J. Japan. Soc. Hort. Sci. 81 (1): 117–122. 2012.
Available online at www.jstage.jst.go.jp/browse/jjshs1


# Effect of Naringin Radical Formed by Peroxidase on Chlorophyll Degradation during Storage of *Citrus nagato-yuzukichi* Fruit

**Naoki Yamauchi[1,2]*, Sukanya Aiamla-or[2], Kuniyasu Eguchi[1]**, Masayoshi Shigyo[1,2] and Catharina Taiko Migita[1,2]**

[1]*Faculty of Agriculture, Yamaguchi University, Yoshida, Yamaguchi 753-8515, Japan*
[2]*The United Graduate School of Agricultural Science, Tottori University, Koyama-Minami, Tottori 680-8553, Japan*

**Free radical formation from flavonoids by peroxidase action and changes in flavonoid levels and peroxidase activity during storage were determined to clarify the involvement of naringin radical in chlorophyll (Chl) degradation in stored green Nagato-yuzukichi (*Citrus nagato-yuzukichi* hort. ex Y. Tanaka) fruit. Chl *a* was degraded by peroxidase-hydrogen peroxide system not in the presence of hesperidin but with naringin. However, radical formation of both naringin and hesperidin, which were the main flavonoids in the flavedo of Nagato-yuzukichi fruit, with peroxidase-mediated oxidation was found by electron spin resonance analysis. The naringin radical, which was formed by a radical forming reagent, potassium ferricyanide, was related to the formation of 13$^2$-hydroxychlorophyll (OHChl) with Chl *a* degradation, whereas the hesperidin radical hardly affected OHChl *a* formation. In addition, superoxide dismutase did not inhibit Chl *a* degradation by naringin radical formed by potassium ferricyanide. In Nagato-yuzukichi fruit, Chl-degrading peroxidase activity increased considerably during storage at 20°C, with a peak value on day 6 of storage in accordance with decrease in Chl content, after which it decreased. The contents of naringin and hesperidin, especially the former, decreased during storage at 20°C, but afterwards increased slightly. These findings suggest that the naringin radical formed by peroxidase could be partly involved in Chl degradation during storage in Nagato-yuzukichi fruit.**

**Key Words:** chlorophyll degradation, *Citrus nagato-yuzukichi*, degreening, flavonoid, naringin radical.

## Introduction

Green Nagato-yuzukichi (*Citrus nagato-yuzukichi* hort. ex Y. Tanaka), which originated in Yamaguchi Prefecture, Japan and belongs to the Yuzu group, is a highly flavoured, acidic citrus, like a lime. The fruit are harvested in late summer in Yamaguchi Prefecture when the peel is still green. It is necessary to retain the green peel as long as possible during transportation and storage to maintain postharvest quality.

An early step in chlorophyll (Chl) *a* degradation seems to be the removal of phytol and the formation of chlorophyllide *a* by chlorophyllase (Amir-Shapira et al., 1987; Shimokawa et al., 1978), followed by the removal of Mg by Mg-dechelating substance (Shioi et al., 1996). Finally, pheophorbide (Pheide) *a* is degraded to fluorescent Chl catabolites, which are primarily colorless catabolites, via a red Chl catabolite reductase (Matile et al., 1999). Recently, Schelbert et al. (2009) found that pheophytin pheophorbide hydrolase (PPH) is involved in the dephytylation of pheophytin (Phein) to form Pheide. In addition, they also suggested that the formation of Phein from Chl could relate to the action of Mg dechelation by a Mg-dechelating substance (Shioi et al., 1996; Suzuki and Shioi, 2002).

Peroxidase mediates *in vitro* Chl degradation in the presence of phenolic compounds such as *p*-coumaric acid, apigenin and naringenin, which have a hydroxyl group at the *p*-position (Kato and Shimizu, 1985; Yamauchi and Eguchi, 2002; Yamauchi and Minamide, 1985). In this reaction, 13$^2$-hydroxychlorophyll (OHChl) *a* is formed as an intermediate. Maunders et al. (1983) reported that OHChl *a* increased with senescence in barley and kidney bean leaves. On the other hand, OHChl *a* in spinach, parsley, Satsuma mandarin, and broccoli decreased with senescence during storage (Yamauchi and Watada, 1991, 1993, 1998; Yamauchi et al., 1997).

