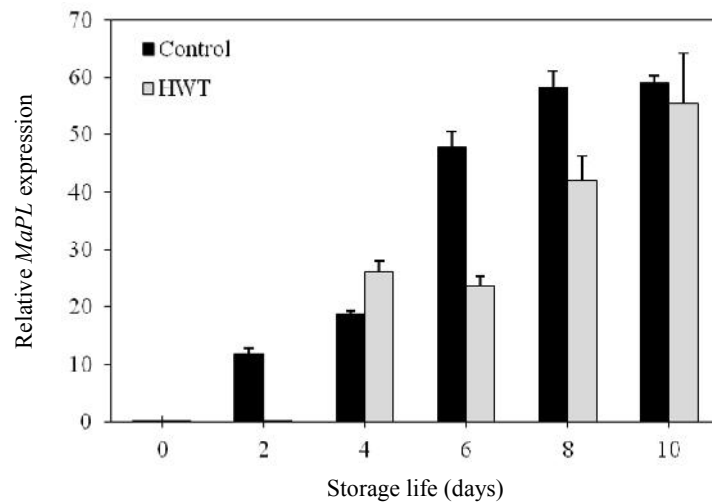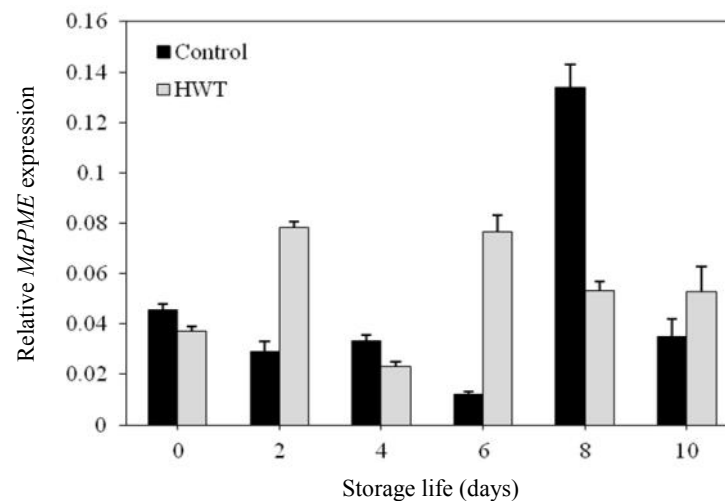

Figure 4.15 Changes in relative *MaPME* expression of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.

3.5 β-1,3 Glucanase (Glu)

Glu catalyzes the hydrolysis of β-1,3 glucosidic linkages in β-1,3 glucans. β-1,3 Glucan is a major structural component of many pathogenic fungi cell walls and also a cell wall constituent of certain cell types in specific plant developmental stages such as pollen tubes and seeds.. The activity of Glu in control fruit rose rapidly in the early stage of ripening and continued to increase throughout the storage period except for a decrease that was observed on day 6 (Figure 4.16). The correlation between Glu activity and firmness was not significant (r = -0.764). Change in Glu activity in treated fruit was similar to untreated fruit. HWT had little effect on Glu level except significant difference between control and treated fruit on day 4 and 8. The transcript abundance of *MaGLU* increased as ripening progressed and reached a maximum on day 8 in both control and treated fruits (Figure 4.17). In HWT fruit, however, the increase in transcript abundance was delayed and began to increase after 4 day of storage. Consequently the maximum expression level was significantly lower than that of unheated, control fruit.

The involvement of Glu in fruit ripening and softening has been proposed. The accumulation of a gene encoding Glu was observed during banana ripening (Clendennen and May, 1997). Studies at both the molecular and biochemical level revealed a possible role of Glu in the ripening process of banana fruit (Peumans et al., 2000; Kesari et al., 2007). Although, Glu is usually present in cell walls in only small proportions; Choudhury et al. (2010) reported the enhanced expression of Glu gene in the ripe peel and pulp tissue of banana which clearly indicated that Glu is ripening regulated. As like banana, in strawberry fruit, two Glu genes were found to be induced during ripening (Shi et al., 2006) thus fruit softening can be influenced by the degradation of Glu. In this study, an increase in Glu activity and gene expression accompanied fruit ripening, but there was no significant correlation with firmness. In contrast, observation of Choudhury et al. (2009) demonstrated that the expression of Glu in banana pulp correlated with enzyme activity and fruit softening rate in 'Rasthali', 'Kanthali' and 'Monthan' bananas. These differences may be explained by differences in softening associated modification between cultivars or differences between pulp and peel.
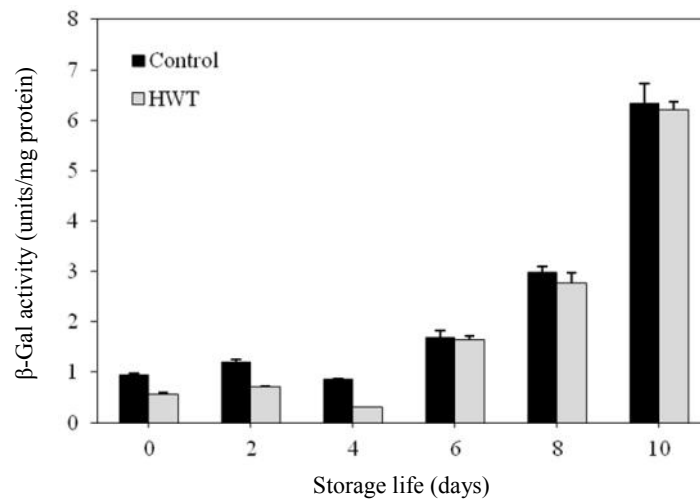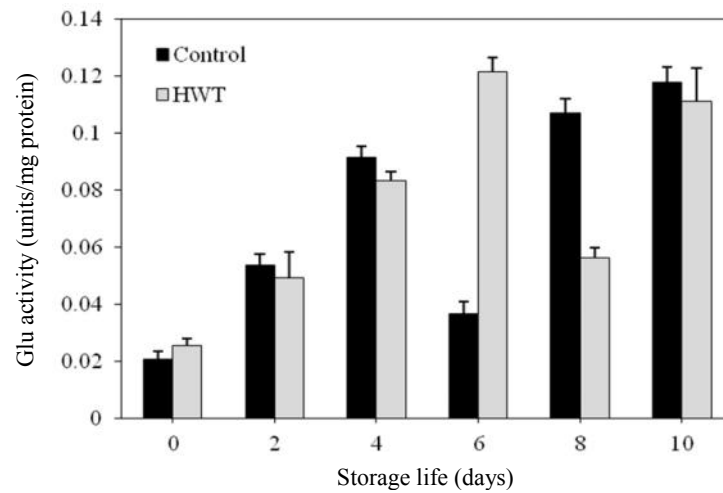
Figure 4.16 Changes in β-1,3 glucanase activity of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.
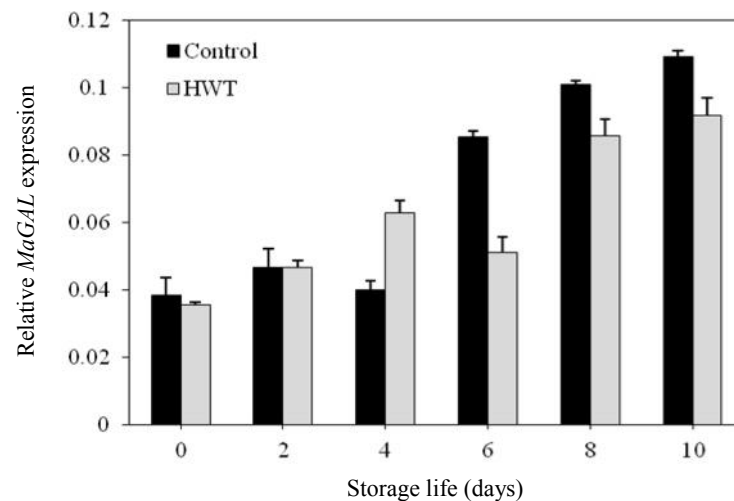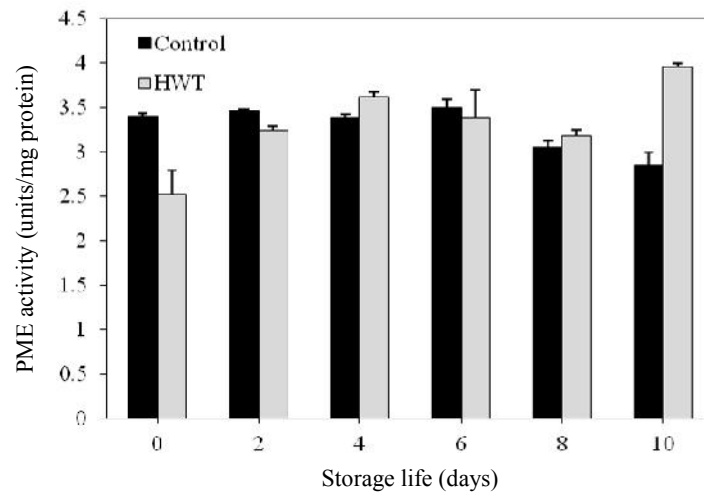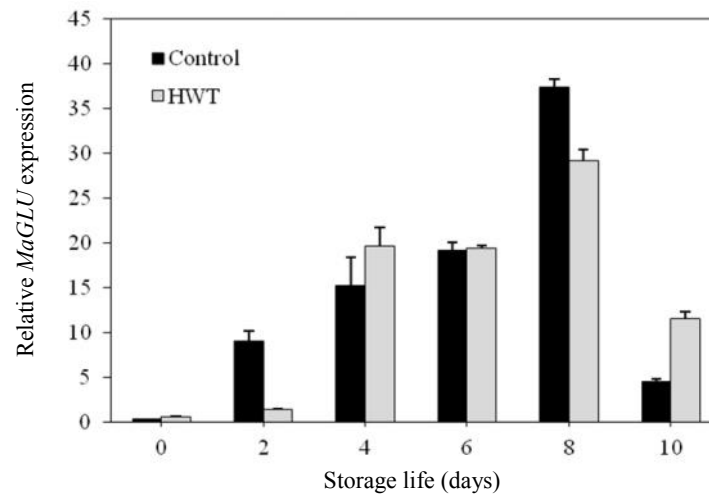

