Characteristics of Starch Degradation in relation to the Physiology of Ripening in Apple Fruit

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CHAPTER 1
RESEARCH BACKGROUND AND PURPOSE

1.1 General introduction

The apple (*Malus domestica*) belongs to the *Pomoideae* subfamily of *Rosaceae*. The *Pomoideae* subfamily is characterized by a basic chromosome number of 17 as compared to other subfamilies of the *Rosaceae* with only 7-9. Pome fruits are commonly described as having a core with fleshy pith within it and a cortex of flesh outside the core line. There are two hypotheses regarding the nature of these tissues, namely the receptacular and the appendicular (Pratt, 1988).

Apples are by far the most important fruits of the deciduous trees. They are widely grown in temperate and increasingly in tropical regions, and figure distinctly in world trade. Apple fruits are primarily grown more for the fresh fruit market more than for processing. In addition, market demand extends throughout the year even though any one cultivar in a particular region has only a limited harvest season, except for some relatively minor tropical produce.

For the fresh consumption of apples, consumers judge the fruit quality based on appearance, texture, and especially on flavor. Harker *et al.* (2003) suggested that the quality of an apple is more important to consumers than its price when prices vary within the expected commercial range. Moreover, it is important that new apple cultivars are easily differentiated in the marketplace and enhanced quality standard is the decision factor in the consumer's selection of fruit items.

1.2 Apple fruit growth and cellular component changes

The growth process of apples can be divided into one short postanthesis stage and two major subsequent stages. About 6-12 days after pollination, short postanthesis occurs where there is a slow increase in the fruitlet weight due to cell division. The first major stage is characterized by a rapid exponential increase of fruitlet weight by cell division and this continues for 6 to 8 weeks after anthesis. After this period, the fruit grows mainly by individual cell expansion (Ryugo, 1988). The cells elongate and continue to expand at a declining rate during the second major stage until harvest. In some cultivars, the seed formation plays an important role in these growth processes. An apple can have 15 or more seeds, but if any locule is lacking seed due to poor pollination, development of this side of the fruit, is arrested in some cultivars.

Brookfield *et al.* (1997) found that the fruit starch concentration of 'Royal Gala' and 'Fuji' actually decreased to reach a minimum value 30 days after anthesis, which then increased during the following 80 days, with the greatest accumulation occurring between 80 and 110 days after anthesis. As the apple enlarged, the water-insoluble dry matter content of the fruit decreased, partly because the starch in the cells is hydrolyzed to glucose. While fruits continue to grow, the cortical parenchyma cells become isodiametric as the cells separate from one another along the middle lamella. Fruit density decreases as the volume of intercellular air spaces increases. The air in these spaces is relatively rich in carbon dioxide and poor in oxygen because of cell respiration and the slow rate of gaseous exchange with the surrounding atmosphere (Ryugo, 1988).

Protein content in the apple fruit decreases to a minimum, two to three weeks before harvest, but increases again as the fruit approaches its full maturity. During this period, the fruit cells produce various kinds of enzymes. The accumulation of mineral elements by the apple is proportional to the rate of increase in dry matter (Ryugo, 1988).

1.3 Fruit maturation and ripening

Maturation is the process leading to physiological or horticultural maturity. Physiological maturity is the stage of development when the fruit will continue ontogeny even if detached. Horticultural maturity is when the fruit meets the criteria laid down by consumers. Ripening refers to the transition period between maturation of fruit and senescence. It is the sum of the processes from the later stages of growth through to the early stages of senescence, resulting in the attainment of eating quality characteristics. Watada et al. (1984) also suggested that the eating quality of the fruit is based on the developmental processes of maturation, ripening, and senescence.

1.3.1 Maturity index

For physiological changes, the rate of respiration per unit fresh weight is high in the early season during the cell division phase of fruit growth, which then declines to a very low level (Bepete and Lakso, 1997). Thereafter, when the fruits reach physiological maturity and ripening processes are initiated, the apples show a marked increase in respiratory activity resulting in increased production of carbon dioxide. This increase in respiratory activity, referred to as the respiration climacteric, precedes the visible symptoms of ripening. Subsequent to this climacteric rise which occurs in apples left attached to the tree and in harvested fruits, respiration rates decline once more (Fig. 1.1).

Ethylene production gradually declines during cell division and expansion. In apples, respiration climacteric ethylene production increases rapidly just prior to the obvious signs of ripening. In climacteric apples, the change in ethylene production may coincide with the change in rate of respiration. Prior to ripening of 'Golden Delicious' apples, ethylene production in the fruit flesh was considerably lower than ethylene in the pedicels and cluster bases. When the fruit started to ripen (157 days after full bloom), ethylene content of the fruit flesh began to increase and was higher than other parts of the apple until 167 days after full bloom. In 'McIntosh' apples, ethylene production started to increase in the fruit flesh, pedicels and cluster base tissues at 132, 136, and 143 days respectively after full bloom (Blanpied, 1972). In addition, Brookfield *et al.* (1997) reported that the internal ethylene concentration of 'Royal Gala' fruit increased slightly between 130 and 140 days after anthesis, after which there was rapid increase concomitant with quick yellowing of the background color as the fruit ripened. For 'Fuji', the internal ethylene concentration increased between 140 and 165 days after anthesis prior to commercial harvest, even though actual values for 'Fuji' had been characteristically low.

Determination of starch hydrolysis by iodine staining (SI) is widely used as a maturity index of the apple by providing an estimation of fruit starch content (Ingle and D'Souza, 1989; Fan et al., 1995; Lau, 1988). The starch index values provide valuable guidance to the level of fruit maturity and the appropriate time to harvest for immediate consumption or long-term storage. Despite the common use of SI to monitor starch loss during fruit maturation, SI varies widely between cultivars and it has been reported that it does not relate well to starch concentration (Fan et al, 1995; Watkins et al., 1993).

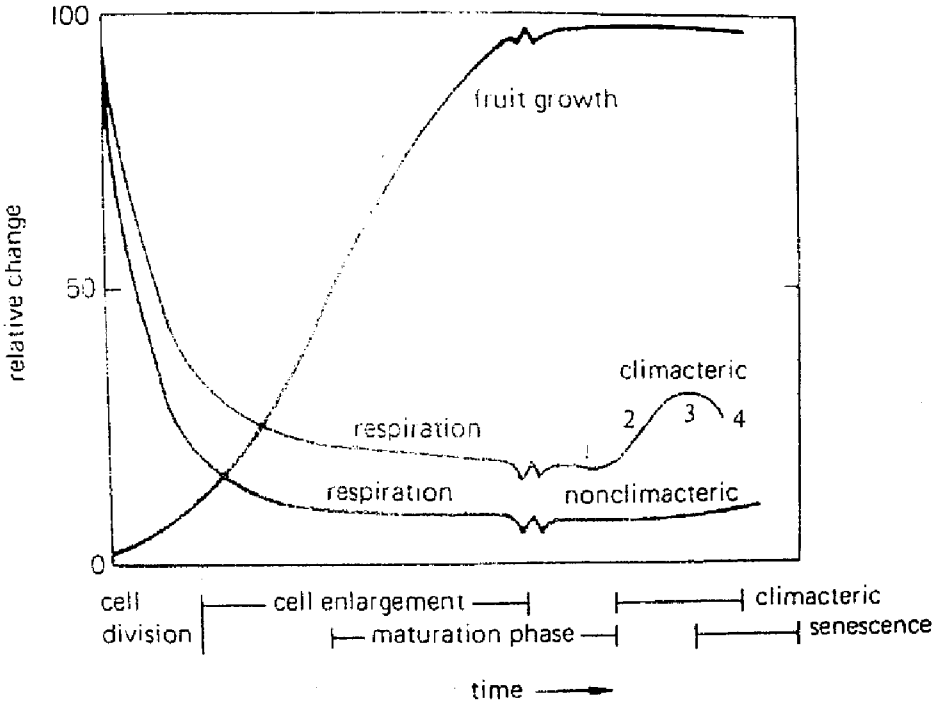

Fig. 1.1 General respiratory pattern of climacteric and non-climacteric fruits during development, maturation, ripening, and senescence (Kays, 1991).

1.3.2 Chemical compositions and eating quality

Many metabolic changes including firmness, starch hydrolysis, sugar accumulation, and the decrease of organic acids and phenolic compounds seem to be the most significant aspects in the determination of fruit eating quality (Fig. 1.2). During ripening, fruits undergo major changes in their chemical and physical states. These changes represent a wide spectrum of synthetic and degradative biochemical processes, of which many occur concurrently or sequentially within the fruit. Alterations in the quality attributes of the fruit are shown in Table 1.1.

Fruit flavor is primarily affected by the composition of sugars, acids, and volatile compounds. Changes in sugars and organic acids are the predominant altering factors of fruit taste. The sucrose, glucose, and fructose are responsible for the sweetness, with some minor contribution from sorbitol. Fructose is the main sugar in mature apples of most cultivars, including 'Golden Delicious' (Pavel and Dejong, 1995) and 'Greensleeves' apples (Defilippi *et al.*, 2004). In the attached fruit, sugars increase via translocation of sorbitol and sucrose from the leaves (Bieleski and Redgwell, 1985; Teo *et al.*, 2006). Upon arrival, sucrose is metabolized by invertase and sucrose synthase, while sorbitol is converted to fructose by sorbitol dehydrogenase. In pome fruits including apples, fruit sweetness is associated with changes in internal sugars which are products derived from the hydrolysis of carbohydrate reserves within the tissues.

Starch is a major substance accumulated during maturation of the apple fruit (Magein and Leurquin, 2000). Ryugo (1988) suggested that starch degradation in the apple starts when the fruit reaches the mature stage. Sugars are accumulated in the fruit cells during ripening, and the fruit becomes sweet, which is an important flavor in determining fruit quality (Kader, 2000). During the ripening of 'Jonagold' apples, the starch decreased ≈3.7 mg g^{-1} fresh weight per week (Lau, 1988). In general, starch hydrolysis begins in the later stages of fruit growth, usually 2 or 3 weeks before the increase of ethylene production in apples (Lau, 1988).

Fruit firmness at an acceptable harvest date of the 'Jonagold' was 73-76 N. Although there was a slight decrease in firmness with a later harvest date, fruits went soft after storage at 0°C in air (Lau, 1988). The firmness of 'Red Delicious' and 'Golden Delicious' apples also decreased during ripening (Blankenship and Unrath, 1988). The firmness at maturity, at optimum harvest date or for long term storage, varies from cultivar to cultivar, e. g. from 68 N for 'McIntosh' to 82 N for 'Deliciuos' in the same season (Lau, 1988), and also from season to season.

In apples, both citric and malic acids show the mid-season rise in concentration but quinic acid concentration falls from the beginning of the season (Knee, 1975). The metabolism of malate by tissue slices increases as apples undergo the climacteric. In addition, the titratable acidity of 'Jonagold' apples decreased ≈11 mg malate 100 mL^{-1} of juice per week during fruit maturation and ripening (Lau, 1988).

An increase in the redness of skin color, and the yellowness of ground color, was observed in 'Jonagold' apples during fruit maturation and ripening (Lau, 1988). Jackson (2003) suggested that anthocyanin synthesis occurs during fruit growth where the color changes during ripening in accordance to the disappearance of chlorophyll *a* and *b*. Carotenoid declines during ripening but xanthophylls increases as mono-and diesters, mainly palmilate and oleate. Galactolipids and associated linolenyl moieties, which are typical chloroplast membrane constituents, are lost on ripening but phospholipids and fatty acyl groups remain constant or increases slightly. Traces of farnasene, thought to be implicated in the development of scold, are present on the surface of pre-climacteric fruits and the amount increases rapidly on ripening. The organic compounds of apple aroma and flavor are synthesized during the climacteric phase.

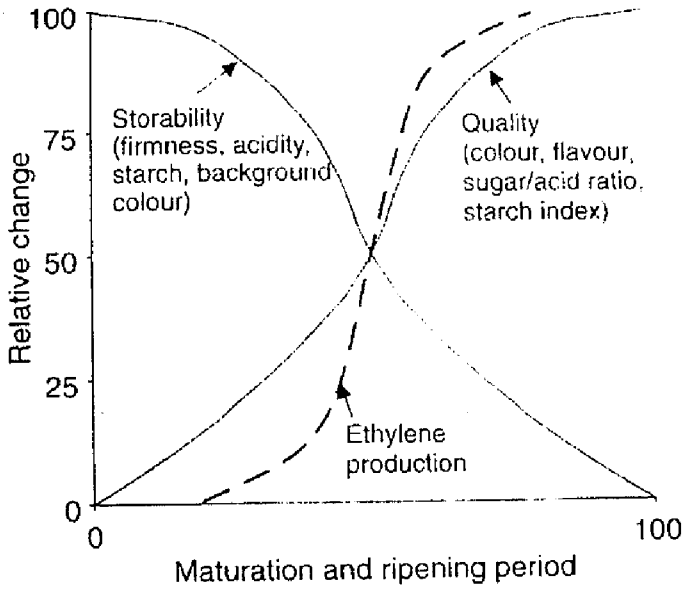

Fig. 1.2 Schematic illustration of the increase in apple-fruit quality during maturation and ripening (Watkins, 2003).

Table 1.1 Physical and chemical alterations that occur during the ripening of fleshy fruit (Kays, 1991).

1. Seed maturation
2. Changes in pigmentation
a. degradation of chlorophyll
b. unmasking of existing pigments
c. synthesis of carotenoids
d. synthesis of anthocyanins
3. Softening
a. changes in pectin composition
b. possible alterations in other cell wall components
c. hydrolysis of storage materials
4. Changes in carbohydrate composition
a. starch to sugar conversion
b. sugar interconversions
5. Production of aromatic volatiles
6. Changes in organic acid
7. Fruit abscission
8. Changes in respiration rate
9. Changes in the rate of ethylene synthesis
10. Changes in tissue permeability
11. Changes in proteins
a. quantative
b. qualitative (enzyme synthesis)
12 Development of surface waxes

1.4 Starch degradation metabolism

Starch plays an important role in the increase of sugar content during fruit ripening. Additionally, as demonstrated by Lau (1988), the starch content of the apple is considered to be a suitable indicator of harvest. In the flesh of the apple, starch accumulation takes place during development and most of the starch granules of the apple disappear at harvest. In 'Jonagold', the total starch accumulation started noticeably in fruitlets only when their mean size exceeds 20 mm in diameter. The starch concentration exceeded 120 mg g^{-1} of dry matter when the fruits were 50 mm in diameter in mid-July.

The starch content of apples during development varied according to the location in the flesh. The most prominent feature was the higher starch content and a lower decrease rate in the outer part of the flesh than in the inner and middle parts (Ohmiya and Kakiuchi, 1990). Brookfield *et al.* (1997) found that all tissues showed a decline in starch concentration with the lowest starch content in the core, and declined to below 0.2 mg· g^{-1} fresh weight in both 'Royal Gala' and 'Fuji' apple cultivars. Starch hydrolysis is accompanied by the appearance of sucrose but the amount of sucrose far exceeded the amount that can be accounted for by starch hydrolysis alone (Whiting, 1970). Sucrose is then slowly hydrolyzed to form more glucose and fructose. The concentration of sugars changes little during storage. Fruit abscission in some apple cultivars always occur at a fixed starch concentration, suggesting a close link between starch content and the natural ripening and senescence processes.

Apple starch consists of two compounds; a straight-chained molecule, amylose (AM), that contains 200-1,000 glucose subunits and amylopectin (AP), a branched-chain molecule that is substantially larger, containing 2,000-200,000 subunits. Amylose has individual glucose molecules linked by α-(1-4) glucosidic bonds, with bonding angles which impart a helical structure to the molecule. Amylopectin has similar α-(1-4) glucosidic bonds between glucose subunits; however, every 20-25 glucose molecules along the chain, there is a branch formed via α-(1-6) glucosidic linkage. AM and AP react differently with I_2-KI solution. AM reacts most efficiently with I_2-KI to produce blue-black pigment (McCready and Hassid, 1943; Fan et al., 1995).

Degradation of starch in plants proceeds in basically two different reactions; hydrolysis and phosphorolysis (Fig. 1.3). Starch phosphorylase is suggested to be involved in the breakdown metabolism of starch (Jackson, 2003). Additionally, hydrolysis of starch in fruits is probably induced by α-amylase, β-amylase, and debranching enzymes (Fig. 1.4). Overview of starch degradation metabolism is shown in Fig. 1.5

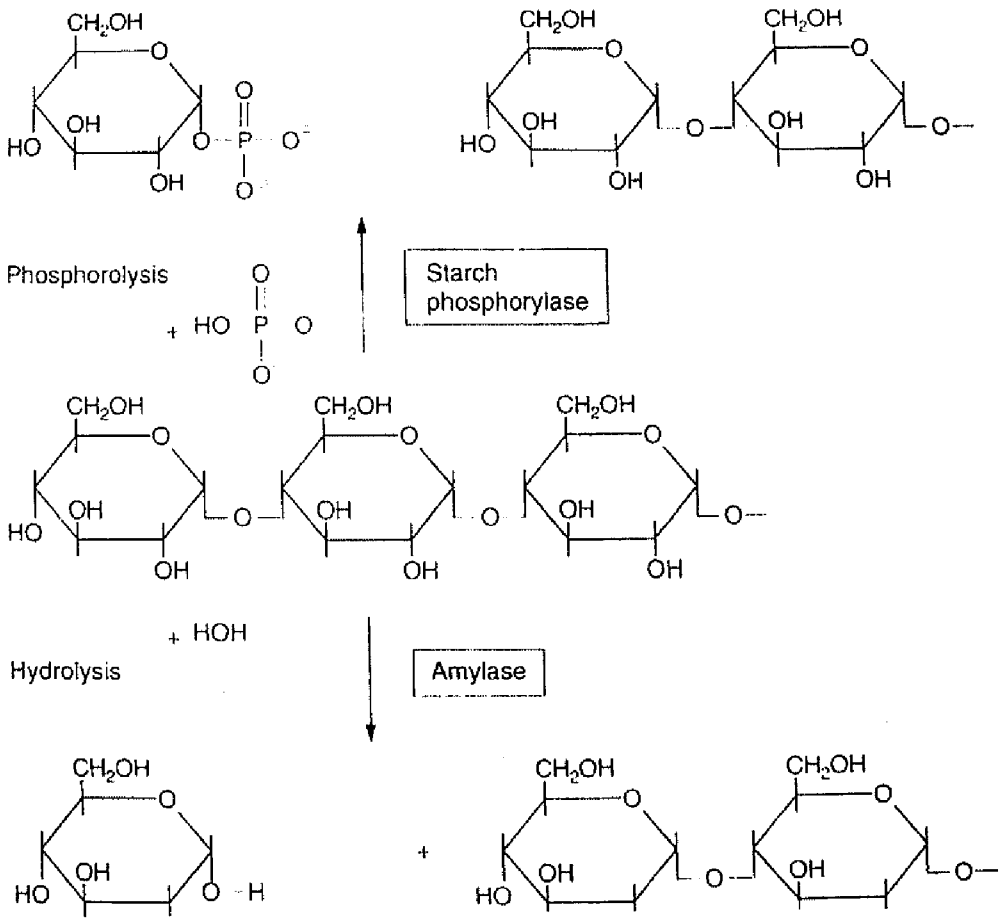

Fig. 1.3 The α-(1-4) linkage in starch molecule can be cleaved by hydrolysis or phosohorolysis (Heldt, 1997).

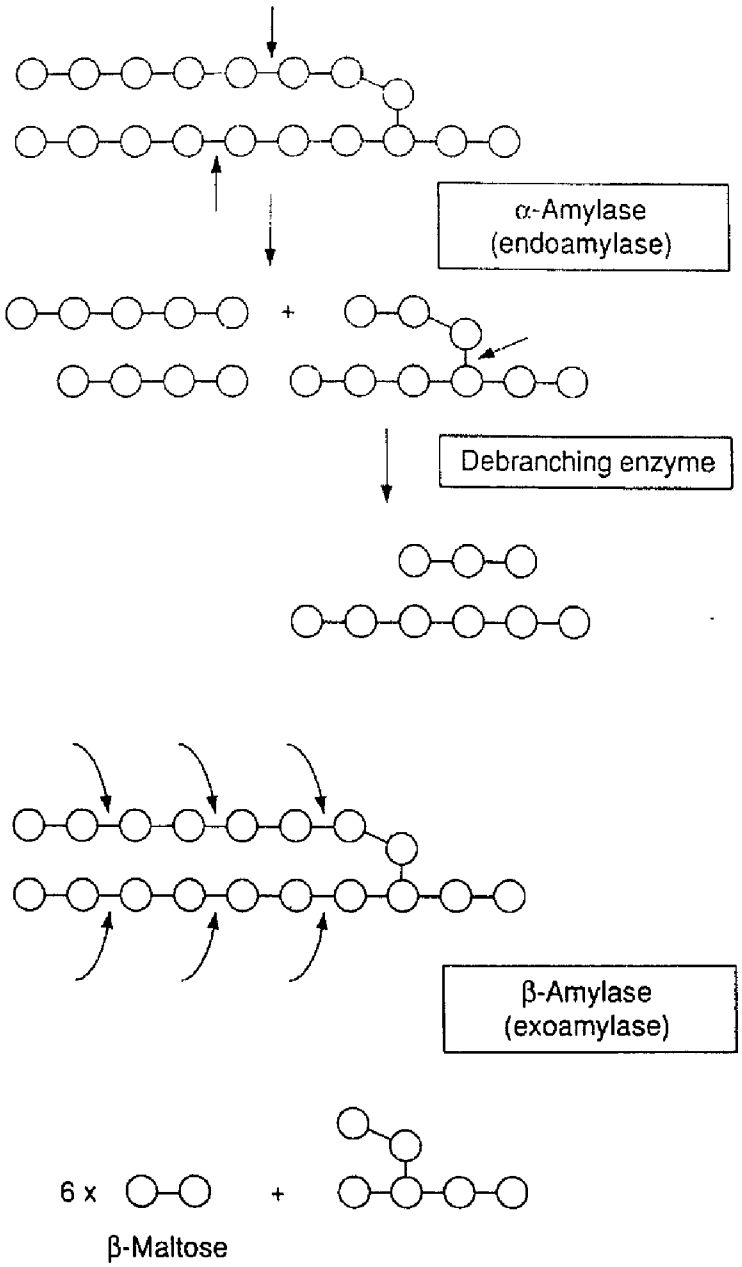

Fig. 1.4 Glucosidic bonds in the interior of starch molecule are hydrolyzed by α-amylases. The debranching enzyme hydrolyses α-(1-6) linkages. β-amylases release disaccharide maltose from the end of starch molecules. Maltose is then further hydrolyzed by enzyme maltase to produce two glucose segments (Heldt, 1997).

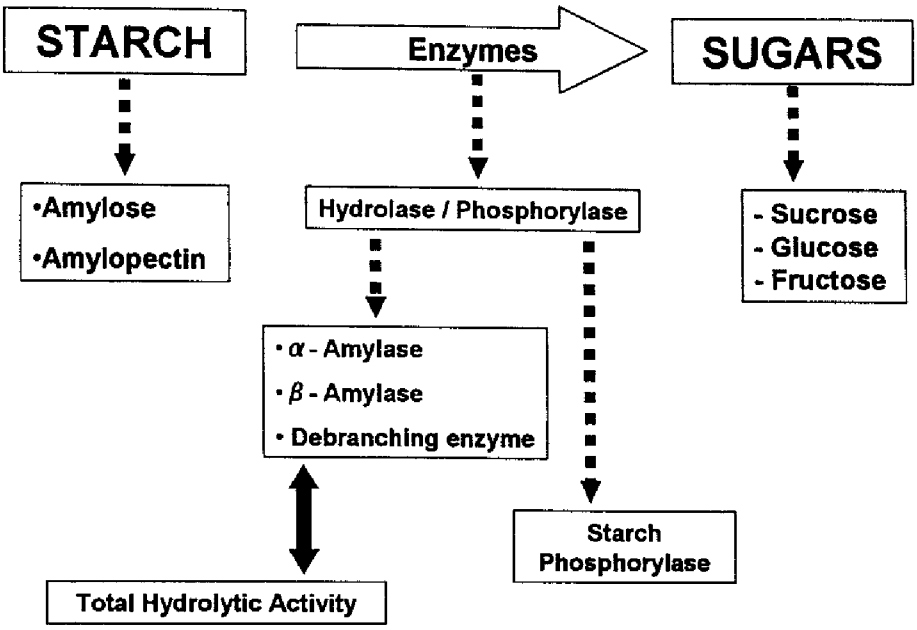

Fig. 1.5 Overview of starch degradation metabolism.

1.5 Factors affecting fruit ripening and starch degradation

1.5.1 Ethylene

For many years, ethylene has been recognized as the hormone that induces ripening of edible fruits. Addition of ethylene to such fruits hastens ripening, and a dramatic increase in ethylene production is closely associated with the initiation of ripening (Taiz and Zeiger, 1998). However, surveys of various fruits have shown that not all of them respond to ethylene.

