Involvement of Flavonoid Oxidation with Chlorophyll Degradation by Peroxidase in Wase Satsuma Mandarin Fruits

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Summary
Effects of flavonoid pigments on chlorophyll (Chl) degradation by Chl degrading peroxidase in the flavedo of Wase satsuma mandarin (Citrus unshiu Marc. var. praecox Tanaka 'Okitsuwase') fruit were studied. Chl was degraded when hydrogen peroxide was added to a reaction mixture containing Chl and a phosphate buffer extract from the flavedo. Chlorophyllide, which was formed by the action of chlorophyllase in the extract, was also degraded while flavonoid content decreased concomitantly. Analyses of the flavonoids with HPLC showed that hesperidin and narirutin were the major flavedo flavonoids; the former decreased significantly, whereas the latter showed almost no change during the Chl degradation reaction. In ethylene-treated fruits, flavedo hesperidin content decreased with the degreening during storage, indicating that flavonoid oxidation by Chl degrading peroxidase could be involved in Chl degradation.

Introduction
In Wase satsuma mandarin fruits, the flesh attains a harvestable stage while the peel is still green. Ethylene is used for the enhancement of peel degreening.

It is well-known that chlorophyllase activity increases concomitantly with a decrease in chlorophyll (Chl) content (Shimokawa et al., 1978; Amir-Shapira et al., 1987). However, chlorophyllide (Chlide), a by-product of chlorophyllase action, is green and has a spectral absorption similar to that of Chl (Yamauchi, 1989). These results indicate that Chl degrading enzymes, together with chlorophyllase, could be involved in Chl degradation. Peroxidase is one such Chl degrading enzyme (Kato and Shimizu, 1985; Yamauchi and Minamide, 1985; Shimokawa et al., 1992). In an in vitro study of parsley leaves, Chl was degraded by a peroxidase-hydrogen peroxide system in the presence of but not in the absence of apigenin, a major flavone of parsley leaves (Yamauchi and Minamide, 1985); peroxidase degraded Chl to colorless, low molecular weight compounds without the accumulation of 13β-hydroxychlorophyll a (Chl a-1) as an intermediate (Yamauchi and Watada, 1994).

We reported in a previous paper that in the satsuma mandarin fruits treated with ethylene, the activities of peroxidase and chlorophyllase increased significantly with the advance of degreening, which indicate that Chls are degraded by chlorophyllase and peroxidase actions: chlorophyllase hydrolyzes Chl to Chlide and phytol; peroxidase degrades Chlide in the presence of flavonoids to colorless compounds (Yamauchi and Hashinaga, 1992). Moreover, Chls were degraded in vitro by peroxidase-hydrogen peroxide system in the presence of naringenin and hesperetin (Yamauchi and Hashinaga, 1992).

In this study, we describe the metabolism of hesperidin and narirutin, major flavonoids in the flavedo of Wase satsuma mandarin fruits, concomitant with Chl degradation by Chl degrading peroxidase to elucidate the involvement of the peroxidase pathway in Chl degradation.

Materials and Methods

Materials
Green Wase satsuma mandarin (Citrus unshiu Marc. var. praecox Tanaka 'Okitsuwase') fruits were treated with 120 ppm ethylene or untreated
for 12 hours in a desiccator (45 l) at 20 °C. After the treatment, five fruits were placed into a polyethylene film bag (16×20 cm in size, 0.03 mm in thickness, having 8, 6 mm diam. holes) and stored at 20 °C. There were 16 bags per treatment. Fruits were removed at scheduled intervals during a 6-day period and the flavedo tissues analyzed.

**Determination of chlorophyll and flavonoid degradation**

Five g of flavedo was homogenized in 20 ml of 20 mM phosphate buffer (pH 7.0). The homogenate was filtered through one layer of Miracloth (CALBIOCHEM) and the filtrate centrifuged at 16,000×g for 15 min; the supernatant was used as the flavedo extract.

The reaction mixture for the determination of Chl degradation contained 1.0 ml flavedo extract, 0.2 ml Chls ethanol solution (300 μg Chl a/ml EtOH), 0.1 ml 1% Triton X-100, 0.1 ml 0.3% H₂O₂, and 1.1 ml 100 mM phosphate buffer (pH 6.0) in a total volume of 2.5 ml. The reaction was carried out for 10 min at 25 °C. Chl degradation was determined spectrophotometrically as the decrease in absorbance at 668 nm as Chl was degraded.

Chls were extracted from spinach leaves and purified by the method of Yoshiura and Iriyama (1979).

**Chlorophyll degrading peroxidase activity**

An acetone powder was prepared by homogenizing 5 g of flavedo tissue in 50 ml cold acetone using a Waring blender; the homogenate was filtered through #2 filter paper (ADVANTEC), and the residue washed with a small volume of cold acetone and diethyl ether and vacuum-dried. The crude enzyme preparation was dissolved by suspending acetone powder (400 mg) in 15 ml of 10 mM phosphate buffer (pH 7.0) for 1 hr at 5 °C. The mixture was filtered through Miracloth and the filtrate centrifuged at 16,000×g for 15 min. The supernatant was used as the crude enzyme solution.

The reaction mixture contained 0.2 ml Chls ethanol solution (300 μg Chl a/ml EtOH), 1.0 ml crude enzyme solution, 0.1 ml 1% Triton X-100, 0.1 ml 0.3% H₂O₂, 0.1 ml 5 mM hesperetin and 1.0 ml 100 mM phosphate buffer (pH 6.0) in a total volume of 2.5 ml. The reaction was carried out for 10 min at 25 °C. The activity was determined spectrophotometrically by reading the decrease in absorbance at 668 nm as Chl was degraded. One unit of the enzyme activity was defined as a 0.01 change in absorbance unit per min.