Figure 4.17 Changes in relative *MaGLU* expression of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.

4. Changes in peel structure of 'Hom Thong' banana fruit affected by hot water treatment.

Anatomical analysis of the peel structure of 'Hom Thong' banana revealed that the peel was composed of three main parts; epidermis, ground tissues, and vascular bundle (Figure 4.18). Epidermis consisted of one layer of small squarish cells without intercellular space (Figure 4.19A). Epidermal cells had thin cell wall and were covered by thin layer of cuticle on the outer surface.

Ground tissue was the main part of banana peel that could be divided into two regions, outer and inner ground tissues. The outer ground tissue consisted of seven to twelve layers of pentagonal to polyhedral parenchyma cells in depth. The arrangement of cells was orderly with small triangular intercellular space. Most of the cells in this zone were found to contain chloroplasts with chlorophylls that provide a green coloration to banana peel. In addition, the inner ground tissue of the peel contained loose parenchyma consisting of isodiametric cells which were less orderly arranged as compared to the outer zone. The formation of air spaces was also observed in the inner ground tissue.

The other part that was found to be distributed throughout the ground tissue was the vascular bundles. They comprised of phloem which was arranged adjacent to the xylem inside (Figure 4.19B). Phloem was made up of small rectangular to pentagonal cells with a thin cell wall, whereas, xylem contained vessels that were large circular cells with thick secondary cell wall. Fiber was located above the phloem and had a polyhedral shape in transverse section (Figure 4.19B). The outer vascular bundles seemed to be more fibrous than the inward vascular bundles, but vascular elements of inward bundles were more prominent.

Microscopic observation of the transverse section of 'Hom Thong' banana revealed an alteration in cellular structure of banana peel during the ripening period. The peel thickness decreased continuously as ripening progressed. The peel of 'Hom Thong' banana was thick ranging from 2.0-3.5 mm. After exposure to hot water at 50 $^{\circ}$C for 10 min, the peel thickness of treated fruits did not differ from control fruits, but

a significant difference in peel thickness was observed on day 4, 6 and 10 of storage at 25 $^{\circ}$C (Figure 4.20). The decline in number of cell layers was found in both control and hot water treated bananas during ripening. However, from day 4, the treated fruits incubated at 25 $^{\circ}$C showed a significantly higher number of cell layers when compared to control fruits from day 4 during incubation at (Figure 4.21). The reduction of peel thickness and cell layer number could be explained by modification occurring in cell wall and the middle lamella during ripening that lead to changes of cell wall structure where the wall of some cells also collapsed, while some cells had joined with other cells. However, the decrease in peel thickness and number of cell layer was slowed down after application of hot water at 50 $^{\circ}$C for 10 min. This incident corresponded to firmness loss that was delayed by HWT.

The epidermis remained to be one layer throughout fruit development. A similar result was reported in 'Poovan' banana (Santhakumari and Krishnamurthy, 1991). In ground tissue, changes in cells became more apparent on day 4 after storage at 25 $^{\circ}$C. There was separation of parenchyma cells in ground tissue which led to increases in air spaces. The size and number of air spaces increased continuously as banana fruit further ripen as previously reported in several fruits including banana (Prabha and Bhagyalakshmi, 1998). The formation of air spaces frequently occurred in inner ground tissue. The arrangement and shape of cells started to change, and the damage of cells were also observed in this zone. In the outer zone, there was no change in arrangement and shape of cells in the early stage, but these changes including air space formation began in the later stage of ripening and included air space formation. The formation of air space suggested that polysaccharide modification in the middle lamella and the cell wall was induced by increased activity of cell wall degrading enzymes which resulted in the damaged cell wall and cell separation, thus forming larger spaces. The common development of air spaces is result of separation of adjacent primary cell wall via the middle lamella. The formation begins at the junction of three or more cells and continues to other parts of cell wall (Evert, 2006). During the air space formation, polymer network that join plant cells are disassembled. The polysaccharide modification in the middle lamella and the cell wall was induced by increased activity of cell wall degrading enzymes

which resulted in the weakening of the middle lamella and cell wall and reduction of adhesion between cells, thus forming larger spaces. The measurement of air space area in peel indicated that the formation of air space increased throughout fruit development. The air space formation was higher in control fruit than in hot water treated fruit but there was no significant difference between control and treated fruits except for day 6 of storage (Figure 4.22). This may related to the inhibition of cell wall degrading enzymes induced by heat treatment.

