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Relationship between Apple Ripening and Browning: Changes in Polyphenol Content and Polyphenol Oxidase

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Changes in the browning of apple juice, polyphenol content, and polyphenol oxidase were examined during apple ripening. The degree of browning of apple juice, the phenol content per gram of flesh, and the polyphenol oxidase (PPO) activity per milligram of protein decreased during ripening. From 4.5 to 6 months after flowering, the amount of PPO per milligram of protein, determined by an enzyme-linked immunosorbent assay, was constant, but its specific activity decreased, suggesting that PPO had denatured. PPO was uniformly distributed in the immature and mature apple, but active PPO was mainly localized around the core in the mature apple.

Keywords: *Apple; Malus pumila; polyphenol oxidase; browning; enzymatic browning; polyphenol; apple ripening; tissue print method; enzyme-linked immunosorbent assay*

INTRODUCTION

Enzymatic browning of raw fruits and vegetables is important for food preservation and processing and is generally considered to be an undesirable reaction because of the unpleasant appearance and concomitant development of an off-flavor. Apple is one of the most popular fruits. Thus, enzymatic browning of this fruit is an important topic from the standpoint of food science and technology. The enzymatic browning of mature apple has been investigated by many authors. It is considered that the polyphenols in apple are oxidized to their corresponding quinones by polyphenol oxidase (EC 1.10.3.1, PPO) and that these quinones are then polymerized with other quinones and amines to form brown pigments.

In mature apple, the degree of enzymatic browning is determined by polyphenol concentration, PPO activity, or both. It depends on the variety of apple that either substrate or PPO activity is a determinant of enzymatic browning (CoSeteng and Lee, 1987). The amount of polyphenol decrease by browning was a good indicator for enzymatic browning (Amiot *et al.*, 1992).

On the other hand, we recently isolated chlorogenic acid oxidase as the main PPO in mature apples to homogeneity by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (Murata *et al.*, 1992), and it was immunochemically studied (Murata *et al.*, 1993). Western blotting analysis has shown that various cultivars of apple have the same PPO. The PPO was mainly located around the core of the fruit, shown by the nitrocellulose tissue print method. This localization of PPO corresponded to intense browning around the core.

There is a little information available on the changes in enzymatic browning during ripening on the tree (Weurman and Swain, 1955; Harel *et al.*, 1966), while the changes in sugars, acids, and amino acids, each having a marked influence on the sensory quality of the fruit, have recently been reported during ripening (Ackermann *et al.*, 1992). There is no report on the

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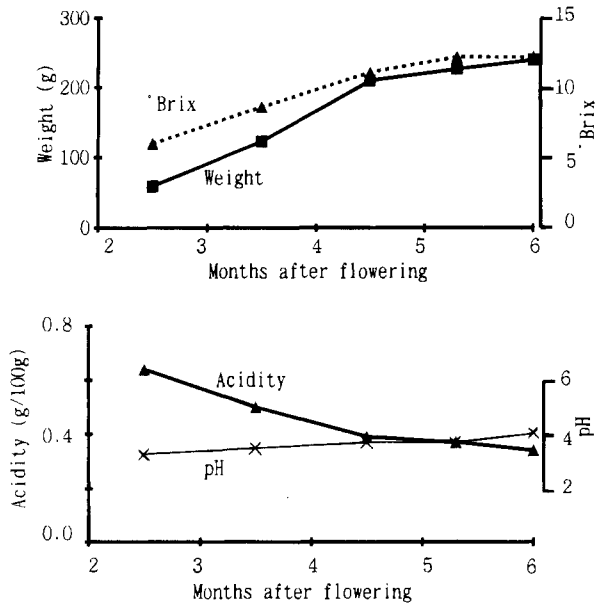

Figure 1. Changes in the weight (g), pH, sugar content (°Brix), and titration acidity (g/100 g) of apple (cv. Fuji) during ripening.

changes in the activity, quantity, and distribution of PPO during apple ripening.

The aim of this study is to follow the fate of important components of the enzymatic browning of apple, polyphenols and PPO, at different stages of fruit development from 2.5 months after flowering to 6 months, the usual harvesting time. Especially the changes in the activity, amount, and distribution of PPO in apple fruit were investigated immunochemically. We also report here the changes in brown color formation, polyphenol content, and remaining polyphenol content after browning, which was determined by HPLC at each ripening step.

