

# Starch degradation characteristics in relation to physiological and biochemical properties during growth and maturation of apple fruit

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## Abstract

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' (early-maturing) and 'Fuji' (late-maturing) apples (*Malus domestica* Borkh.). Different starch degradation characteristics during the growth and maturation processes were observed between 'Tsugaru' and 'Fuji'. By iodine staining, the loss of starch in 'Tsugaru' was observed earlier than in 'Fuji'. The different degradation patterns of starch were also demonstrated through the observations on 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 'Fuji', starch degraded gradually throughout growth and maturation process and was clearly degraded at 170 DAFB with a low level of ethylene production and decreased respiration. In both the 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 in both cultivars. 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, sugars from the degradation of accumulated starch and sugar translocation seem to influence mainly the sweetness quality as the fruit ripens.

**Key words:** Amylopectin, amylose, *Malus domestica* Borkh., starch degradation, total hydrolytic activity.

## 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 as a maturity index of an apple to provide an estimation of the starch content (Fan *et al.*, 1995; Ingle and D'Souza, 1989; 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 (Fan *et al.*, 1995; McCready and Hassid, 1943). 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.

Knowledge of the relationship between these properties would be valuable for 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 'Fuji', which produces very low amounts of ethylene.

## Materials and methods

Two cultivars of apples, 'Tsugaru' fruits from a 6-year-old tree, and 'Fuji' fruits from a 31-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 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); and the 'Fuji' fruits on 95,

110, 125, 140, 155, 170 DAFB. Fifteen fruits of each cultivar per harvesting crop were used. The last harvest of each cultivar was a week before commercial harvest.

### Measurements

**Respiration rate, ethylene production and starch rating:** To determine CO<sub>2</sub> 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 chromatography (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°C. To determine the ethylene content, 1 mL of the headspace gas was removed and injected into a gas chromatography (model GC-8A, Shimadzu Co., Ltd., Japan) 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°C, respectively.

The starch distribution was measured by dipping an apple slice taken from the equatorial region in I<sub>2</sub>-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.

**Starch, AM, and AP contents:** Samples were taken by a cork borer (1 cm  $\phi$ ), 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<sub>2</sub> and stored at -80°C 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 Leurquin (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 thoroughly mixed and then incubated at 20°C for 30 min. 10 mL distilled H<sub>2</sub>O was added to each tube; the supernatant was mixed and centrifuged at 3,800 $\times$ 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  $\mu$ L of I<sub>2</sub>-KI (2 mg I<sub>2</sub> and 20 mg KI per mL H<sub>2</sub>O) 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, Ltd., Japan).

AM (Sigma Chemical Co., St. Louis) 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<sub>AM606</sub> = 6.88, A<sub>AM530</sub> = 4.54, A<sub>AP606</sub> = 5.00, A<sub>AP530</sub> = 6.99, and a typical standard curve with an r<sup>2</sup> 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.

**Sugar content:** For sugar determination, 100 mg 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°C. The homogenates were centrifuged at 15,000 $\times$ 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 resolubilized 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 Co., Ltd., Japan). De-gassed, distilled, de-ionized water at 1 mL min<sup>-1</sup> and 80°C was used as the mobile phase. A refractive index detector (RI-98, Laboratory System Co., Ltd., Tokyo, Japan) was used to quantify sugar content following the separation. Sugar content was determined by comparison with standard samples of known concentration of glucose, fructose, sucrose, and sorbitol.

**Activity of total hydrolytic enzyme:** The total hydrolytic activities of 'Tsugaru' and 'Fuji' (inner, middle, and outer parts) were investigated by the method described by Steup (1990). Briefly, apple flesh (approximately 5 g fresh weight) was homogenized in 30 mL ice-cold grinding medium which consisted 50 mM 2-(N-morpholino)ethane-sulphonic acid (Mes), brought to pH 6.0 with NaOH, 5mM CaCl<sub>2</sub>, and 5% (v/v) glycerol. The homogenate was filtered through several layers of Miracloth and the filtrate was centrifuged for 15 min at 40,000 $\times$ g. The supernatant was passed through a Sephadex G-25 gel which had 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°C. At 5 min, the incubation mixture was added to an equal volume of alkaline colour 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 colour reagent, treated as above). The alkaline colour 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<sub>2</sub>O 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 colour 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 colour reagent and processed as described above. A typical standard curve of maltose has an r<sup>2</sup> value of 0.99 (Fig. 7A).