Received; June 13, 2011. Accepted; August 31, 2011.
* Corresponding author (E-mail: yamauchi@yamaguchi-u.ac.jp).
** Present address: Hiroshima Branch Office, Agro-Kanesho Co., Ltd., Naka-ku, Hiroshima 730-0805, Japan.

Moreover, the activity of peroxidase related to Chl degradation showed a sharp increase with senescence in these crops, suggesting that peroxidase could be partly involved in Chl degradation.

We reported previously that sucrose laurate ester treatment at 50°C effectively reduced the degreening of green Nagato-yuzukichi fruit due to the suppression of the enhancement of Chl-degrading peroxidase (Yamauchi et al., 2003). In Chl degradation by peroxidase in Nagato-yuzukichi fruit, a radical of naringin, which is one of the main flavonoids, is formed. This radical might be involved in Chl degradation, judging from the inhibitory effects of various chemicals such as nitroblue tetrazolium (NBT), superoxide dismutase (SOD), and Tiron (Yamauchi and Eguchi, 2002).

This paper reports electron spin resonance (ESR) analysis of free radical formation from flavonoid by peroxidase action and changes in flavonoid levels and peroxidase activity during storage to clarify the involvement of naringin radical in Chl degradation in stored green Nagato-yuzukichi fruit.

## Materials and Methods

*1. Plant material and storage*

Green Nagato-yuzukichi fruit were harvested in late summer in an orchard in Nagato, Yamaguchi Prefecture and then transported to the laboratory of Horticultural Science, Yamaguchi University. The fruit were kept for 3 days at room temperature as a pretreatment (5% weight loss). Four fruit per perforated polyethylene-film bag (20 × 14 cm, 0.04 mm thick, with two 6 mm holes) were stored at 5 or 20°C for 15 days. Three bags of each treatment were removed at scheduled intervals during the 15-day period so that the fruit flavedo could be analyzed. All analyses were performed using triplicate samples and the data are presented as the mean and SE (n = 3).

*2. Preparation of fruit flavedo extract*

Fruit flavedo (2.5 g) was ground in liquid nitrogen using a mortar and pestle and homogenized in 20 mL of 20 mM phosphate buffer (pH 7.0). The homogenate was filtered through one layer of Miracloth (Calbiochem, USA) and the filtrate centrifuged at 16,000 × g for 15 min at 4°C. The supernatant was passed through a PD-10 column (GE Healthcare, USA) to remove low molecular-weight substances. The eluant was used as flavedo extract.

*3. ESR analysis of flavonoid radical*

The reaction mixture for the flavonoid radical assay contained 20 µL flavedo extract, 0.08% Triton X-100, 10 mM naringin and/or hesperidin, 0.012% hydrogen peroxide and 68 mM phosphate buffer (pH 6.0) in a total volume of 100 µL. Approximately 60 µL of the reaction mixture was placed in a high quality quartz flat cell. All the experiments were conducted at room temperature and ESR measurement was performed using an EPR spectrometer (E500, Bruker, Germany). The experimental conditions were as follows: microwave frequency, 9.8 GHz; microwave power, 10 mW; field modulation amplitude, 4 gauss; averaged scans, 50.

*4. Chlorophyll-degrading peroxidase assay*

Flavedo tissues (2.5 g) were homogenized in 20 mL of 0.1 M phosphate buffer (pH 7.5), containing 10 mM cysteine and 500 mg Polyclar AT (Wako, Japan). The homogenate was filtered through one layer of Miracloth and centrifuged at 16,000 × g for 15 min at 4°C. Granular ammonium sulfate was added to the supernatant at 0°C to give 90% saturation. One hour later, the sample was centrifuged at 16,000 × g for 15 min at 4°C. The pellet was suspended in 90% saturated ammonium sulfate (pH 7.0) and stored at −20°C. An aliquot of the solution was diluted three-fold with 20 mM phosphate buffer (pH 7.0). The solution was passed through a PD-10 column to remove low molecular-weight substances. The eluant was used as crude enzyme.