Treatment with ethylene induces the fruit to produce additional ethylene and its action can be described as autocatalytic. In climacteric fruits including the apple, ripening is associated with an increased production of ethylene, and ethylene treatment can accelerate ripening. When the unripe fruits are treated with ethylene, the onset of the climacteric rise is accelerated. When non-climacteric fruits are treated in the same way, the magnitude of the respiratory rise increases as a function of the ethylene concentration, but the treatment does not stimulate the production of endogenous ethylene and does not accelerate ripening.

In general, ethylene enhances the taste and flavor of fruits by stimulating ripening (Watada, 1986). Defilippi *et al.* (2004) suggested that the regulation of flavor metabolism in apples by ethylene is complex. Sugars (mainly sucrose and fructose) and organic acids are under ethylene regulation. On the other hand, the metabolism of phenolic compounds may at least be considered as an ethylene-independent process in the last stages of fruit development. Moreover, ester accumulation is clearly regulated by ethylene.

In climacteric fruits, ethylene is known to trigger many physiological and biochemical changes during fruit ripening, and to induce ethylene production, respiration rate, and fruit senescence (Pratt and Goeschl, 1969). Starch to sugar conversion has also been suggested as one aspect of fruit ripening stimulated by ethylene (Kader, 1985; Watkins, 2003). Significant correlations between starch loss and the increase of internal ethylene concentration (IEC) was observed in 'Golden Delicious', 'Red Delicious', and 'Jonagold' apples (Lau *et al.*, 1986; Ingle and D'Sonza, 1989; Lau, 1988). However, starch degradation of 'Fuji', 'Gala' and 'Delicious' apples were observed prior to any increase in IEC (Brookfield *et al.*, 1997; Blankenship and Unrath, 1988). Although starch degradation has been studied in many fruits (Fan *et al.*, 1995; Garcia and Lajolo, 1988; Zhang and Wang, 2002), there is no clear result about the role of ethylene in starch degradation of the apple. Additionally, different apple cultivars may have different responses to ethylene.

1.5.2 1-Methylcyclopropene (1-MCP)

1-MCP is a new tool for the management of ethylene action and/or production by climacteric fruit including apples. It is a gas with a molecular weight of 54 and a formula of C_4H_6. This compound is an ethylene action inhibitor that prevents plant tissues from responding to ethylene by combining with ethylene receptors. It improves storage life and prevents other physiological disorders in many apple cultivars (Sisler and Serek, 1997; Fan *et al.*, 1999).

It has been shown that 1-MCP inhibited ethylene production in 'Fuji' and effectively reduced ethylene production of 'Braeburn' apples (Argenta *et al.*, 2001). In general, 1-MCP reduces respiration rates or delays increases in respiration. Respiration has been inhibited via treatment of 1-MCP in 'Fuji' (Fan and Mattheis, 1999b), 'Granny Smith' and 'Red Delicious' (Fan *et al.*, 1999).

The de-greening of 'Fuji' apples was inhibited by 1-MCP (Fan and Matthis, 1999b). Although 1-MCP-treated 'Red Chief' apples had a greener background color than untreated fruits, the chlorophyll fluorescence measurements indicated that loss of chloroplast function was largely independent of ethylene (Mir *et al.*, 2001).

The formation of volatile compounds in apples is differentially inhibited by 1-MCP (Fan and Matteis, 1999a). The 'Anna' apple treated with 1-MCP retained more volatiles associated with newly harvested apples and lesser amounts of volatiles associated with ripening apples. The untreated apples developed a more ripe and fruity volatile composition than the 1-MCP-treated apples (Lurie *et al.*, 2002). The total volatiles formation was inhibited in 'McIntosh' and 'Delicious' (Rupasinghe *et al.*, 2000).

The apples were shown to maintain their firmness after 1-MCP treatment, with data presented on 'Delicious', 'Granny Smith', 'Fuji', Ginger Gold', 'Gala', 'Idared', 'Jonagold', and 'McIntosh' (Rupasinghe *et al.*, 2000; Fan *et al.*, 1999a; Watkins *et al.*, 2000; Mir *et al.*, 2001). Moreover, 1-MCP treatment maintained apple firmness better than the controlled atmosphere (CA) storage (Mir *et al.*, 2001). In addition, Watkins *et al.* (2000) found that the combination of 1-MCP and CA was better than either one alone.

Watkins *et al.* (2000) found that the titratable acidity of the 'Law Rome', 'Delicious', 'Empire', and 'McIntosh' was always higher in 1-MCP-treated fruit during air storage. 1-MCP maintained the titratable acidity in 'Red Delicious', 'Granny Smith', 'Fuji', 'Jonagold', 'Ginger Gold', and 'Gala' apples (Fan *et al.*, 1999). In contrast, 1-MCP did not affect the titratable acidity in 'Red Chief' apples during storage at several temperatures (Mir *et al.*, 2001).

Soluble solids were higher in 1-MCP-treated apples (Fan *et al.*, 1999), but remained unaffected by 1-MCP in some cultivars (Rupasinghe *et al.*, 2000; DeEll *et al.*, 2002). The contrasting results among different apples are notable and may be due to different cultivars or varied experimental conditions used. Watkins *et al.* (2000) found differences in the response of apple cultivars, with 1-MCP-treated 'McIntosh' and 'Law Rome' fruits having lower soluble solids and 'Delicious' and 'Empire' having higher soluble solids than untreated fruits.

1.6 Purpose of research

'Fruit ripening' refers to the changes in the fruit that make it ready for consumption. In the apple fruit, such changes typically include starch hydrolysis, sugar accumulation, softening, and the decrease of organic acids and phenolic compounds. Anthocyanins and carotenoids often accumulate in the epidermis of fruits. Ripening occurrence is therefore important for fruit products as it also correlates with the development of eating quality. Ethylene has been known as the hormone that accelerates the ripening of fruit. Addition of ethylene to fruits increases ripening, and a dramatic increase in ethylene production is closely associated with the initiation of ripening.

In the apple fruit, starch is observed to be the major accumulated carbohydrate which is then degraded to provide sugars associated to fruit sweetness as fruit ripens. Ethylene has been reported to be involved in the ripening of the apple fruit; however, the action of ethylene on starch degradation and sugar accumulation during ripening is not clear. The response of fruits to ethylene at different growth stages with different levels of accumulated starch is not well-studied. In addition, the characteristics of starch degradation may vary according to cultivar variations and their physiological properties. Therefore, the relationship between fruit ripening and starch degradation characteristics, in relation to ethylene in fruit tissues at different developmental stages, and cultivars was studied in this research.

From this research, it is expected that the relationship between ethylene, 1-MCP treatments, and starch degradation will provide a clearer understanding about the effects of ethylene on the ripening and the characteristics of starch breakdown in apples. Additionally, the understanding of the effects of ethylene and 1-MCP on sugar accumulation provides basic data for further studies about starch to sugar conversion during ripening. It may be used in post-harvest practices to improve fruit eating quality. Moreover, changes in physiological properties, and chemical changes including amylose, amylopectin, and sugar content in fruit flesh during growth and maturation of apple fruits provide basic knowledge for further use not only for fruit physiologists and biochemists, but also for fruit growers.

1.7 Experimental scope

1.7.1 The effects of ethylene on ripening and starch degradation of two apple cultivars (cv. *Tsugaru* and *Fuji*), harvested at immature and mature stages, were evaluated. 1-methylcyclopropene (1-MCP) was used as an ethylene-action inhibitor (Chapter 2).

1.7.2 The effect of ethylene and 1-MCP on starch degradation pattern and sugar accumulation in each particular zone of fruit flesh and the physiological changes during storage of immature and mature 'Tsugaru' were examined (Chapter 3).

1.7.3 Characteristics of starch degradation including changes in amylose and amylopectin contents, and sugar accumulation in each particular flesh zone of on-tree apple fruits (cv. *Tsugaru*, *Golden Delicious*, *Fuji*, and *Orin*) were investigated during growth and maturation. Physiological aspects including respiration rate and ethylene production were also measured (Chapter 4).

CHAPTER 2

STARCH DEGRADATION OF DETACHED APPLE FRUIT IN RELATION TO RIPENING AND ETHYLENE

2.1 Introduction

Starch is the primary carbohydrate in unripe fruit. As fruit ripens, starch is degraded, and the sugar content (soluble solid concentration) increases, providing the sweetness associated with the taste of ripe fruit (Blankenship and Unrath, 1988; Brookfield et al., 1997; Dinar and Stevens, 1981; Prabha and Bhagyalakshmi, 1998). Additionally, sweetness is one of the important attributes in apples, affecting the final flavor and increasing the fruit quality and its price; therefore, starch accumulation during growth, and starch degradation during ripening are important phenomena.

The net loss of starch starts at the beginning of the fruit ripening process on and off the tree (Brookfield et al., 1997; Lau, 1988; Magein and Leurquin, 2000). After harvesting apple fruit, a rapid increase in ethylene production and the respiration rate of the fruit is simultaneously observed with the loss of the starch content. In addition, ethylene has been suggested to be involved in stimulating the conversion of starch to sugar (Kader, 1985; Watkins, 2003); however, Blankenship and Unrath (1988) reported that the starch conversion process of 'Golden Delicious' was independent of ethylene production. While there are some studies of the physiology of starch degradation, the relationship between starch degradation and other physiological changes, such as respiration and ethylene production, is still unclear. Moreover, further research is required to clarify the role of ethylene in starch degradation during the development of apple fruit.

The ethylene action inhibitor, 1-methylcyclopropene (1-MCP) (Sisler and Serek, 1997), has been shown to increase post-harvest life and maintain fruit quality. It reduces ethylene production and the respiration rate, maintains fruit firmness, and reduces the loss of starch content in apples (Fan et al., 1999; Pre-Aymard et al., 2003). Although there are studies showing that 1-MCP might have inhibitory effects on the starch degradation process during ripening by blocking ethylene action, there is still debate about the effect of 1-MCP on the starch metabolism of apple fruit.

The objective of this study was to investigate the physiology of starch degradation in relation to ripening and ethylene in 'Tsugaru', which produces high amounts of ethylene, and 'Fuji', which produces very low amounts of ethylene.

2.2 Materials and Methods

Apple fruits

Two cultivars of apples, early-maturing 'Tsugaru' fruit from a 5-year-old tree and late-maturing 'Fuji' fruit from a 30-year-old tree grafted on Marubakaido (*Malus prunifolia* Borkh.) rootstock, were obtained from the experimental orchard of the Faculty of Agriculture and Life Science, Hirosaki University, Japan. Fruits of each cultivar were harvested at two maturing stages. Immature fruits were harvested at 70 days after full bloom (DAFB) for 'Tsugaru' and 116 DAFB for 'Fuji'. Mature fruits were harvested at 106 DAFB for 'Tsugaru' and 148 DAFB for 'Fuji'. The fruits of each harvesting crop were separated into three groups for treatment with ethylene or 1-MCP, and a control.

Treatments

For ethylene treatment, pure ethylene gas (2.8 mL, GL Sciences Inc., Japan) was injected into a closed container (28 L) to produce a final concentration of 100 $\mu L \cdot L^{-1}$ of ethylene. The container was then kept at 25°C for 24 h. For 1-MCP treatment, 1 g of SmartFresh™ (0.14% A.I., Rohm and Haas, Japan) powder was placed in a flask inside a container and 25 mL distilled water was then added with a syringe through the cap and a rubber hose into the flask, which produced 22.3 $\mu L \cdot L^{-1}$ of 1-MCP. The fruit was treated with 1-MCP at 25℃ for 24 h. After the treatments, all fruits were kept at 25°C in a storage room for ripening.

Measurements

1. Physiological Analyses

To determine CO_2 production, each fruit was weighed and sealed in 1.4 L plastic boxes for 2 h, and 1 mL of headspace gas was injected into a gas chromatograph (model GC-18A, Shimadzu, Kyoto, Japan) equipped with a molecular sieve column (60/80 mesh, GL Sciences, Tokyo, Japan), and a thermal conductivity detector. Helium was the carrier gas. The injector, oven, and detector temperatures were set at 60℃. To determine the ethylene content, 1 mL of the headspace gas was removed and injected into a gas chromatograph (model GC-8A, Shimadzu, Kyoto, Japan) equipped with an activated alumina column (30/60 mesh, GL Sciences, Tokyo, Japan) and flame ionization detector. Nitrogen was the carrier gas. The injector, oven, and detector temperatures were set at 120, 100, and 120℃, respectively.

2. Chemical Analysis

The fruit flesh was sampled, immediately frozen in liquid nitrogen, and kept at –80°C until starch analysis. The method of determination was that of Pharr and Sox (1984) and Wang et al. (1997). Briefly, 200 mg of the sample was ground and extracted three times with 4 mL of 80% (v/v) ethanol at 80°C, each for 30 min, and then the suspension was centrifuged. The remaining pellet was dried and used for starch determination. Two milliliters of 0.2 M KOH was added to the pellet and the suspension was boiled for 30 min. After cooling to room temperature, the pH of the suspension was adjusted to 4.5 with 1 M CH_3COOH and then 600 units of amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3) (Sigma Chemical Co., MO, USA) dissolved in 0.2 M NaAc-HAc buffer was added to hydrolyze the starch to glucose by incubation in a 55°C water bath for 1 h. The reaction was stopped by immersing the sample tubes in boiling water for 10 min, and the digest was then centrifuged. The supernatant was filtered, and injected into an HPLC for glucose determination. The HPLC was operated under the following conditions: column: SCR101C (7.9 I.D. × 300 mm, Shimadzu LC, Shimadzu Co., Ltd.); mobile phase: water 0.5 $mL \cdot min^{-1}$; post column mixture: 1 M NaOH, 0.25 $mL \cdot min^{-1}$; column temperature: 80°C, detector: PAD.

Data analysis

Analysis of Variance (ANOVA) with Completely Randomized Design (CRD) using chemical treatments as a factor was performed using SPSS (SPSS, IL, USA), and Tukey's multiple-range test was used to test significant difference at the 95% confidence level of each variable.

2.3 Results

The effect of ethylene and 1-MCP on starch degradation in immature apple fruit

The loss of starch content in immature 'Tsugaru' occurred over 4-day storage, and almost all of the starch in fruits of all treatments was degraded within 8-day storage. The starch content of ethylene-treated fruit was lower than the control on day 4 after harvest with significant difference ($P \leq 0.05$); however, no difference in starch content between 1-MCP-treated fruit and the control was observed (Fig. 2.1A). The respiration rate decreased, remaining low during storage with no obvious differences among treatments (Fig. 2.1B). Ethylene production in fruits of all treatments was less than 0.005 $\mu L \cdot L^{-1}$ during storage (data not shown).

In immature 'Fuji', the starch content decreased in proportion to the storage time. The starch degradation of 1-MCP-treated fruits was consistently low, but it did not definitely differ from the control (Fig. 2.2A). The respiration rate of all the fruits decreased during storage, with 1-MCP treatment being lowest at days 4-8. There was no difference in the respiration rate between ethylene-treated fruit and the control (Fig. 2.2B). Ethylene production was too low to calculate reliably with our measuring system during investigation of the fruits of all treatments.

The starch content of the immature 'Tsugaru' positively correlated with the respiration rate at $r = 0.798$ (Table 2.1). Also, in the immature 'Fuji', the respiration rate showed a significant correlation with the starch content ($r = 0.912$).

The effect of ethylene and 1-MCP on starch degradation in mature apple fruit

The starch content of ethylene-treated 'Tsugaru' fruit decreased from day 4 to day 12, and was significantly different from 1-MCP-treated fruit ($P \leq 0.05$). Ethylene treatment enhanced starch degradation and lower starch content was observed in ethylene-treated fruit on day 4. The remaining starch content of 1-MCP-treated fruit was significantly higher than the control fruit, while the loss of starch in 1-MCP-treated fruit was observed during the investigation (Fig. 2.3A). Ethylene treatment promoted ethylene production and the respiration rate on days 4-8. 1-MCP treatment completely inhibited both changes on days 8-12 (Fig. 2.3B, C; $P \leq 0.05$).

In mature 'Fuji', the starch content drastically decreased 4 days after treatment. Although the starch content of 1-MCP-treated fruit was slightly higher than the control on day 8, it did not differ from the control on day 4 or 12. The starch content of ethylene-treated fruit was not significantly different from the control during the experiment (Fig. 2.4A). The ethylene production of 1-MCP-treated fruit remained low. It was significantly lower than the control fruit on day 8, but there was no difference when comparing ethylene treatment with the control (Fig. 2.4B). The respiration rate increased in the control and ethylene-treated fruit; however, no difference between these treatments was observed (Fig. 4C; $P \leq 0.05$).

The increased ethylene production of the mature 'Tsugaru' obviously correlated with the starch content (Table 2.1; $r = -0.698$). In addition, the starch content and a significant negative correlation with the respiration rate was observed (Table 2.1; $r = -0.783$). However, in the mature 'Fuji', the correlation coefficients between the starch content and ethylene production or respiration rate were very low.

2.4 Discussion and conclusion

The starch content decreases in association with fruit ripening on and off the tree (Brookfield et al., 1997; Lau, 1988; Magein and Leurquin, 2000; Watkins, 2003), and the starch content of the fruit is used as a maturity index of apple fruit. The loss of apple starch seems highly related to fruit climacteric and physiological changes such as increasing ethylene production and respiration rate; however, there is no clear report examining how the maturity of fruit affects starch degradation and fruit ripening physiology.

In the case of immature fruit, the starch of 'Tsugaru' and 'Fuji' rapidly decreased after harvest, while respiration gradually fell and ethylene production was low. Moreover, the effects of ethylene and 1-MCP on starch degradation were very small; the starch disappeared within 8 days after harvest for 'Tsugaru' and 12 days for 'Fuji', irrespective of the treatment. Although the fruit at this stage produced a low basal rate of ethylene, 1-MCP had no effect on starch degradation. These results showed that starch degradation at this stage did not relate to the climacteric of the fruit and ethylene. It is also supported by the results of immature 'Jonagold', a mid-maturing cultivar, degradation of starch and changes of respiration and ethylene production was not induced by treatments of ethylene or 1-MCP (data not shown). Thus, it seems that immature fruit irrespective of cultivars could not respond to endogenous and exogenous ethylene.

Although it has been reported that the respiration rate of immature fruit decreases after harvest (McGlasson and Pratt, 1964; Zauberman and Schiffmann-Nadel; 1972); there is no clear explanation for this change. As fruits treated with ethylene had a higher respiration rate and lower starch content, it seems that starch degradation is partially related with respiration.

For mature fruit, the relation between starch degradation and the climacteric was different between 'Tsugaru' and 'Fuji'. In mature 'Tsugaru', ethylene treatment promoted the respiration rate and ethylene production, and 1-MCP treatment retarded both changes. In 'Fuji', although the respiration rate was inhibited by 1-MCP treatment, ethylene treatment had no effect on the production of ethylene and respiration. Moreover, although 1-MCP treatment clearly retarded starch degradation in 'Tsugaru', the effect was very low in 'Fuji'. It was suggested that starch degradation in 'Tsugaru' was related to the fruit climacteric, and ethylene has an important role in this metabolism. It seems that ethylene is not involved in the starch degradation of 'Fuji'. The starch content of 'Tsugaru' increased with the growth of fruit, and the level of starch in 'Tsugaru' was much higher than that of 'Fuji'. The different efficacy of 1-MCP on starch degradation between mature 'Tsugaru' and 'Fuji' might be due to this different starch level, but further research is needed.

Blankenship and Unrath (1988) found that starch degradation started before the increase in the internal ethylene concentration and suggested that ethylene is not the only factor inducing fruit ripening. Autio and Bramlage (1982) reported that pre-harvest treatment of the inhibitor of ethylene synthesis (AVG) in 'Early McIntosh', 'McIntosh', 'Cortland', and 'Royal Red Delicious' fruits retarded ethylene production, but there was no difference in the ripening aspects, including the starch content. Although 1-MCP strongly inhibits ethylene action, it has no effect on starch degradation during storage (Pre-Aymard et al., 2003; Rupasinghe et al., 2000; Watkins, 2006). Although these results suggested that ethylene plays a small role in starch degradation, results of this study showed that ethylene is partially involved in starch degradation in mature 'Tsugaru'.

It is well known that changes in the respiration rate and ethylene production are different among cultivars. While 'Tsugaru' produces a high amount of ethylene during ripening, 'Fuji' is known as a cultivar which produces a small amount of ethylene during ripening on the tree. It can be presumed that the role of ethylene in fruit ripening physiology, such as starch degradation, might differ between 'Tsugaru' and 'Fuji', because the starch content decreases during ripening before harvest in both cultivars.

It has been suggested that the loss of starch in the apple fruits is based on the action of enzymes, such as amylase (Beck and Ziegler, 1989; Frenkel et al., 1968; Garcia and Lajolo, 1988; Jackson, 2003; Zhang and Wang, 2002); however, the factors inducing the gene expression of the enzymes and enzyme activities are still unknown. From this study, it is particularly interesting that the degradation of starch during the immature stage occurred without increases in the respiration rate and ethylene production. It seems that the detachment of fruit from the tree and cessation of carbohydrate translocation into the fruit induced the physiological change and starch degradation (Irving et al., 1999). While the mechanism is not known, it has long been recognized that the tree factor including genetic control and enzyme action influences fruit ripening and may also trigger the onset of starch degradation.

2.5 Summary

The physiology of starch degradation in relation to ripening and ethylene was investigated using 'Tsugaru' (early-maturing) and 'Fuji' (late-maturing) apples (*Malus domestica* Borkh.). Fruits were harvested at immature and mature stages, and treated with ethylene and 1-methylcyclopropene (1-MCP). In immature fruit of both cultivars, starch content rapidly decreased during storage at 25℃, and 1-MCP had little effect on this change. Ethylene treatment slightly stimulated the degradation of starch, but differences in starch among treatments were small. The respiration rate gradually decreased and ethylene production remained low during storage irrespective of the treatments and cultivars. These results showed that fruit at this stage could not respond to endogenous and exogenous ethylene for inducing the climacteric, and starch degradation did not relate to the climacteric or ethylene. In mature 'Tsugaru', 1-MCP treatment significantly inhibited ethylene production and reduced the respiration rate and starch degradation. The effects of 1-MCP and ethylene on starch degradation in mature 'Fuji' were small, and starch content decreased drastically in all treatments, although 1-MCP significantly inhibited ethylene production and the respiration rate. It is suggested that ethylene is partially involved in starch degradation in mature 'Tsugaru', but not in 'Fuji'. These results showed that the role of ethylene in starch degradation differs between cultivars and their harvested stages, relating to ripening and physiological characteristics of the fruit.