Cells in ground tissue were damaged and collapsed with a prolonged ripening period. At the end of storage, a serious damage of cells was found in the innermost region, close to banana pulp. Damage and change in the arrangement of cells in ground tissue were delayed by the application of hot water. Apparent cell collapse was more severe in control fruit than in treated fruit after storage at 25 ^{o}C for 10 days (Figure 4.23). This result could be explained by an inhibition of polysaccharide component change after hot water application especially pectin fraction which is one of the main component in the middle lamella and primary cell wall and also an important adhesive material between cells. The study of Ratule et al. (2007) in 'Berangan' banana (*Musa* cv. Berangan AAA) demonstrated that banana fruit ripened at 37 ^{o}C retains more integrity of peel cell wall, especially in the middle lamella in comparison to banana fruit ripened at 25 ^{o}C.
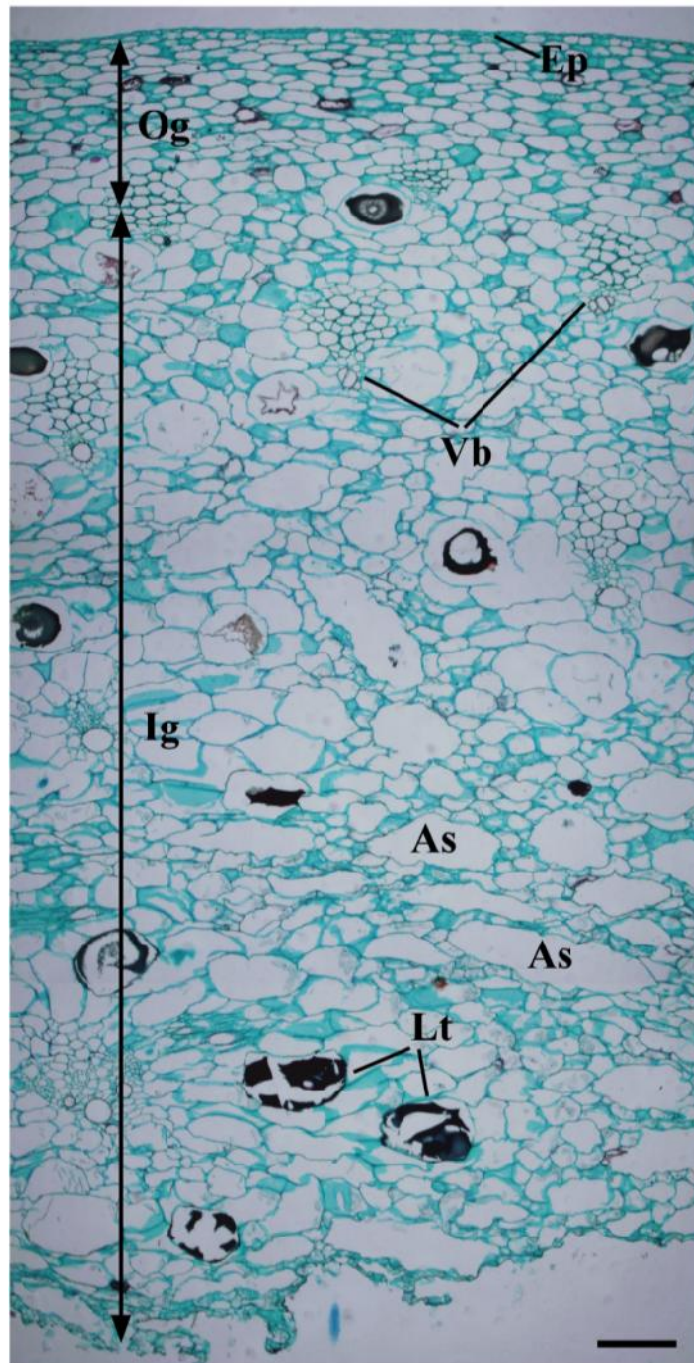
Figure 4.18 Cross section showing peel structure of 'Hom Thong' bananas at mature green stage. Abbreviations: Ep, Epidemis; Og, Outer ground tissue; Ig, Inner ground tissue; Vb, Vascular bundle; As, Air space; Lt, Laticiferous cell. Scale bar = 0.2 mm.
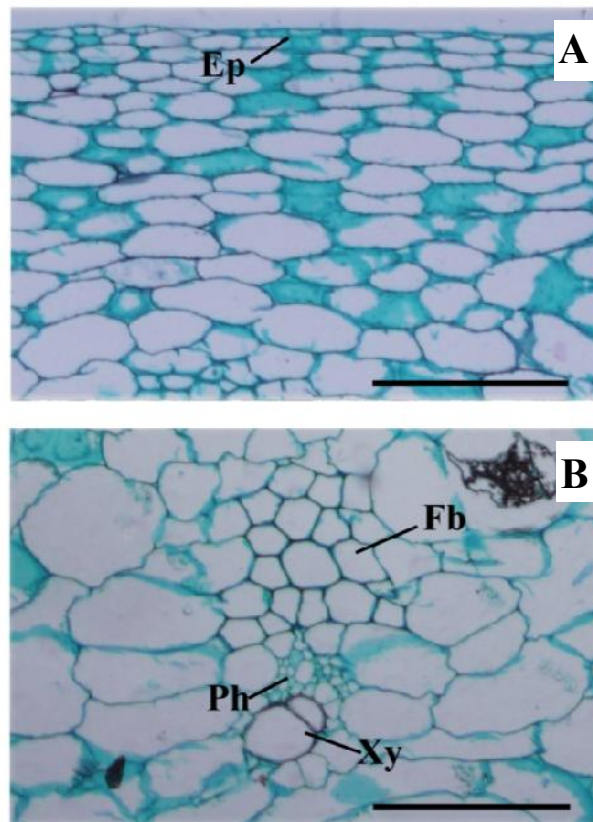
Figure 4.19 Cross section of 'Hom Thong' bananas at mature green stage, Epidermis (A) and Vascular bundle (B). Abbreviations: Ep, Epidermis; Xy, Xylem; Ph, Phloem; Fb, Fiber. Scale bars: A = 0.1 mm; B = 0.05 mm.
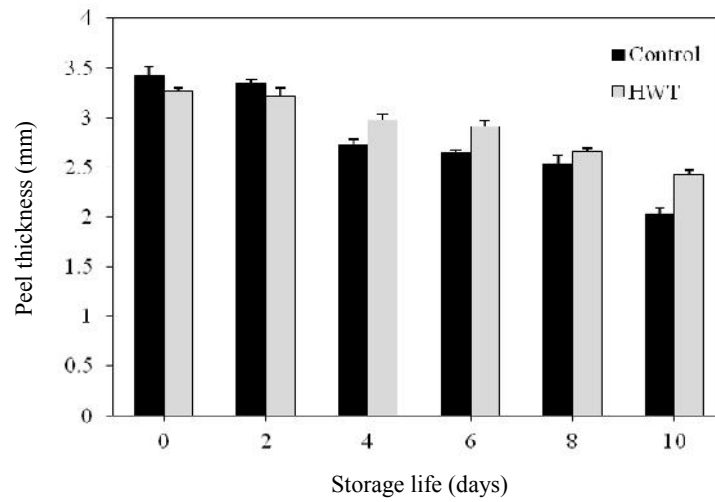
Figure 4.20 Changes in peel thickness of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.
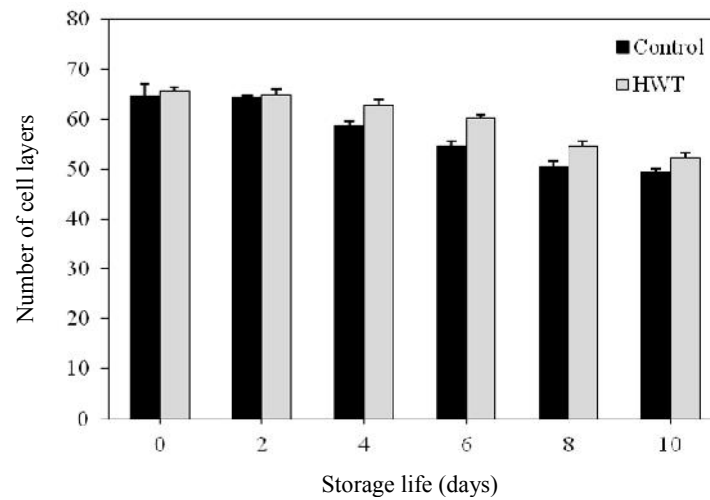
Figure 4.21 Changes in cell layer numbers of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.
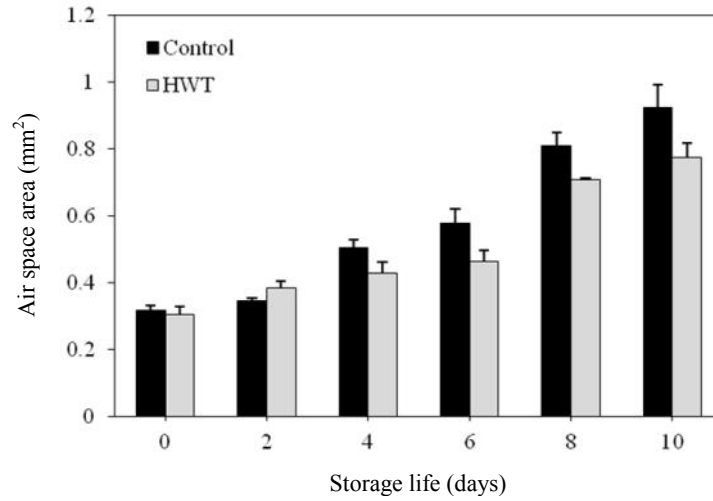
Figure 4.22 Changes in air space area of control and hot water treated (50 °C, 10 min) banana fruits during the storage of fruits at 25 °C. Data represents the mean ± standard error.
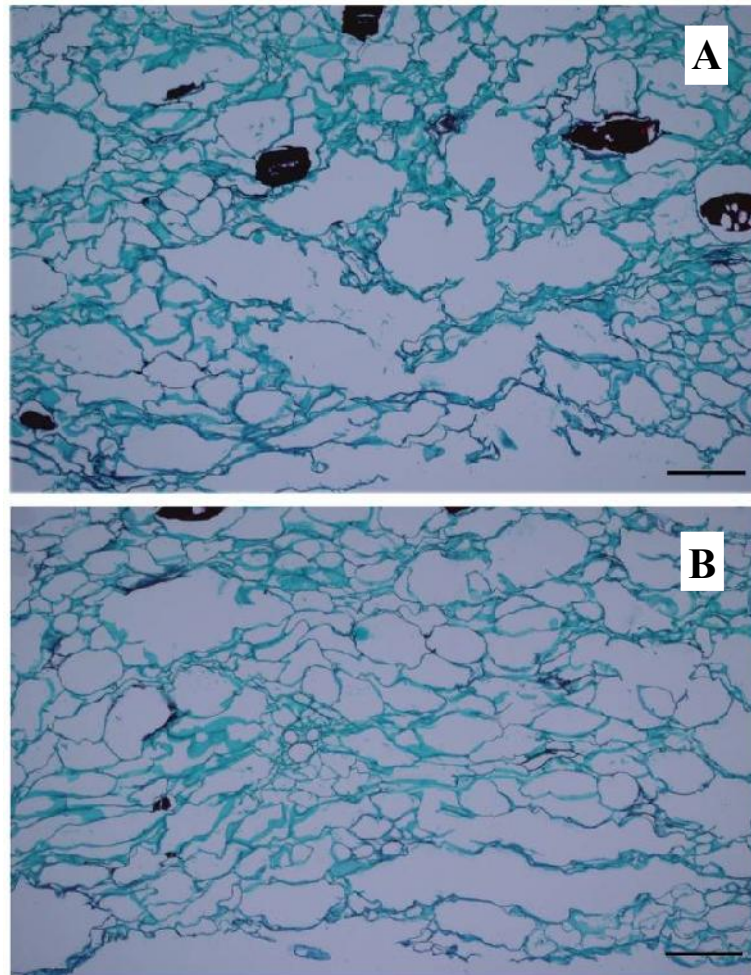
Figure 4.23 Cross section of banana peel showing changes in inner ground tissue of control (A) and hot water treated (50 °C, 10 min) (B) banana fruits during the storage of fruits at 25 °C for 10 days. Scale bar = 0.2 mm.

