<Postharvest Biology and Technology>

Inhibitory effect of sucrose laurate ester on degreening in *Citrus nagato-yuzukichi* fruit during storage

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Abstract

An inhibitory effect of sucrose laurate ester (SLE) on the degreening of Nagato-yuzukichi (*Citrus nagato-yuzukichi* hort. ex Y. Tanaka) fruit was determined. SLE treatment suppressed the degreening of the fruit during storage at 20℃ more efficiently than the treatment with any other sucrose fatty acid ester, such as myristate, palmitate or stearate. SLE, itself did not have an inhibitory effect on the chlorophyllase and chlorophyll (Chl)-degrading peroxidase activities, but laurate, which was de-esterified from SLE, had a significant effect. Laurate inhibited both enzyme activities more effectively than any other fatty acid, such as caprylate, caprate, myristate, palmitate or stearate. The fruit flavedo extract had an activity to decompose SLE to laurate and sucrose, and treatment of the fruit with laurate significantly suppressed degreening during storage at 20℃ as well. These results indicate that the suppression of degreening in SLE-treated Nagato-yuzukichi fruit could be in part due to the formation of laurate from SLE by esterase, such as a lipase, which is present in the flavedo, and the formed laurate might be involved effectively in the inhibition of Chl-degrading enzyme activities. Moreover, the suppression of degreening by SLE treatment could be owing to the inhibition of degreening by laurate in addition to the coating effect of SLE.

Keywords: chlorophyll degradation; *Citrus nagato-yuzukichi* ; degreening; lauric acid; sucrose laurate ester

1. Introduction

Nagato-yuzukichi (*Citrus nagato-yuzukichi* hort. ex Y. Tanaka) fruit, which belong to the Yuzu (*Citrus junos*) group, are harvested in late summer in Yamaguchi Prefecture, Japan, when the rind is still green. The quality characteristics are a highly flavored, acidic citrus fruit. For the maintenance of postharvest quality, it is necessary to retain the green peel as long as possible.

Coating treatment is known to maintain the quality of stored horticultural crops by suppressing water loss, improving in strength of peel tissue and retaining volatile components, and control the ripening by modifying CO_2 and O_2 concentrations inside the fruit (Baldwin, 1994, 2003). Waxes such as carnauba and paraffin, oils, gums, polysaccharides and the rest have been used as a coating reagent. Sucrose fatty acid esters are also used as edible coating reagents for keeping quality in horticultural produce and effectively delay the degreening of Kabosu (*Citrus sphaerocarpa*) and banana fruits. Murata (1989) demonstrated that the treatment of sugar fatty acid esters delayed the degreening of the flavedo tissue in green Kabosu fruit. Momen et al. (1997) found that treatment with sucrose laurate ester (SLE), sucrose palmitate ester and sucrose stearate ester delayed the degreening of ethylene-treated banana fruit. We also reported in a previous paper (Yamauchi et al., 2003) that *Citrus nagato-yuzukichi* fruit treated with SLE at 50℃ for 3 min effectively reduced the degreening of flavedo tissue during storage at 20℃ and that the degreening control by SLE treatment at 50℃ could be due to the formation of modified atmosphere conditions by coating. In addition, SLE treatment reduced the enhancement of the activities in chlorophyll (Chl)-degrading enzymes such as

chlorophyllase and Chl-degrading peroxidase, especially the latter, which could imply that SLE has control over the Chl degradation of fruit flavedo in addition to having an effect on the concentration of internal gas by coating.

This study deals with the inhibitory role of SLE on degreening in Nagato-yuzukichi fruit during storage.

2. Materials and Methods

2.1. Plant materials

Green Nagato-yuzukichi (*Citrus nagato-yuzukichi* hort. ex. Y. Tanaka) fruit were harvested in summer at Hagi Citrus Research Station. The fruit were kept for 4 days at room temperature as a pretreatment (4~5% weight loss from the peel) and then transported to the laboratory of Horticultural Science, Yamaguchi University.