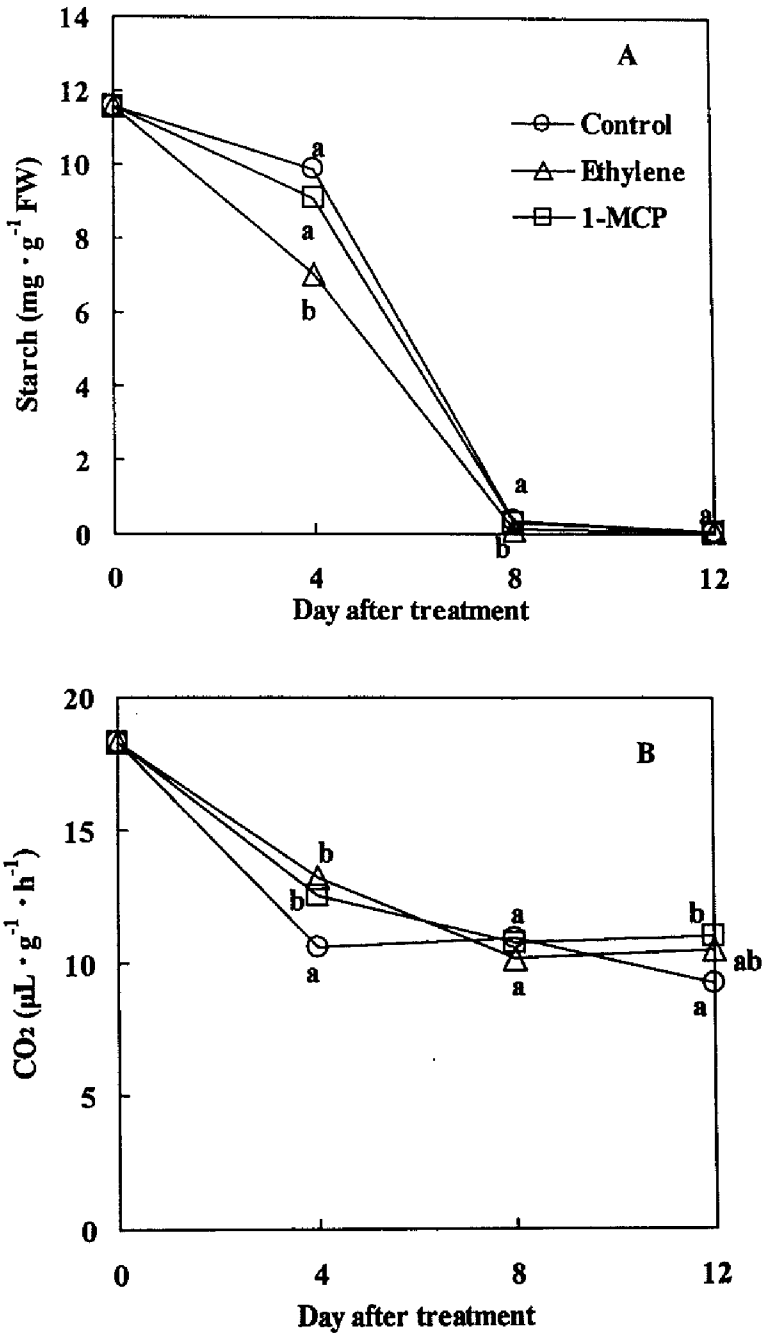

Fig. 2.1 Effects of ethylene and 1-MCP treatments on the starch content (A) and respiration rate (B) of immature 'Tsugaru' apples. Each value is the mean of five replicates. Means with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

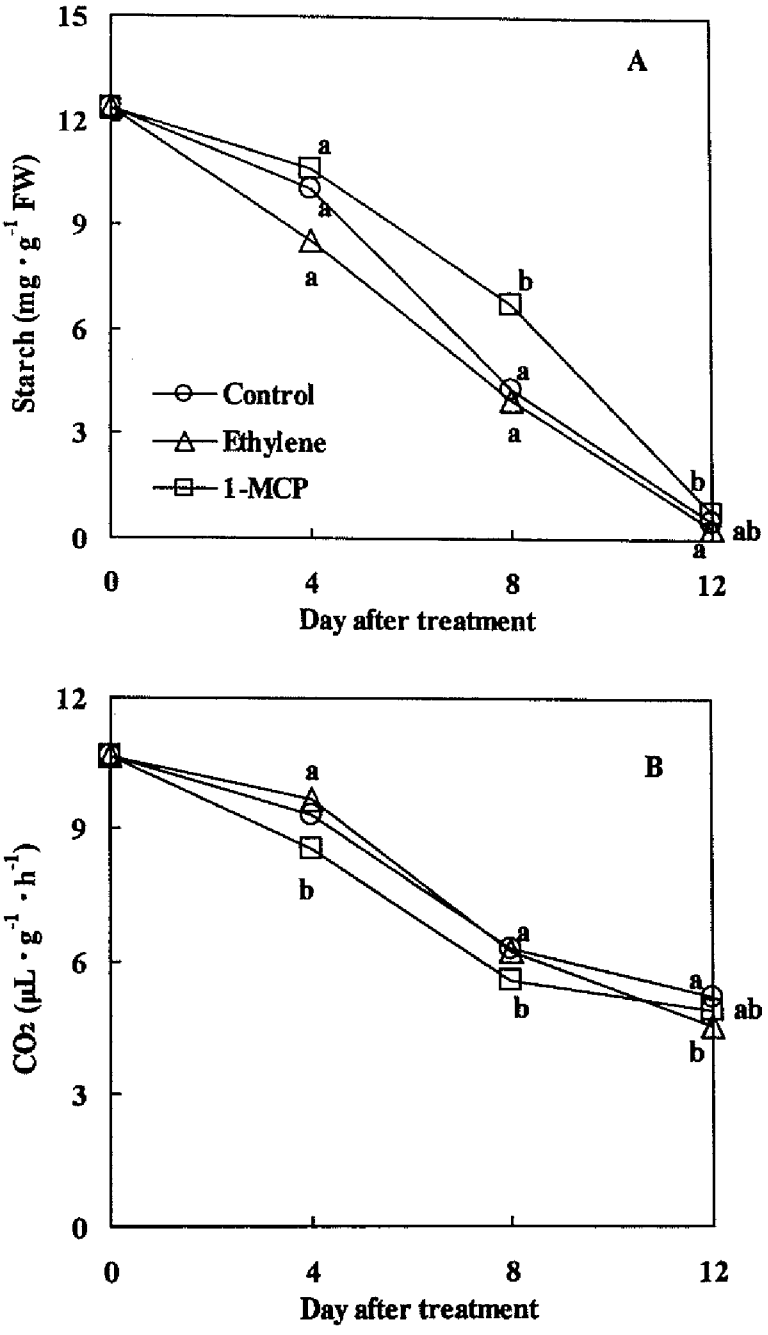

Fig. 2.2 Effects of ethylene and 1-MCP treatments on the starch content (A) and respiration rate (B) of immature 'Fuji' apples. Each value is the mean of five replicates. Means with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

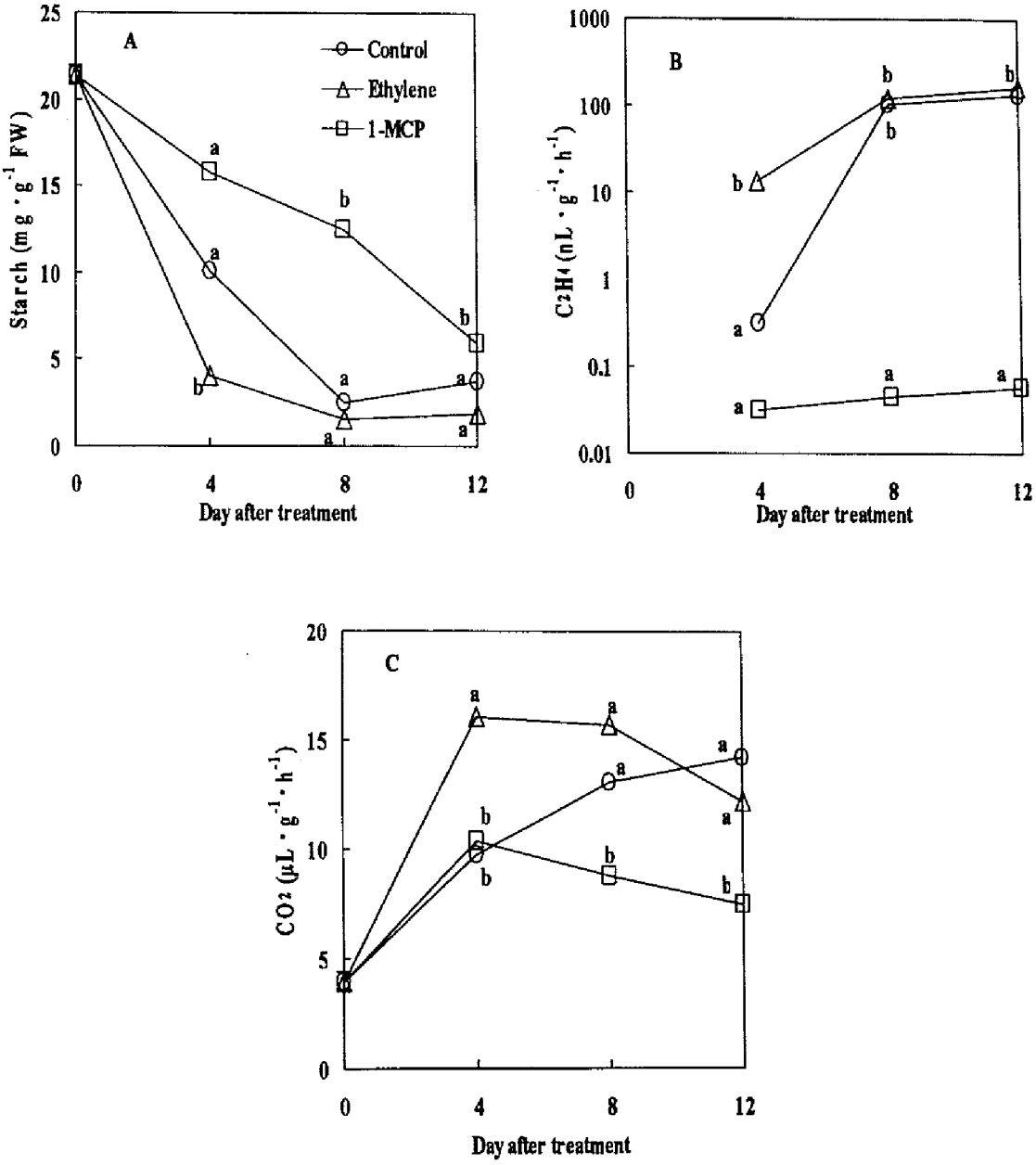

Fig. 2.3 Effects of ethylene and 1-MCP treatments on the starch content (A), ethylene production (B), and respiration rate (C) of mature 'Tsugaru' apples. Each value is the mean of four replicates. Means with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

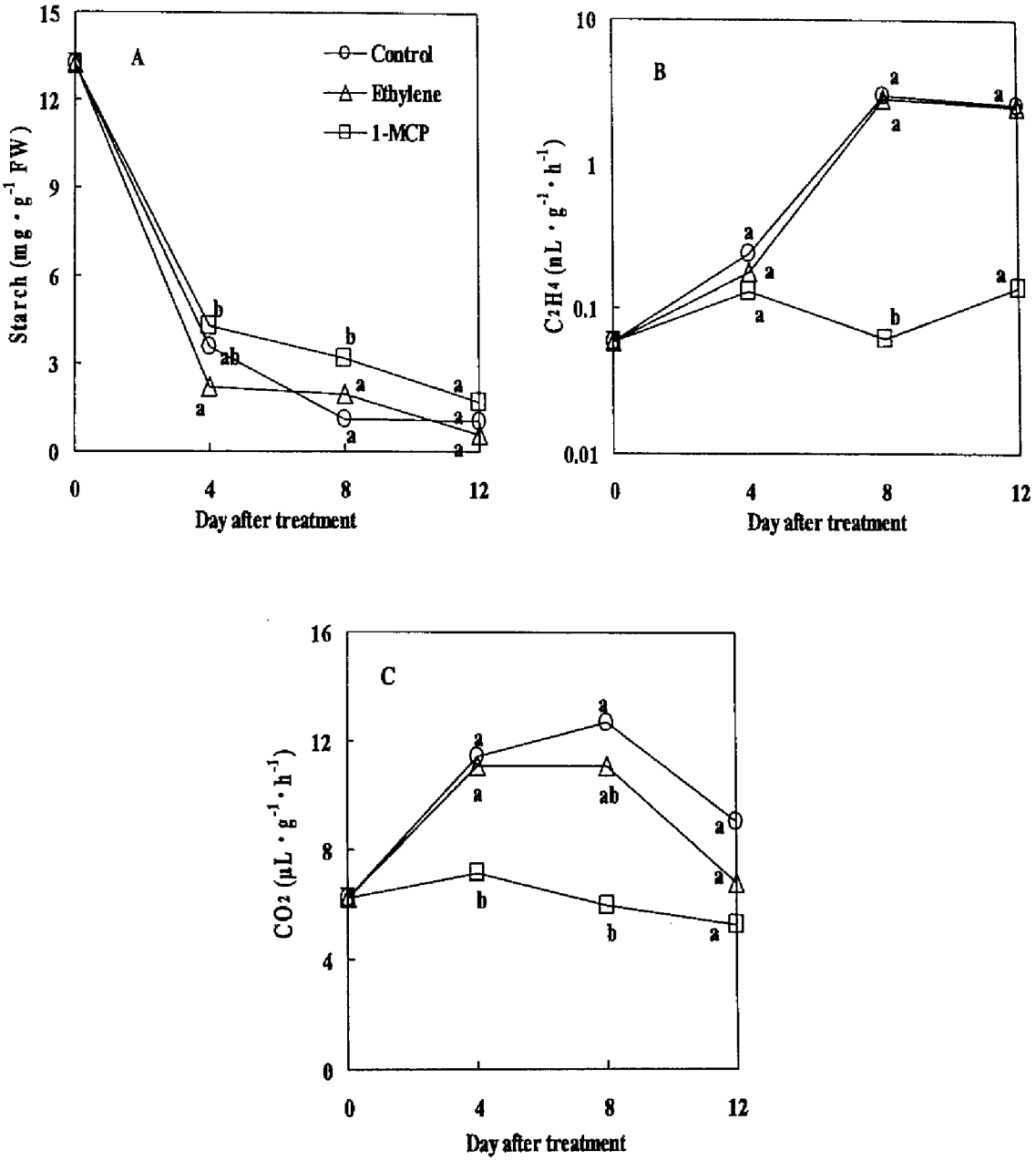

Fig. 2.4 Effects of ethylene and 1-MCP treatments on the starch content (A), ethylene production (B), and respiration rate (C) of mature 'Fuji' apples. Each value is the mean of four replicates. Means with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

Table 2.1 Correlation coefficients of starch content, respiration rate and ethylene production in 'Tsugaru' and 'Fuji'.

Stage	Cultivar	Respiration rate	Ethylene production
Immature	'Tsugaru'	0.798**	0.237
	'Fuji'	0.912**	-
Mature	'Tsugaru'	-0.783**	-0.698**
	'Fuji'	-0.376	-0.371

** indicates the significant difference at $P \leq 0.01$.

CHAPTER 3

STARCH TO SUGAR CONVERSION OF TWO DIFFERENT MATURATION STAGES OF 'TSUGARU' APPLE FRUIT IN RELATION TO ETHYLENE AND 1-METHYLCYCLOPROPENE

3.1 Introduction

Fruit sweetness is one of the major determinants of fruit quality and also in the assessment of its market value. It reflects the concentration of sucrose, glucose, fructose, and sorbitol in the fruit flesh. In the early stages of maturation, accumulated starch is progressively degraded to increase sweetness, thus affecting fruit taste during ripening (Brookfield et al., 1997; Lau, 1988; Magein and Leurquin, 2000; Warrington, *et al.*, 1999). Until now, ripening and starch to sugar conversion process has not been clearly reported. As the apple is generally harvested at the mature stage and it ripens up until the time of consumption, the changes in starch and sugar compositions during the ripening process after harvest may greatly affect the sweetness of the fruit. In addition, when sugar translocation has ceased after harvest, sweetness quality of the detached fruit seems to be influenced mainly by accumulated starch in fruit cells and accumulated cellular sugar at the time of harvest.

Ethylene has been suggested to stimulate the conversion of starch to sugar (Kader, 1985; Watkins, 2003). Additionally, 1-MCP has been recommended as a valuable tool for post-harvest handling (Fan et al., 1999; Pre-Aymard et al., 2003). Ethylene and 1-MCP have been shown to affect starch degradation and physiological properties during storage according to cultivars and their harvesting dates, as described in Chapter 2.

However, the effects of ethylene and 1-MCP on fruit sweetness and individual sugar content of the detached fruit flesh during ripening has not been well-studied. In addition, a study on the changes in sugar content of the detached apple fruit may provide more understanding on starch degradation and also increase knowledge helpful for further fruit quality improvement and commercial use. Therefore, in this study, the changes of sugar concentration in different flesh zones of the fruit treated with ethylene or 1-MCP were investigated. 'Tsugaru' fruits harvested at two different developmental stages were used, as they were shown to respond well to ethylene and 1-MCP and had the most the predominant simultaneous increase of sugars with starch degradation as observed in a previous study (Chapter 2).

3.2 Materials and Methods

Apple fruits

'Tsugaru' fruits were obtained from the experimental orchard of the Faculty of Agriculture and Life Science, Hirosaki University. Fruits were harvested at two maturing stages in the year 2006 on August 8 (80 days after full bloom (DAFB)), and September 7 (110 DAFB). The fruits of each harvest crop were separated into three groups for treatment with ethylene or 1-MCP, and the control.

Treatments

For the ethylene treatment, pure ethylene gas (4.0 mL, GL Sciences Inc., Japan) was injected into a closed container (40 L) to produce a final concentration of 100 $\mu L \cdot L^{-1}$. The container was then kept at 25°C for 24 h. For the 1-MCP treatment, 0.13 g of SmartFresh™ (0.14% A.I., Rohm and Haas Co., Japan) powder was placed in a flask within a container and 25 mL distilled water

was then added with a syringe through the cap and rubber hose into the flask, which produced 2 $\mu L \cdot L^{-1}$ of 1-MCP. The fruit was treated with 1-MCP at 25℃ for 24 h. After the treatments, all fruits were kept at 25℃ in a storage room for ripening.

Measurements

1. Respiration rate, ethylene production, and starch rating

The determination of CO_2 production and ethylene content was done with the methods described in Chapter 2.

Starch distribution was measured by dipping an apple slice taken from the equatorial region in I_2-KI solution (10 g/25 g in 1 L distilled water), and the starch-iodine rating was done using the generic starch-iodine index chart for comparison (Watkins, 2003). This method uses a 1 to 8 scale, with 1 = all starch and 8 = no starch.

2. Sugar content

For sugar determination, 100 mg of dried sample was extracted three times, each 20 min, with 2 mL of 80% (v/v) ethanol at 80℃. The homogenates were centrifuged at 15,000×g for 10 min to give ethanol-soluble and ethanol-insoluble fractions. The ethanol soluble fractions were pooled and evaporated to dryness with a concentrator and resolublized in 2 mL of de-ionized water. The soluble fraction was then filtered. Glucose, fructose, sucrose, and sorbitol were separated with a high-performance liquid chromatograph (HPLC) (Shimadzu, Tokyo, Japan). De-gassed, distilled, de-ionized water at 1 $mL \cdot min^{-1}$ at 80℃ was used as the mobile phase. A refractive index detector (RID-10A; Shimadzu) was used to quantify sugar content following the separation. Recovery rate was determined by comparison with standard samples of known concentration of glucose, fructose, sucrose, and sorbitol.

Data analysis

Data analysis was done as described in Chapter 2.

3.3 Results

Starch rating and iodine staining

The determination of starch content by starch rating values and iodine staining in the flesh of immature 'Tsugaru' is shown in Fig. 3.1. From the starch rating values, starch degradation was observed in all fruits. Starch loss of the ethylene-treated fruit was significantly greater than the control and the 1-MCP-treated fruit on day 7-10 after treatment. Starch loss of the 1-MCP-treated fruit did not differ from the control (Fig. 3.1A). Iodine staining also showed rapid loss of starch in the fruit treated with ethylene (Fig. 3.1D).

For the mature 'Tsugaru', the loss of starch in the fruit treated with ethylene was greater than the other treatment. However, it did not differ from the control at day 10 (Fig. 3.2A). Although there was no difference in the starch rating of 1-MCP treatment and control on day 4 and 10 statistically, iodine staining showed a greater amount of remaining starch in the fruit treated with 1-MCP compared to the other two treatments (Fig. 3.2C).

Physiological aspects

Respiration rate of all immature 'Tsugaru' fruits decreased during storage, irrespective of treatments, and the difference among treatments was not clear (Fig. 3.3). Ethylene production of immature fruits was too low to be determined accurately with our measuring system in this investigation for all fruits.

In the mature fruits, respiration rate of 1-MCP-treated fruit decreased slightly and was the lowest in this investigation. The respiration rate of the ethylene-treated fruit and control increased between days 4-7 and then dropped at day 10. There was no difference in respiration rates between control and ethylene treatment groups (Fig. 3.4A). Although ethylene production of the

ethylene-treated fruit increased between days 4-10, it was not significantly different from the control at day 4 and 10. Production of ethylene in the 1-MCP-treated fruit was observed to be at a low level and was significantly lower than ethylene treatment and control (Fig. 3.4B).

Sugar contents

In the immature 'Tsugaru' fruit, total sugar content in fruits of all treatments increased slightly during storage. However, differences among flesh zones were small. The amount of total sugar in all zones of the ethylene-treated fruit increased significantly at day 4 while there was no observable difference from day 0 in both control and 1-MCP (Fig. 3.5). For individual sugar contents, sucrose content of the control decreased slightly at day 4, and then increased greatly at day 7 and 10 (Fig. 3.6). Sucrose content of the ethylene-treated and 1-MCP-treated fruits at day 7-10 seemed not different from day 0; however sucrose content was lower than in control. The glucose content of all fruits obviously increased during storage from day 0-10, with the highest amount in the inner cortex (Fig. 3.7). There was no observable difference in the fructose content among treatments and fructose content was stable throughout the investigation period (Fig. 3.8). Sorbitol content was observed to be at a low level in the tissues of all fruits. However, a distinct increase in sorbitol content was found in the fruit treated with ethylene at day 10 after treatment (Fig. 3.9).

Sugar contents of the mature 'Tsugaru' are shown in Fig. 3.10 to Fig. 3.14. The total sugar content of the control increased between days 4-7, but decreased at day 10 back to a level similar to day 0 (Fig. 3.10). Total sugar content in fruits treated with 1-MCP or ethylene increased slightly at day 4, but dropped sharply between days 7-10. For the individual sugars, an increase in sucrose content was observed between days 4-7. However, the increase of sucrose content in the 1-MCP treated fruit was significantly inhibited between

days 7-10 (Fig. 3.11). The glucose content of all treatments increased between days 4-10 and was highest in the inner tissue zone (Fig. 3.12). There was an inhibitory effect of ethylene and 1-MCP on glucose accumulation on day 7 and 10, respectively. However, the glucose content was generally higher than day 0. The increase in fructose content was found between days 4-7, followed by a decrease at day 10 in all tissue zones in the control. On the other hand, fructose content of 1-MCP and ethylene treatment changed slightly during storage and ethylene seemed to decrease the fructose content of all tissues between days 7-10 (Fig. 3.13). The sorbitol content of all tissues was very low during investigation period and differences among treatments were small (Fig. 3.14).

3.4 Discussion and conclusion

As starch degradation occurs when the fruit ripens, the level of sugar accumulation in the mature fruit was observed to be higher than in the immature one. Ethylene has been suggested to be involved in the starch to sugar conversion (Kader, 1985; Watkins, 2003) and it has been demonstrated that ethylene induced starch degradation of the apple fruit differently according to cultivars and their harvested stages (Thammawong and Arakawa, 2007). However, there are few reports that examined the relationship between sugar accumulations in each particular fruit flesh zone and the occurrence of starch degradation in the detached fruit. Moreover, the effects of 1-MCP and ethylene on the starch to sugar conversion in fruit cells still need to be clarified.

In immature 'Tsugaru', changes of physiological aspects including respiration rate and ethylene production were not induced by ethylene. 1-MCP treatment also did not inhibit starch degradation nor respiration rate (Fig. 3.1, 3.3). The total sugar content changed slightly during degradation of starch and

there was little difference among treatments. Although the results show that glucose content of the ethylene-treated fruit was higher than other treatments after harvesting, there was no increase in respiration and ethylene production observed. These results supported earlier conclusion that immature fruits do not respond to exogenous ethylene used for inducing fruit climacteric, and that starch degradation including sugar accumulation do not relate to the climacteric or ethylene (Chapter 2). Although the starch degradation metabolism of immature 'Tsugaru' does not seem to be induced by ethylene, the results of this study suggest that the degradation of accumulated starch in the immature fruit provides glucose as a main product during storage.

For mature 'Tsugaru', a significant inhibitory effect of 1-MCP on starch degradation and physiological aspects of the apple was observed in this study. As 1-MCP has been shown to increase post-harvest life and maintain fruit quality (Fan et al., 1999; Pre-Aymard et al., 2003), previous studies had suggested that treatment of 1-MCP inhibits ripening of the apple fruit by preventing or delaying the increase in ethylene production associated with the climacteric ripening stage (Dauny and Joyce, 2002; Defilippi et al., 2004; Fan et al., 1999; Moran and McManus, 2005; Rupasinghe et al., 2000). Inhibitory effects of ethylene and 1-MCP were also found in the accumulation of sucrose and fructose. Although it has been reported that 1-MCP has an effect on soluble solid concentrations (SSC) in apple fruits, SSC in fruits treated with 1-MCP can be lower, higher or equal to those in untreated fruits (Bai et al., 2002; Dauny and Joyce, 2002; DeEll et al., 2002; Fan et al., 1999; Moran and McManus, 2005; Saftner et al., 2003; Watkins et al., 2000). Additionally, it is particularly interesting to note that although there was an increase in physiological aspects such as respiration rate and ethylene production simultaneously with starch loss in fruits treated with ethylene; this did not induce accumulation of sugar components.

From these results, there was no increase in total sugar content in the immature fruit although degradation of starch was observed during storage. In addition, although there was an increase in total sugars between days 4-7 in the mature fruit, it dropped at day 10. Since living cells of harvested fruits respire continuously, the ability to respire is an essential component of metabolic processes that occur in live products (Kays, 1991). From a postharvest point of view, respiration is a central process that mediates the release of energy through the breakdown of carbon compounds and the formation of carbon skeletons is necessary for maintenance and synthetic reaction after harvest. In the mature fruit, growth is decreased and it proceeds to ripening and senescence stages after harvest. Therefore, sugars from starch degradation are important for determining fruit flavor and might be greatly utilized in respiration and cell maintenance. However, a high rate of cell elongation and expansion is observed in immature fruits (Ryugo, 1988). Kays (1991) also suggested that during rapid growth of plant products, hexose sugars are often not completely oxidized to carbon dioxide but only proceed partway through the respiratory system pathway, yielding carbon skeletons. All these suggest that sugar products from the degradation of accumulated starch in immature fruits might be used mainly as respiratory substrates to produce energy and carbon skeletons that can be used in other synthetic reactions in the continuation of cell growth after harvest.