MATERIALS AND METHODS

Plant Materials. Apples (*Malus pumila* cv. Fuji) were obtained from the Morioka Branch of the Fruit Tree Research Station (Iwate, Japan), Fuji being the most popular cultivar in Japan. The apples were picked in July (2.5 months after flowering), August (3.5 months), September (4.5 months), October (5.3 months), and November (6.0 months) during 1992 and 1993 from the same tree. The tree flowered on May 13, 1992, and May 16, 1993. The apples were stored in a cool room at 4 °C, and their extracts were prepared within a week after harvesting.

Weight, pH, Sugar Content, and Acidity. Ten apples at each ripened stage were weighed. An apple was peeled, cored, weighed, and homogenized with the same weight of distilled water in a juicer (National MJ 410G, Osaka, Japan) for 30 s. The filtrate was used for the measurement of pH, titration acidity, and sugar content, which was determined by a refractometer (Atago, Tokyo, Japan). The endpoint of titration acidity was determined by pH meter (pH 7.0). The acidity is expressed as grams of malic acid per 100 g of apple. Three apple extracts were prepared at each ripened stage, and each extract was measured three times.

Measurement of the Browning of Apple Juice. A cold apple was peeled, cored, weighed, and then homogenized with the same weight of cold distilled water in a juicer for 30 s. The homogenate was incubated on a reciprocating shaker (about 100 oscillations/min; Yamato BT-46, Tokyo, Japan) at 30 °C for 0, 30, and 60 min and 24 h, the same volume of a 4% metaphosphoric acid solution being added to the apple juice sample to stop browning. Reflectance measurements (L, a, and b values) were determined by a TC-3600 color difference meter (Tokyo-Denshoku, Tokyo, Japan) to evaluate the degree of browning. Saturation and color difference were respectively calculated as $(a^2 + b^2)^{1/2}$ and $(\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$ after 24 h of incubation, each measurement being taken three times. Three apple juice samples were prepared at each ripened stage.

Extract Preparation for Polyphenol Determination. A cold apple was peeled, cored, weighed, and homogenized with about 2 volumes of cold methanol in a juicer for 30 s and the juice shaken for 10 min. The homogenate was filtered, and the residue was extracted twice by methanol in the same way. The combined filtrate was concentrated *in vacuo*, and an aliquot of the concentrate was used for determining total phenol. The concentrate was further extracted by ethyl acetate three times, the extract being dried by Na_2SO_4 and concentrated *in vacuo*. The crude paste obtained was dissolved in methanol, passed through Sep-Pak C_{18} (Waters/Millipore, Milford, MA) and Chromatodisk (0.45 μm, Kurabo, Osaka, Japan), and then subjected to high-performance liquid chromatography (HPLC). Three apple extracts were prepared at each ripened stage.

Polyphenol Determination. Each extract was measured three times. Total phenol was calorimetrically determined according to the method of Weurman and Swain (1955), using the phenol reagent. Chlorogenic acid was used as the standard, and chlorogenic acid, (+)-catechin, and (−)-epicatechin were determined by HPLC. The HPLC system was as follows: pump, Hitachi L-6000 (Tokyo, Japan); column, Capcell-Pak C_{18} (Shiseido, Tokyo, Japan; 4.6 i.d. × 250 mm); detector, Hitachi L-4200 (280 nm wavelength); recorder, Hitachi D-2000 chromatointegrator; eluent, CH_3CN and 5% aqueous acetic acid (4:96) for 0−25 min and then 8:92 for 26−50 min; flow rate, 1.0 mL/min.

Polyphenol Determination after Browning. An apple was peeled, cored, weighed, and then homogenized with the same weight of distilled water in a juicer for 30 s. The homogenate was stirred for 30 min at 30 °C, and then 2 volumes of methanol was added. The phenol remaining after browning was extracted with ethyl acetate and analyzed as already described, three apples being analyzed at each ripened stage.

Preparation of Polyphenol Oxidase (PPO) from Apple. An apple was peeled, cored, weighed, and then homogenized with twice the weight of cold acetone in a juicer for 30 s, this whole procedure being conducted at 0−4 °C. The homogenate was filtered, and the residue was washed several times with cold acetone. The washed residue was dried *in vacuo* and stored as an acetone powder at −20 °C until it was used. Three acetone powders were prepared at each ripened stage. Each acetone powder (0.5 g) was extracted with 20 mL of a 0.1 M KH_2PO_4/Na_2HPO_4 buffer (pH 7.2) containing 1.0% Triton X-100 by homogenizing with a mortar and pestle for 10 min. After filtering, the residue was extracted again with 20 mL of the buffer. The combined filtrate was centrifuged for 30 min at 12000g, and the supernatant was used as crude PPO.