**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.

## Results

**Fruit maturity:** Starch index value, respiration rate, and ethylene production were investigated during the growth and maturation

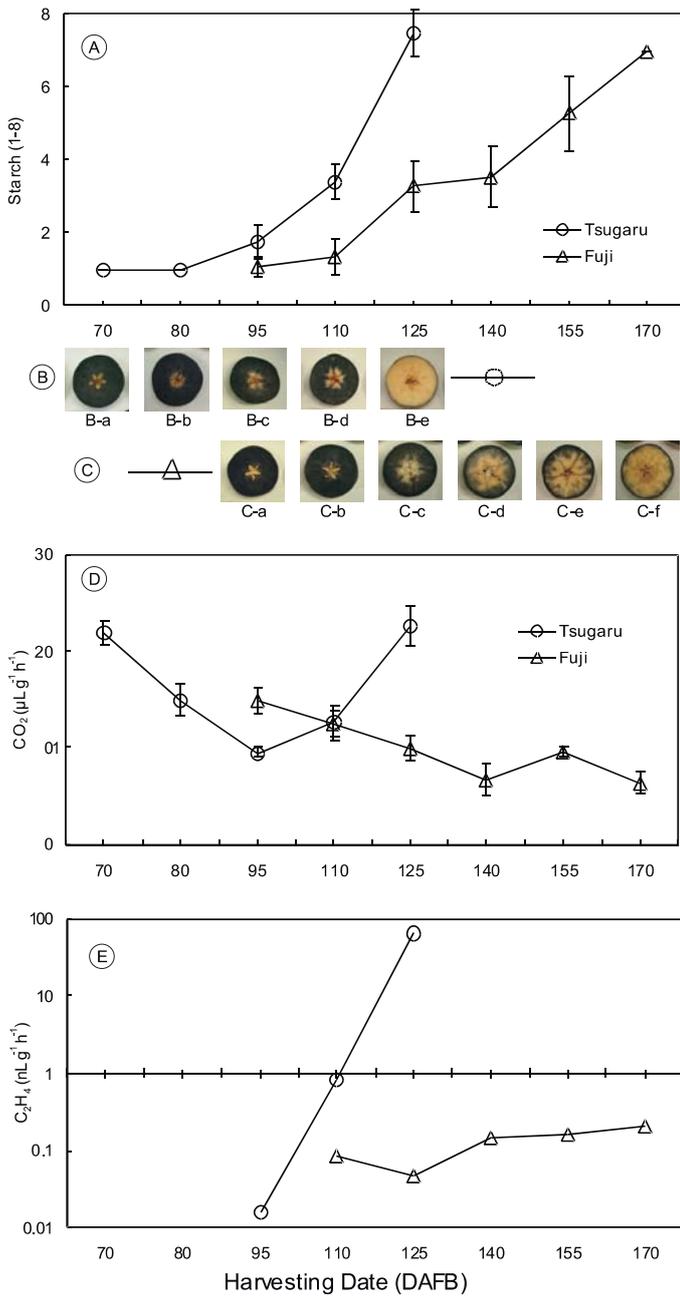


Fig. 1. Starch rating scores (A), iodine staining patterns (B; 'Tsugaru' and C; 'Fuji'), respiration rate (D), and ethylene production (E) of 'Tsugaru' and 'Fuji' apples. Each value is the mean of five replicates.

processes of the fruit samples. The starch degradation of fruit flesh and different degradation patterns between 'Tsugaru' and 'Fuji' 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 content of 'Fuji' degraded gradually throughout growth and maturation and obvious loss was observed at 170 DAFB (Fig. 1A).