On the overall, physiological aspects of the ripening process in detached apple fruits responded to treatment of ethylene and 1-MCP differently, in accordance to the maturation stage at harvest. Moreover, during degradation of starch, accumulation of sugars in the fruit during storage was not induced by the actions of ethylene or 1-MCP.

3.5 Summary

A study was done to evaluate the effects of ethylene and 1-MCP on sugar accumulation during storage of 'Tsugaru' apples in the ripening process. Fruits were harvested at immature and mature stages, and treated with ethylene and 1-methylcyclopropene (1-MCP). Sucrose, glucose, fructose, and sorbitol contents in different flesh zones indicated as inner, middle, and outer tissue zones were determined. In immature 'Tsugaru', degradation of starch seemed to mainly produce glucose. However, any difference in the sugar contents between ethylene-treated and untreated fruit was observed only at day 4 after treatment and total sugar content (sucrose, glucose, and fructose) of all tissue zones changed only slightly during storage. In addition, physiological aspects of the ripening process in the immature fruit were not affected by ethylene and 1-MCP. This supports previous suggestions that the physiological aspects and accumulation of sugar in the immature fruit were not affected by ethylene and 1-MCP. In the mature fruit, ripening aspects were inhibited by 1-MCP; however it seemed to reduce the accumulation of sugar only at day 7 after treatment. Additionally, although there was an increase in changes of starch loss and physiological aspects of fruits treated with ethylene, the sugar content changed slightly between day 4-7 and then dropped at day 10. Moreover, exogenous ethylene did not induce sugar accumulation of mature 'Tsugaru' fruit during storage.

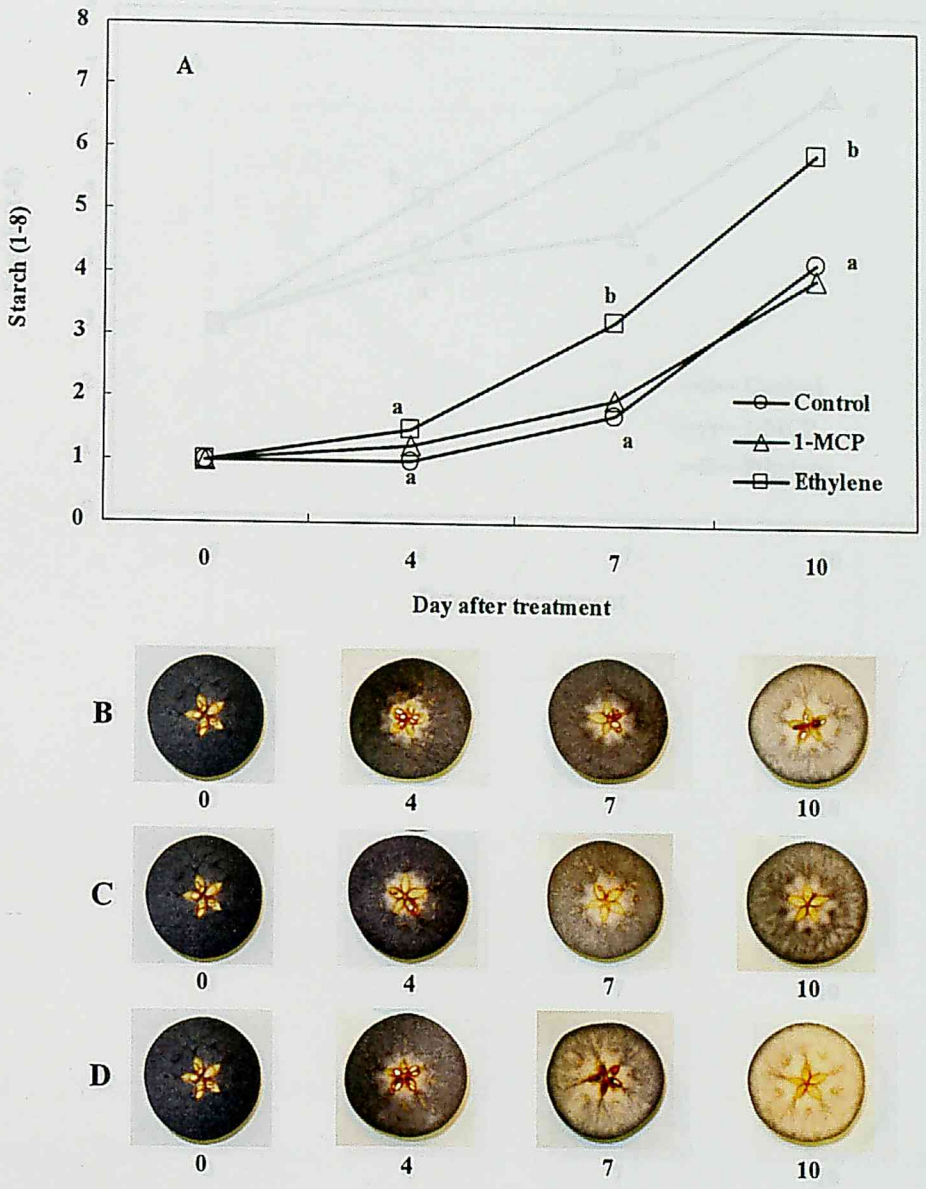

Fig. 3.1 Starch rating scores (A) and iodine staining of immature 'Tsugaru' (harvested at 80 DAFB); control (B), 1-MCP treatment (C), and ethylene treatment (D) during storage at 25℃.

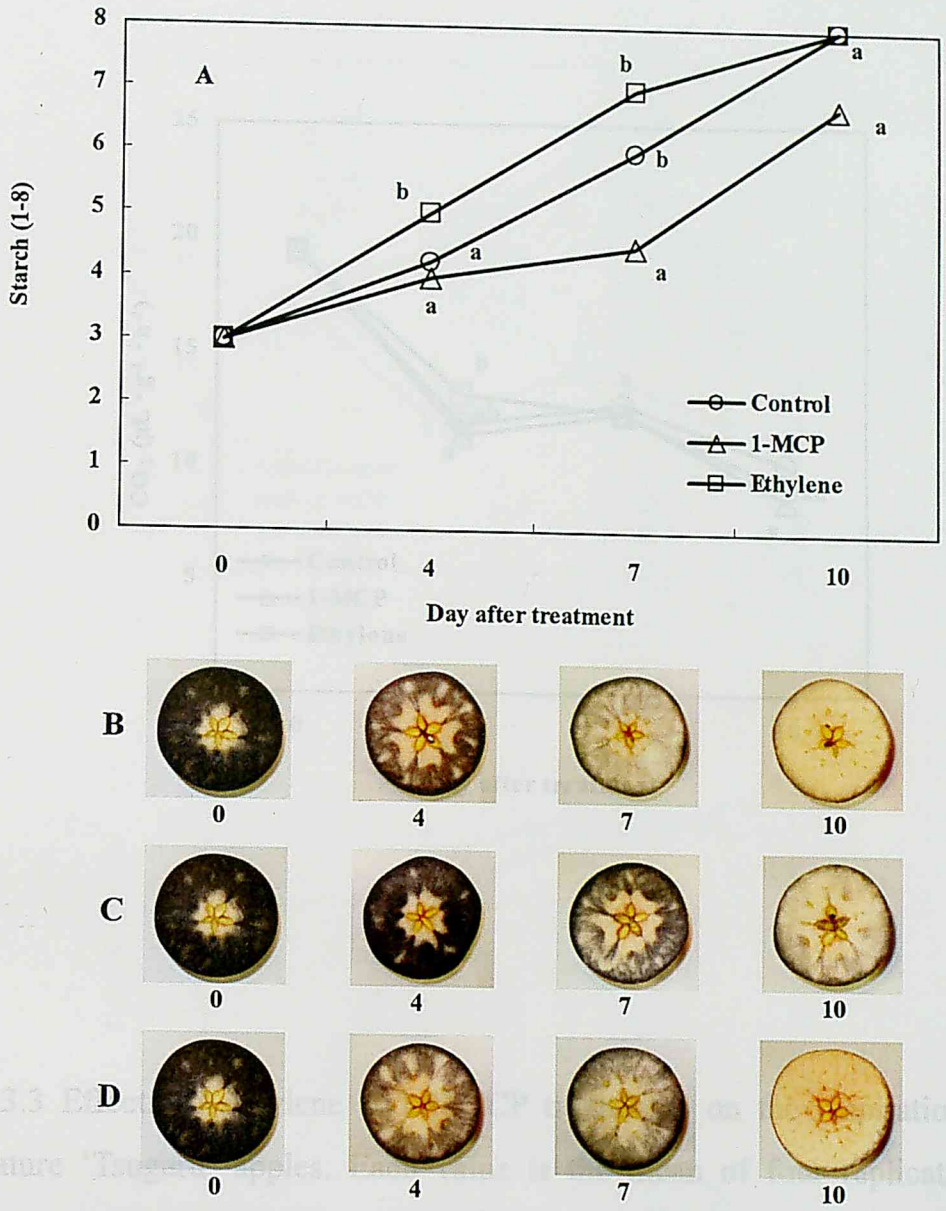

Fig. 3.2 Starch rating scores (A) and iodine staining of mature 'Tsugaru' (harvested at 110 DAFB); control (B), 1-MCP treatment (C), and ethylene treatment (D) during storage at 25℃.

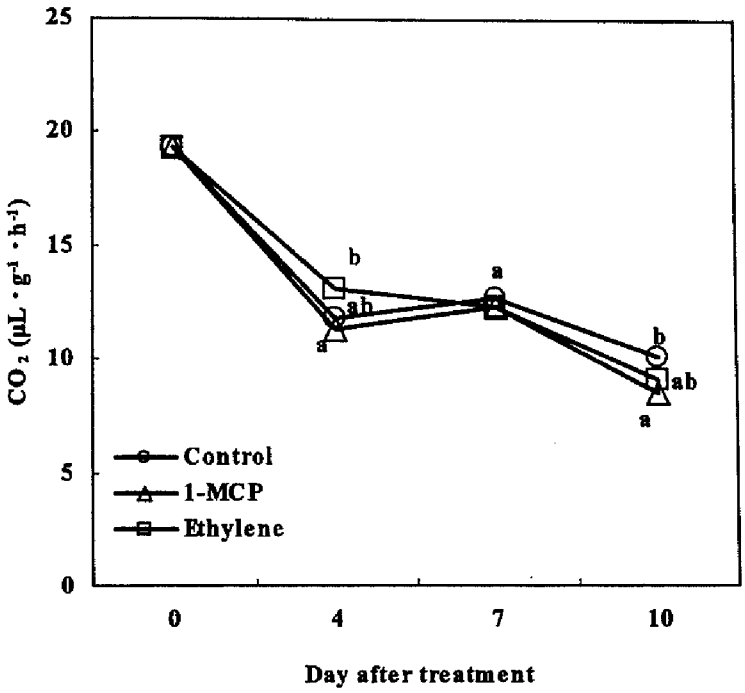

Fig. 3.3 Effects of ethylene and 1-MCP treatments on the respiration rate of immature 'Tsugaru' apples. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

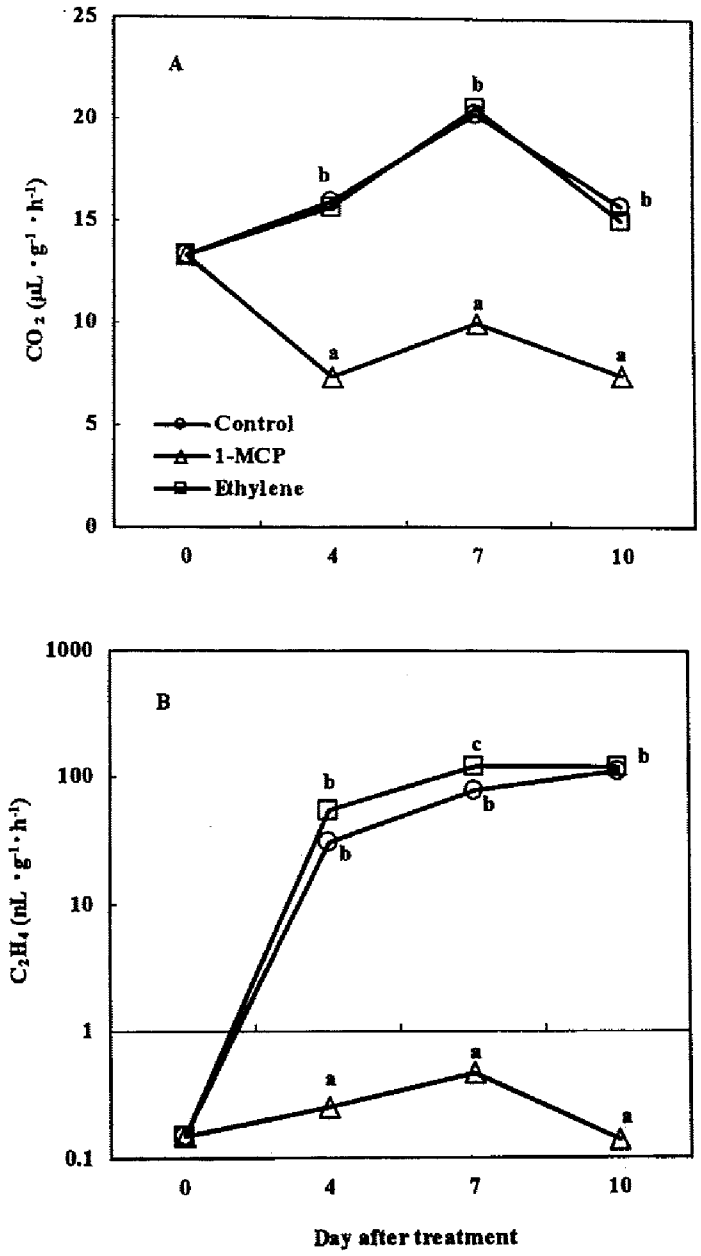

Fig. 3.4 Effects of ethylene and 1-MCP treatments on the respiration rate (A) and ethylene production (B) of mature 'Tsugaru' apples. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

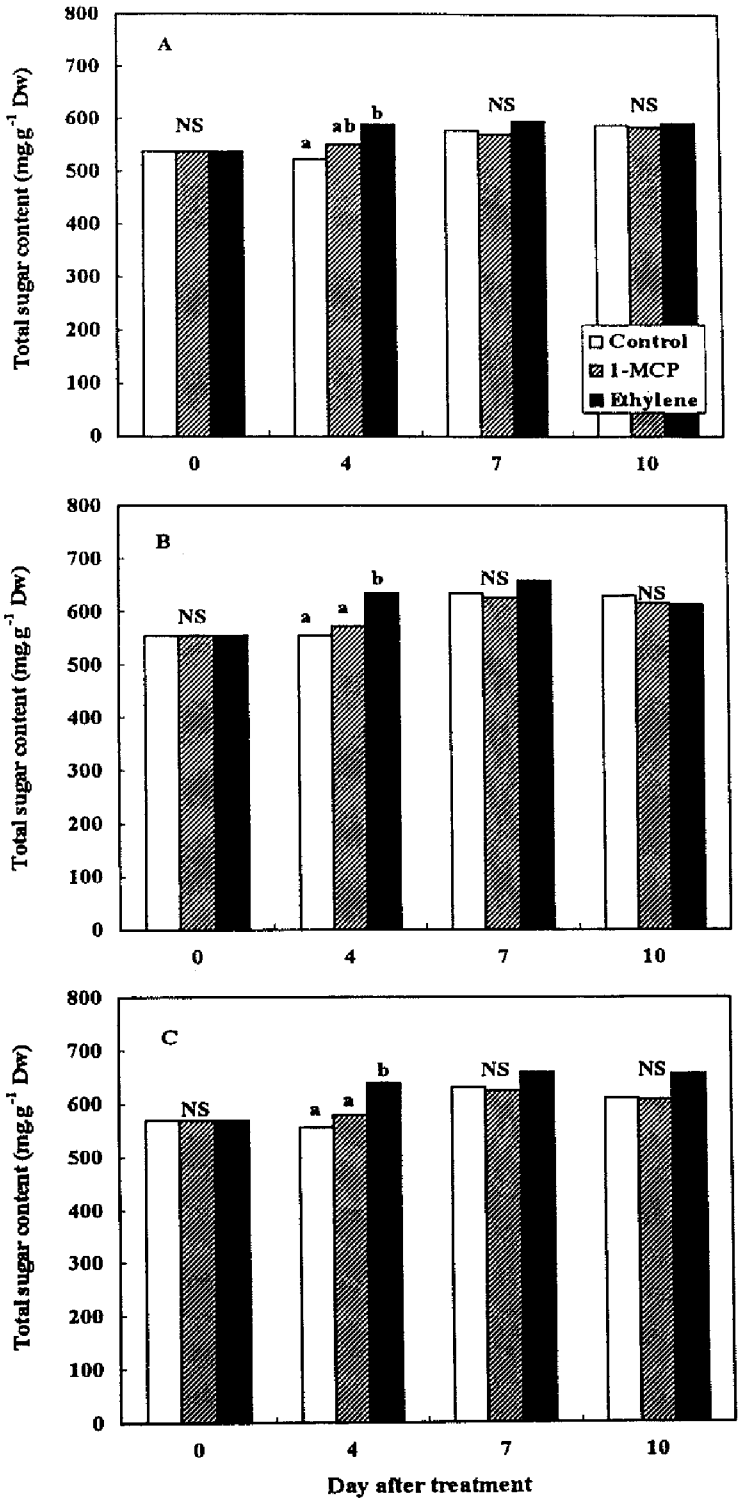

Fig. 3.5 Total sugar content of inner (A), middle (B), and outer (C) tissues of control, and fruits treated with 1-MCP and ethylene of immature 'Tsugaru' apples during storage. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

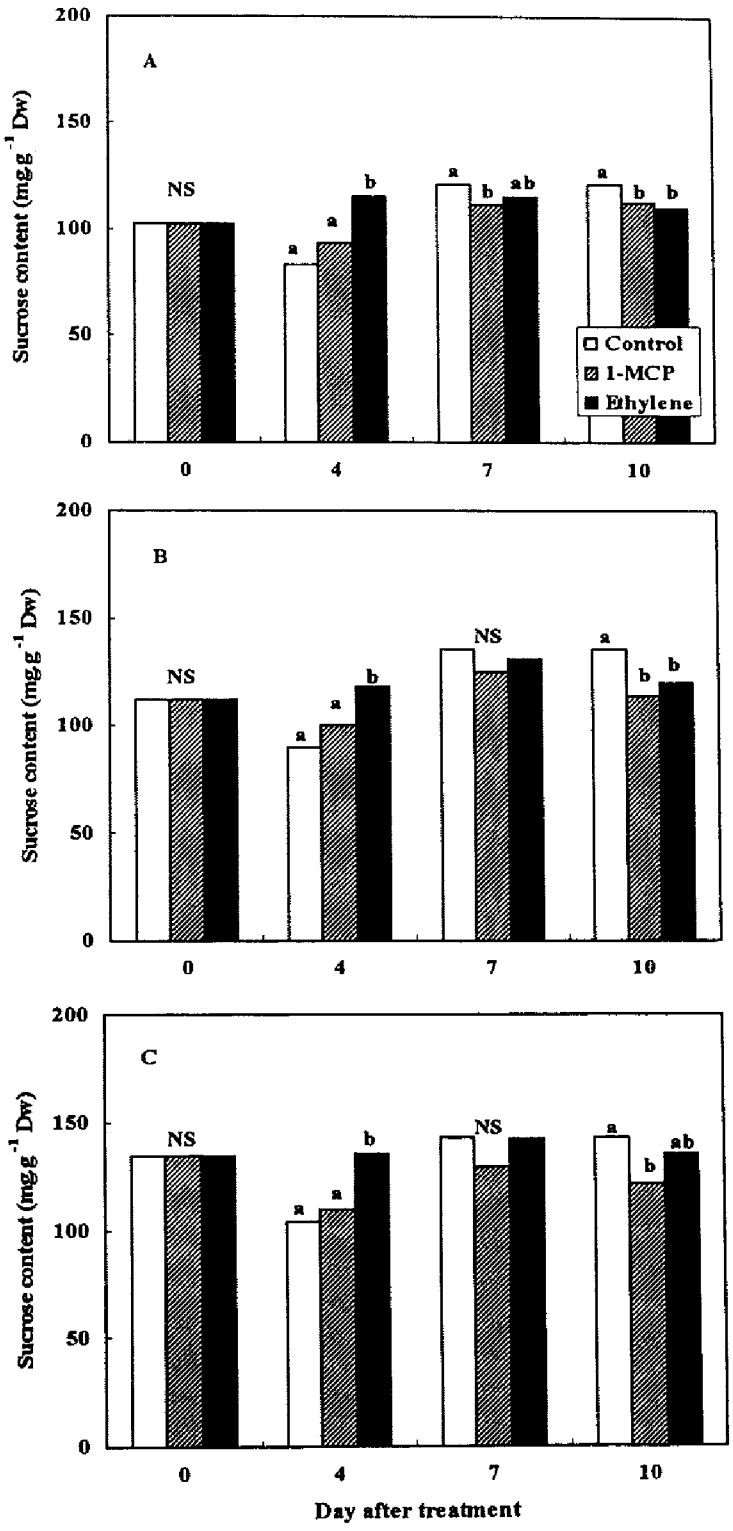

Fig. 3.6 Sucrose content of inner (A), middle (B), and outer (C) tissues of control, and fruits treated with 1-MCP and ethylene of immature 'Tsugaru' apples during storage. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

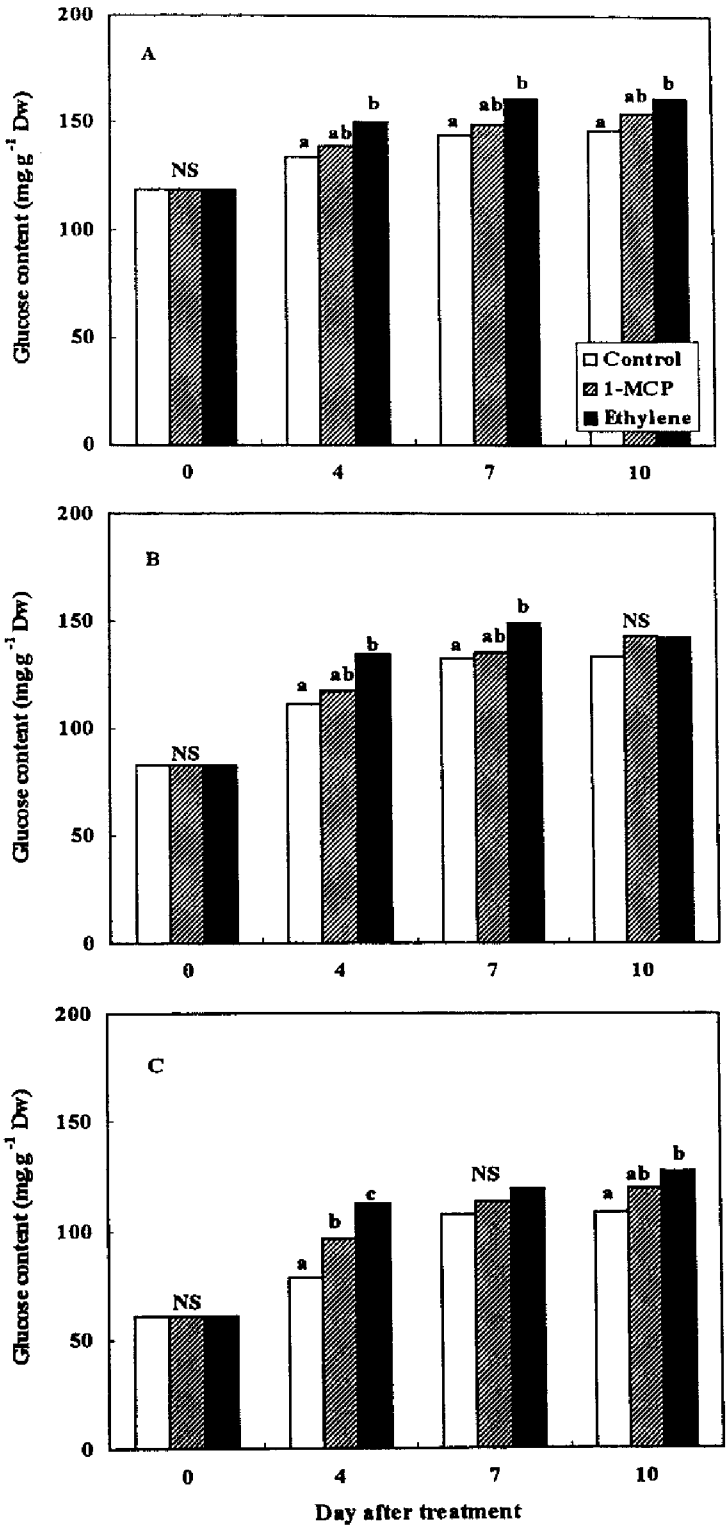

Fig. 3.7 Glucose content of inner (A), middle (B), and outer (C) tissues of control, and fruits treated with 1-MCP and ethylene of immature 'Tsugaru' apples during storage. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