2.2. Surface color and chlorophyll assays

The surface color of the fruit was measured using color difference meter (Nippon-denshoku NF777). For Chl analysis, one g flavedo segments obtained from 5 fruit was immersed in *N, N*-dimethylformamide for 1 day at 5℃ in the dark, and Chl was measured by reading the absorbance at 664 nm and 647 nm according to the method of Moran (1982).

2.3. Sucrose fatty acid ester treatment and storage

The fruit were treated with 2% sucrose fatty acid esters (SLE; sucrose laurate ester, SME; sucrose myristate ester, SPE; sucrose palmitate ester and SSE; sucrose stearate ester) or 0.1% sodium laurate at ambient temperature for 3 min and then dried for one day at ambient temperature. After drying, five fruit per perforated polyethylene-film bag (20 x 14 cm, 0.04 mm thick, with two 6 mm holes) were stored at 20℃ for 20 days. All analyses were conducted using triplicate samples, and the data are presented as mean and SE (n = 3).

2.4. Chlorophyll-degrading enzyme assay

For the enzyme assay, an acetone powder (300 mg) was suspended in 8 ml of a 5 mM phosphate buffer (pH 7.0) containing 1% CHAPS for chlorophyllase activity or without it for peroxidase activity. The suspension was stirred for 1 hour at 0℃ and then filtered through Miracloth (Calbiochem). Afterwards, the filtrate was centrifuged at 16,000 x g for 15 min at 4℃. The supernatant was used as a crude enzyme extract.

Chlorophyllase activity was determined by a modification of the method of Amir-Shapira et al. (1987). The reaction mixture contained 0.3 ml enzyme solution, 0.2 ml Chl *a* (Tama Chemical) acetone solution (500 μg ml-1) and 0.5 ml 100mM phosphate buffer (pH 7.0). The mixture was incubated in a water bath at 25℃ for 1 hour, and the enzyme reaction was stopped by the addition of 4 ml acetone. The remaining Chl *a* was extracted with 4 ml hexane and assayed by reading the absorbance by Chl *a* at 663 nm.

Chl-degrading peroxidase was determined as described by Yamauchi et al. (1997). The reaction mixture contained 0,1 ml enzyme solution, 0.1 ml 1% Triton

X-100, 0.2 ml Chl a acetone solution (500 μg ml-1), 0.1 ml 5 mM naringin, a flavonoid of the enzyme substrate included in the Nagato-yuzukichi fruit flavedo (Yamauchi and Eguchi, 2002), 0.1 ml 0.3% hydrogen peroxide, 0.4 ml distilled water and 1.5 ml 100 mM phosphate-citrate buffer (pH 4.5). The activity was determined spectrophotometrically by measuring the decrease of Chl *a* at 668 nm at 25℃.

One unit of those enzymes was defined as a change of 1 μg Chl *a* degradation per min. The enzyme protein content was assayed by the method of Bradford (1976).

2.5. Laurate formation from sucrose laurate ester by flavedo tissue or lipase

The flavedo tissue (2.5 g) was homogenized in 20 ml of a 100 mM phosphate buffer (pH 7.5) containing 5 mM dithiothreitol and 250 mg Polyclar AT. The homogenate was filtered through Miracloth and centrifuged at 16,000 x g for 20 min at 4℃. The supernatant was passed through a PD-10 desalting column (GE Healthcare Bioscience) to remove low-molecular-weight substances. The elute was used as a flavedo extract. Five ml of a 100 mM phosphate buffer (pH 7.0) containing 2% SLE was added to the flavedo extract (2.5 ml) , the flavedo tissue segments (2.5 g, 5 x 5 mm) or 2.5 ml of a lipase solution (Sigma-Aldrich, 150 Units ml^{-1}) and the mixture was incubated at 25℃. After the incubation, 3 ml of a hexane solution was added to the reaction mixture (2 ml) and stirred vigorously. The hexane layer and the aqueous layer which were separated into two phases, were used for lauric acid and sucrose analyses, respectively.