Starch rating, by iodine staining, was quantified at five and six stages of growth and maturation for 'Tsugaru' and 'Fuji', respectively (Fig. 1B, 1C). The starch-staining pattern of 'Tsugaru' showed slight loss of starch at the core area between 80 and 95 DAFB (Fig. 1B-b, 1B-c). Starch degradation was observed all over the fruit flesh, with starch content of the core area completely lost at 110 DAFB (Fig. 1B-d) and almost all starch completely lost at 125 DAFB (Fig. 1B-e). Contrarily, starch in 'Fuji' was lost

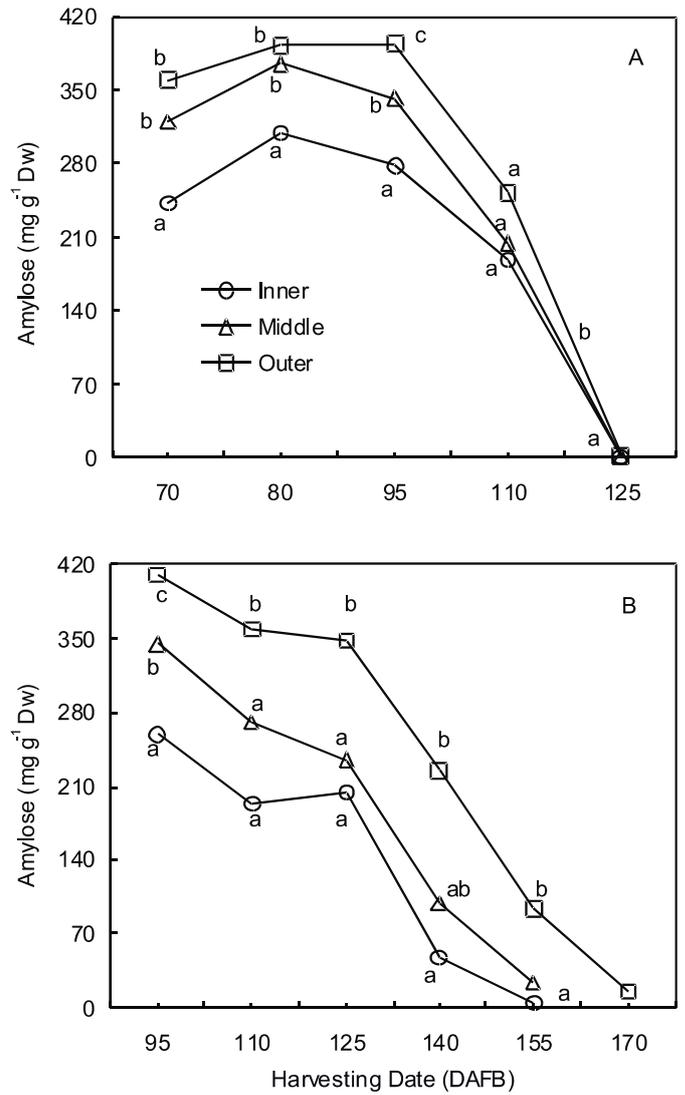


Fig. 2. Amylose content of 'Tsugaru' (A) and 'Fuji' (B) apples during growth and maturation. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at  $P=0.05$  by Tukey's multiple-range test.

gradually during growth and maturation. Starch of the core area was completely degraded while some loss of starch in the middle cortex was observed at 125 DAFB (Fig. 1C-c). Almost all starch was degraded at the last harvest at 170 DAFB (Fig. 1C-f).

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. However, in 'Fuji', respiration rate decreased gradually and low level of ethylene production was observed during investigation period (Fig. 1D, 1E).

**AM and AP contents:** AM content of the two 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 to 110 DAFB. Almost all starch was degraded at 125 DAFB (Fig. 2A).