Chl-degrading peroxidase was determined as described by Yamauchi et al. (1997). The reaction mixture contained 0.25 mL enzyme solution, 0.2 mL Chls acetone solution (Chl *a* 400 µg·mL$^{-1}$), 0.1 mL of 1% Triton X-100, 0.1 mL of 5 mM naringin, 0.1 mL of 0.3% hydrogen peroxide, and 1.75 mL of 0.1 M phosphate-citrate buffer (pH 4.5). The activity was determined spectrophotometrically by measuring the decrease of Chl *a* at 668 nm at 25°C. One unit of enzyme activity was defined as a change of 1.0 µg Chl *a* degradation per min. The enzyme protein content was assayed by the method of Bradford (1976).

*5. Chlorophyll quantification and preparation*

The content of Chl, including Chls *a* and *b*, in flavedo tissue was determined using *N,N*-dimethylformamide (Moran, 1982). Chls were extracted from spinach (*Spinacia oleracea* L.) leaves using acetone, and Chls were partially purified by adding 1,4-dioxane and distilled water to the acetone extract. The mixture was then allowed to stand until a precipitate formed. The mixture was then centrifuged at 15,000 × g for 10 min, and the pellet was dissolved in acetone (Yoshiura and Iriyama, 1979). Chl *a* was separated using sugar powder column chromatography (Perkins and Roberts, 1962). OHChl *a*, a derivative of Chl *a* oxidized at position 13$^2$, was prepared by adding peroxidase (horseradish, Sigma-Aldrich, USA) in the presence of hydrogen peroxide and *p*-coumaric acid to Chl *a* solution (Kaewsuksaeng et al., 2007).

*6. Chlorophyll degradation by flavonoid radical*

Using a radical-forming reagent, potassium ferricyanide (Srivatsan et al., 2003), Chl degradation by naringin or hesperidin radical was estimated. The reaction mixture contained 0.1 mL Chl *a* acetone solution (500 µg·mL$^{-1}$),

0.1 mL of 2% Triton X-100, 0.1 mL of 5 mM naringin or hesperidin, 0.1 mL distilled water, and 2.0 mL of 100 mM phosphate buffer (pH 6.0). The reaction was carried out for 10 min at 25°C and stopped by the addition of 5 mL acetone. The acetone solution was filtered through a 0.5 μm DISMIC filter (Advantec, Japan) and then Chl *a* and OHChl *a* were analyzed by HPLC.

*7. Flavonoid and chlorophyll analyses by HPLC*

Flavonoids from fruit flavedo (5.0 g) were extracted with hot ethanol (70% final concentration) for 15 min. Lipid-soluble compounds like Chls were removed from the flavedo extract using n-hexane, the extract was filtered through a 0.5 μm DISMIC filter and then the filtrate analyzed for flavonoids by HPLC using a slightly modified procedure of Yamauchi et al. (1997). An aliquot of filtrate (50 μL) was injected onto the HPLC fitted with a LiChrospher C18 column (4 × 250 mm, Merck, USA). The column was eluted with a linear gradient of 40% to 70% methanol over 50 min, then kept isocratic for an additional 10 min; the flow rate was 0.6 mL·min$^{-1}$. The absorbance (monitored at 283 nm) distinguished naringin and hesperidin by their retention times and absorption spectra compared to standards.

Chl *a* and OHChl *a* were analyzed by HPLC according to the method of Eskin and Harris (1981). An aliquot (50 μL, 80% acetone solution) was injected onto the HPLC fitted with a LiChrospher C18 column (4 × 250 mm, Merck), using two solvents: A, methanol : water (80 : 20, v/v) and B, ethyl acetate in a gradient. Solvent B was added to solvent A at a linear rate until a 50 : 50 mixture was attained at the end of 20 min. The 50 : 50 mixture was then used isocratically for an additional 20 min; the flow rate was 1 mL·min$^{-1}$. The absorbance, which was monitored at 665 nm, distinguished Chl *a* and OHChl *a* by their retention times and absorption spectra using standards.