The results in this study showed the structural and compositional changes in cell wall during 'Hom Thong' banana ripening (Figure 4.24). Pectin that is the main component in cell wall matrix and the middle lamella is modified by action of pectin degrading enzymes including PME, PG and PL. PME removed methyl groups from galacturonic acid that makes polygalacturonic acid susceptible for further degradation by PG and PL leading to pectin solubilization. The solubilization originated from polymer tightly integrated to cell wall or linked to hemicelluloses. Changes in polysaccharide compositions and glycosyl linkage, decreased distribution of molecular mass and increased depolymerization of pectin were also reported during banana ripening (Duan et al., 2008). The modification occurred in pectin polysaccharides contributed to loosening and disintegration of cell wall.

The breakdown of hemicelluloses was observed during ripening of 'Hom Thong' banana. The hemicellulose modification and depolymerization in cell wall were responsible for softening during banana ripening (Cheng et al., 2009). The degradation of hemicellulose polysaccharides that hydrogen bond to microfibrils and cross-link between cellulose microfibrils might partially disrupt the cellulose-hemicellulose network leading to reduction in cell wall rigidity. Loosening of the cellulose-hemicellulose network could also be part of cell wall swelling. The degradation of both pectin and hemicellulose was cooperatively involved in banana fruit softening.

In addition, loss of cell wall galactose that is catalyzed by β-Gal is one of the cell wall compositional changes during ripening. Galactose is present in side chain of RG-I, xyloglucan, glucuronoarabinoxylan, and RG-II. Loss of galactan side chain might increase cell wall porosity and alter cell wall rigidity and intercellular adhesion. The modification in polysaccharide components thus affected cell wall structure resulting in textural change in 'Hom Thong' banana.
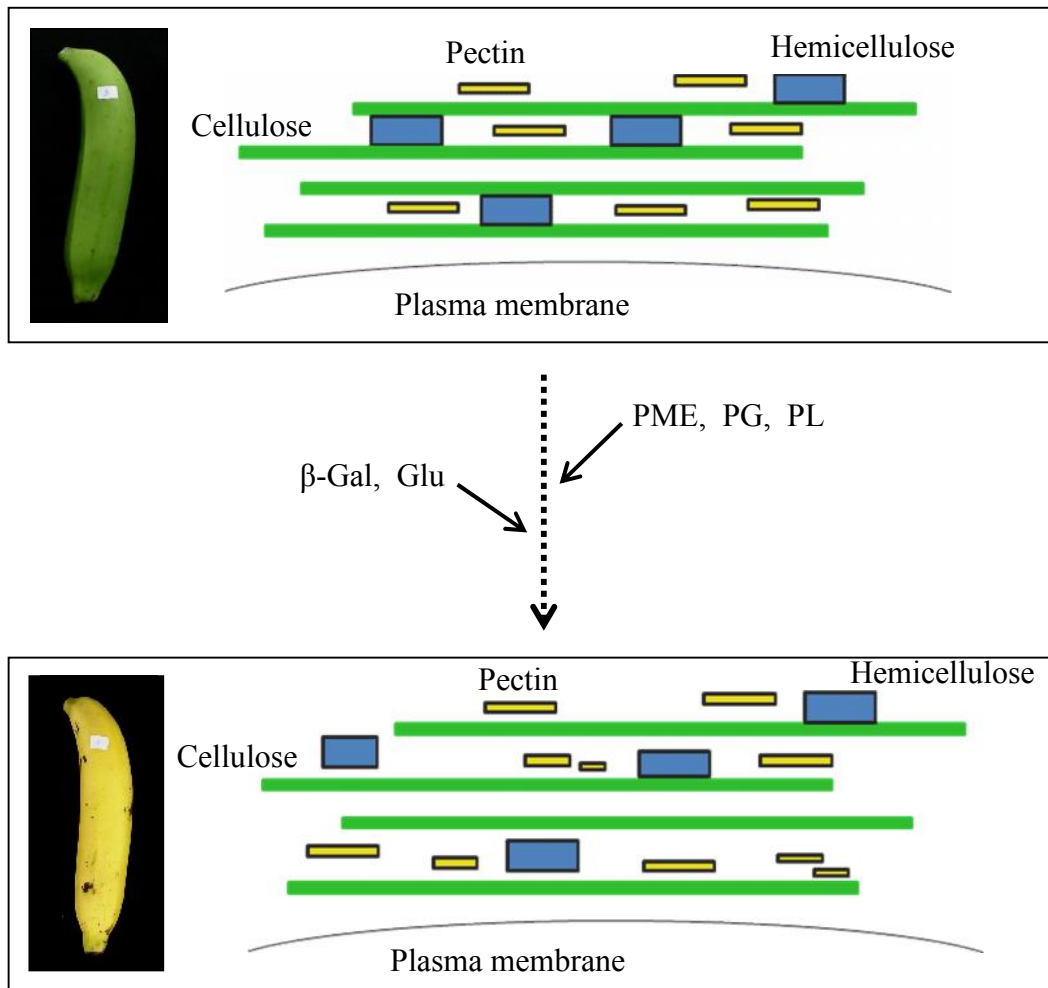
Figure 4.24 Model of cell wall components describing changes in pectin (yellow), hemicelluloses (blue) and cellulose (green) during ripening of 'Hom Thong' banana.