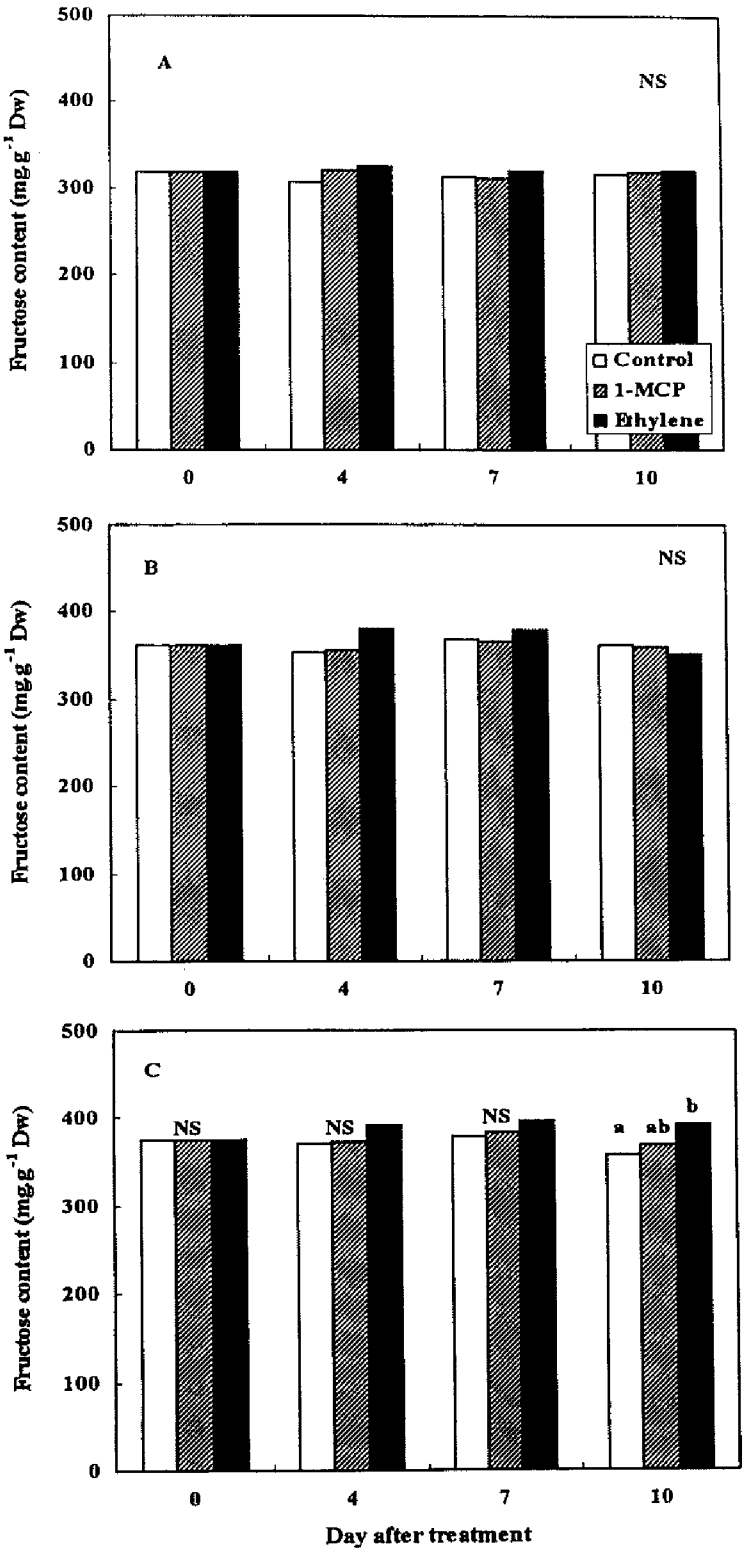

Fig. 3.8 Fructose content of inner (A), middle (B), and outer (C) tissues of control, and fruits treated with 1-MCP and ethylene of immature 'Tsugaru' apples during storage. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

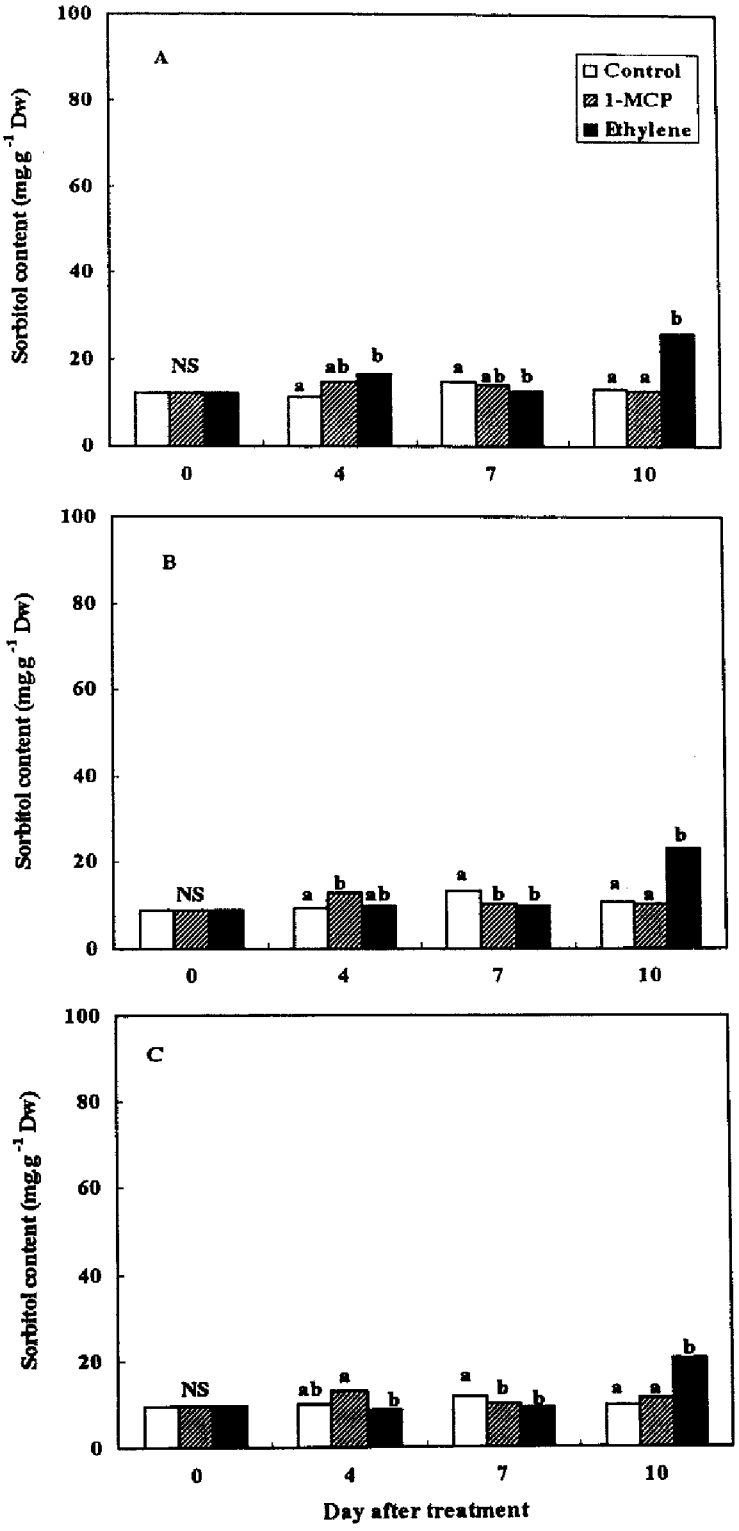

Fig. 3.9 Sorbitol content of inner (A), middle (B), and outer (C) tissues of control, and fruits treated with 1-MCP and ethylene of immature 'Tsugaru' apples during storage. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

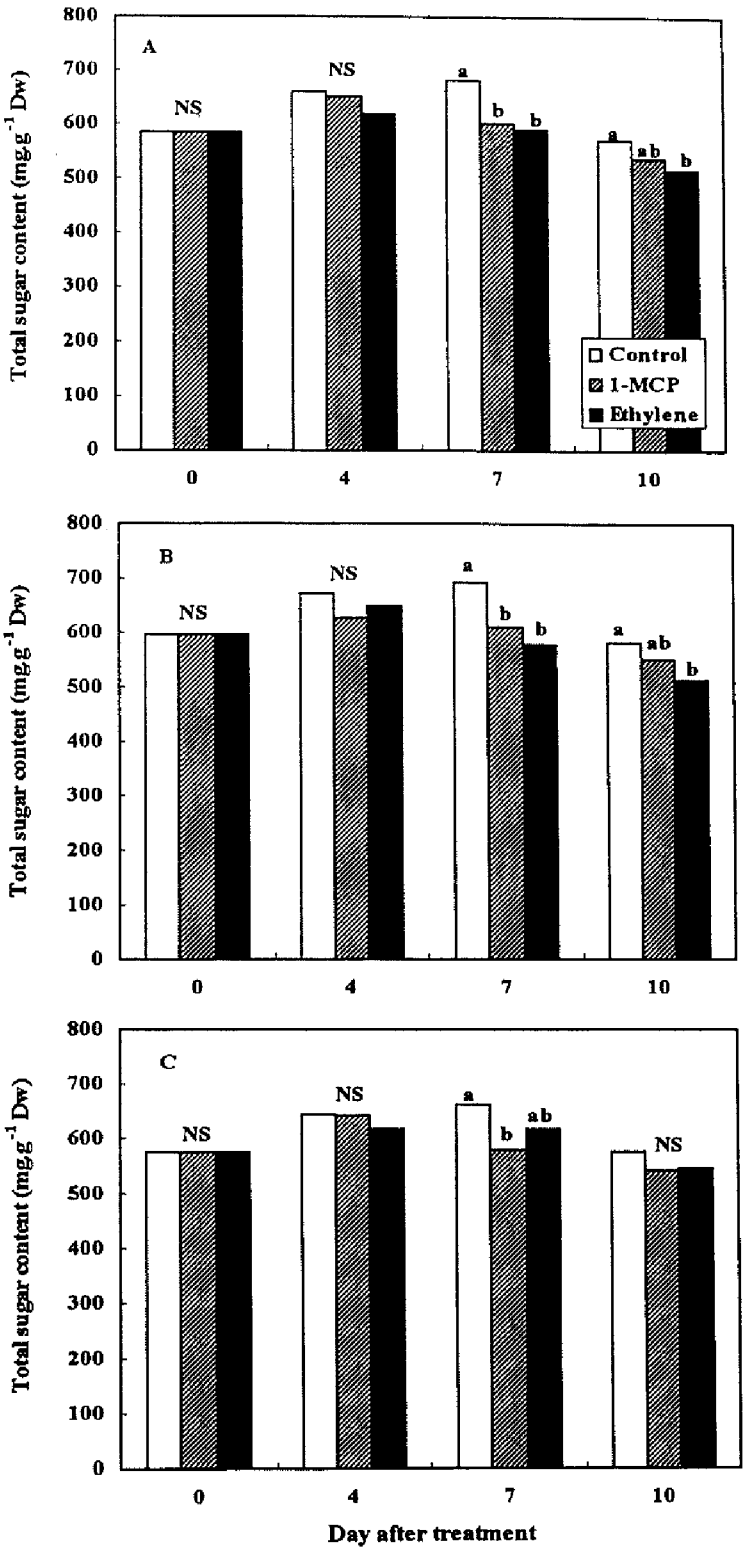

Fig. 3.10 Total sugar content of inner (A), middle (B), and outer (C) tissues of control, and fruits treated with 1-MCP and ethylene of mature 'Tsugaru' apples during storage. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

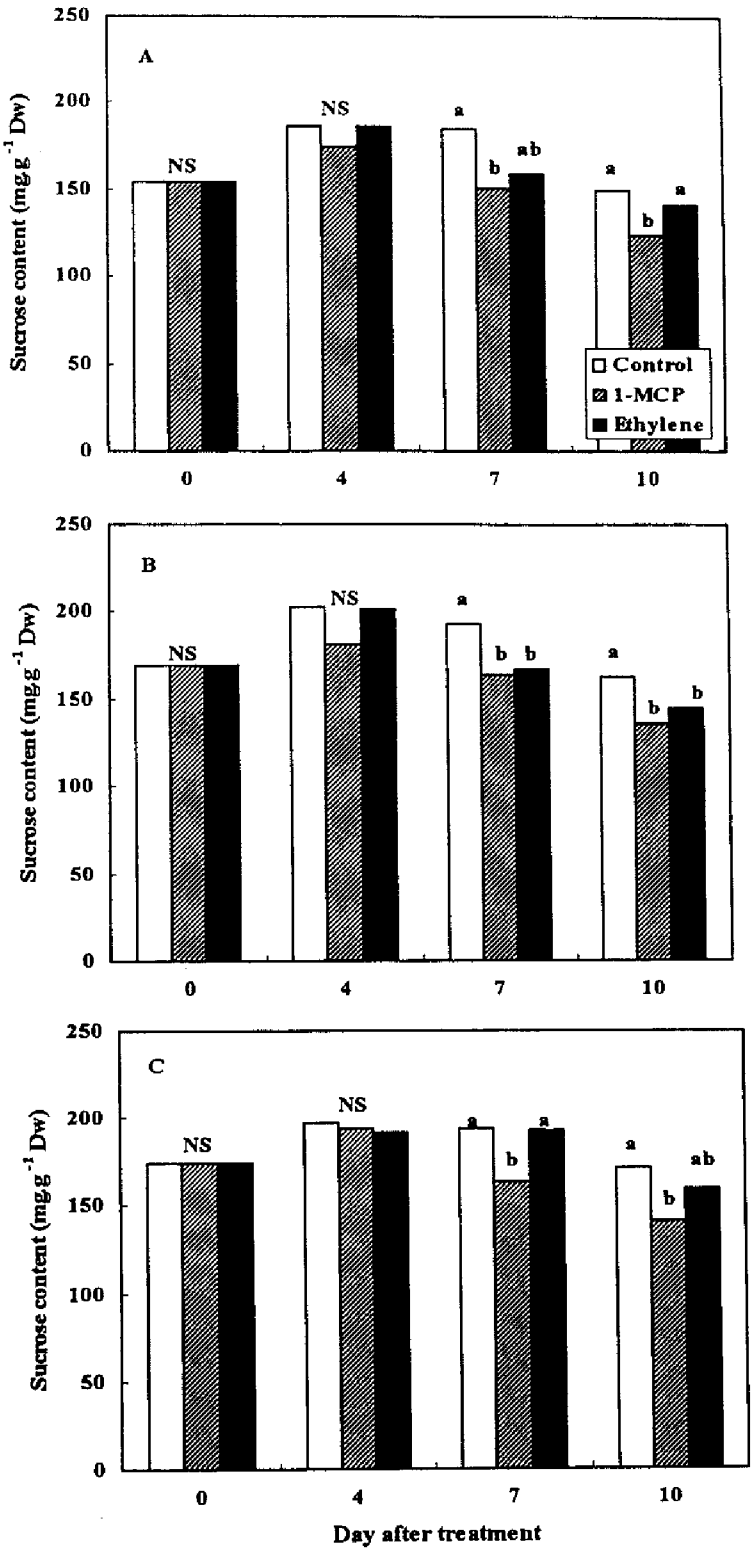

Fig. 3.11 Sucrose content of inner (A), middle (B), and outer (C) tissues of control, and fruits treated with 1-MCP and ethylene of mature 'Tsugaru' apples during storage. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

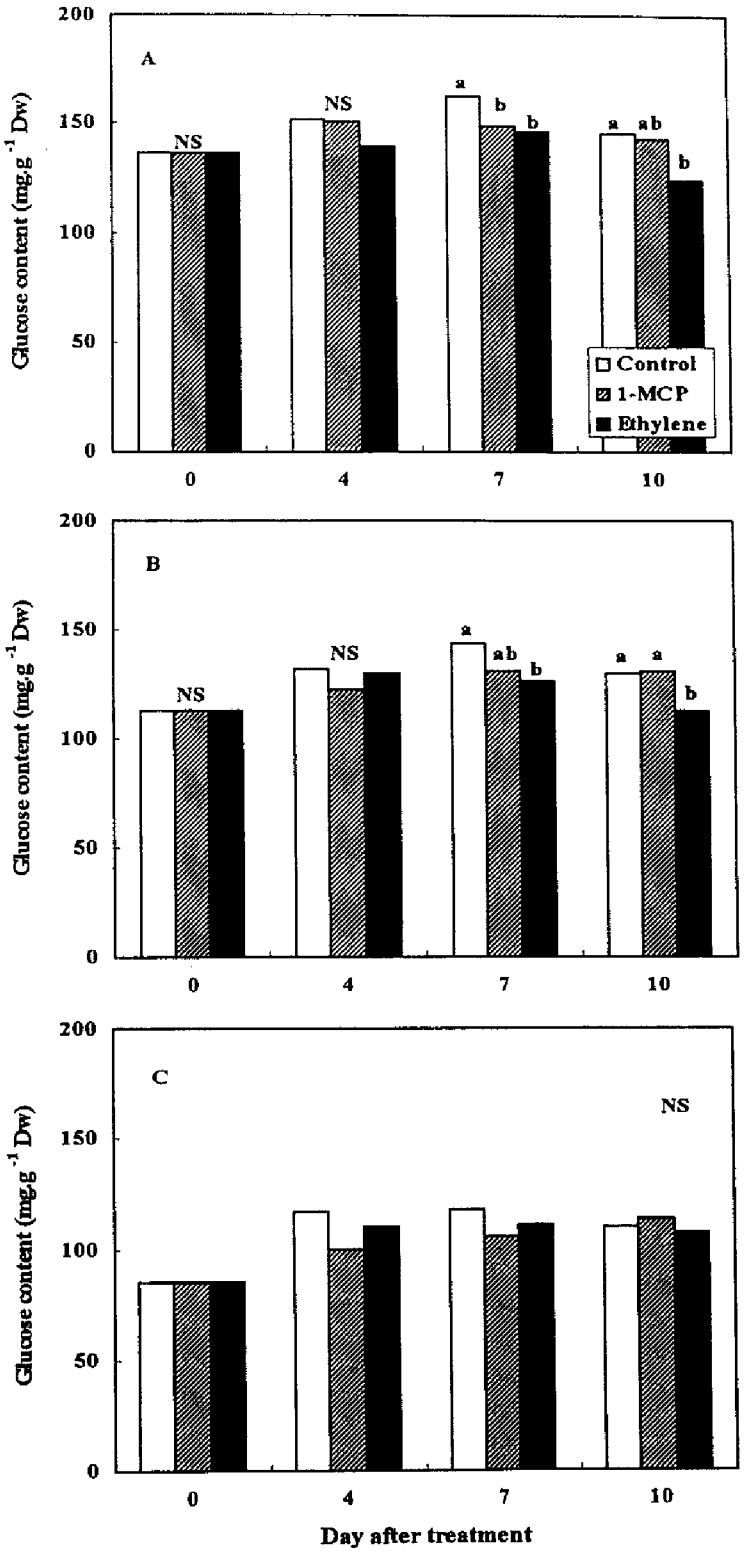

Fig. 3.12 Glucose content of inner (A), middle (B), and outer (C) tissues of control, and fruits treated with 1-MCP and ethylene of mature 'Tsugaru' apples during storage. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

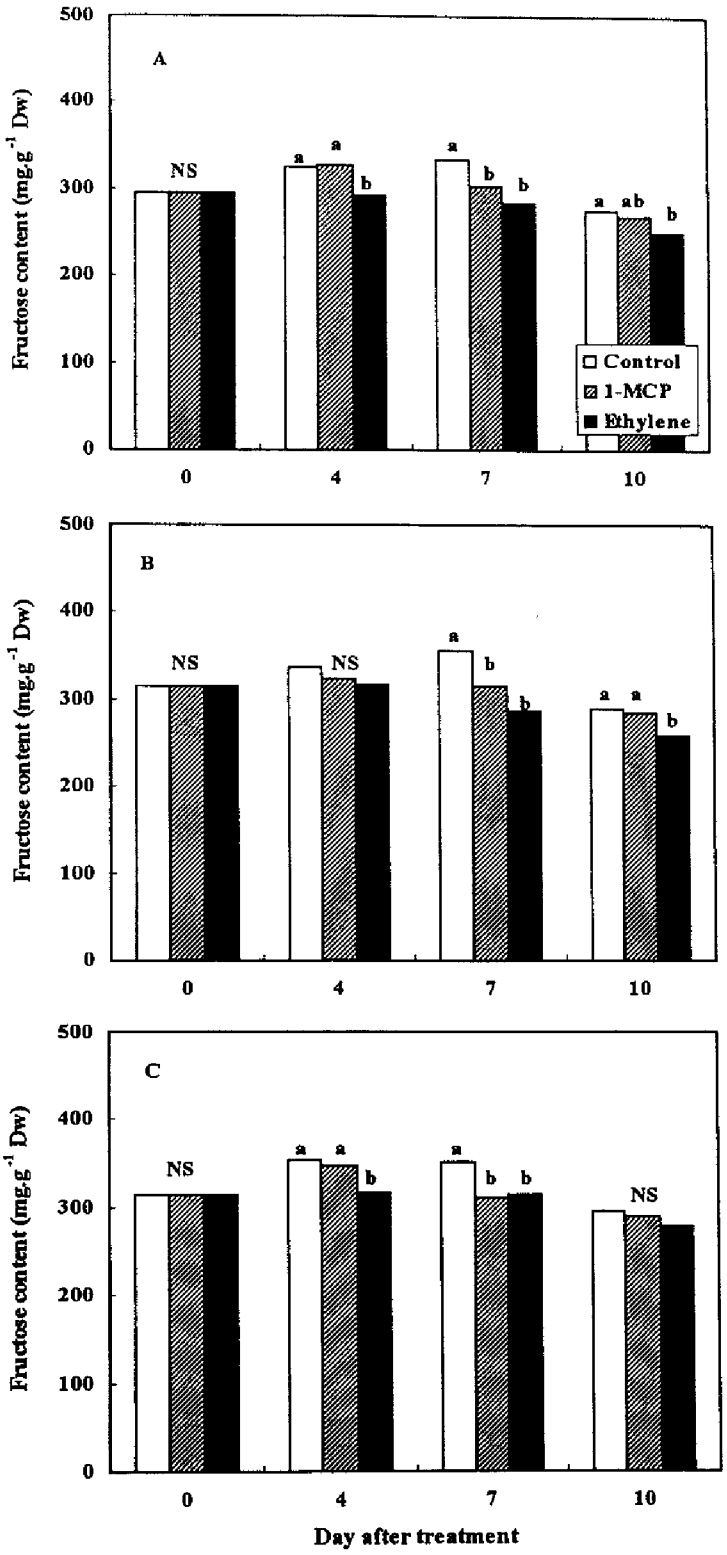

Fig. 3.13 Fructose content of inner (A), middle (B), and outer (C) tissues of control, and fruits treated with 1-MCP and ethylene of mature 'Tsugaru' apples during storage. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

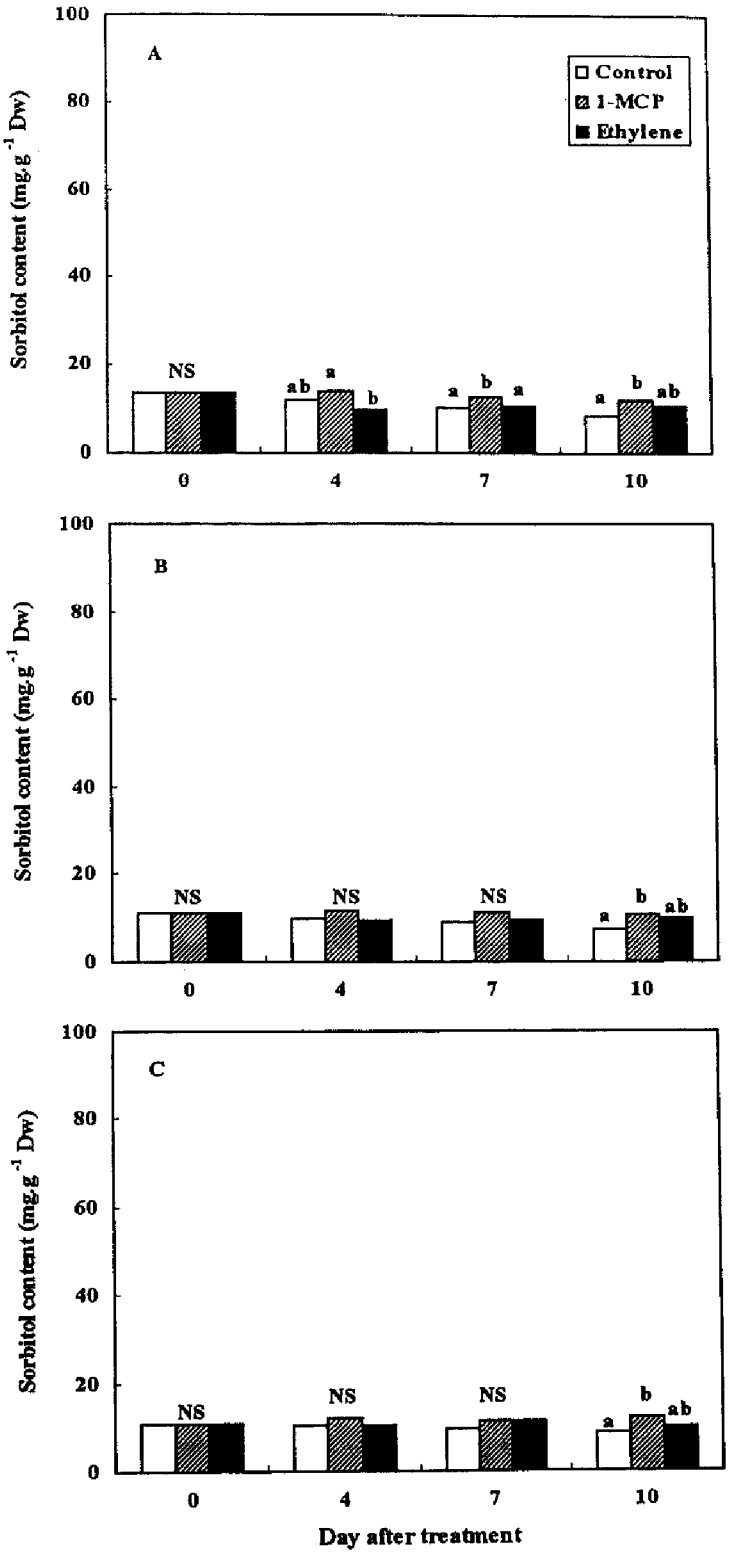

Fig. 3.14 Sorbitol content of inner (A), middle (B), and outer (C) tissues of control, and fruits treated with 1-MCP and ethylene of mature 'Tsugaru' apples during storage. Each value is the mean of four replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

CHAPTER 4

CULTIVAR VARIATIONS AND STARCH DEGRADING PATTERN IN RELATION TO PHYSIOLOGICAL AND BIOCHEMICAL PROPERTIES DURING GROWTH AND MATURATION OF APPLE FRUIT

4.1 Introduction

As starch is degraded when the fruit ripens (Blankenship and Unrath, 1988; Dinar and Stevens, 1981; Prabha and Bhagyalakshmi, 1998), determination of starch hydrolysis by iodine staining (SI) is widely used, and commonly reported as a maturity index of an apple to provide an estimation of the starch content (Ingle and D'Souza, 1989; Fan *et al.*, 1995; Lau, 1988). The starch index value provides valuable guidance to the level of fruit maturity and to the appropriate time of harvesting for immediate consumption or for long-term storage. Despite the common use of SI to monitor starch loss during fruit maturation, SI results varied widely between cultivars and it has been reported that it does not relate well to starch concentration (Fan *et al.*, 1995; Watkins *et al.*, 1993).