## Results and Discussion

*1. Chlorophyll degradation by peroxidase regarding flavonoid radical*

Figure 1 shows the ESR spectra of naringin and hesperidin radicals, As was apparent from these spectra, a radical was formed from naringin by the peroxidase, which was included in the flavedo extract, in the presence of hydrogen peroxide (Fig. 1a). Hesperidin was also oxidized by peroxidase to form a radical (Fig. 1b), and each radical level was in about equal amounts; however, the radical was formed slightly in the control without the addition of the flavedo extract. Furthermore, radical formation increased greatly when both naringin and hesperidin were contained in the reaction mixture (Fig. 1c). In a previous paper (Yamauchi and Eguchi, 2002), Chl was markedly degraded with naringin oxidation but hardly with hesperidin oxidation by peroxidase, and Chl degradation was suppressed by 35% in the presence of both naringin and hesperidin. These findings note that Chl might not be degraded by hesperidin radical but by naringin radical. Naringin radical also acts on hesperidin to form its radical since Chl degradation by naringin radical was inhibited by the addition of hesperidin, and the radical-forming level in the presence of both flavonoids was higher than that in the presence of each flavonoid alone.

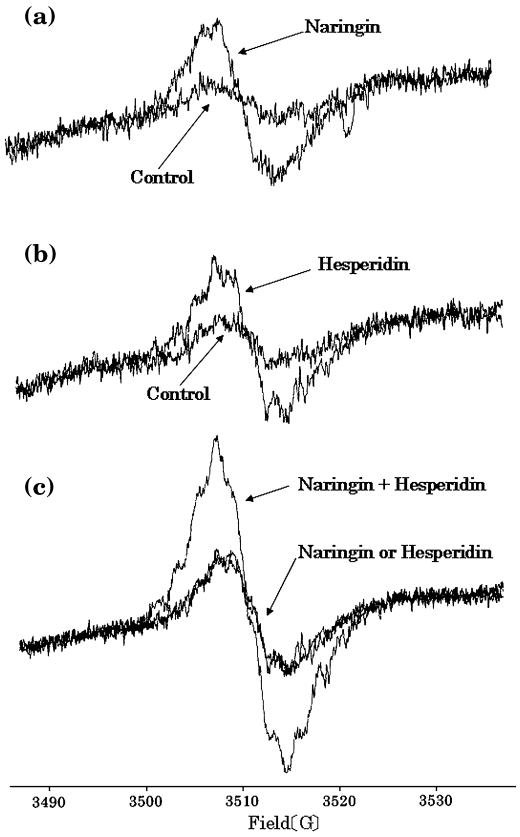


**Fig. 1.** ESR spectra of flavonoid radicals formed by the peroxidase action in the flavedo extract. The reaction mixture contained flavedo extract, Triton X-100, naringin and/or hesperidin, $H_2O_2$, and phosphate buffer (pH 6.0) and in the control, no flavedo extract was included in the mixture. (a) naringin radical formation, (b) hesperidin radical formation, (c) radical formation from naringin and hesperidin.

Adachi and Shimokawa (1995) reported that Chl *a* oxidation by peroxidase prepared from radish cotyledons was inhibited by the addition of both superoxide anion and radical scavengers, which suggests that the superoxide anion plays an important role in enzymatic Chl *a* bleaching reactions. We also found that in peroxidase-mediated Chl degradation using flavedo extract of Nagato-yuzukichi fruit, superoxide anion scavengers, such as NBT, Tiron, and $MnCl_2$, effectively inhibited Chl degradation but SOD did not have any inhibitory effect on Chl degradation (Yamauchi and Eguchi, 2002). Therefore, using a radical-forming reagent, potassium ferricyanide, Chl *a* degradation and the resulting derivative formation relating to naringin or hesperidin radical was determined. As is apparent in Figure 2, OHChl *a* accumulated greatly with Chl *a*

degradation in the presence of naringin radical. On the other hand, hesperidin radical was barely involved in OHChl *a* formation, although Chl *a* was slightly broken down (Fig. 3). Furthermore, after adding SOD (Sigma-Aldrich, USA, 100 Units in the reaction mixture) to the reaction mixture including naringin, Chl *a* degradation by naringin radical was not inhibited (data not shown).