The effect of hot water treatment on softening during banana ripening was described according to the model shown in Figure 4.25. Exposure of 'Hom Thong' banana to hot water at 50 °C for 10 min could reduce gene expression and enzyme activity of PME, PG and PL and also reduced β-gal activity during pre-ripe stage (0-2 days of storage at 25 °C). PME action is a pre-requisite before PG and PL degradation. It is then possible that the reduction in PME activity found in treated fruit could decrease the activity of PG and PL.

When ripening began, decrease in gene expression and enzyme activity of PL and β-gal were detected in treated fruit including Glu activity. Decreased activity of PL and β-gal from ripe stage (4-6 days of storage at 25 °C) and PG, PL and β-gal from pre-ripe stage could cause the delay in conversion of covalently bound pectin into water soluble pectin. Water-insoluble pectin is generally converted to water-soluble form during fruit ripening and this change contributes to loosening and disintegration of cell wall leading to textural changes. Slowing down in structural alteration of heated banana peel observed in this study could be explained by lower pectin solubilization. So, the reduction in peel thickness and number of cell layer and the formation of air space were delayed as compared to unheated banana peel. These resulted in enhanced firmness.

In over-ripe stage (8-10 days of storage at 25 °C), gene expressions and activity of all enzymes were reduced in treated fruit. The reduction in activity of all enzymes except for Glu was responsible for decreased level of water soluble pectin and increased level of HCl soluble pectin. In addition to pectin, increase of cellulose content was observed in this stage. It might be due to reduction in Glu activity. The inhibition of cell wall modification especially pectin fraction produced by HWT could delay cell damage and collapse occurring in ground tissue of banana peel leading to maintenance of 'Hom Thong' banana firmness.
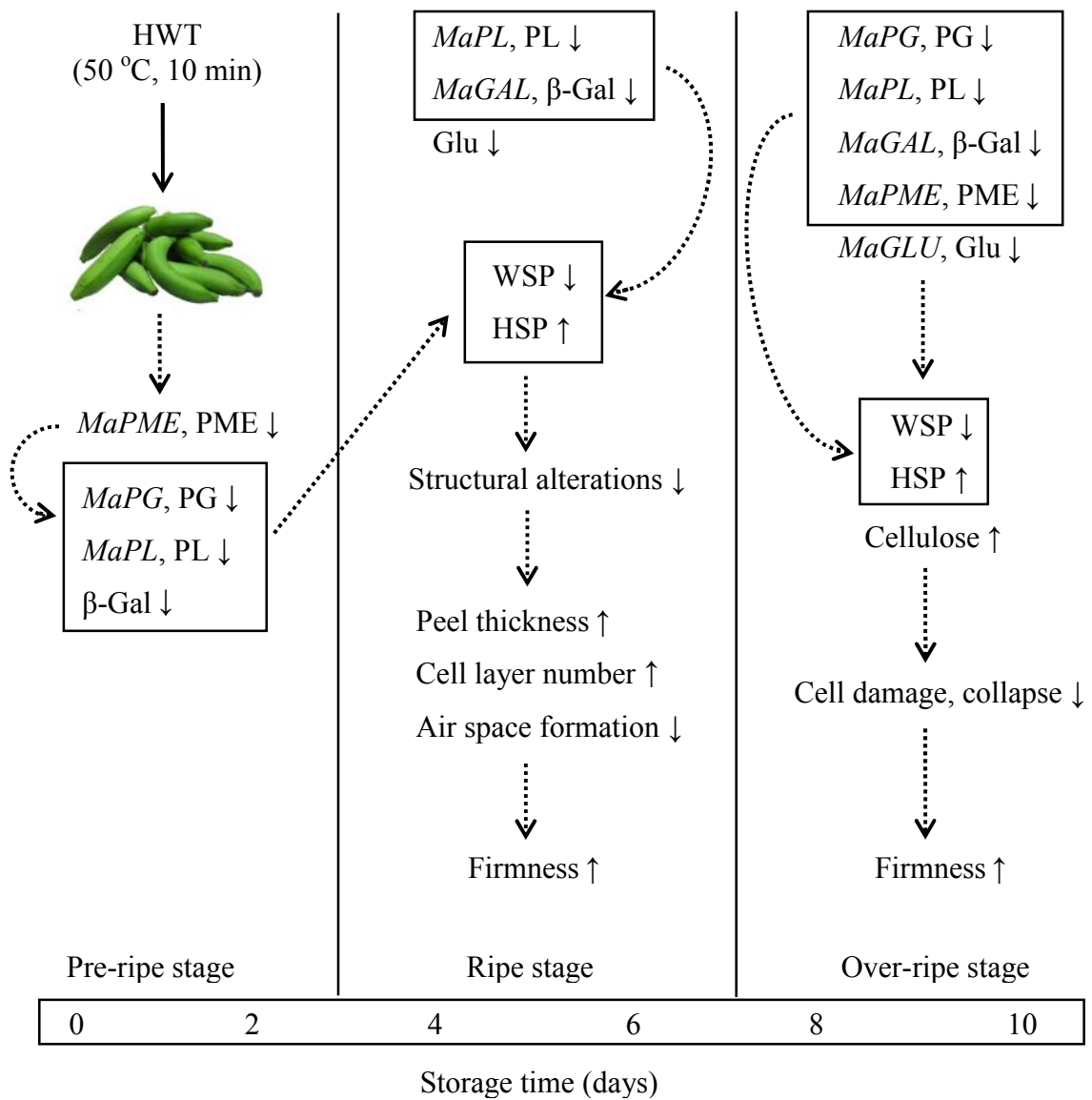
Figure 4.25 Model describing effects of hot water treatment on gene expressions, activity of cell wall enzymes and fruit softening of 'Hom Thong' bananas during storage at 25 °C for 10 days.

CHAPTER V

CONCLUSION

The investigation of hot water treatment (HWT) on changes in cell wall compositions, the modification in activities of cell wall degrading enzymes and the expression of genes involved in cell wall degradation of 'Hom Thong' banana demonstrated that postharvest application of hot water at 50 ^{o}C for 10 min was effective in preventing banana fruit softening. Firmness of hot water treated fruit was maintained at a higher level during the storage of fruits at 25 ^{o}C.

Determination of cell wall fractions found that HWT delayed the increase in WSP content after 6 days of storage and retained HSP amount from day 8 during storage, but had little effect on changes in ESP level. Loss of hemicellulose and cellulose content in heated fruit tended to be slower than that in unheated fruit but there was no significant difference.