PPO Activity and Protein Assay. PPO activity was measured by the spectrophotometric method at 325 nm to detect chlorogenic acid as the substrate (Fujita and Tono, 1988). The reaction solution consisted of 0.8 mL of a McIlvaine buffer prepared by mixing 0.1 M citric acid with 0.2 M Na_2HPO_4 (pH 4.0), 0.1 mL of 0.5 mM chlorogenic acid, and 0.1 mL of the enzyme solution. A decrease in absorbance of 0.1/min at 325 nm and 30 °C is defined as 1 unit of PPO, that is, chlorogenic acid oxidase activity. Each measurement was take three times, and three apples were analyzed at each ripened stage.

Protein was determined according to the Lowry method (Lowry *et al.*, 1951) with bovine serum albumin as the standard.

Enzyme-Linked Immunosorbent Assay (ELISA) for Apple PPO. The serially diluted crude enzyme solution (100 μL) was added to each well of a polystyrene microplate (Maxisorp Nunc, Roskilde, Denmark) and kept overnight at 4 °C. After three washes with phosphate-buffered saline (PBS: 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, 8.0 g/L NaCl, and 0.2 g/L KCl) containing 0.05 g/100 mL Tween 20, 350 μL/well of a blocking solution (2 g/100 mL of bovine serum albumin in PBS)