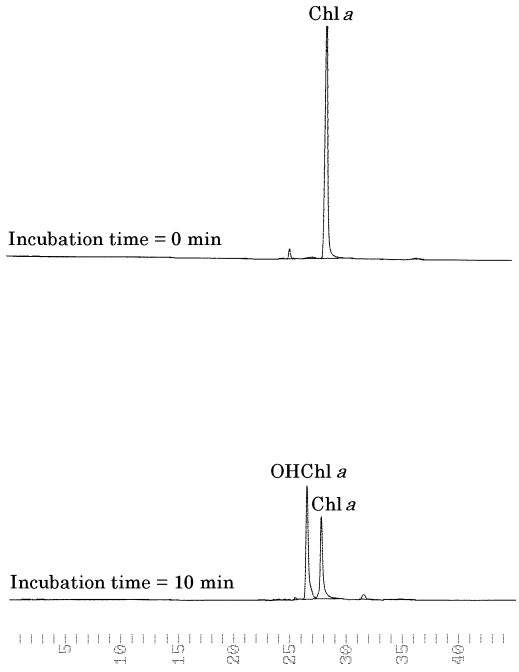


**Fig. 2.** Chlorophyll *a* degradation in the presence of naringin radical formed by potassium ferricyanide. The reaction was carried out for 10 min at 25°C, and Chl *a* and OHChl *a* were analyzed by HPLC. Chl, chlorophyll; OHChl, $13^2$-hydroxychlorophyll.

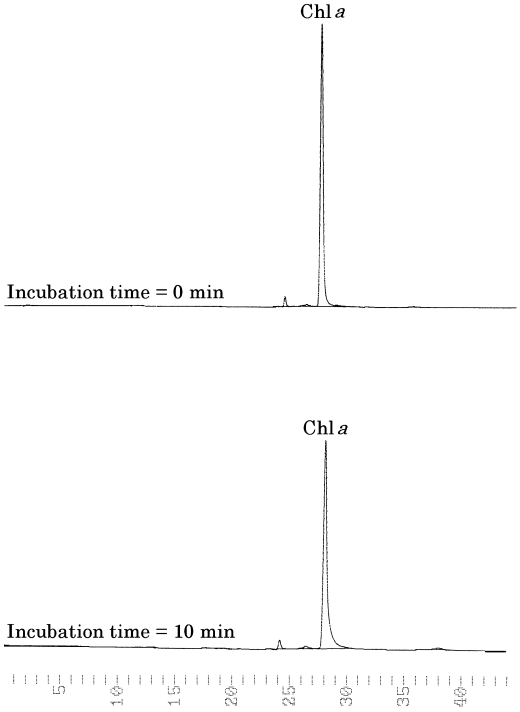


**Fig. 3.** Chlorophyll *a* degradation in the presence of hesperidin radical formed by potassium ferricyanide. The reaction was carried out for 10 min at 25°C, and Chl *a* and OHChl *a* were analyzed by HPLC. Chl, chlorophyll.