According to enzyme activity and gene expression analysis, hot water treated fruit showed a significantly lower level in PG activity and relative *MaPG1* expression at 2 and 8 days of storage. Treatment with hot water decreased PL activity during day 2 to day 6 and reduced *MaPL1* mRNA abundance throughout storage at 25 ^{o}C except for day 4. The activity of β-Gal was reduced after hot water application up to 4 days. The expression of *MaGAL* was unaffected by HWT in early stage but decreased expression was detected from day 4 to the end of storage. HWT caused a reduction in PME activity immediately after heating but did not affect the activity thereafter. Transcript level of *MaPME1* was lower in treated fruit on day 0, 4 and 8. Significant difference in Glu activity between control and treated fruit was observed at 4 and 8 day of storage. The increase in *MaGLU* transcript abundance was delayed by heat treatment and maximum expression level in treated fruit was lower comparing to control fruit.

Furthermore, an association between structural changes in cell wall of banana fruit and HWT was found. A slow alteration in peel structure of hot water treated banana fruits was revealed as compared to control fruits. Cell damage and collapse in banana peel were delayed by HWT. The peel of treated fruit was thicker than that of untreated control particularly in later stage of ripening. Higher number of cell layers was detected in treated fruit after 4 days of storage. HWT could also decrease the formation of air spaces in banana peel.

These results suggest that loss of firmness was correlated to cell wall degrading enzymes. PG, PL and β-Gal were more closely related to peel softening than PME and Glu in 'Hom Thong' banana. Treatment with 50 ^{o}C hot water consistently reduced activities and gene expressions of PL and β-Gal. PG activity was also reduced. Hot water treatment had little effect on PME and Glu enzyme activities and their gene expressions. The effect of HWT on activities and gene expressions of the enzymes associated cell wall degradation caused compositional changes by delaying solubilization of cell wall polymer especially pectin fractions. Subsequently, alteration in banana peel structure was slowed down leading to maintenance of 'Hom Thong' banna firmness during fruit ripening.

This research gained further insight of hot water effect on alteration of cell wall structure and composition of banana fruit during postharvest storage that associated with softening retardation. A better understanding of the molecular and biochemical processes occurring in the banana fruit during softening should aid in developing other successful treatments and could be applied to other fruits in order to delay fruit ripening and maintain fruit quality according to cell wall metabolism. In addition, the identification of numerous cell wall degrading enzymes with enzyme activity and gene expression patterns related to fruit softening provided us with good candidates for molecular breeding schemes to improve banana cultivars.

Nevertheless, fruit softening of banana during ripening is a complex processes. Any individual enzyme is not sufficient to bring about cell wall modification, but fruit softening is coordinated by multiple isoforms and may also involve posttranscriptional regulation. Even though the parameters analyzed in this study

provided some information how hot water treatment delay banana softening, but they could not clarify the entire mechanism. Other factors that contribute to fruit texture softening cannot be overlooked, enzymes which depolymerize xyloglucan and non-xyloglucan matrix glycans, non-enzymatic mechanisms or modification in cell wall neutral sugars. Xyloglucan endotransglycosylase/hydrolase, α-L-arabinofuranosidase, and expansin were investigated in relation to banana softening at molecular and biochemical level. Pectin acetylesterase, endo-1,4-β-glucanase, and β-xylanase were also reported in several fruits during ripening. Therefore, these factors including the additional genes encoded cell wall enzymes that were not investigated in this study should be further examined in future study.

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APPENDICES

APPENDIX A

Chemical solutions

1. 1 M Tris-HCl pH 8.2

Tris base   60.57 g

Dissolve Tris base in water and adjust pH to 8.2 with concentrated HCl and then bring total volume to 500 ml before autoclave.

2. 0.5 M EDTA pH 8.0

Na_2EDTA 186.1 g

NaOH pellet   20 g

Dissolve Na_2EDTA in water and adjust pH to 8.0 with NaOH and then bring total volume to 1,000 ml before autoclave.

3. 10 M LiCl

LiCl   42.39 g

Dissolve LiCl in DEPC water and bring total volume to 100 ml before autoclave.

4. 3 M Na acetate pH 4.5, 5.2

Anhydrous Na-acetate   24.624 g

Dissolve anhydrous Na-acetate in DEPC water and adjust pH to 4.5 or 5.2 with gracial acetic acid and then bring total volume to 100 ml before autoclave.

5. 10 mg/ml Ethidium bromide

Ethidium bromide	1 g
Water	100 ml

Stir for several hours and store in dark bottle at 4 ^{o}C.

6. 0.1% DEPC water

DEPC	1 ml
Nanopure water	1,000 ml

Stir very well for 24 h or overnight in the fume hood and then autoclave.

7. DNS reagent

DNS	10 g
NaOH	16 g
Na-K tartrate	300 g
Water	1,000 ml

Store in dark bottle at room temperature.

8. 0.2 M Sodium phosphate buffer pH 7.0

0.2 M $NaH_2PO_4.H_2O$	39 ml
0.2 M Na_2HPO_4	61 ml

Mix solutions, adjust pH to 7.0 and bring total volume to 1,000 ml.

9. Anthrone reagent

Anthrone	2 g
Conc. H_2SO_4	100 ml

This should be made up fresh before use.

10. Enzyme extraction buffer (Lohani et al., 2004)

0.2 M Na-Pi buffer pH 7.0	100 ml
0.02 M EDTA	7.44 g
1% Triton X-100	10 g
0.02 M Cysteine-HCl	2.423 g
1 mM PMSF*	0.1742 g
Total volume	1,000 ml

* Add before use

APPENDIX B

RNA extraction (Asif et al., 2000)

1. Extraction buffer

1 M Tris-HCl	100 ml
NaCl	81.816 g
0.5 M EDTA	40 ml
CTAB	20 g
Total volume	1,000 ml

Add 2-mercaptoethanol (1 µl 2-mercaptoethanol : 1 ml extraction buffer) before use.

2. Protocol

- Grind 0.5 g dry weight of banana peel with mortar and pestle

- Add 10 ml extraction buffer (65 ^{o}C) and transfer solution to centrifuge tube

- Incubate at 65 ^{o}C for 1 h (gentle vortex every 15 min)

- Add 10 ml chloroform* and shake

- Centrifuge at 12,000 x g for 15 min at 20 ^{o}C

- Pipette aqueous phase to new centrifuge tube

- Re-extract with an equal volume of chloroform

- Centrifuge at 12,000 x g for 15 min at 20 ^{o}C

- Pipette aqueous phase to new centrifuge tube

- Add 10 M LiCl to a final concentration of 3 M

- Store at 4 ^{o}C overnight to allow RNA to precipitate

- Centrifuge at 17000 x g for 20 min at 4 ^{o}C to recover RNA

- Dissolve pellet in 500 ml DEPC water

- Extract with phenol, phenol : chloroform (1:1), chloroform sequentially

- Pipette aqueous phase to microcentrifuge tube

- Add 1/30 volume of 3 M Na acetate pH 5.2 and 0.1 volume of absolute ethanol

- Mix well and keep on ice for 30 min

- Centrifuge 12,000 x g for 25 min at 4 ^{o}C

- Pipette clear supernatant to new tube (discard pellet)

- Add 3 M Na acetate to final concentration 0.3 M and 3 volume of absolute ethanol

- Store at -80 ^{o}C for 3 h or overnight

- Centrifuge 12,000 x g for 20 min at 4 ^{o}C

- Wash pellet with 70% ethanol

- Re-suspend in 100 µl DEPC water

- Quantify RNA by nanodrop (blank with DEPC water)

* Chloroform is used as mixture of chloroform : isoamyl alcohol (24:1)

Denaturing agarose gel for RNA separation (Michelle Jones Lab)

1. **Reagents**

    1.1 **Agarose gel (50 ml gel box)**

Agarose	0.5 g
10X MOPS	5 ml
37% Formaldehyde	10 ml
DEPC water	35 ml

    1.2 **Denaturation buffer**

Deionized formamide	500 µl
37% Formaldehyde	170 µl
10X MOPS	100 µl
EtBr	1-2 µl

    Note: This should be made up fresh before each use. Make up in a microcentrifuge tube.