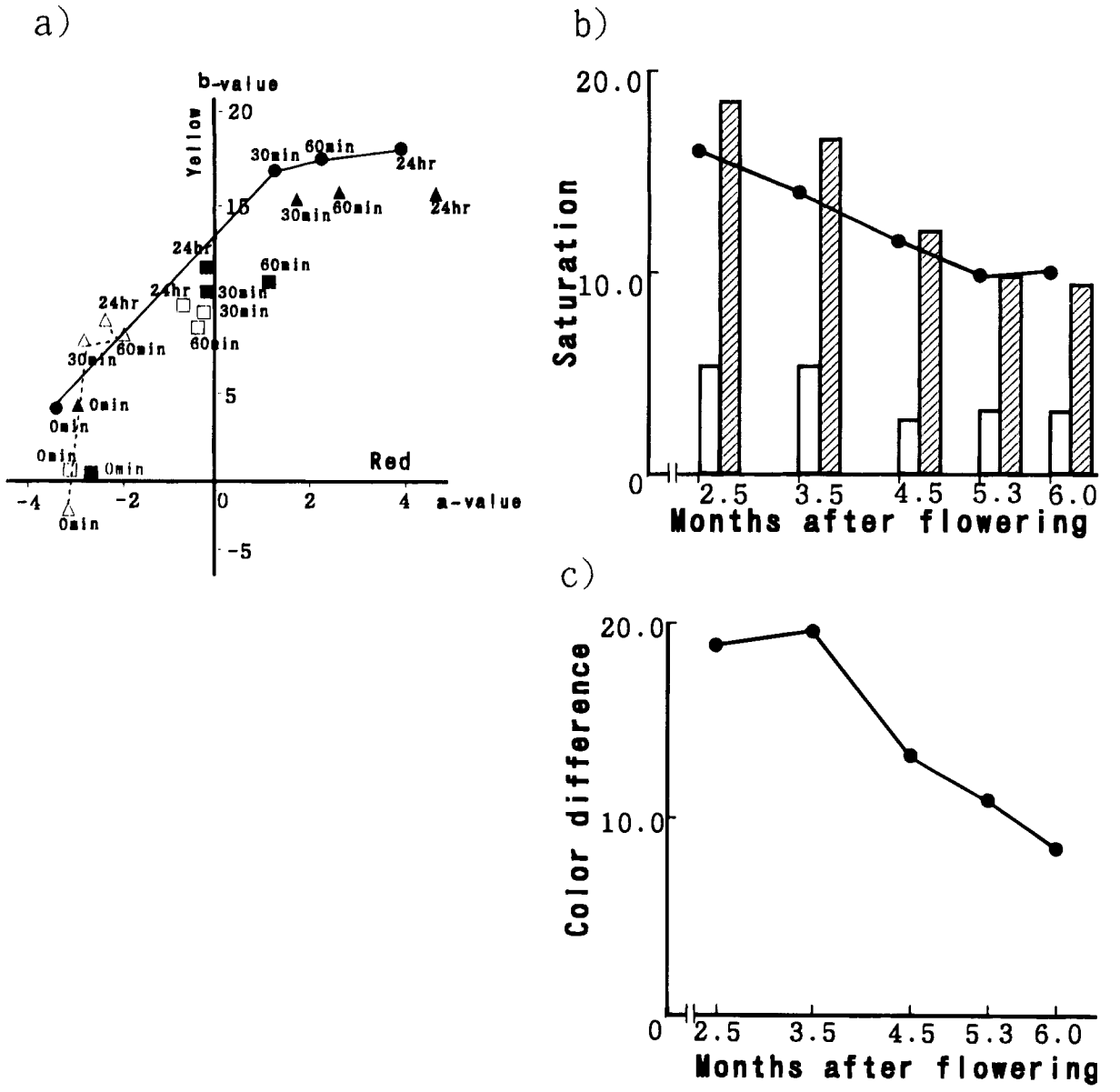

Figure 2. Changes in *a* and *b* values (a), saturation (b), and color difference (c) of apple (cv. Fuji) juice by browning during ripening. Apple juice was browned by incubation for 24 h. (a) Apple juice from fruit at the respective stage after flowering (●, 2.5 months; ▲, 3.5 months; ■, 4.5 months; □, 5.3 months; △, 6.0 months) browned by incubation for 0, 30, and 60 min and 24 h. (b) Open bars, saturation of apple juice; slashed bars, saturation of browned apple juice; ●, difference in saturation between unbrowned and browned apple juice. (c) Color difference between unbrowned and browned apple juice.

was added over a 2-h period at room temperature. The microplate wells were washed three times, 50 μL of anti-PPO antibody (Murata *et al.*, 1993) diluted with PBS (1:2000) was added, and the wells were incubated for 2 h at room temperature. The microplate was then washed three more times, and 50 μL of biotinylated goat anti-mouse IgG antibody (Dako Japan, Kyoto, Japan) diluted 3:2000 in PBS containing 0.05% Tween 20 (PBST) was added to each well. The microplate was again washed three times, 50 μL of a streptavidin–alkaline phosphatase conjugate (Dako Japan) was added to each well, and the wells were incubated for 1 h at room temperature. The plate was washed twice more, and after a final wash of the plate with a substrate buffer consisting of 2 M diethanolamine–HCl (pH 9.8) and 0.5 M $MgCl_2$, 100 μL of a substrate solution (10 mM 4-nitrophenyl phosphate in the substrate buffer) was added to each well. The absorbance at 405 nm was measured by a microplate reader.

Electrophoresis and Western Blotting. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli *et al.* (1970). Crude PPO containing about 5 μg of protein was denatured by incubation in boiling water for 5 min with 2% SDS and 0.05 M β-mercaptoethanol. The gel was transferred onto a nitrocellulose membrane (0.45 μm, Advantec, Tokyo, Japan) with a semidry KS-8460 blotter (Malysor, Tokyo, Japan) for 20–30 min, using a transfer solution consisting of 25 mM tris(hydroxymethyl)aminomethane, 192 mM glycine, and 20% methanol. The nitrocellulose blot was blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBST) for 90 min. The primary antibody against apple PPO was diluted 1:2000 in PBST and incubated overnight at 4 °C. An anti-mouse IgG peroxidase conjugate (Dako Japan) was diluted 1:1000 in PBST and incubated for 1 h. Detection of the peroxidase on protein blots was performed by adding 0.06% diaminobenzidine, 0.03% $NiCl_2$, and 1.0 μL/mL H_2O_2 as substrate.

Nitrocellulose Tissue Print. Nitrocellulose tissue prints were obtained by following the method of Reid *et al.* (1990) as described previously (Murata *et al.*, 1993). An apple was cut vertically. The surface of the section was washed with water or left unwashed and then blotted onto a nitrocellulose membrane by finger pressure for 1 min; the nitrocellulose membrane had been soaked beforehand in a 10 mM phosphate buffer. PPO was detected as described for Western blotting. The membrane was blocked with 5% skim milk in PBST, the primary antibody was diluted 1:500, and the anti-mouse IgG peroxidase conjugate was diluted 1:1000 in PBST.