Starch concentration of fruit flesh differed between tissue zones, with the highest starch content in the outer cortex and the lowest content in the core (Brookfield *et al.*, 1997; Ohmiya and Kakiuchi, 1990). Apple starch consists of amylose (AM) and amylopectin (AP) which react differently with I_2-KI solution. AM is reacted most efficiently with I_2-KI to produce blue-black pigment (McCready and Hassid, 1943; Fan *et al.*, 1995). The different degradation patterns of starch in the fruit cortex may be due to the different ratio of AM and AP. However, the relationship of starch degradation pattern and AM or AP content in the fruit flesh has not been clearly explained.

Although maturity indices of the apple such as respiration rate, ethylene production and starch rating had been extensively studied (Dinar and Stevens, 1981; Kader, 1985; Prabha and Bhagyalakshmi, 1998; Watkins, 2003), other aspects including AM and AP composition, sugar content and the enzyme actions necessary to understand starch degradation mechanisms and quality development of fruit flesh are still poorly understood. To know the relationship between these properties could be very valuable to the improvement of fruit quality, not only for plant physiologists but also for pomologists and fruit growers. Therefore, the objective of this study was to investigate the degradation mechanism of starch among different tissue zones of the apple fruit flesh during growth and maturation in relation to the physiological and biochemical properties using 'Tsugaru', which produces large amounts of ethylene, and 'Golden Delicious', 'Fuji', and 'Orin' which produces very low amounts of ethylene.

4.2 Materials and Methods

Apple fruits

Four cultivars of apples, 'Tsugaru', 'Golden Delicious', 'Fuji', and 'Orin' fruits were obtained from the experimental orchard of the Faculty of Agriculture and Life Science, Hirosaki University, Japan. Fruits were picked on five to six different days from July to November 2005, at 10-15 days intervals. The 'Tsugaru' fruits were harvested on 70, 80, 95, 110, 125 days after full bloom (DAFB); the 'Golden Delicious' fruits on 80, 95, 110, 125, 140, 155 DAFB, and the 'Fuji' and 'Orin' fruits on 95, 110, 125, 140, 155 DAFB. Fifteen fruits of each cultivar per harvesting crop were used. The last harvest of each cultivar was a week before commercial harvest.

Measurements

1. Respiration rate, Ethylene production, and Starch rating

To determine CO_2 production, each fruit was weighed and sealed in 1.4 L plastic boxes for 2 h, and 1 mL of headspace gas was injected into a gas chromatograph (model GC-18A, Shimadzu Co., Ltd., Japan) equipped with a molecular sieve column (60/80 mesh, GL Sciences, Inc.), and a thermal conductivity detector. Helium was the carrier gas. The injector, oven, and detector temperatures were set at 60℃. To determine the ethylene content, 1 mL of the headspace gas was removed and injected into a gas chromatograph (model GC-8A, Shimadzu Co., Ltd.) equipped with an activated alumina column (30/60 mesh, GL Sciences, Inc.) and a flame ionization detector. Nitrogen was the carrier gas. The injector, oven, and detector temperatures were set at 120, 100, and 120℃, respectively.

The starch distribution was measured by dipping an apple slice taken from the equatorial region in I_2-KI solution (10 g/25 g in 1 L distilled water), and the starch-iodine rating was done using the generic starch-iodine index chart for comparison (Watkins, 2003). This method uses a 1 to 8 scale, with 1 = all starch and 8 = no starch.

2. Starch, AM, and AP contents

Samples were taken by a cork borer (1 cm ø), and were peeled, cored, and cut. The fruit flesh between the core and the outer skin were separated into three equal portions, with each sample containing 3-5 g of fruit flesh. The tissues were frozen in liquid N and stored at -80℃ before being freeze-dried. Samples were reweighed after freeze-drying, and then ground in a TI-100 vibrating sample mill (Heiko Sample Mill, Heiko Seisakusho Ltd., Japan).

Measurements of starch, AM, and AP were obtained through methods applied in Fan *et al.* (1995) and Magein and Leurguin (2000). Briefly, 1 mL of 18% HCl was added to each tube of ground sample (60 to 80 mg). The ground cortex tissue and 18% HCl were thoughly mixed and then incubated at 20℃ for 30 min. 10 mL distilled H_2O was then added to each tube; the supernatant was mixed and centrifuged at 3800×g for 10 min. Supernatant of 0.2 to 1.0 mL was used, and 5.0 mL of 1.8% HCl was added to each tube. After mixing, 200 μL of I_2-KI (2 mg I_2 and 20 mg KI per ml H_2O) solution was added. Each sample was mixed, and 10 min later, absorbance was measured at 530 nm (for AP) and 606 nm (for AM) using the spectrophotometer (U-2000 Spectronic, Hitachi, Japan).

AM (Sigma Chemical, St. Louis, USA) and AP (MP Biomedicals, Inc., Ohio) standards were dissolved in 18% HCl for 30 min and then diluted 10-fold. The two standards were mixed in various ratios, and absorbance was measured at 530 and 606 nm to generate a standard curve. Absorbance coefficients (A), for AM and AP standards, were $A_{AM606} = 6.88$, $A_{AM530} = 4.54$, $A_{AP606} = 5.00$, $A_{AP530} = 6.99$, and a typical standard curve with an r^2 value of 0.92 was obtained. The amount of AM and starch concentration (SC) in the samples were calculated using equations derived by Magel (1991); AP concentration can then be obtained by subtracting the AM concentration from the SC.

3. Sugar content

For sugar determination, one hundred milligrams of the dried sample was extracted three times, each over a duration of 20 min, with 2 mL of 80% (v/v) ethanol at 80℃. The homogenates were centrifuged at 15,000×g for 10 min to give ethanol-soluble and ethanol-insoluble fractions. The ethanol soluble fractions were pooled and evaporated to dryness with a concentrator, and resolublized in 2 mL of de-ionized water. The soluble fraction was then filtered. Glucose, fructose, sucrose, and sorbitol were separated with a high-performance

liquid chromatograph (HPLC) (Shimadzu, Kyoto, Japan). De-gassed, distilled, de-ionized water at 1 $mL \cdot min^{-1}$ and 80℃ was used as the mobile phase. A refractive index detector (RID-10A; Shimadzu) was used to quantify sugar content following the separation. Recovery rate was determined by comparison with standard samples of known concentration of glucose, fructose, sucrose, and sorbitol.

4. Activity of total hydrolytic enzyme

The total hydrolytic activities of 'Tsugaru' and 'Fuji' (inner, middle, and outer parts) were investigated by the method applied from Steup (1990). Briefly, apple flesh (approximately 5 g fresh weight) was homogenized in 30 mL ice-cold grinding medium which consist of 50 mM 2-(N-morholino)ethane-sulphonic acid (Mes), brought to pH 6.0 with NaOH, 5mM $CaCl_2$, and 5% (v/v) glycerol. The homogenate was filtered through several layers of Miracloth and the filtrate was centrifuged for 15 min at 40000×g. The supernatant was passed through a Sephadex G-25 gel which has been previously equilibrated with grinding medium.

The incubation mixture contained 1 mL 2% (w/v) soluble starch, 0.9 mL grinding medium, and 0.1 mL of the filtered supernatant. A blank was prepared by mixing 2% (w/v) soluble starch with an equal volume of grinding medium. Mixtures were incubated at 30℃. At 5 min, the incubation mixture was added to an equal volume of alkaline color reagent, mixed thoroughly, and heated for 5 min in a boiling water bath. Samples were then cooled to room temperature and stored for at least 30 min. Absorbance at 546 nm was measured against a reference (2 mL blank plus 2 mL alkaline color reagent, treated as above). The alkaline color reagent was prepared by dissolving 1 g 3,5-dinitrosalicylic acid in a mixture of 40 mL 1 N NaOH and approximately 30 mL H_2O at an elevated temperature. Solid potassium sodium tartrate (3g) was added and dissolved. After

cooling to room temperature, the mixture was brought to a final volume of 100 mL.

For calibration purposes, varying amounts of maltose were reacted with the alkaline color reagent. Samples (2 mL each) which contained 0-2.0 mM maltose, 0.2 mL 2% (w/v) soluble starch and grinding medium were prepared. Each sample was mixed with 2 mL alkaline color reagent and processed as described above. A typical standard curve of maltose has an r^2 value of 0.99 (Fig. 4.10A).

Data analysis

Analysis of Variance (ANOVA) with Completely Randomized Design (CRD) using tissue zones as a factor was performed using SPSS (SPSS, IL, USA), and Tukey's multiple-range test was used to test significant difference at the 95% confidence level of each variable.

4.3. Results

Fruit maturity

Starch index value, respiration rate, and ethylene production were investigated during the growth and maturation processes of the four apple cultivars. The starch degradation of fruit flesh and different degradation patterns among cultivars were observed by monitoring the starch index values. Starch in 'Tsugaru' started degrading at 95 DAFB, and there was great loss at 110 to 125 DAFB, but the starch level of other three cultivars degraded gradually throughout the growth and maturation processes and obvious loss was observed at 155 DAFB in 'Golden Delicious' and at 170 DAFB in 'Fuji' and 'Orin' (Fig. 4.1A).

Starch rating, by iodine staining, of 'Tsugaru' was quantified at five stages of growth and maturation and that of 'Golden Delicious', 'Fuji', and 'Orin' were done at six stages (Fig. 4.1B, 4.1C, 4.1D, 4.1E). The starch-staining pattern of 'Tsugaru' showed slight loss of starch at the core area between 80 and 95 DAFB. Starch degradation was observed all over the fruit flesh, with starch content of the core area completely lost at 110 DAFB and almost all starch completely lost at 125 DAFB (Fig. 4.1B-125). Contrarily, in 'Golden Delicious', starch of core area started degrading at 110 DAFB and completely lost at 125 DAFB. Starch of all regions continuously lost until 155 DAFB. However, it seemed that the remaining starch in fruit flesh was still high at this stage (Fig. 4.1C). In 'Fuji' and 'Orin', starch of the core area was completely degraded while some loss of starch in the middle cortex was observed at 125 DAFB. Almost all starch in 'Fuji' was degraded at 170 DAFB, but 'Orin' still showed higher remaining starch in the outer cortex at the last harvest (Fig. 4.1D, 4.1E).

The respiration rate of 'Tsugaru' decreased initially, followed by a steep increase between 110 and 125 DAFB. Ethylene production of 'Tsugaru' also increased significantly between 110 to 125 DAFB, demonstrating the climacteric rise of the fruit (Fig. 4.2). However, in the other tree cultivars, respiration rate decreased gradually and low level of ethylene productions was observed during the study period (Fig. 4.2).

AM and AP content

AM content of the four cultivars was highest in the outer cortex and lowest in the inner cortex ($P \leq 0.05$). The changes in AM content were observed simultaneously in 3 different fruit tissues; inner, middle, and outer parts. AM content of 'Tsugaru' changed slightly at the first two harvest dates, and dropped significantly between 95 to110 DAFB. Almost all starch was degraded at 125 DAFB (Fig. 4.3A). However, AM content of 'Golden Delicious', 'Fuji', and 'Orin' was highest at the first harvest and then decreased gradually during the investigation period (Fig. 4.3B, 4.3C, 4.3D). AP content was highest in the outer cortex, but the difference between the middle and inner cortex was not clear. In 'Tsugaru', AP content changed slightly the first three harvest dates and then lost rapidly between 110 to 125 DAFB; however, there was no significant difference in AP content among tissue zones (Fig. 4.4A; $P \leq 0.05$). AP contents of 'Golden Delicious', 'Fuji', and 'Orin' were highest at the first harvest and then decreased gradually during investigation period (Fig. 4.4B, 4.4C, 4.4D).

Sugar content

Total sugar content (sucrose, glucose, and fructose) of 'Tsugaru', 'Golden Delicious', 'Fuji', and 'Orin' tended to increase during growth and maturation (Fig. 4.5). Although the difference among tissue zones was not clear, the predominant increase of total sugar content was observed in 'Tsugaru' between 110 and 125 DAFB (Fig. 4.5A). The individual sugar component of the four apple cultivars were shown in Fig. 4.6, 4.7, 4.8, and 4.9. For the individual sugar content of 'Tsugaru', sucrose content of all tissues was high between 110 and 125 DAFB, and was highest in the outer cortex (Fig. 4.6A). Glucose content increased slightly, and was highest in the inner tissue zone (Fig. 4.6B). Fructose and sorbitol contents changed slightly and the difference among tissue zones was not clear (Fig. 4.6C, 4.6D).

In 'Golden Delicious', sucrose content increased during growth and maturation. The highest sucrose content was observed in the outer cortex (Fig. 4.7A). The glucose content of the inner cortex was higher than the middle and outer cortex, but the changes of glucose content among harvest dates were small (Fig. 4.7B). The fructose and sorbitol contents changed slightly during growth and maturation (Fig. 4.7C, 4.7D).

In 'Fuji', total sugar and sucrose contents tended to increase during maturation (Fig. 4.8A). The glucose and fructose contents changed slightly with the highest content in the inner cortex (Fig. 4.8B, 4.8C). The sorbitol content of 'Fuji' was increased and it was higher than other cultivars. The highest sorbitol content of the inner cortex was observed between 155 and 170 DAFB (Fig. 4.8D).

The increase in sucrose content during investigation period was also observed in 'Orin' (Fig. 4.9A). The glucose and fructose content changed slightly, and the sorbitol content increased between 140-170 DAFB (Fig. 4.9B, 4.9C, 4.9D).

Total hydrolytic activity

The total hydrolytic activity of 'Tsugaru' was higher than 'Fuji'. It changed slightly between 70 to 95 DAFB, but dropped significantly at 110 DAFB. In 'Fuji', the total hydrolytic activity changed slightly and decreased gradually throughout the growth and maturation processes. There was a small difference observed in the tissue zones among cultivars (Fig. 4.10B, 4.10C; $P \leq 0.05$).

4.4. Discussion and conclusion

From a previous study, the role of ethylene in starch degradation of a detached apple fruit was shown to differ between cultivars and their harvest stages, and is related to ripening and physiological characteristics of the fruit (Thammawong and Arakawa, 2007). Although starch degradation of the detached fruit has been studied in various apple cultivars, the characteristics of starch degradation in the fruit flesh on the tree during growth and maturation is not well studied. Moreover, the relationship between cultivar variation and starch degradation mechanism, which includes the role of physiological aspects and cellular components such as starch and sugar content, and enzyme activity in each tissue zone of the fruit flesh, is still unclear.

Starch index value can be used as a tool to indicate fruit maturity based on the level of starch degradation and hence indicate the appropriate time for harvesting (Kingston, 1992; Knee *et al.*, 1989; Lau, 1988; Watkins, 2003). However, the characteristics of starch patterns shown by iodine staining vary according to growing conditions, canopy environment, seasonal changes, and cultivar variation (Reid *et al.*, 1982; Smith *et al.*, 1979; Watkins *et al.*, 1982; Watkins *et al.*, 1993). From the results, the loss of starch in 'Tsugaru' (early-maturing cultivar) was rapid and complete between 110 to 125 DAFB (Fig. 4.1B). However, iodine staining of other three cultivars showed slight changes in starch degradation until commercial harvesting began. This difference between cultivars suggests that iodine staining is recommended more for determining the maturation in late-maturing cultivars such as 'Golden Delicious', 'Fuji', and 'Orin' rather than the early-maturing 'Tsugaru'.

During starch degradation, physiological aspects of the ripening characteristics were observed to differ between 'Tsugaru' and 'Fuji'. In 'Tsugaru', starch content changed slightly between 70-80 DAFB, but loss of starch content occurred rapidly between 95 to 125 DAFB with simultaneous increases in the rate of respiration and ethylene production (Fig. 1A, 1D, 1E, 2A). On the other hand, in 'Golden Delicious', 'Fuji', and 'Orin', degradation of starch occurred gradually throughout growth and maturation processes with a low level of ethylene production and decreased respiration. As ethylene has been suggested to play a role in stimulating physiological changes and in the conversion of starch to sugar (Kader, 1985; Watkins, 2003), the rapid change of starch content in 'Tsugaru' might be due to the induction of internal ethylene production and respiration rate. Furthermore, the response of the apple fruit to endogenous and exogenous ethylene for climacteric induction and starch degradation varied according to cultivar variations (Thammawong and Arakawa, 2007).

The changing patterns of AM and AP content of the 'Tsugaru' and 'Fuji' showed the highest accumulated starch in the outer cortex and lowest in the inner cortex. The action of starch degrading enzymes has been suggested to play a role in the loss of starch in the apple fruit (Beck and Ziegler, 1989; Frenkel *et al.*, 1968; Garcia and Lajolo, 1988; Jackson, 2003; Zhang and Wang, 2002). Since it has been suggested that starch hydrolysis generally proceeds from the core (carpel) towards the skin (Poapst *et al.*, 1959), the degradation of AM and AP content in both cultivars was observed simultaneously in 3 different regions of the cortex tissue. However, there was only small difference in the total hydrolytic activity among different tissue zones in 'Tsugaru' and 'Fuji' cultivars (Fig. 4.10B, 4.10C). This suggests that starch degradation began simultaneously rather than preferentially in any one tissue zone. The important factors affecting starch degradation patterns are probably the amount of starch in the tissue and the rate of degradation. If starch concentration in the core region is very low, the lack of

iodine staining will occur sooner rather than later during fruit maturation and ripening (Fig. 4.11). Additionally, this supports the hypothesis that different degradation patterns between 'Tsugaru' and other three cultivars during maturation might be due to the physiological aspects of the ripening effect such as rate of respiration and ethylene production.

As the sweetness of a ripe apple fruit is associated to its cellular sugar components, sugar from the degradation of accumulated starch and sugar translocated from the leaves were both taken into account. Jackson (2003) suggested that starch hydrolysis is generally accompanied by the appearance of sucrose which is then slowly hydrolyzed to produce more glucose and fructose. However, Whiting (1970) suggested that the amount of sucrose far exceeded the amount produced by starch degradation alone. From the results, the total sugar content (sum of sucrose, glucose, and fructose) increased during maturation. The individual fructose content was the highest in the fruit flesh of both cultivars; it might have accumulated from the conversion of translocated sorbitol from leaves by sorbitol dehydrogenase (SDH, EC 1.1.1.14) (Bieleski and Redgwell, 1985; Teo *et al.*, 2006). There was a predominant increase of sucrose content that occurred simultaneously with the loss of starch. Sugar content increased significantly in 'Tsugaru' between 110 and 125 DAFB while a gradual increase during growth and maturation was observed in 'Golden Delicious', 'Fuji', and 'Orin'. As such, it can be assumed that the high amount of sucrose in 'Tsugaru' may be due to the hydrolysis of accumulated starch in the fruit tissue. However, an attached fruit may also gain sucrose from the sugar translocation process as it is transported to the fruit sink together with sorbitol. The simultaneous accumulation of sugar from translocation during starch degradation in the maturation process is clearly supported by the occurrence of sorbitol translocation in 'Fuji' (Fig. 4.8D). To clarify this phenomenon, however, further study of sugar translocation and individual enzyme expression of each cultivar

are required. Moreover, in order to improve fruit eating quality, the accumulation of sugar from both starch hydrolysis and translocation should be taken into consideration.

Cultivar variations and their individual physiological and chemical characteristics of the fruit development and maturation process seem to account conjointly for the degradation pattern of starch in the fruit flesh. 'Tsugaru', an early-maturing cultivar with a short growth and maturation period, produces a high amount of ethylene and has increased rate of respiration in order to induce degradation metabolism of starch and sugar production to develop fruit sweetness as the fruit ripen. However, in 'Golden Delicious', 'Fuji', and 'Orin', gradual starch degradation occurs simultaneously with low levels of respiration and ethylene production, and sugar translocation seemed to be the main factor in sweetness development.

In addition, during degradation of starch was observed in fruit flesh, the changing pattern of sugar was different between the attached and detached 'Tsugaru' fruit (Chapter 3). The contents of total sugar, sucrose, and glucose of the attached fruit tended to increase during investigation period (Fig. 4.5A, 4.6). For the detached fruit (control), total sugar, sucrose, glucose, and fructose contents in the immature fruits increased slightly during storage, however those in the mature fruits was increased between days 4-7 and then decreased greatly at day 10. From this point, it can be assumed that the sugar products from the starch hydrolysis in the detached fruits might be used as respiratory substrates to produce energy and intermediate carbon compounds for cell maintenance as discussed in Chapter 3. However, in the mature attached fruits, the increases of respiration rate and amount of sugar contents were observed until the last harvest. This might be due to the great accumulation of sugars from the starch degradation together with sugars from the continuation of translocation process.

4.5 Summary

Fruit maturity indices, i.e. respiration rate and ethylene production, amylose (AM) and amylopectin (AP) content, total hydrolytic activity, and sugar content were investigated during the growth and maturation of 'Tsugaru', 'Golden Delicious', 'Fuji', and 'Orin' apples (*Malus domestica* Borkh.). Different starch degradation characteristics during the growth and maturation processes were observed among cultivars. By iodine staining, the loss of starch in 'Tsugaru' was observed earlier than in 'Golden Delicious', 'Fuji', and 'Orin'. The different degradation patterns of starch were also demonstrated by the AM and AP content. In 'Tsugaru', AM and AP degraded rapidly between 95 to 110 days after full bloom (DAFB) and almost all starch were lost rapidly at 125 DAFB with simultaneous increases in rate of respiration and production of ethylene. However, in 'Golden Delicious', 'Fuji', and 'Orin', starch degraded gradually throughout growth and maturation process and was clearly degraded at 155 or 170 DAFB with a low level of ethylene production and decreased respiration. This indicates that the level of starch loss and its degradation characteristics depend on cultivar variation, and could be affected by physiological aspects. In all cultivars, content of AM and AP were highest in the outer cortex and lowest in the inner cortex. Starch degradation was observed simultaneously in 3 different tissue zones and there was little difference in the total hydrolytic activity among tissue zones between 'Tsugaru' and 'Fuji'. These results suggest that starch hydrolysis in the apple flesh began simultaneously rather than preferentially in any one tissue zone. For sugar content, although differences among tissue zones were not clear, it increased distinctly with loss of starch content. Moreover, sugar from the degradation of accumulated starch and sugar translocation both seem to mainly influence the sweetness quality as the fruit ripens.