2. **Protocols**

    - Mix agarose with DEPC water and boil in the microwave, cool to 65 ^{o}C.

    - Add the 10X MOPS and 37% formaldehyde in the hood.

    - Pour gel into box and allow it to set up.

    - Pipette desired amount of sample into a microcentrifuge tube.

    - Bring up to volume with DEPC water and mix in denaturing buffer 1:3.

    - Heat this at 65 ^{o}C in heat block for 10 min and quick cool on ice.

    - Centrifuge for 30 sec. and load.

    - Run at about 70 volts.

DNase treatment (Promega, Madison, WI)

Procedure

- Add 1/10 volume of DNase buffer to RNA sample and mix gently.

- Add 2 µl RQ1 RNase free DNase and mix gently and incubate at 37 $^{\circ}$C for 30 min.

- Add 1/10 volume of DNase inactivation reagent and incubate 65 $^{\circ}$C for 10 min.

- Quantify RNA by nanodrop (blank with DEPC water)

cDNA synthesis (Omniscript Reverse Transcriptase Kit, Qiagen, Valencia, CA)

1. **Making 2 µg RNA**

   Concentration of RNA from nanodrop = X ng/µl

   Making RNA 2 µg = (2/X) x 1000 µl

2. **Reverse-transcription reaction components**

Master mix	Volume/reaction	
10X buffer RT	2	µl
dNTP Mix (5 mM each dNTP)	2	µl
Oligo-dT primer (10 µM)	2	µl
RNase inhibitor (10 units/µl)	1	µl
Omniscript reverse transcriptase	1	µl
Template RNA		
2 µg RNA	12	µl
RNase free water		
Total volume	20	µl

3. **Protocols**

   - Pipette 8 µl master mix into microcentrifuge tube.

   - Add template RNA and mix by vortexing for no more than 5 sec.

   - Centrifuge briefly to collect residual liquid.

   - Incubate at 37 ^{o}C for 60 min.

   - Store reaction on ice and proceed directly with real-time PCR or store reaction at -20 ^{o}C for long term storage

Real-time PCR primer design (Michelle Jones Lab)

1. **Rules for designing**

   - 18-30 nucleotides

   - 40-60% GC content

   - Primer Tm values should not be lower than the reverse transcription reaction temperature (e.g. 50 ^{o}C)

   - PCR product length 100-200 bp

   - Avoid complementarity of 2 or more bases at the 3' ends of primer pairs to reduce primer-dimer formation

   - Avoid mismatches between the 3' end of the primer and the target template sequence

   - Avoid runs of 3 or more Gs or Cs at the 3' end

   - Avoid a 3' end T

   - Avoid complementary sequences within a primer sequence and between the primers of a primer pair

2. **Detailed instructions**

   - Go to IDT/SciTools, PrimerQuest:
   http://www.idtdna.com/Scitools/Applications/Primerquest/

   - Copy and paste sequence into 'Sequence' field or enter accession number and click 'Get sequence'.

   - Select 'PCR primers' under Design for and 'Real-time PCR' under parameter set.

   - At top of PrimerQuest, move into 'Advanced' screen.

   - Under 'Primer conditions' change primer GC content to minimum = 40% and maximum = 60%. Keep all other values at default settings. Note, the product length default is 100-200 bp.

- Click 'Calculate' at bottom of PrimerQuest page.

- Evaluate the results based on above criteria and calculate results for each primer pair. BLAST each primer sequence to determine specificity.

Finding optimal annealing temperature (Michelle Jones Lab)

1. **Reaction component**

Master mix	Volume/reaction	
5X Go Tag buffer	10	μl
2.5 mM dNTP	4	μl
Nanopure water	30.75	μl
5 μM Forward primer	1.5	μl
5 μM Reverse primer	1.5	μl
Go Tag (5 units/μl)	0.5	μl
cDNA template	2	μl
Total volume	50	μl

2. **Thermal cycle (Gradient PCR)**

- 95 °C   x 2 min

- 95 °C   x 30 sec
- 56-62 °C   x 30 sec   } 35 cycles
- 72 °C   x 30 sec

- 72 °C   x 5 min

- 10 °C   forever

Gel extraction (QIAquick Gel Extraction Kit, Promega, Madison, WI)

Protocol

- Excise DNA fragment from agarose gel.

- Put gel slice in microcentrifuge tube and weigh.

- Add 3 volume QG buffer to 1 volume gel (100 mg 100 µl).

- Incubate at 50 ^{o}C for 10 min. Vortex tube every 2-3 min.

- After gel has dissolved completely (color of the mixture is yellow), add 1 gel volume of isopropanol to the sample and mix well.

- Apply the sample to the QIAquick column and centrifuge at 13,000 rpm for 1 min.

- Discard flow-through.

- Add 500 µl QC buffer to the column and centrifuge at 13,000 rpm for 1 min.

- To wash, add 750 µl PE buffer to the column and centrifuge at 13,000 rpm for 1 min.

- Discard flow-through and centrifuge at 13,000 rpm for 1 min to remove residual wash buffer.

- Place the column into 1.5 ml centrifuge tube.

- To elute DNA, add 30 µl EB buffer (heating EB buffer to 65 ^{o}C before use).

- Let the column stand for 1 min and centrifuge for 1min.

- Quantify DNA (blank with EB buffer).

Real-time PCR (Promega, Madison, WI)

1. Setting up the reaction

Master mix	Volume/reaction	
SYBR Green mix	10	µl
Water	6.6	µl

Sub-mix	Volume/reaction	
Master mix	16.6	µl
5 µM Forward primer	1.2	µl
5 µM Reverse primer	1.2	µl

Sub-sub-mix	Volume/reaction	
Sub-mix	19	µl
cDNA	1	µl

2. Procedure

- Thaw all supplies on ice.

- Map out plate set up.

- Set real time PCR plate in a plate holder and place in ice.

- Pipette 20 µl for each sub-sub mix into designated well of the real time PCR plate (running each cDNA in triplicate)

- Seal plate with sealing tape and keep on ice.

3. Setting up IQ cycler

- Activate Protocol. Select, create or edit protocol and save.

- Activate Plate. Select, create or edit plate and save. Set up No Template Controls, Standards and Unknowns. Select dilution series and units for the standards. Select 20 µl for reaction volume, plate for vessel and film for seal. Select fluorophore.

- Activate Run Set. File-Create New Run Set. Select protocol and plate to be included; double check to ensure the correct sets. Save and place plate in machine.

- Select run protocol under Run Set.

- The initiate run screen will appear. Select collect well factors from experimental plate. Select Begin Run.

- Save run data for using for data analysis.

4. Analyzing data

- Set threshold value.

- Set analysis to 'PCR baseline subtracted'

- Select one target gene set by clicking on 'Select wells' and de-highlighting those samples that do not want to analyze.

- Analyze melt curve. If there is more than one peak, the primers are detecting more than one product; primers should be redesigned and checked for specificity.

- Click on 'Results' when analysis is complete.

- Determine relative expression of each target gene by calculating the starting quantity relative to normalizing gene (actin).

$$\text{Relative expression of gene X} = \frac{\text{(Starting quantity gene X)}}{\text{(Staring quantity actin)}}$$

Table C.1 Changes in firmness in control and hot water treated (50 °C, 10 min) bananas during the storage of fruits at 25 °C. Data represents the mean ± standard error.