However, AM content of 'Fuji' was highest at the first harvest (95 DAFB) and then decreased gradually during the investigation

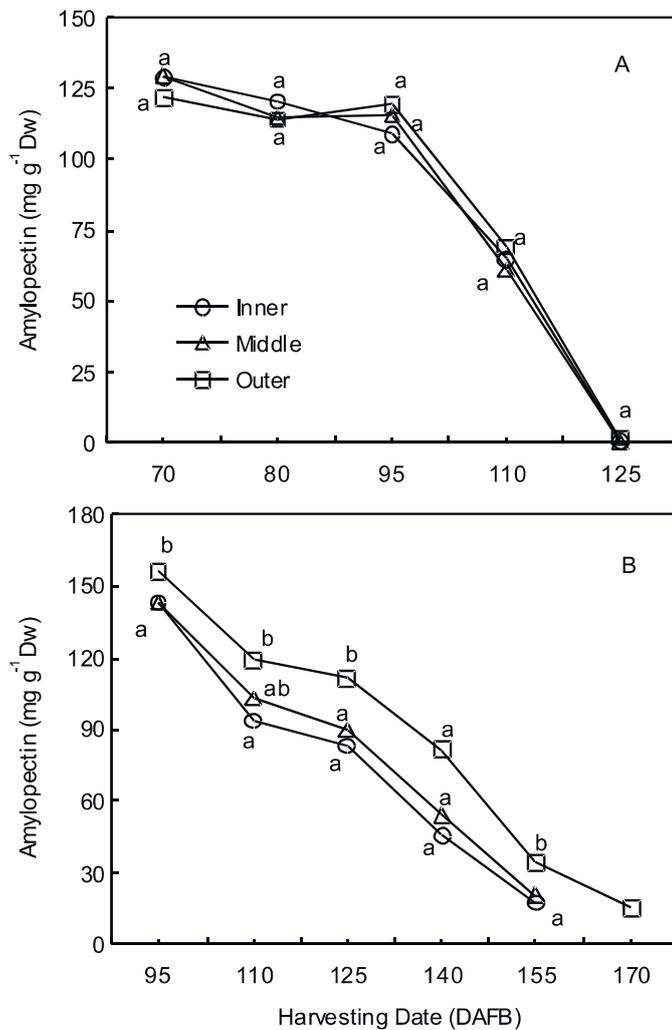


Fig. 3. Amylopectin content of 'Tsugaru' (A) and 'Fuji' (B) apples during growth and maturation. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at  $P=0.05$  by Tukey's multiple-range test.

period (Fig. 2B). AP content also decreased during growth and maturation; however, there was no significant difference in AP content among tissue zones in 'Tsugaru' (Fig. 3;  $P \leq 0.05$ ).

**Sugar content:** Total sugar content (sum of sucrose, glucose, and fructose) of 'Tsugaru' and 'Fuji' gradually increased during growth and maturation (Fig. 4). The individual sugar component of these two cultivars was shown in Fig. 5 and Fig. 6. Although the difference among tissue zones was not clear, total sugar content of 'Tsugaru' increased during growth and maturation, and it increased greatly between 110 and 125 DAFB (Fig. 4A). For the individual sugar content of 'Tsugaru', sucrose content was high between 110 and 125 DAFB, and was highest in the outer cortex (Fig. 5A). Glucose content increased slightly, and was highest in the inner tissue zone (Fig. 5B). Fructose and sorbitol contents changed slightly and the difference among tissue zone was not clear (Fig. 5C, 5D).

In 'Fuji', total sugar and sucrose contents tended to increase during maturation (Fig. 4B, 6A). The glucose and fructose contents changed slightly with the highest content in the inner cortex (Fig. 6B, 6C). The sorbitol content of 'Fuji' was higher than 'Tsugaru', and the highest sorbitol content of the inner cortex was observed between 155 and 170 DAFB (Fig. 6D).