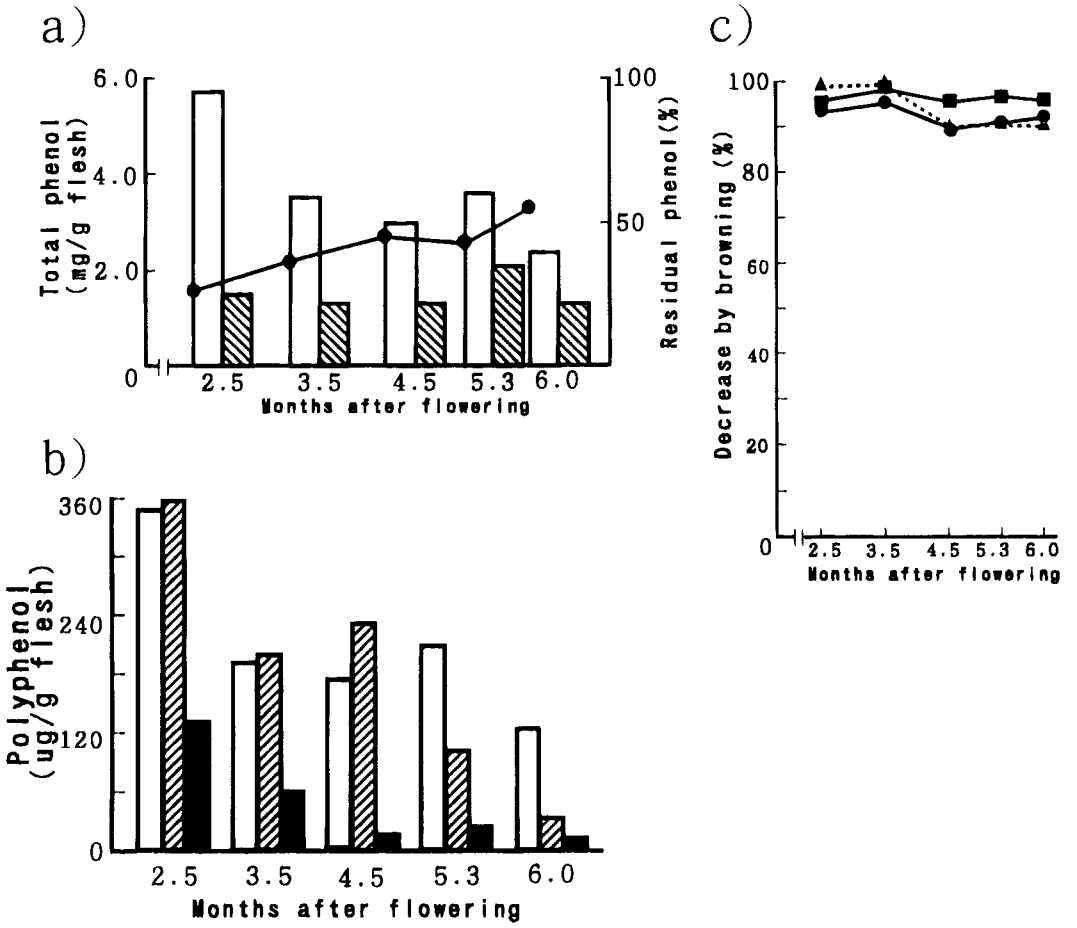

Figure 3. Changes in polyphenols and polyphenols remaining after the browning of apple (cv. Fuji) during ripening. Apple juice was browned by 30 min of incubation. (a) Total phenol determined by the phenol reagent before and after browning: open bars, phenol before browning; slashed bars, remaining phenol after browning; ●, residual phenol percent after browning. (b) Chlorogenic acid (open bars), (−)-epicatechin (slashed bars), and (+)-catechin (solid bars) determined by high-performance liquid chromatography before browning. (c) Ratio of decrease in chlorogenic acid (●), (−)-epicatechin (■), and (+)-catechin (▲) by browning.

RESULTS AND DISCUSSION

The results of the analyses on apples picked in 1992 and 1993 were almost identical; therefore, the results from the 1993 crop are mainly shown here.

Weight, pH, Sugar Content, and Titration Acidity. As can be seen in Figure 1, the weight of the apple increased by about 5 times during the period from 2.5 to 6 months after flowering. The pH value changed from 3.4 to 4.0, and the titration acidity decreased from 0.64 to 0.34 g/100 mL. The sugar content increased from 6.0 to 12.2 °Brix. The apple 4.5 months after flowering began to turn reddish, and those 5.3 months after flowering contained nectar when cut. Apples are usually harvested for consumption 6 months after flowering.