2.6. Lauric acid and sucrose assays

The hexane solution, which was dehydrated by the addition of anhydrous sodium sulfate, was concentrated with a rotary evaporator, and the dried residue was then dissolved with 0.25 ml of benzene. HCl-methanol (5%, v/v, 0.5 ml) was added to the aliquot in a vial and heated to methylate the lauric acid at 100℃ for 2 hours after the vial was sealed. Afterwards, both 1.5 ml of hexane and 4 ml of 2% $KHCO_3$ were added to the aliquot and the mixture was kept at 5℃ for one day. The hexane layer or the $KHCO_3$ layer was used for the analysis of lauric acid or sucrose.

Lauric acid methyl ester in the separated hexane layer was identified and measured by GC-MS (Hewlett Packard, GC 5890 and Mass selective detector 5972) and GC (Hitachi G-3900) with FID, respectively, by a modified procedure of Takano and Koike (2000). A capillary column (GC-MS: HP-5MS, 0.25 mm i.d. x 30 m, GC: DB-1, 0.25 mm i.d. x 30 m) was used in a temperature-gradient mode (0 – 3 min at 120℃; 3 – 23 min linear gradient from 120 to 220℃, followed by 2 min at 220℃). The temperature at the injection port or detector was 250℃; and the injection volume was 5 μl. Margaric acid was used as an internal standard for GC analysis.

For sucrose analysis, ethanol was added to the $KHCO_3$ solution to adjust to 70% at the final concentration, and the ethanol solution was filtered with DISMIC filter (0.45 μm, ADVANTEC). The filtrate was analyzed by HPLC according to a slightly modified procedure of Yamashita et al. (1993). A Hitachi Model L-7100 pump and L-7490 RI detector were used for HPLC analysis. Sucrose was separated on a LiChrosphere NH_2 column (MERCK), 4 x 250 mm,

using acetonitrile : water (85 : 15, v/v) solvent. The solvent was kept isocratic for 30 min. The flow rate was 0.8 ml min^{-1}, and the injection volume 20 μl. The identification of sucrose was based on the retention time using the standard.

3. Results

3.1. Effects of sucrose fatty acid esters on the degreening of Nagato-yuzukichi fruit during storage

As shown in Table 1, the level of the Hue angle (surface color) in the fruit flavedo treated with SLE, SME, SPE and SSE decreased by 10%, 11%, 13% and 17% with degreening after 20 days of storage at 20℃, respectively. The change in the Hue angle in the control fruit was similar to that in the fruit treated with SSE. The Chl *a* content in the fruit flavedo treated with SLE was also remained during storage higher than that with any other treatment. Thus, SLE treatment was more effective to keep the green color of the flavedo than any other treatment.

3.2. Inhibitory effect of laurate on chlorophyll-degrading enzyme activities

In a previous paper (Yamauchi et al., 2003), Chl degradation in Nagato-yuzukichi fruit treated with SLE at 50℃ for 3 min was more efficiently suppressed than that with SLE at ambient temperature for 3 min. This finding, together with the result obtained here, indicates that SLE, which is infiltrated into the flavedo tissue, might have an inhibitory action on Chl degradation. The

effect of SLE on Chl-degrading enzyme activities was determined, but the chlorophyllase and Chl-degrading peroxidase activities were hardly inhibited by the addition of SLE to the reaction mixture (data not shown). The effect of laurate on Chl-degrading enzyme activities was then measured.

As shown in Table 2 (Expt. 1), laurate inhibited Chl-degrading peroxidase activity up to 64%, while the control did not, whereas no other fatty acid showed an inhibitory action. On the other hand, regarding chlorophyllase activity, laurate, myristate and palmitate, particularly the former, showed an inhibitory effect, while stearate did not have that effect. Caprylate and caprate also had an inhibitory effect, but the level of inhibition in both fatty acids was lower than that in laurate (Table 2, Expt. 2).