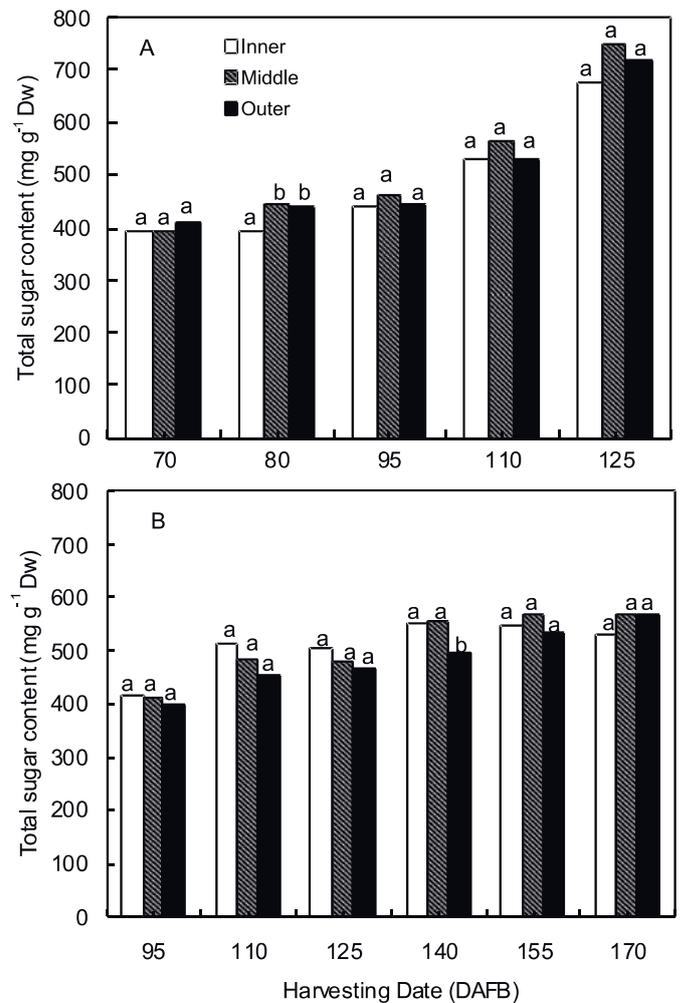


Fig. 4. Total sugar content of 'Tsugaru' (A) and 'Fuji' (B). Each value is the mean of five replicates. Mean values with the same letter are not significantly different at  $P=0.05$  by Tukey's multiple-range test.

**Total hydrolytic activity:** The total hydrolytic activity of 'Tsugaru' changed slightly between 70 to 95 DAFB, but dropped significantly at 110 DAFB. In 'Fuji', however, 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 both cultivars (Fig. 7B, 7C;  $P \leq 0.05$ ).