Change in Browning of Apple Juice during Ripening. Apple juice diluted with the same amount of water was prepared to estimate browning susceptibility, water being added to make the preparation of juice easier. The diluted juice was then incubated at 30 °C with shaking until it turned brown, the degree of browning being measured by a color difference meter. Amiot *et al.* (1992) have shown that the browning susceptibility could be adequately determined by simultaneously measuring the soluble pigment by its absorbance at 400 nm and the insoluble brown pigment by L^* value. We did not separate the soluble and insoluble pigments in this study, both being applied to the color difference meter as pulpy apple juice for simplicity. When an immature apple was cut, its surface quickly turned brown, while this happened more slowly in mature fruit. In the juice of an immature apple, for example one 2.5 months after flowering, both the *a* value, corresponding to red, and the *b* value, corresponding to yellow, increased markedly up to 30 min. After 30 min, the *a* value increased more gradually up to 24 h (Figure 2a). On the other hand, in the juice of a mature apple 6 months after flowering, the *a* value increased little but the *b* value did increase. When the color difference of apple juice at the sequential ripened stages is compared after 24 h of incubation, it decreased by about half during ripening (Figure 2c), which corresponds to the observation that a mature apple turned brown much more slowly than a young fruit. The saturation also decreased during ripening (Figure 2b), although the ratio of decrease was less than that in the color difference.

Change in Polyphenols. Figure 3a shows the change in total phenol per gram of flesh determined by the phenol reagent. Total phenol quickly decreased up to 3.5 months after flowering, but after that, it was almost constant or decreased slightly. Further, three polyphenols, chlorogenic acid, (−)-epicatechin, and (+)-catechin, were analyzed, because individual phenolic compounds have been shown to vary in their browning rate (Oleszek *et al.*, 1989). Chlorogenic acid is the major polyphenol in mature apples (Spanos and Wrolstad, 1992). Catechin is the major contributor to browning,

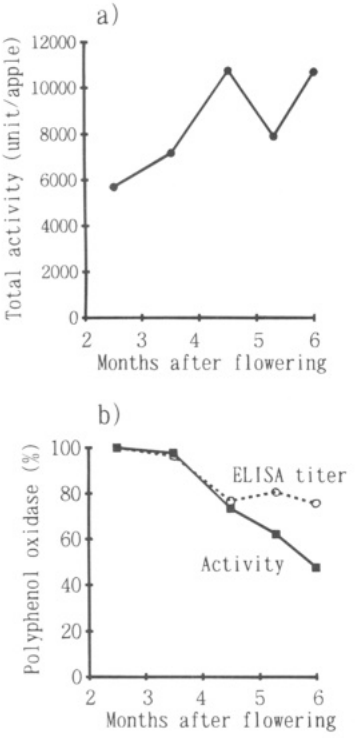

Figure 4. Changes in polyphenol oxidase (PPO) activity and enzyme-linked immunosorbent assay (ELISA) titer during ripening: (a) total activity (units/apple); (b) activity (units/mg of protein) and ELISA titer (titer/mg of protein) expressed as a percentage of the control value (apple 2.5 months after flowering).

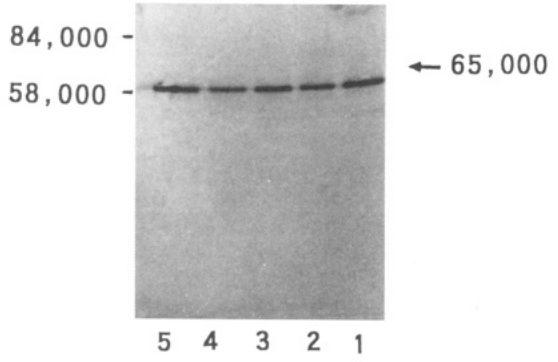

Figure 5. Western blotting analysis of polyphenol oxidase (PPO) of apple (cv. Fuji) during ripening: lane 1, 2.5 months after flowering; lane 2, 3.5 months; lane 3, 4.5 months; lane 4, 5.3 months; lane 5, 6.0 months.

because its oxidative product showed higher intensity of color than that of chlorogenic acid (Oszmianski and Lee, 1990). All three polyphenols decreased during ripening (Figure 3b), although immature apples contained more catechin than chlorogenic acid, being the major phenol in mature apples. The decrease in phenol content during apple development appears to be the general trend as previously reported (Harel *et al.*, 1966; Burda *et al.*, 1990).