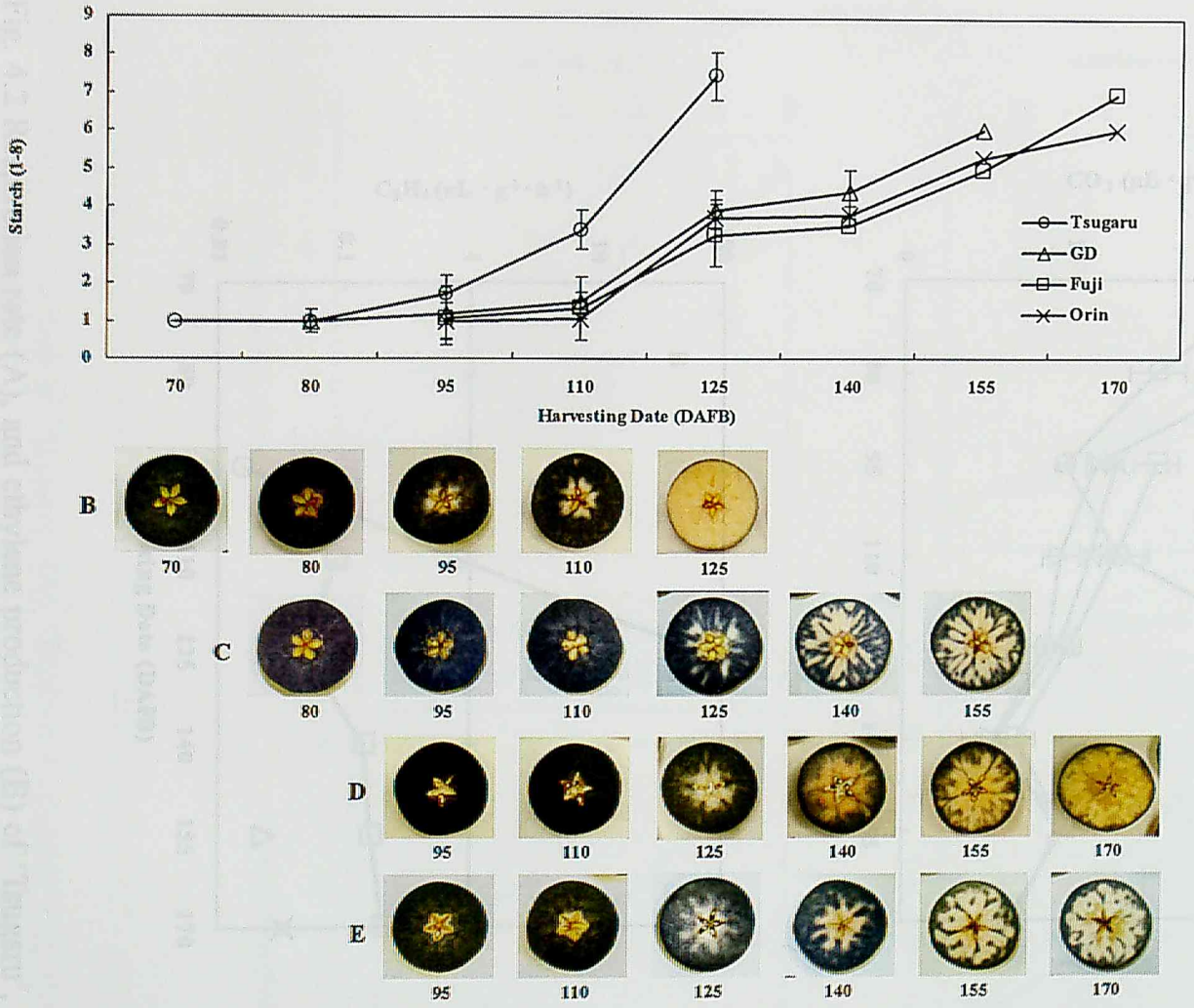

Fig. 4.1 Starch rating scores (A) of four cultivars, and iodine staining of 'Tsugaru' (B), 'Golden Delicious (C), 'Fuji' (D), and 'Orin' (E).

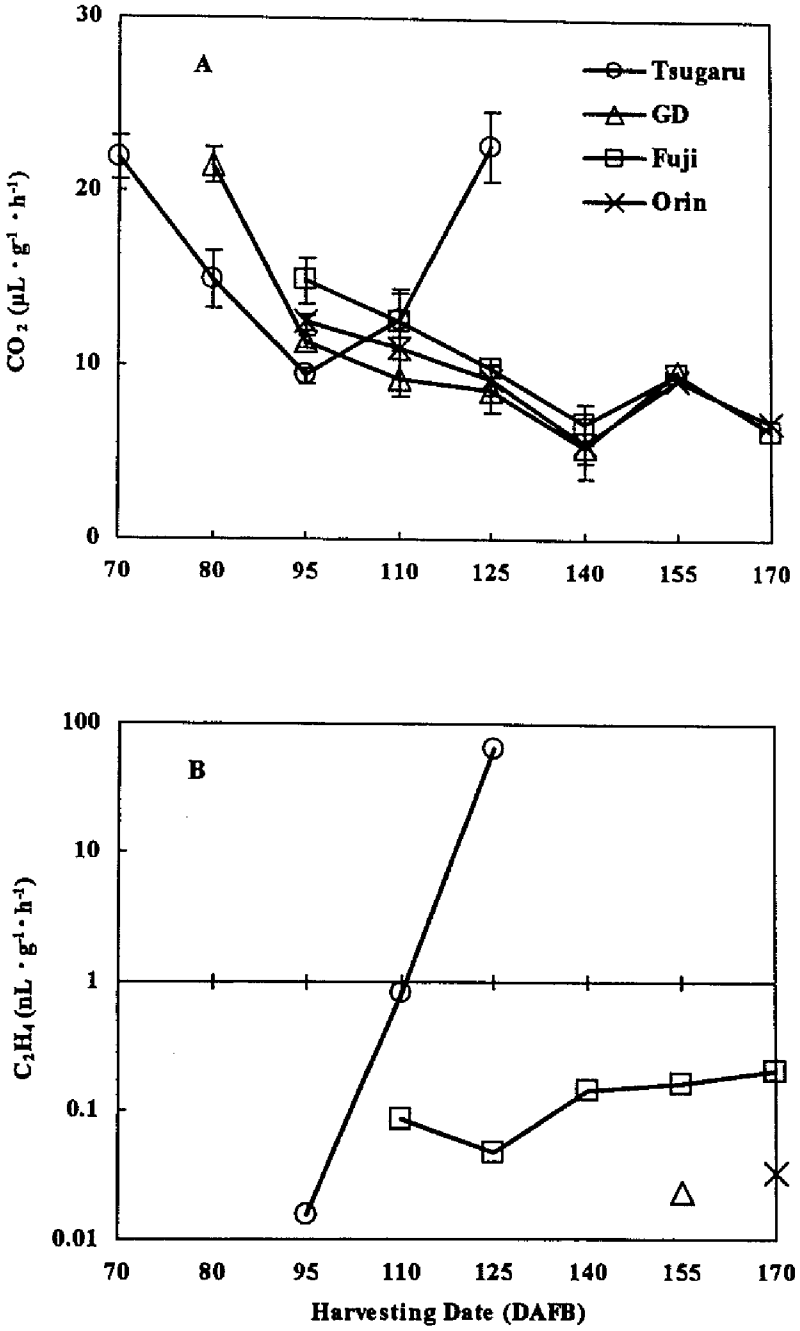

Fig. 4.2 Respiration rate (A), and ethylene production (B) of 'Tsugaru', 'Golden Delicious', 'Orin', and 'Fuji' apples. Each value is the mean of five replicates.

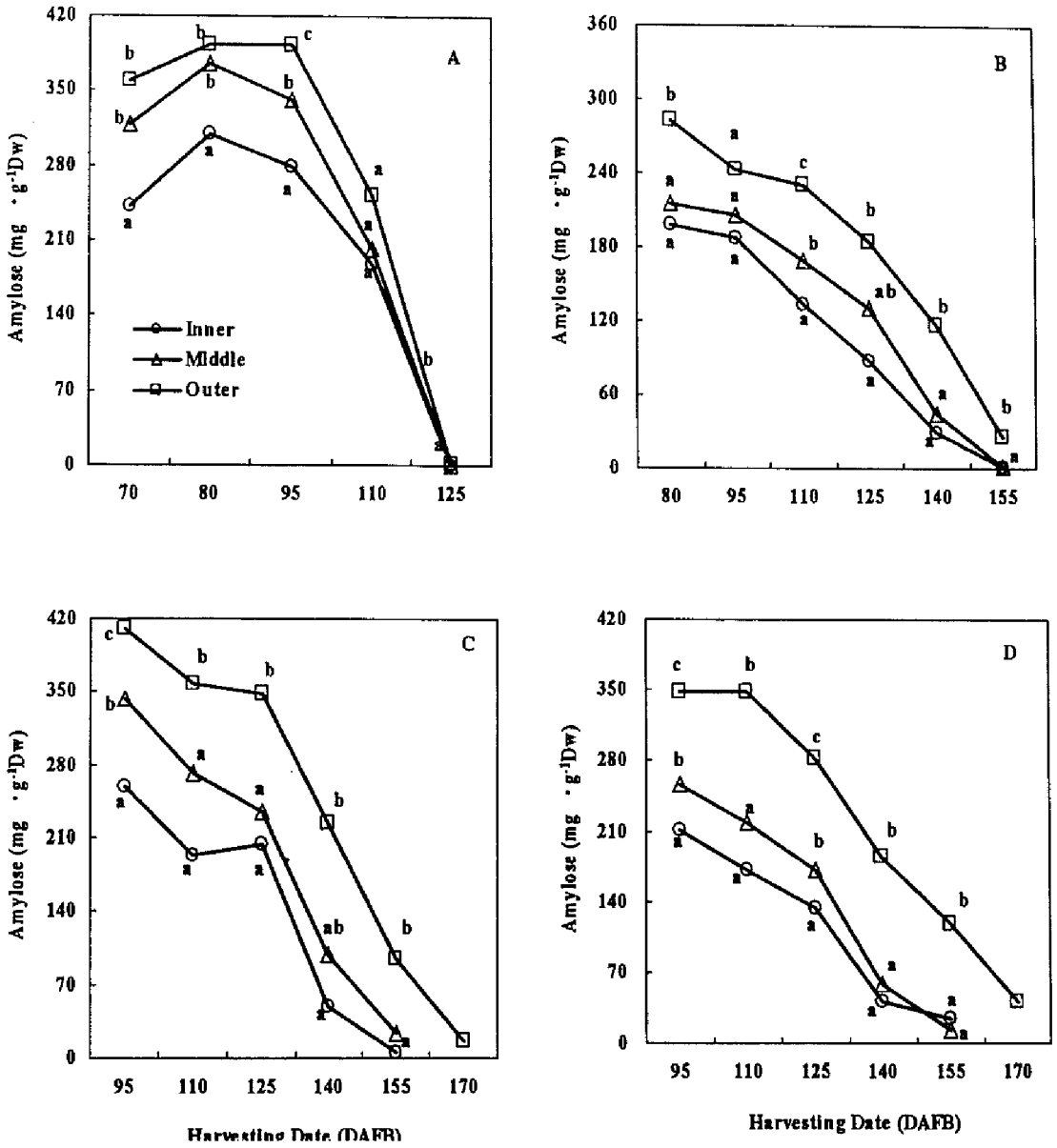

Fig. 4.3 Amylose content of 'Tsugaru' (A), 'Golden Delicious' (B), 'Orin' (C), and 'Fuji' (D) apples during growth and maturation. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

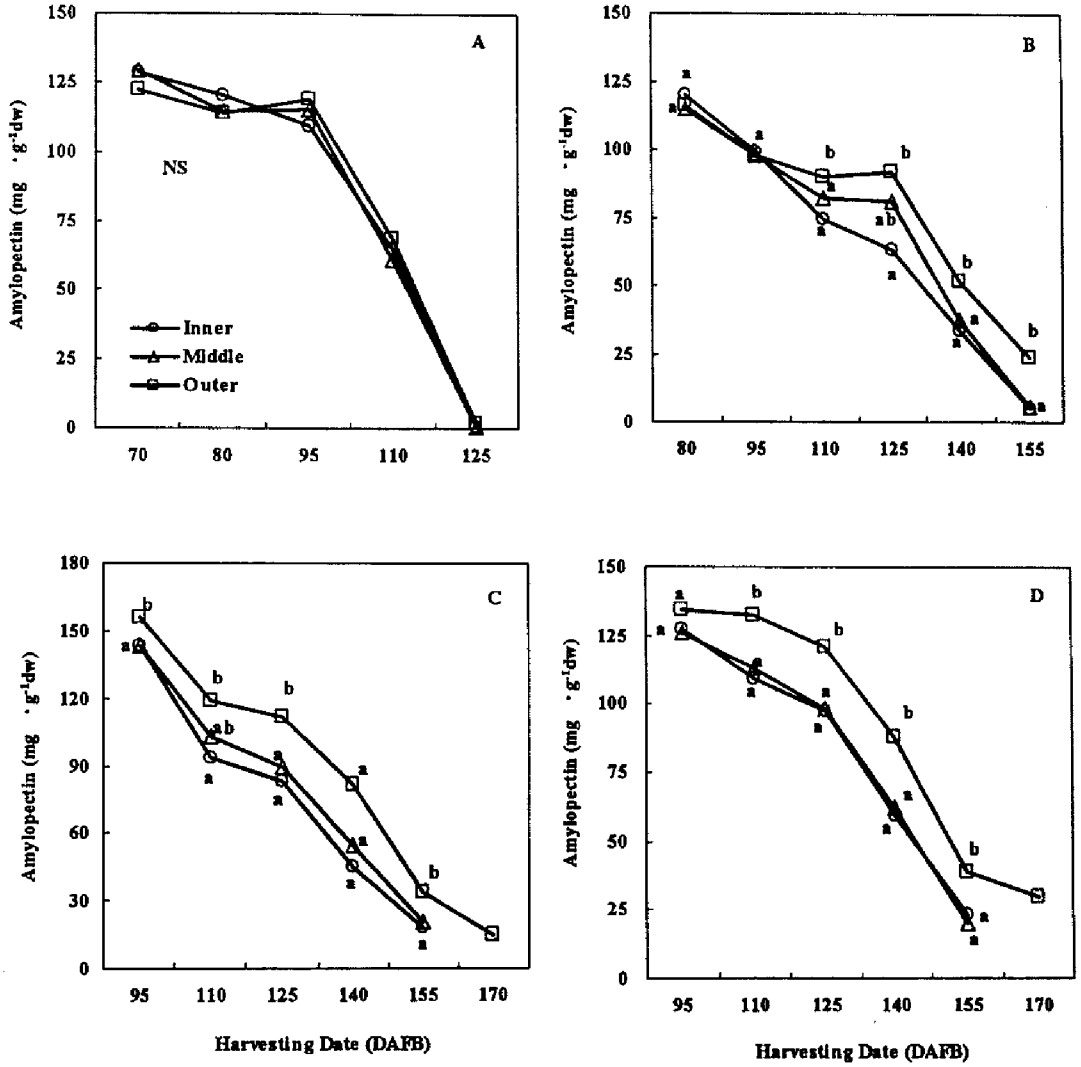

Fig. 4.4 Amylopectin content of 'Tsugaru' (A), 'Golden Delicious' (B), 'Orin' (C), and 'Fuji' (D) apples during growth and maturation. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

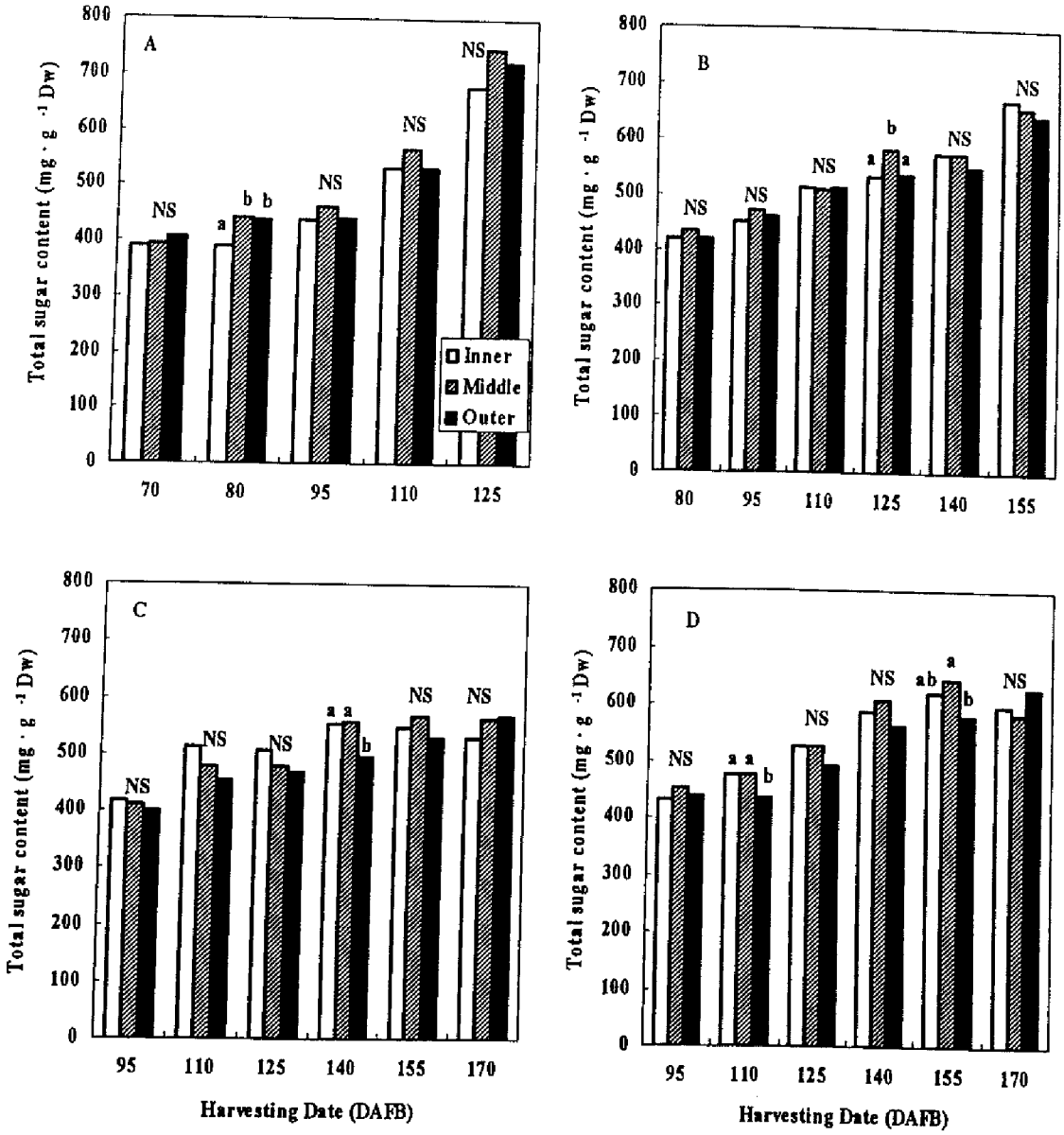

Fig. 4.5 Total sugar content of 'Tsugaru' (A), 'Golden Delicious' (B), 'Fuji' (C), and 'Orin' (D) during growth and maturation. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

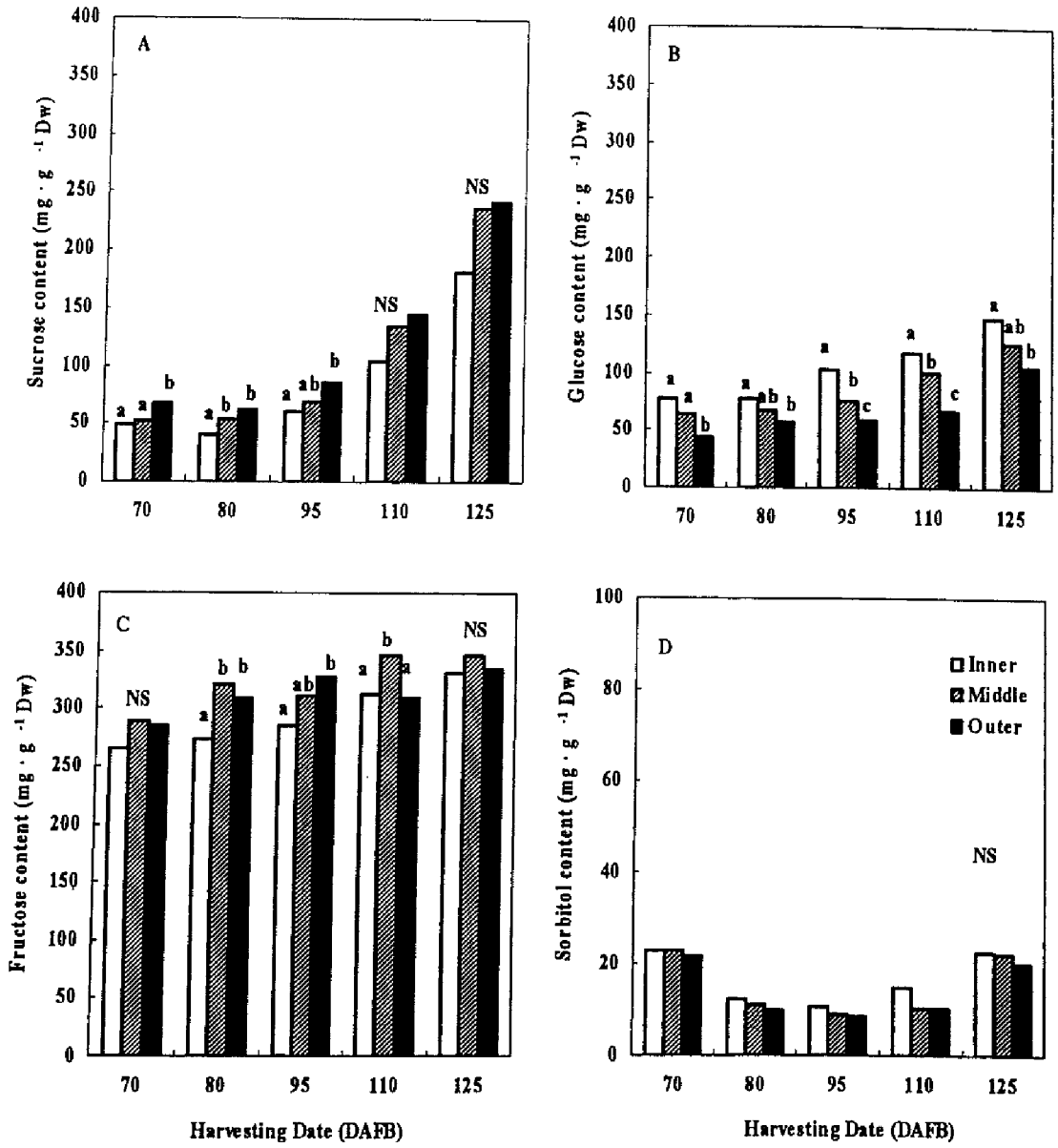

Fig. 4.6 Sucrose (A), glucose (B), fructose (C), and sorbitol (D) contents of 'Tsugaru' during growth and maturation. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