## Discussion

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

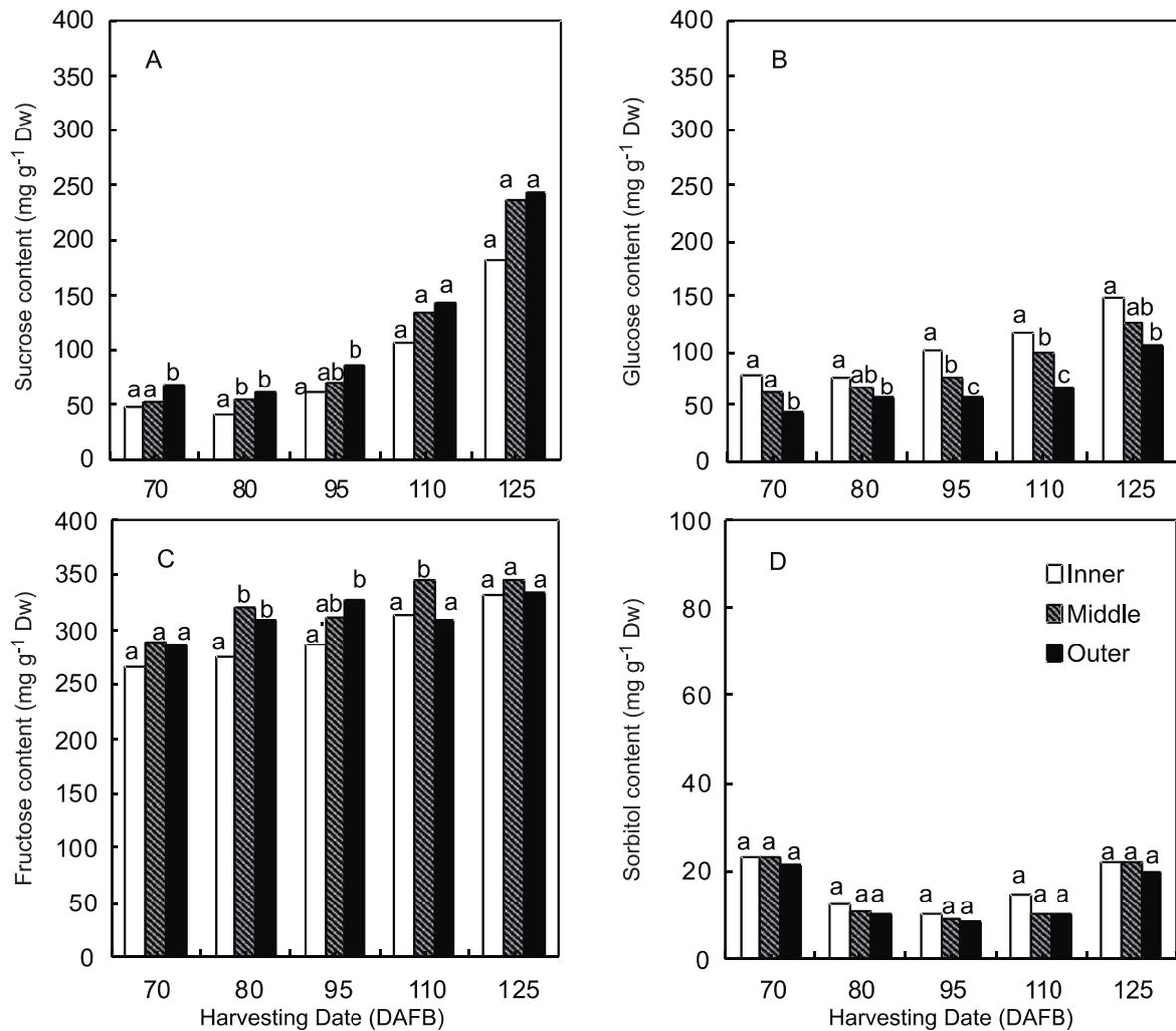


Fig. 5. Sucrose (A), glucose (B), fructose (C), and sorbitol (D) content of 'Tsugaru' during growth and development. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at  $P=0.05$  by Tukey's multiple-range test.

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 our results, 'Tsugaru' and 'Fuji' revealed different patterns of starch loss. The loss of starch in 'Tsugaru' (early-maturing cultivar) was rapid and complete between 110 to 125 DAFB (Fig. 1B). However, iodine staining of 'Fuji' 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 of late-maturing cultivars such as 'Fuji' 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 'Fuji', 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. 7B, 7C). 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