Change in Polyphenols Remaining after Browning. The amount of polyphenols remaining after the browning of apple juice was also examined. The decrease in the amount of polyphenols due to browning showed a good correlation with the degree of enzymatic browning (Amiot *et al.*, 1992). The amount of phenols remaining after browning was almost constant, while the ratio of phenols remaining after browning increased slightly (Figure 3a). This suggests that phenols which are not involved in browning were constant during ripening. The ratio of the decrease in chlorogenic acid, (−)-epicatechin, and (+)-catechin by browning was more than 90% and almost constant at each stage (Figure 3c). This result corresponds to the observation that they were good substrates for PPO (Janovitz-Klapp *et al.*, 1990; Murata *et al.*, 1992) and oxidative browning (Oszmianski and Lee, 1990) and suggests that there was no change in the substrate specificity of apple PPO during ripening.

Change in Polyphenol Oxidase (PPO). Figure 4 shows the changes in the activity and ELISA titer of PPO during ripening. Total PPO activity per apple increased during the period from 2.5 to 4.5 months after flowering and then fluctuated. PPO activity per milligram of protein consistently decreased during ripening. However, the amount of PPO estimated by the ELISA titer per protein was characteristic, decreasing up to 4.5 months after flowering and then remaining constant. It should be pointed out that this change of PPO in 4.5 months after flowering was identical both in 1992 and in 1993. The result of Western blotting analysis shows only the same band during ripening, the molecular weight being 65 000 (Figure 5). These results strongly suggest that apple PPO began to be inactivated or denatured without any major degradation of polypeptides 4.5 months after flowering.

Change in the Distribution of PPO. The distribution of PPO was examined by the tissue print method (Figure 6). The membrane for the immature apple section could be strongly and uniformly visualized, irrespective of washing the membrane, this result remaining up to 4.5 months after flowering. This shows the uniform distribution of PPO and its tight binding to the plastid in an apple cell. The section of mature apple not washed could be much more strongly visualized than that washed with water. The section not washed showed relatively uniform distribution of the enzyme, while the section washed with water showed enzyme distribution only around the core of the apple, indicating that most of the PPO in mature apple had been dissolved and washed out. This result should be attributable to the solubilization and denaturation of PPO, except that around the core, from 4.5 months after flowering, because browning most strongly occurred around the core of a mature apple. The denaturation of PPO started 4.5 months after flowering, and the enzyme became extremely unstable when it was solubilized from the plastid.

The juice of an immature apple turned brown much more strongly than that of a mature apple. This result seems to be due to the effect of a decrease in such polyphenols as chlorogenic acid, (−)-epicatechin, and (+)-catechin, being good substrates of PPO, and a decrease and denaturation of PPO during ripening. The intensity of browning in a mature apple is determined by the amount of phenolic compounds, PPO activity (Vamos-Vigyazo *et al.*, 1976) or both (Harel *et al.*, 1966; CoSeteng and Lee, 1987). In this study, both polyphenol concentration and PPO activity per milligram of protein decreased, and the intensity of browning in apple juice was reduced during ripening. The degree of decrease in each component of enzymatic browning might be dependent on such factors as cultivation condition and characteristics of cultivar.

Harel *et al.* (1966) showed that catechol oxidase activity, catechol content, and the rate of browning of apple slices dropped during apple fruit development,