3.3. Formation of laurate from sucrose laurate ester by flavedo extract

Figure 1 shows the decomposition of SLE by the flavedo extract. SLE was decomposed by the flavedo extract and both the lauric acid and sucrose levels increased gradually during incubation at 25℃. The increase of the lauric acid level was parallel with that of the sucrose level. As shown in Fig. 2, SLE was also degraded by flavedo tissue segments and the laurate level decreased gradually followed by a sharp increase in the lauric acid level.

3.4. SLE decomposition by lipase

Lipase is known to be a kind of esterase and hydrolyzes a lipid to fatty acid and glycerol (Bier, 1955). As shown in Fig. 3, SLE was decomposed to laurate by

lipase and formed laurate, which was accumulated during incubation.

3.5 Suppression of degreening by laurate treatment

The effect of laurate on flavedo degreening in Nagato-yuzukichi fruit was determined (Fig. 4). As would be expected, laurate treatment suppressed, in particular, the degreening of the flavedo during storage at 20℃; furthermore, the laurate treatment at 50℃ for 3 min was more effective than that at ambient temperature.

4. Discussion

Edible coating reagents containing sugar and/or fatty acid are used for quality maintenance of *Citrus* fruit (Murata, 1989; Baldwin, 1994; Perez-Gago et al., 2002). Murata (1989) found that the sucrose fatty acid ester effectively inhibited the occurrence of chilling injury and degreening in Kabosu (*Citrus sphaerocarpa*) fruit. Polysaccharide coating containing sucrose fatty acid ester maintained the quality of orange and lime fruits during storage (Baldwin, 1994). Perez-Gago et al. (2002) demonstrated that treatment with edible hydroxypropyl methylcellulose-lipid composite coatings containing fatty acid and glycerol extended the postharvest shelf-life of Fortune mandarins. In addition, in apple fruit, sucrose ester-based coatings formulated with antioxidants such as diphenylamine, n-propyl gallate and ascorbyl palmitate, also prevented the occurrence of superficial scald, which is well-known as a physiological disorder appearing during long-term storage at low temperature

(Bauchot et al., 1995). Thus, edible coating reagents, such as sugar fatty acid esters, could be useful for maintaining freshness and controlling physiological disorders in fruit, such as degreening and chilling injury.

In Nagato-yuzukichi fruit, degreening of fruit flavedo during storage was reduced more effectively by treatment with SLE than by any other treatment with a sucrose fatty acid ester. As reported in a previous paper (Yamauchi et al., 2003), SLE treatment at 50℃ for 3 min suppressed the degreening more strongly than SLE treatment at ambient temperature, and the enhancement of Chl-degrading peroxidase activity was appreciably suppressed in the fruit treated with SLE at high temperature, particularly when compared with the fruit treated with SLE at ambient temperature. In contrast to the change of Chl-degrading peroxidase, chlorophyllase activity in the control fruit decreased with degreening during storage at 20℃, whereas that activity in SLE-treated fruit with or without high temperature showed a sharp decline. In this study, we observed that laurate reduced both the chlorophyllase and Chl-degrading peroxidase activities and that inhibition on peroxidase activity was more apparent. Furthermore, we also found that laurate could inhibit the reaction of peroxidase-mediated Chl degradation through its role in uncompetitive inhibition (data not shown).

SLE was decomposed by the flavedo extract and the flavedo tissue segment to form laurate and sucrose. We also demonstrated that lipase broke down SLE to form laurate as apparent in Fig. 3. Both the flavedo extract and the flavedo tissue segment play a role in SLE hydrolysis, and lipase-like enzyme in the flavedo tissue, which plays a role in lipid hydrolysis, might participate in the decomposition of SLE to form laurate. In addition, the laurate treatment at

ambient temperature and at 50℃ for 3 min, especially the latter, suppressed degreening of the flavedo at 20℃, which suggests that the laurate had infiltrated considerably more into the flavedo tissue at high temperature than it had during the treatment at ambient temperature.