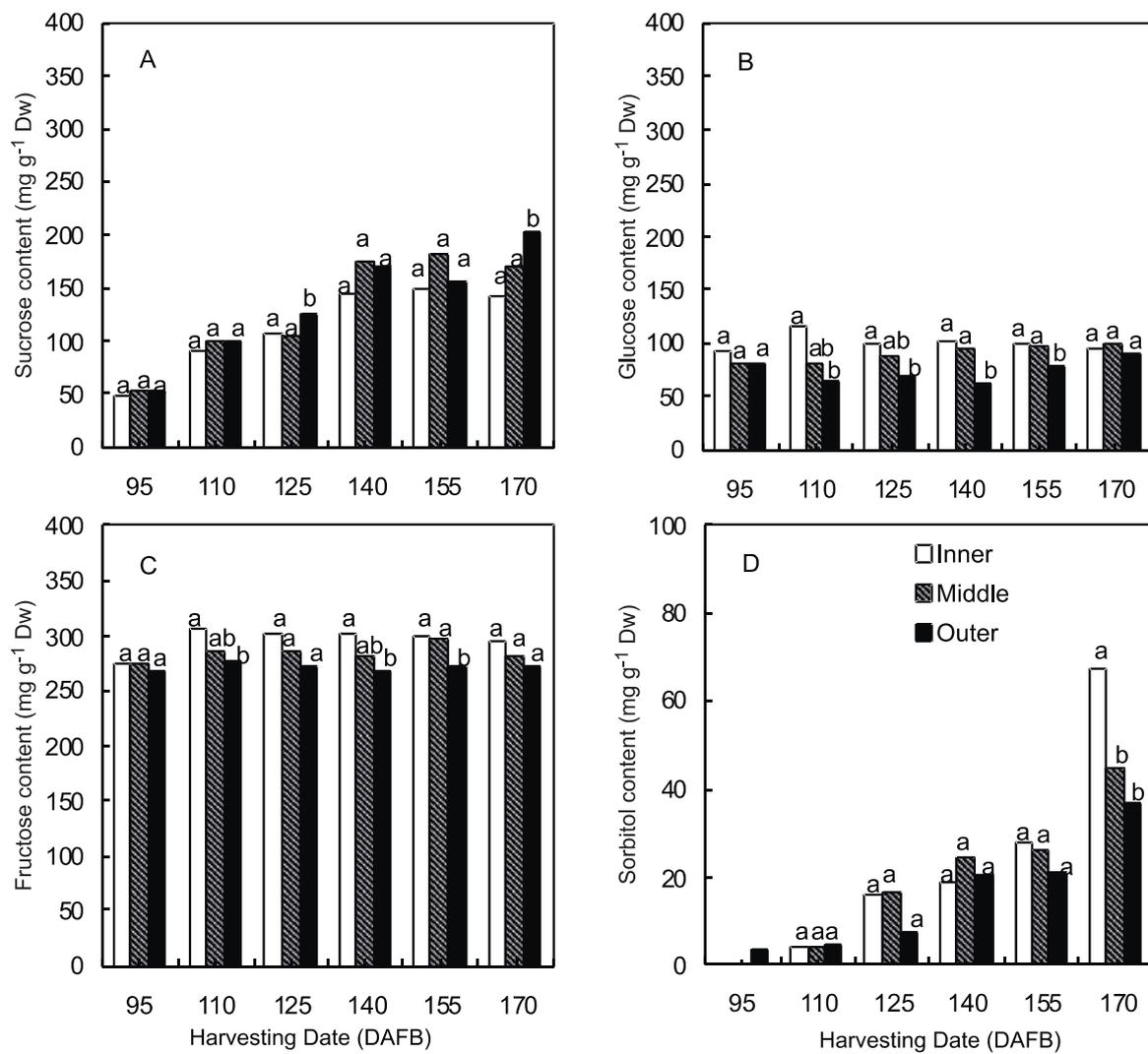


Fig. 6. Sucrose (A), glucose (B), fructose (C), and sorbitol (D) content of 'Fuji' during growth and development. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at  $P=0.05$  by Tukey's multiple-range test.

is very low, the lack of iodine staining will occur sooner rather than later during fruit maturation and ripening. Additionally, this supports the hypothesis that different degradation patterns between 'Tsugaru' and 'Fuji' 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. Our results revealed that the total sugar content (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) (Bialeski 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 'Fuji'. 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. 6D). 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.