Parameter	Storage time (days)					
	0	2	4	6	8	10
Firmness (N)						
Control	0.890±0.012	0.836±0.010	0.773±0.031	0.657±0.027	0.556±0.021	0.437±0.026
HWT 50°C	0.877±0.001	0.836±0.009	0.828±0.011	0.739±0.015*	0.658±0.037*	0.570±0.038*

The asterisks within column for each parameter in same day indicate significant difference ($P<0.05$)

Table C.2 Changes in cell wall compositions in control and hot water treated (50 °C, 10 min) bananas during the storage of fruits at 25 °C. Data represents the mean ± standard error.

Parameter	Storage time (days)					
	0	2	4	6	8	10
Water soluble pectin (µg/mg AIS)						
Control	24.50±1.51	17.79±0.83	27.55±0.48	46.24±0.64	67.64±0.92	75.49±0.96
HWT 50°C	28.52±1.22	17.85±0.83	26.63±1.83	32.78±1.28*	53.92±1.50*	82.41±3.75
EDTA soluble pectin (µg/mg AIS)						
Control	9.71±0.42	4.71±0.41	6.83±1.30	7.43±0.56	10.75±0.62	11.36±0.00
HWT 50°C	10.08±0.25	5.91±0.42	7.20±0.43	8.33±0.82	9.89±0.50	11.24±0.81
HCl soluble pectin (µg/mg AIS)						
Control	13.26±0.29	15.55±0.48	23.44±0.43	28.04±0.60	25.90±0.50	24.32±0.43
HWT 50°C	12.17±0.64	13.39±0.69	25.53±0.57*	27.99±0.62	30.81±0.66*	27.01±0.67*
Hemicellulose (µg/mg AIS)						
Control	330.70±11.54	388.65±5.66	298.70±6.74	160.69±6.50	117.43±3.09	156.23±3.14
HWT 50°C	304.62±6.02	402.13±4.35	302.44±5.18	172.88±4.16	96.23±2.55*	155.44±3.40
Cellulose (µg/mg AIS)						
Control	887.33±5.22	1358.73±18.43	1351.92±25.92	1125.96±40.27	1115.55±15.52	1004.54±8.63
HWT 50°C	870.05±20.39	1381.65±20.11	1351.80±27.48	1132.83±27.96	1132.53±21.55	1087.29±20.12*

The asterisks within column for each parameter in same day indicate significant difference ($P<0.05$)

Table C.3 Changes in activity of cell wall degrading enzymes in control and hot water treated (50 °C, 10 min) bananas during the storage of fruits at 25 ^{o}C. Data represents the mean ± standard error.

Enzyme activity	Storage time (days)					
	0	2	4	6	8	10
Polygalacturonase (units/mg protein)						
Control	0.013±0.001	0.036±0.006	0.019±0.001	0.063±0.005	0.094±0.004	0.108±0.015
HWT 50°C	0.012±0.001	0.027±0.002	0.030±0.004	0.058±0.004	0.066±0.005*	0.112±0.005
Pectate lyase (units/mg protein)						
Control	0.035±0.003	0.068±0.002	0.078±0.003	0.096±0.004	0.128±0.003	0.173±0.005
HWT 50°C	0.038±0.004	0.056±0.003*	0.060±0.002*	0.085±0.003	0.171±0.006*	0.181±0.012
β-galactosidase (units/mg protein)						
Control	0.94±0.026	1.20±0.050	0.86±0.023	1.68±0.155	2.98±0.107	6.33±0.384
HWT 50°C	0.57±0.032*	0.71±0.027*	0.30±0.008*	1.65±0.081	2.76±0.206	6.22±0.142
Pectin methylesterase (units/mg protein)						
Control	3.39±0.047	3.47±0.019	3.38±0.042	3.51±0.082	3.06±0.068	2.85±0.138
HWT 50°C	2.53±0.266*	3.24±0.049*	3.62±0.053*	3.39±0.316	3.18±0.071	3.96±0.037*
Glucanase (units/mg protein)						
Control	0.021±0.003	0.054±0.004	0.092±0.004	0.037±0.004	0.107±0.005	0.118±0.006
HWT 50°C	0.026±0.002	0.050±0.009	0.083±0.003	0.121±0.005*	0.056±0.004*	0.111±0.012

The asterisks within column for each parameter in same day indicate significant difference (*P<0.05*)

Table C.4 Changes in relative gene expression in control and hot water treated (50 °C, 10 min) bananas during the storage of fruits at 25 ^{o}C. Data represents the mean ± standard error.

Relative gene expression	Storage time (days)					
	0	2	4	6	8	10
Polygalacturonase						
Control	0.027±0.003	0.136±0.005	0.054±0.003	0.123±0.008	0.110±0.007	0.014±0.003
HWT 50°C	0.032±0.002	0.006±0.001*	0.182±0.007*	0.150±0.005*	0.063±0.002*	0.038±0.002*
Pectate lyase						
Control	0.018±0.002	11.818±0.894	18.799±0.582	47.950±2.608	58.188±2.959	58.949±1.319
HWT 50°C	0.002±0.000*	0.001±0.000*	26.102±1.870*	23.518±1.980*	42.134±4.145*	55.605±8.625
β-galactosidase						
Control	0.039±0.005	0.047±0.006	0.040±0.003	0.085±0.002	0.101±0.001	0.109±0.002
HWT 50°C	0.037±0.001	0.047±0.002	0.063±0.004*	0.051±0.005*	0.086±0.005*	0.092±0.005*
Pectin methylesterase						
Control	0.046±0.002	0.029±0.004	0.034±0.002	0.012±0.001	0.134±0.009	0.035±0.007
HWT 50°C	0.037±0.002*	0.079±0.002*	0.023±0.002*	0.077±0.006*	0.053±0.004*	0.053±0.010
Glucanase						
Control	0.268±0.027	9.026±1.176	15.284±3.106	19.163±0.871	37.365±0.989	4.530±0.208
HWT 50°C	0.505±0.089	1.375±0.123*	19.663±2.075	19.408±0.338	29.239±1.159	11.473±0.860*

The asterisks within column for each parameter in same day indicate significant difference ($P<0.05$)

Table C.5 Changes in peel thickness, number of cell layer and air space area in control and hot water treated (50 °C, 10 min) bananas during the storage of fruits at 25 $^{\circ}$C. Data represents the mean ± standard error.

Parameter	Storage time (days)					
	0	2	4	6	8	10
Peel thickness (mm)						
Control	3.421±0.122	3.347±0.034	2.716±0.060	2.651±0.017	2.529±0.094	2.030±0.056
HWT 50°C	3.264±0.038	3.218±0.077	2.976±0.058*	2.910±0.063*	2.657±0.037	2.426±0.045*
Number of cell layer						
Control	64.56±2.48	64.36±0.42	58.75±0.82	54.72±0.94	50.31±1.18	49.31±0.76
HWT 50°C	65.58±0.74	64.83±1.22	62.78±1.05*	60.06±0.87*	54.61±1.07*	52.33±0.98*
Air space area (mm^2)						
Control	0.317±0.014	0.344±0.008	0.505±0.023	0.579±0.043	0.811±0.039	0.923±0.070
HWT 50°C	0.305±0.022	0.384±0.020	0.428±0.035	0.462±0.034*	0.709±0.053	0.776±0.041

The asterisks within column for each parameter in same day indicate significant difference ($P<0.05$)

BIOGRAPHY

Miss Nuankamol Amnuaysin was born on March 28, 1983 in Chachoengsao Province. She graduated with a Bachelor's degree in Biology from Faculty of Science, Silpakorn University in 2004. Then, she continued on her Master degree in Department of Botany, Faculty of Science, Chulalongkorn University from 2005 to 2007. The study in Bachelor's degree and Master degree was supported by the Ministry of Education and the Institute for the Promotion of Teaching Science and Technology (IPST) under the Development and Promotion of Science and Technology Talents Project (DPST). This scholarship also supported her study in Biological Science program, Faculty of Science, Chulalongkorn University for the degree of Doctor of Philosophy from 2008 to 2011.