In conclusion, we suggest that the enzyme included in the flavedo tissue, which is an esterase, such as a lipase, related to lipid hydrolysis, could be involved in SLE degradation and that the formed laurate could partially inhibit Chl degradation during storage. Moreover, the suppression of degreening by SLE could be due to the inhibition of degreening by laurate in addition to the coating effect of SLE.

Acknowledgements

We are grateful to Mitsubishi-Kagaku Foods Corporation for supplying sucrose fatty acid esters and the Hagi Citrus Research Institute for supplying the Nagato-yuzukichi fruit.

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Figure legends

Fig. 1. Formation of lauric acid and sucrose from sucrose laurate ester by flavedo extract. The vertical bars represent SE (n=3). Reaction mixture: 2.5 ml flavedo extract (0.2 g ml^{-1}) + 5 ml 2% sucrose laurate ester – phosphate buffer (100 mM phosphate buffer, pH 7.0), 25℃

Fig. 2. Formation of lauric acid from sucrose laurate ester by flavedo tissue fragment. The vertical bars represent SE (n=3). Reaction mixture: 2.5 g flavedo tissue fragment + 5 ml 2% sucrose laurate ester – phosphate buffer (100 mM phosphate buffer, pH 7.0), 25℃

Fig. 3. Formation of lauric acid from sucrose laurate ester by lipase. The vertical bars represent SE (n=3). Reaction mixture: 2.5 ml lipase solution (150 Units ml^{-1}) + 5 ml 2% sucrose laurate ester – phosphate buffer (100 mM phosphate buffer, pH 7.0), 25℃

Fig. 4. Changes in surface color of laurate and heat-treated Nagato-yuzukichi fruit during storage at 20℃. The vertical bars represent SE (n=3). 0.1% laurate: The fruit was treated with 0.1% laurate at ambient temperature (0.1% Laurate) or 50℃ (HT + Laurate) for 3 min. HT: heat treatment.

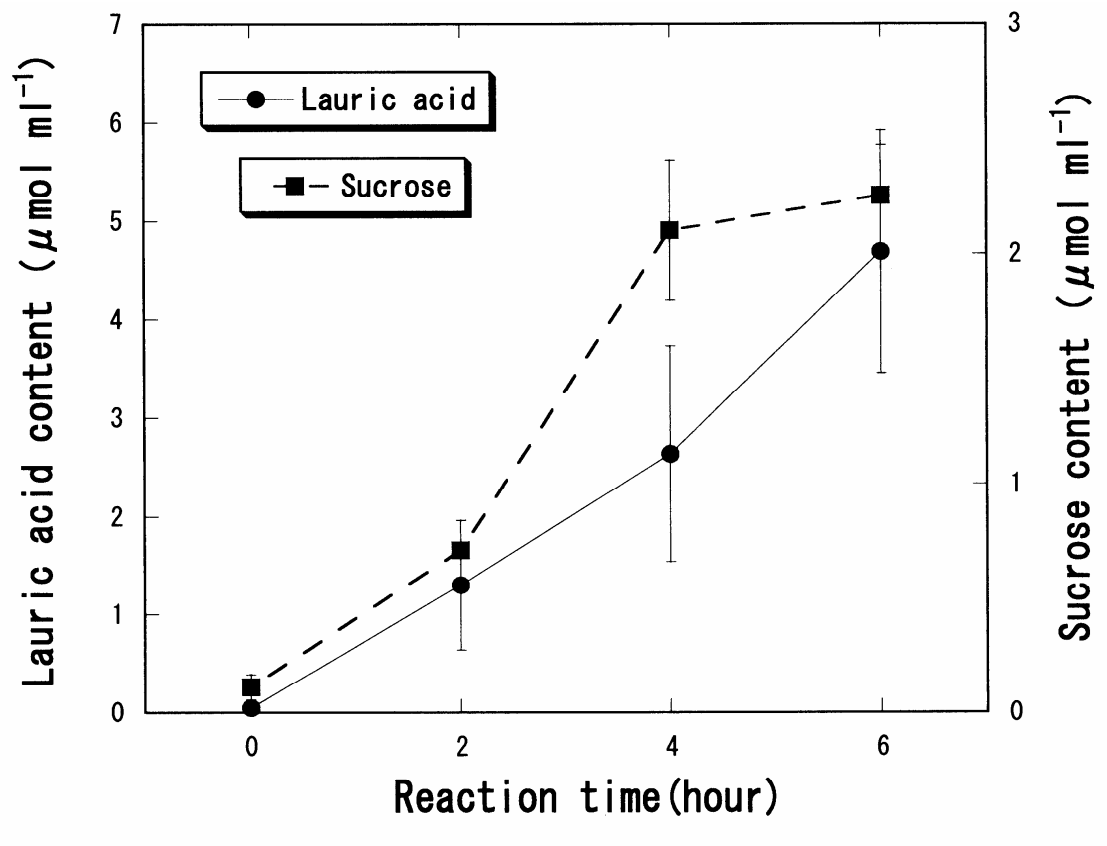
Lauric acid
Sucrose
Lauric acid content (μmol ml^{-1})
Sucrose content (μmol ml^{-1})
Reaction time(hour)
0
1
2
3
4
5
6
7
0
1
2
3
0
2
4
6