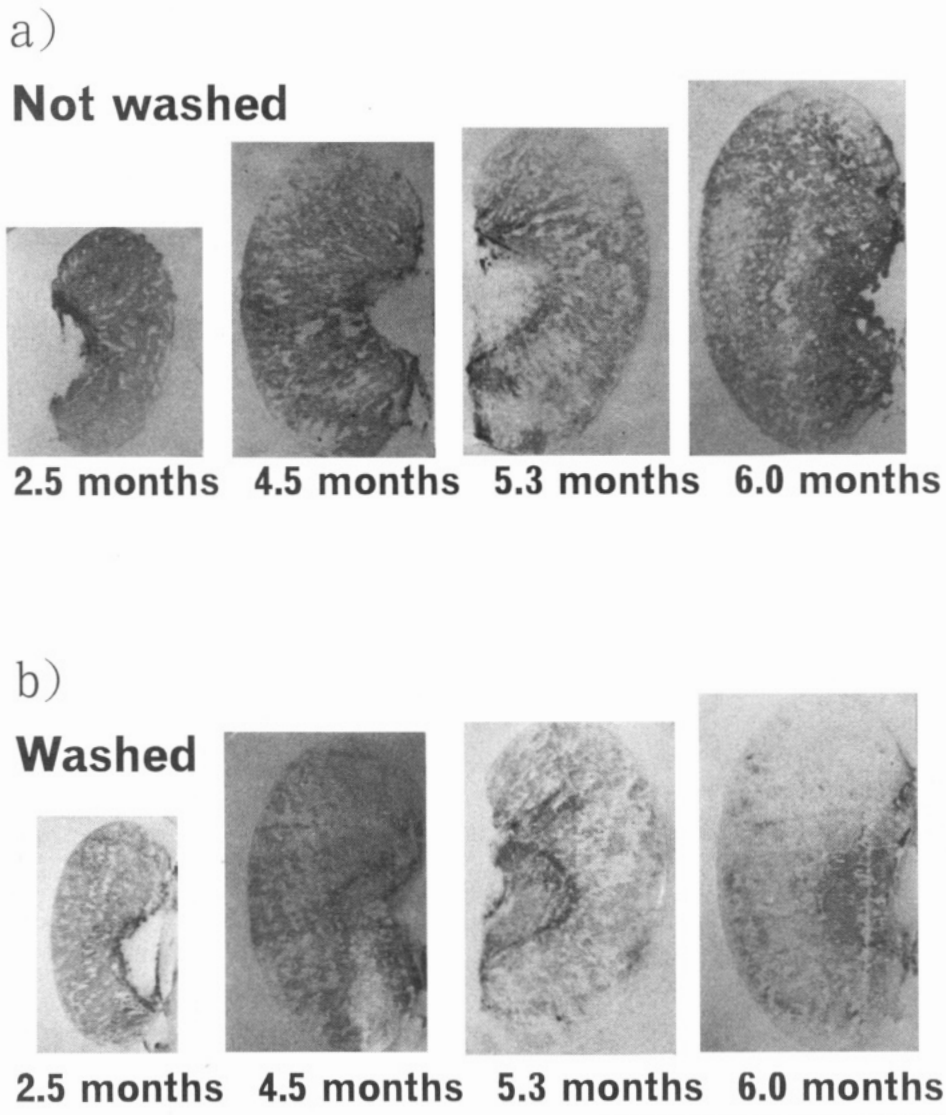

Figure 6. Change in the distribution of polyphenol oxidase of apple (cv. Fuji) visualized by the nitrocellulose tissue print method during ripening: (a) vertical tissue sections unwashed and blotted onto a nitrocellulose membrane soaked in a 10 mM phosphate buffer (pH 7.2); (b) vertical sections washed with water and blotted onto a membrane soaked in a 10 mM phosphate buffer (pH 7.2). The membrane was stained by the apple PPO antibody and peroxidase-conjugated secondary antibody.

although each ratio of decrease was not identical. Burda *et al.* (1990) also showed that catechin, the major contributor of enzymatic browning, rapidly decreased during ripening of apple. These results corresponded to our results.

Harel *et al.* (1966) also showed that catechol oxidase activity in particulate fraction decreased sharply during ripening, while a slight amount of soluble enzyme in a mature apple appeared. This result suggests that PPO is solubilized during ripening. The effect of washing on browning of mushrooms was recently reported (Choi *et al.*, 1994). It was shown that some PPO and polyphenols were washed out and that the rate of browning was reduced. Our result obtained by the tissue print method also showed that most of the PPO in mature apple was solubilized and washed out.

In this study, the changes in quantity, quality, and distribution of polyphenols and PPO of apple were determined with development of fruit maturity. The following conclusions are presented. About 90% of polyphenols such as catechins and chlorogenic acid were consumed in the enzymatic oxidation at every step of the development. The immature apple turned strongly and uniformly brown, while the mature one browned weakly and only around the core. Both polyphenol content and PPO activity in the immature apple were much higher than those in the mature apple, and active PPO was mainly localized near the core in the mature apple, while it was uniformly distributed in immature apple. It is strongly suggested that PPO is solubilized and denatured at the later stage of development, although enough active PPO to cause browning still remains around the core.

ABBREVIATIONS USED

PPO, polyphenol oxidase, ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.05% Tween 20.

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