Peroxidase is known to be related to the oxidation of many kinds of phenolic compounds, monophenols, diphenols and flavonoids (Kato and Shimizu, 1985; Yamauchi and Watada, 1994). Kato and Shimizu (1985) demonstrated that phenolic compounds such as *p*-coumaric acid, which has a hydroxyl group at the *p*-position, could be involved in peroxidase-mediated Chl degradation. We reported in a previous paper that Chl in parsley extract was degraded by the peroxidase-hydrogen peroxide system. An apigenin, which has a hydroxyl group at the *p*-position, in the extract was oxidized by peroxidase, and then Chl was degraded by the oxidized apigenin (Yamauchi and Minamide, 1985; Yamauchi et al., 1980). In Nagato-yuzukichi fruit, naringin, hesperidin and neohesperidin levels decreased with peroxidase-mediated Chl degradation and only naringin, which has a hydroxyl group at the *p*-position, was involved in Chl degradation by peroxidase. We also found that peroxidase-mediated Chl degradation was efficiently inhibited by the addition of radical scavengers, ascorbate and n-propyl gallate, and superoxide anion scavengers, NBT, Tiron, and $MnCl_2$. However, SOD hardly inhibited peroxidase-mediated Chl degradation, suggesting that the phenoxy radical formed by peroxidase might relate predominantly to Chl degradation (Yamauchi and Eguchi, 2002). In this study, we found by ESR analysis that hesperidin radical as well as naringin radical was formed by the peroxidase-hydrogen peroxide system, and the level of radical formation was enhanced in the presence of both flavonoids. OHChl *a* accumulated with Chl *a* degradation by naringin radical, which is formed in the presence of potassium ferricyanide, a radical-forming reagent. In addition, the formation of naringin radical was not suppressed by the addition of SOD. A possible explanation for peroxidase-mediated Chl degradation is that only naringin radical effectively degrades Chl to colorless, low-molecular weight compounds through the formation of OHChl *a*, an intermediate of peroxidase-mediated Chl *a* degradation, although both naringin and hesperidin radicals are formed by peroxidase action (Fig. 4).

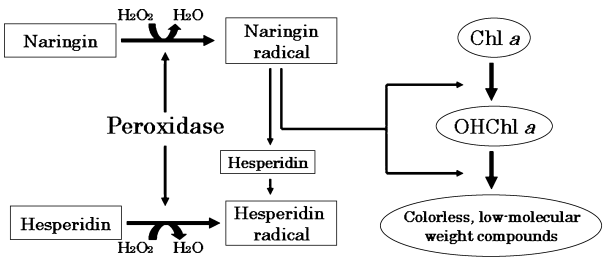


**Fig. 4.** *In vitro* chlorophyll *a* degradation with flavonoid oxidation by peroxidase. Chl, chlorophyll; OHChl, $13^2$-hydroxychlorophyll.

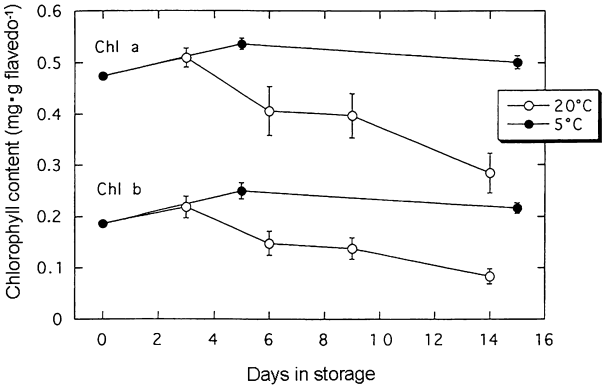


**Fig. 5.** Changes in chlorophyll content of the flavedo in Nagato-yuzukichi fruit during storage. Vertical bars represent SE (n = 3). Chl, chlorophyll.

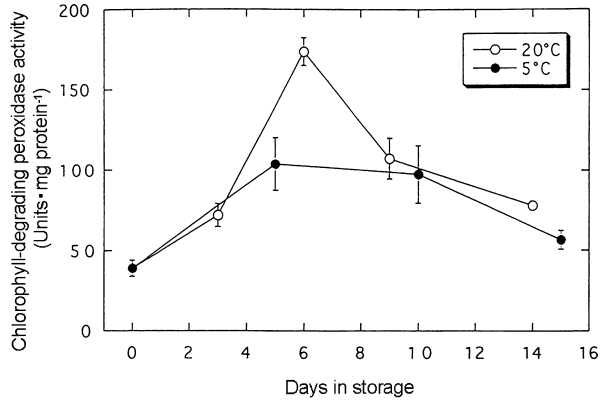


**Fig. 6.** Chlorophyll-degrading peroxidase activities in stored Nagato-yuzukichi fruit. Vertical bars represent SE (n = 3). Unit: Chlorophyll *a* degradation μg·min$^{-1}$.