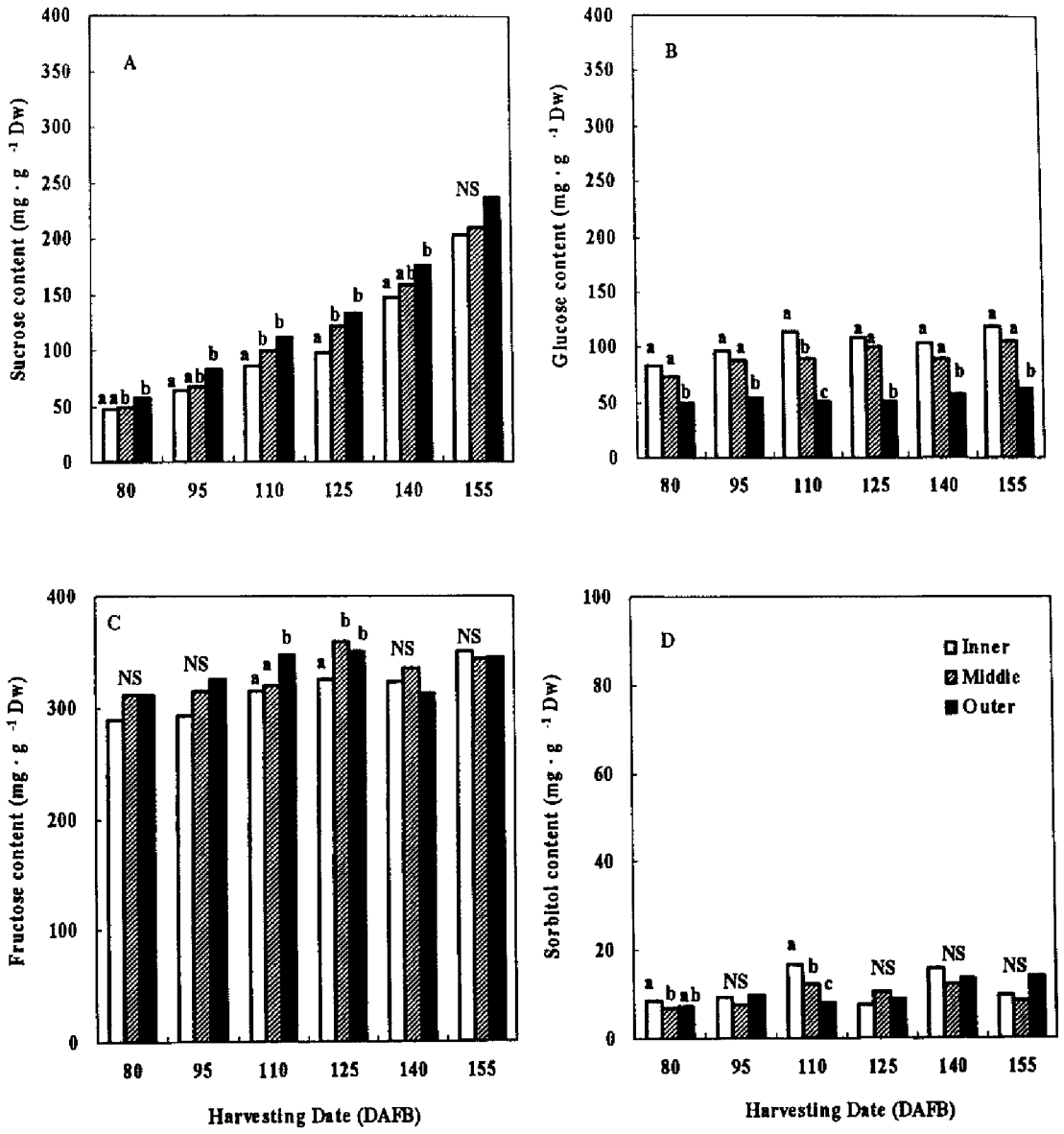

Fig. 4.7 Sucrose (A), glucose (B), fructose (C), and sorbitol (D) contents of 'Golden Delicious' during growth and maturation. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

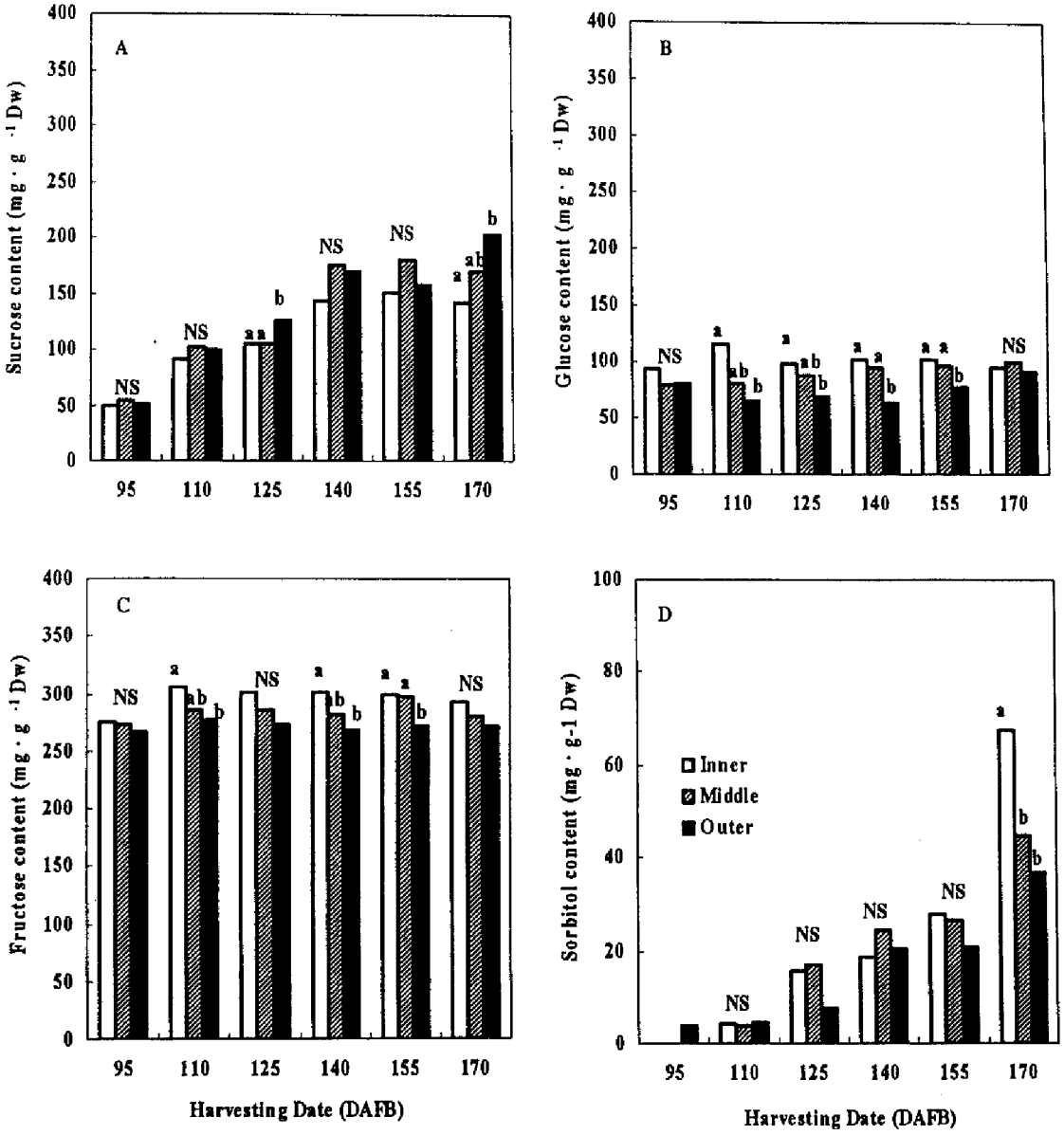

Fig. 4.8 Sucrose (A), glucose (B), fructose (C), and sorbitol (D) contents of 'Fuji' during growth and maturation. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

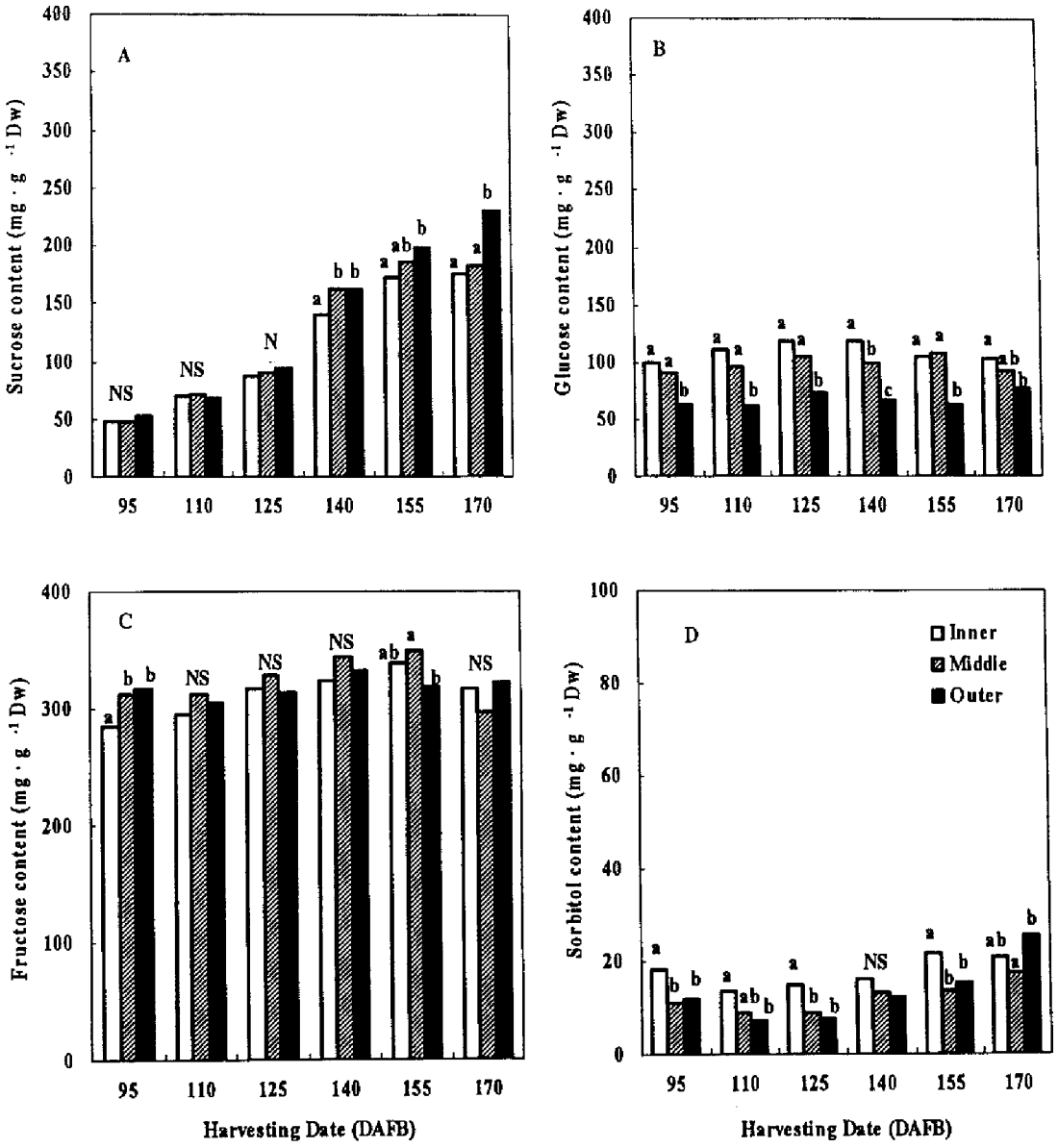

Fig. 4.9 Sucrose (A), glucose (B), fructose (C), and sorbitol (D) contents of 'Orin' during growth and maturation. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

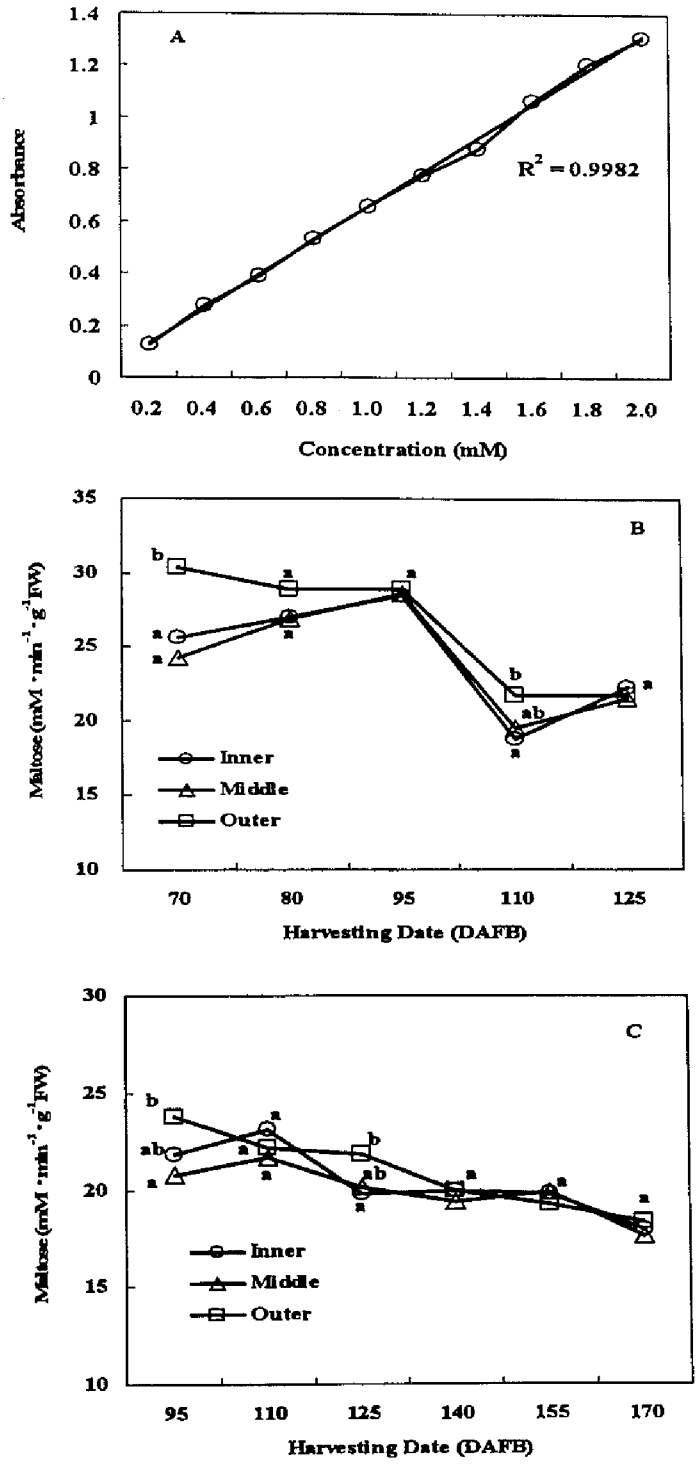

Fig. 4.10 Standard curve of maltose at different concentrations (A), and total hydrolytic activity of 'Tsugaru' (B) and 'Fuji' (C) during growth and development. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

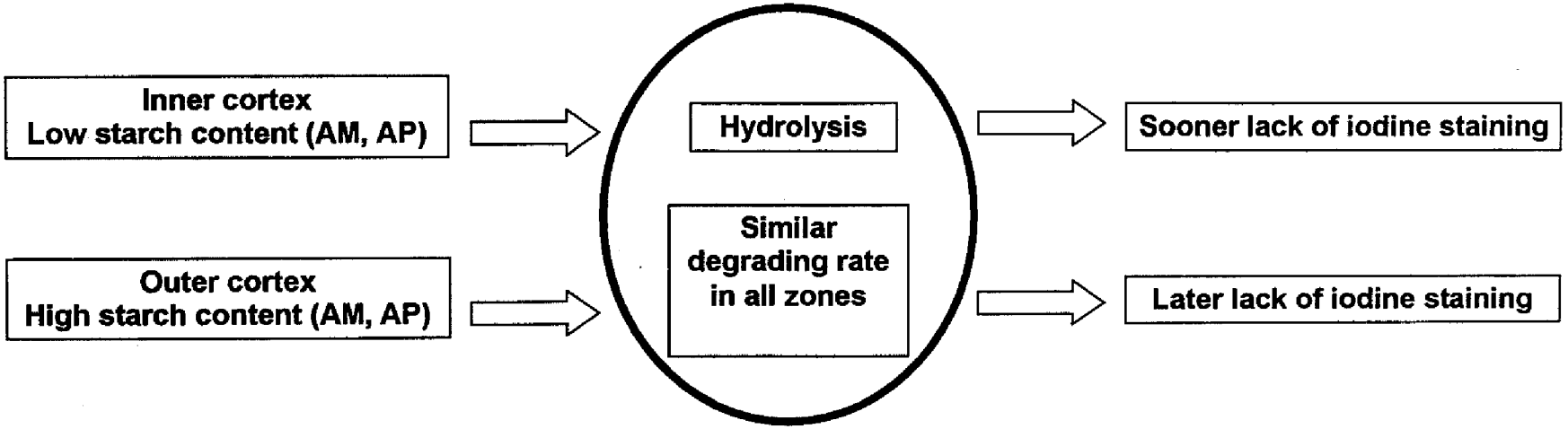

Fig. 4.11 Characteristics of starch degradation pattern in apple fruit flesh.

CHAPTER 5
OVERALL DISCUSSION AND CONCLUSION

Ethylene is known to trigger many physiological and biochemical changes during fruit ripening (Pratt and Goeschl, 1969). This study examined starch degradation of detached apple fruits in relation to the effects of ethylene during ripening using 'Tsugaru' (early-maturing) and 'Fuji' (late-maturing) apples (*Malus domestica* Borkh.). Fruits were harvested at immature and mature stages, and treated with ethylene and 1-MCP. The results showed that in immature fruit of both cultivars, although starch content decreased rapidly during storage at 25℃, 1-MCP had little effect on this. Ethylene treatment stimulated the degradation of starch slightly, but any differences in starch content among different treatments were small. The respiration rate decreased gradually and ethylene production remained low during storage, irrespective of treatments and cultivars. The results suggest that fruits at the immature stage do not respond to both endogenous and exogenous ethylene, and starch degradation does not relate to the climacteric or ethylene. Taiz and Zeiger (1998) suggested that when non-climacteric fruits are treated with ethylene, the magnitude of the respiratory rise increases as a function of ethylene concentration, but the treatment does not stimulate production of endogenous ethylene nor accelerate ripening. Response of immature fruit to exogenous ethylene might be similar to that of the non-climacteric. In mature 'Tsugaru', 1-MCP treatment significantly inhibited ethylene production and reduced respiration rate and starch degradation. The effects of 1-MCP and ethylene on starch degradation in mature 'Fuji' were small, and starch content decreased drastically in all treatments, although 1-MCP had significantly inhibited ethylene production and respiration rate. It is suggested that ethylene is partially involved in starch degradation in mature 'Tsugaru', but not in 'Fuji'. The mature 'Fuji' has been classified as a climacteric fruit. However, the low levels of respiration rate and ethylene production suggest that regulation

of starch degradation metabolism in mature 'Fuji' might be similar to that of immature 'Fuji'. Starch to sugar conversion has also been suggested as another aspect of fruit ripening stimulated by ethylene (Kader, 1985; Watkins, 2003). However, the role of ethylene in starch degradation of detached apple fruit differs between cultivars and their harvest stages.

In addition, the study on the effects of ethylene and 1-MCP on sugar accumulation during storage of immature and mature 'Tsugaru' apples suggest that ripening physiological aspects and accumulation of sugar in immature fruits are not affected by ethylene and 1-MCP. Total sugar content changed only slightly during degradation of starch and there was little difference among treatments. Although the results showed that glucose content of the ethylene-treated fruit was higher than other treatments after harvest, there was no increase in rate of respiration or ethylene production. These results again support the hypotheses that immature fruits could not respond to exogenous ethylene for inducing fruit climacteric, and starch degradation including sugar accumulation did not relate to the climacteric or ethylene (Chapter 2). Although starch degradation of the immature 'Tsugaru' fruit did not seem to be induced by ethylene, the result of this study suggests that the degradation of accumulated starch in the immature fruit provides glucose as a main product during storage. In the mature fruit, there was an inhibitory effect of ethylene and 1-MCP on the accumulation of sugar. Soluble solids were higher in 1-MCP-treated apples (Fan *et al.*, 1999). However, they were neither unaffected nor inhibited by 1-MCP in some cultivars (Rupasinghe *et al.*, 2000; DeEll *et al.*, 2002; Watkins *et al.*, 2000). In addition, as hexose sugar is a substrate for respiration metabolism and fruit respires continuously after harvest (Kays, 1991), sugars from starch degradation in the detached fruit might be used mainly in respiration to produce energy and other carbon compounds for cell maintenance and necessary synthetic reactions during storage.

The effect of ethylene on starch degradation and sugar accumulation in detached apple fruits differed between cultivars and their harvest stages, in relation to the ripening characteristics of the fruit. Additionally, a study on the characteristics of starch hydrolysis and changes in physiological and chemical aspects in different flesh zones of on-tree fruits was conducted using 'Tsugaru', 'Golden Delicious', 'Fuji', and 'Orin' apples. It is suggested that, even in attached fruits, simultaneous increases in ethylene and respiration observed during starch degradation of 'Tsugaru', and changes in amylose, amylopectin, total hydrolytic activity, and sugar compositions cumulatively account for the different degradation patterns of starch in fruit flesh among different cultivars. Moreover, as starch degradation of the apple fruit proceeds from the core towards the outer cortex (Poapst et al., 1959), the contents of amylose and amylopectin were highest in the outer cortex and lowest in the inner core area and also, similar degradation patterns of starch and total hydrolytic activity were observed in all tissue zones. This supports the theory that starch degradation of the apple fruit seemed to begin simultaneously rather than preferentially in any one tissue zone (Brookfield *et al.*, 1997). In addition, although there was no clear difference among tissue zones, an increase in the sugar content of 'Tsugaru' occurred simultaneously with the loss of starch, but in the other three cultivars, only slight changes were observed during growth and maturation. In late-maturing cultivars such as 'Fuji', the high sorbitol content in all tissues suggests that sugar translocation may be involved in the development of sweetness in attached apple fruits. Moreover, the increase in total sugar content may be due to conversion of translocated sorbitol to fructose by SDH during growth and maturation (Bieleski and Redgwell, 1985; Teo et al., 2006). To clarify this phenomenon, further studies on sugar translocation and individual enzyme expression of each cultivar are required. In order to improve fruit eating quality, further studies on starch degradation metabolism should take physiological properties and the accumulation of sugars from both starch hydrolysis and translocation during

growth, maturation, and ripening into consideration. Moreover, this research shows that there were differences in starch degradation mechanisms and in the responses of fruit physiology to exogenous ethylene between fruits of varying harvest dates. The further study at the molecular level could clarify the specific action of ethylene on starch degradation metabolism and the differential response of fruits to ethylene as observed between varying cultivars and development stages.

SUMMARY

In an unripe apple, starch is a major carbohydrate accumulated in the fruit. As the fruit ripens, starch is then degraded to provide sugars associated with fruit sweetness. The relationship between fruit ripening and starch degradation characteristics of the apple fruit was studied in this research. Starch degradation of the detached fruit in relation to ripening and ethylene was studied in 'Tsugaru' (early-maturing) and 'Fuji' (late-maturing) fruits with treatments of ethylene or 1-methylcyclopropene (1-MCP), an ethylene inhibitor. Moreover, in order to understand starch to sugar conversion during ripening of detached fruits, effects of ethylene and 1-MCP on sugar accumulation during storage of immature and mature 'Tsugaru' apples were evaluated. In addition, since simultaneous increase in ethylene and respiration rate were observed during starch hydrolysis in on-tree fruits and cultivar variations seem to influence degradation pattern of fruit starch, characteristics of the changes in physiological aspects, starch (amylose and amylopectin), sugar contents, and total hydrolytic activity of attached apples were investigated during development and maturation processes.

For the relationship between fruit ripening and starch degradation, the physiology of starch degradation in relation to ripening and ethylene was investigated using 'Tsugaru' (early-maturing) and 'Fuji' (late-maturing) apples (*Malus domestica* Borkh.). Fruits were harvested at immature and mature stages, and treated with ethylene and 1-MCP. The results showed that fruits at the immature stage could not respond to endogenous and exogenous ethylene for inducing the climacteric, and starch degradation did not relate to the climacteric or ethylene. For the mature fruits, the results suggest that ethylene is partially involved in starch degradation in mature 'Tsugaru', but not in 'Fuji'. In addition, the role of ethylene in starch degradation of detached apple fruits differs between cultivars and their harvest stages.

For starch to sugar conversion during ripening, effects of ethylene and 1-MCP on sugar accumulation during storage of immature and mature 'Tsugaru' apples were evaluated. In immature 'Tsugaru', ripening physiological aspects of the immature fruit were not affected by ethylene or 1-MCP. Although increased glucose content was found in the ethylene-treated fruit, accumulation of total sugar does not seem to be induced by ethylene. In the mature fruit, ripening aspects were inhibited by 1-MCP; however it seemed to reduce the accumulation of sugar only at day 7 after treatment. Although observed changes in the ripening aspects and starch loss of the ethylene-treated fruit were greater than the untreated fruit, exogenous ethylene did not induce sugar accumulation during storage of mature 'Tsugaru' fruit.

In addition, cultivar variations and the characteristics of starch hydrolysis and sugar accumulation in different flesh zones were also investigated in on-tree fruits using 'Tsugaru', 'Golden Delicious', 'Fuji', and 'Orin' apples, harvested on five to six different dates. Iodine staining and changes in amylose and amylopectin contents revealed different starch degradation patterns among cultivars. Additionally, this suggests that the climacteric of fruits seemed to influence the loss of starch in 'Tsugaru', but not in later-maturing cultivars such as 'Golden Delicious', 'Fuji', and 'Orin'. In view of each particular flesh zone, although the content of amylose and amylopectin was highest in the outer cortex and lowest in the inner core area, similar degradation patterns of starch and total hydrolytic activity were observed in all tissue zones. Starch degradation of the apple fruit seemed to begin simultaneously rather than preferentially in any one tissue zone. Although there was no clear difference observed among tissue zones, there was a simultaneous increase in sugar content of 'Tsugaru' with the loss of starch, but in the other three cultivars, only slight changes were observed during growth and maturation. In late-maturing cultivars such as 'Fuji', sugar translocation may be involved in sweetness development in attached apple fruits.

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