Enzyme protein content was assayed by the method of Lowry et al. (1951).

**Determination of surface color and flavonoid content**

Surface color of the fruits was measured by using a color difference meter (Nippon-denshoku ND 300–A).

Flavonoids from flavedo tissue (2.0 g) were extracted with hot 71.4% methanol (final 70% methanol) for 2 hrs. The extract was filtered through a 0.45 μm DISMIC filter and the filtrate was analyzed by HPLC. The conditions for HPLC analysis of flavonoid are outlined in Fig. 3.

**Results**

**In vitro degradation of chlorophylls and flavonoids in flavedo extract**

Chls were degraded when H₂O₂ was added to a reaction mixture containing Chls and flavedo extract (Fig. 1 and 2). With a boiled extract, Chls were not degraded upon addition of H₂O₂ to the reaction mixture (Fig. 1). Chlide which is formed by the action of chlorophyllase on the extract was also degraded (Fig. 2).

Kamiya and Esaki (1969) and Sawabe et al. (1989) reported that hesperidin and narirutin are the major flavedo flavonoids. In our test, the former decreased significantly while the latter remained unchanged during the Chl degradation reaction (Fig. 3).

**Changes in flavonoids and chlorophyll degrading peroxidase in the flavedo of satsuma mandarin fruits treated or untreated with ethylene during storage**

Wase satsuma mandarin fruits treated with
ethylene remained green after the first day of storage at 20 °C. Afterward, the color of the peel turned markedly yellow. In contrast, fruit treated with air remained green during the first 2 days of storage. The surface color (L \cdot b/|a|) of ethylene-treated fruit turned yellow rapidly as Chl degradation occurred (Fig. 4). During storage at 20 °C, hesperidin content in the flavedo of ethylene-treated fruit decreased significantly in contrast to the control (Fig. 5). Narirutin content in the flavedo of fruit treated with ethylene or ambient air remained almost constant during storage.

Chl degrading peroxidase activity in the flavedo increased markedly with ethylene treatment and then remained at a high level during storage (Table 1).

**Discussion**

In ethylene-treated citrus fruit, chlorophyllase activity increased concomitantly with a decrease in Chl content (Shimokawa et al., 1978; Amir-Shapira et al., 1987). We also observed that chlorophyllase activity in stored spinach leaves increased concomitantly with a loss of Chls and the formation of Chlide a (Yamauchi and Watada, 1991). Contrarily, Aljuburi et al. (1979) noted that chlorophyllase activity heightened with the in-
crease in Chl content during regreening of Valencia oranges. Recently, Minguez-Mosquera and Gallardo-Guerrero (1996) demonstrated that in the initial growth period of olive fruits, chlorophyllase activity increased with the formation of Chls and that small amounts of Chlides were detectable. Thus, chlorophyllase may be involved in both anabolic and catabolism of Chls. In addition, Chlide formed by chlorophyllase remains green, suggesting that other Chl degrading enzymes together with chlorophyllase could degrade Chl in stored satsuma mandarin fruits.

In parsley leaves, Chls were degraded by an in vitro peroxidase-hydrogen peroxide system in the presence but not in the absence of apigenin, a major flavone of parsley leaves (Yamauchi and Minamidate, 1985). It is known that in the in vitro peroxidase-hydrogen peroxide system, Chls are degraded in the presence of phenolic compounds, especially those which have a \( p \)-hydroxy group (Kato and Shimizu, 1985; Yamauchi and Minamidate, 1985; Yamauchi and Watada, 1994). In this study, both Chl and Chlide were degraded by the in vitro Chl degrading peroxidase-hydrogen peroxide system concurrent with a decrease in hesperidin, a major flavonoid of satsuma mandarin fruits (Kamiya and Esaki, 1969; Sawabe et al., 1989). A decrease in hesperidin content in flavedo of ethylene-treated fruit accompanied its degreening process during storage at 20 °C. We propose from these results that the oxidation of hesperidin by Chl degrading peroxidase could be related to Chl degradation of flavedo tissue.

Shimokawa et al. (1978, 1992) determined the actions of chlorophyllase and Chlide a peroxidase,
which degrade Chlide a in the presence of 2, 4-dichlorophenol and hydrogen peroxide, in ethylene-treated satsuma mandarin fruits. Moreover, Yanagisako et al. (1994) reported that Chl was degraded to pyropheophorbide through Chlide in crude enzyme extract from satsuma mandarin fruits. We found that Chl degrading peroxidase as well as chlorophyllase may play a role on Chl degradation in satsuma mandarin fruits. To elucidate the relationship between Chl degrading peroxidase and chlorophyllase actions, additional studies are necessary.

In a previous paper (Yamauchi and Hashinaga, 1992), we observed that both hesperetin, an aglycone of hesperidin, and naringenin, an aglycone of naringin, were involved in Chl degradation in the in vitro system using horseradish peroxidase; naringenin being more reactive than hesperetin. In the system using Chl degrading peroxidase prepared as an acetone powder from the flavedo of satsuma mandarin fruit, hesperidin but not naringin accompanied Chl degradation. This suggests that the substrate affinity of satsuma mandarin Chl degrading peroxidase is different from that of the horseradish peroxidase. More study is also needed to support this speculation.

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**Literature Cited**