*2. Peroxidase activity and flavonoid changes in stored Nagato-yuzukichi fruit*

As shown in Figure 5, Chls *a* and *b* contents in Nagato-yuzukichi fruit decreased greatly after 6 days of storage at 20°C, whereas they showed almost no change at 5°C. Chl-degrading peroxidase activity increased considerably during storage at 20°C, peaked on day 6, and then decreased (Fig. 6). On the other hand, activity also increased slightly during storage at 5°C. Moreover, the level of naringin and hesperidin, the main flavonoids of the flavedo, was determined in relation to peroxidase-mediated Chl degradation during storage. The contents of naringin and hesperidin, especially the former, decreased for the first 6 days of storage at 20°C and afterwards increased slightly (Fig. 7); however, they increased gradually during storage at 5°C.

Chl seems to show a decline with senescence in stored horticultural produce, concomitantly with the enhancement of peroxidase activity (Kaewsukusaeng et al., 2011; Ketsa et al., 1999; Martínez et al., 2001; Yamauchi and Watada, 1991). In lime fruit, Chl-degrading peroxidase activity increased considerably with the degreening of peel flavedo during storage at 25°C (Kaewsukusaeng et

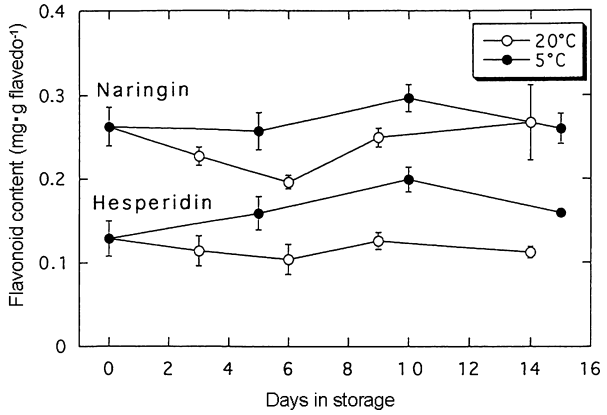


**Fig. 7.** Changes in flavonoid content of the flavedo in Nagato-yuzukichi fruit during storage. Vertical bars represent SE (n = 3).

al., 2011). We reported that Chl-degrading peroxidase activity was enhanced concomitantly with the yellowing of florets in stored broccoli (Aiamla-or et al., 2010; Funamoto et al., 2002). In this study, we estimated that Chl-degrading peroxidase activity increased greatly for the first 6 days of storage at 20°C and that the level of naringin, which could act as a substrate of peroxidase, decreased for the first 6 days of storage, suggesting that naringin radical formed by peroxidase could be highly related to Chl degradation of stored Nagato-yuzukichi fruit. These results indicate that peroxidase might be involved in Chl degradation with the degreening of Nagato-yuzukichi fruit during storage. Martínez et al. (2001) demonstrated that phenolic compounds extracted from strawberry fruit at the small green stage effectively inhibited peroxidase-mediated Chl degradation and the effect decreased with ripening. We also found that hesperidin had an inhibitory effect on Chl degradation by peroxidase-mediated naringin oxidation (Yamauchi and Eguchi, 2002). In this study, hesperidin content decreased with degreening during storage, suggesting that the decline of hesperidin might be involved in the suppression of peroxidase action.

In conclusion, the naringin radical formed by peroxidase, which is present in the Nagato-yuzukichi flavedo extract, is involved in Chl *a* degradation and Chl *a* could be degraded to colorless low-molecular weight compounds through OHChl *a* as an intermediate. In Nagato-yuzukichi fruit, Chl degradation could relate to both the enhancement of peroxidase activity and the decline of the naringin level during storage.

## Acknowledgements

We thank Dr. Cindy B. S. Tong, University of Minnesota, for reading this manuscript.

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