Overall, physiological and biochemical characteristics of the fruit development and maturation process, including respiration rate, ethylene production, AM and AP contents, and sugar content, 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, produced a high amount of ethylene and had increased rate of respiration in order to induce degradation of starch and production of sugar to develop fruit sweetness as the fruit ripened. However, in the late-maturing 'Fuji', gradual starch

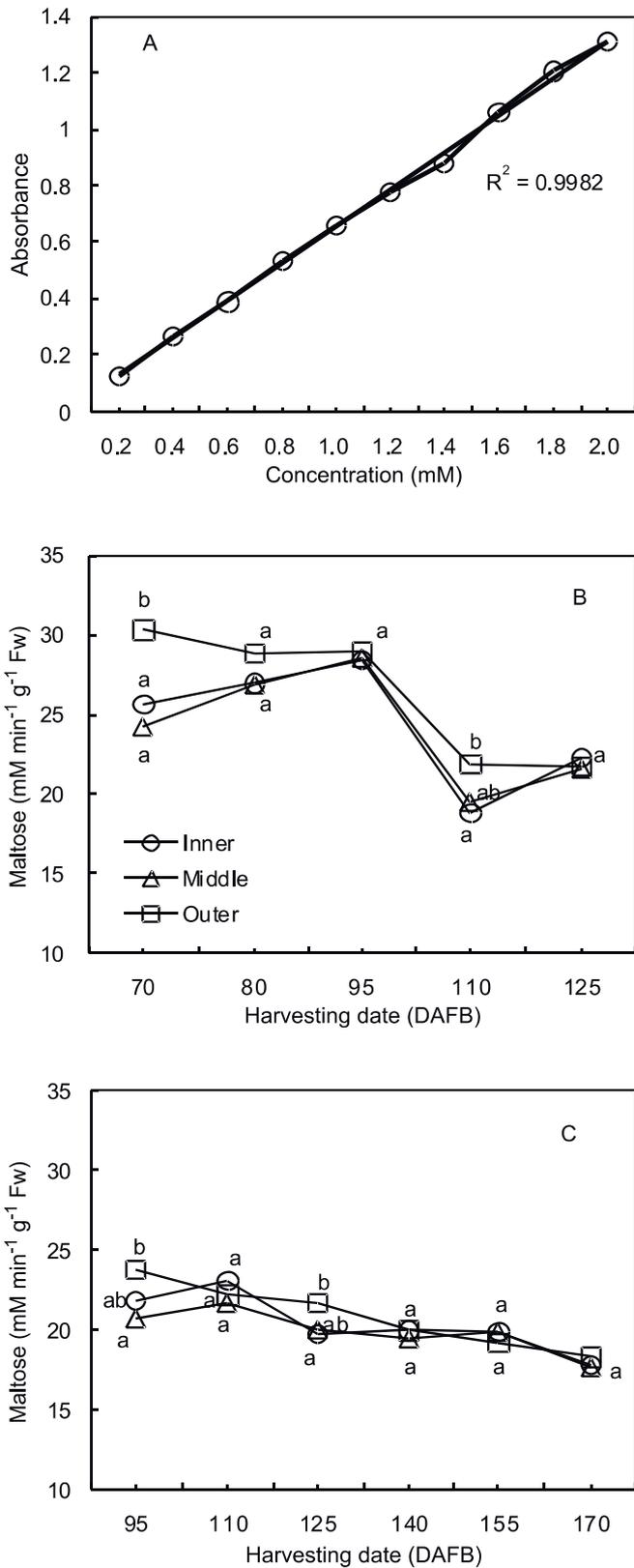


Fig. 7. Standard curve of maltose at different concentrations (A), and total hydrolytic activity of 'Tsugaru' (B) and 'Fuji' (C) during growth and development. Each value is the mean of five replicates. Mean values with the same letter are not significantly different at  $P=0.05$  by Tukey's multiple-range test.

degradation occurred simultaneously with low levels of respiration and ethylene production, and sugar translocation seemed to be the main factor in sweetness development.

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