Fig. 1.

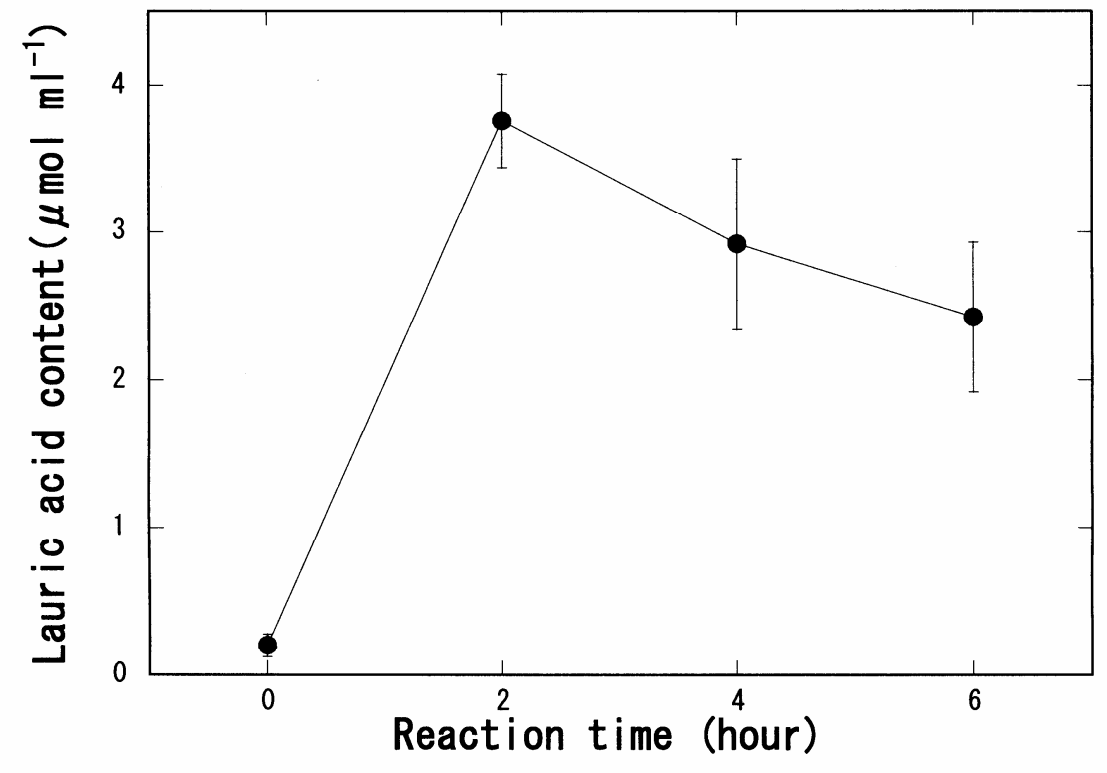

Fig. 2.

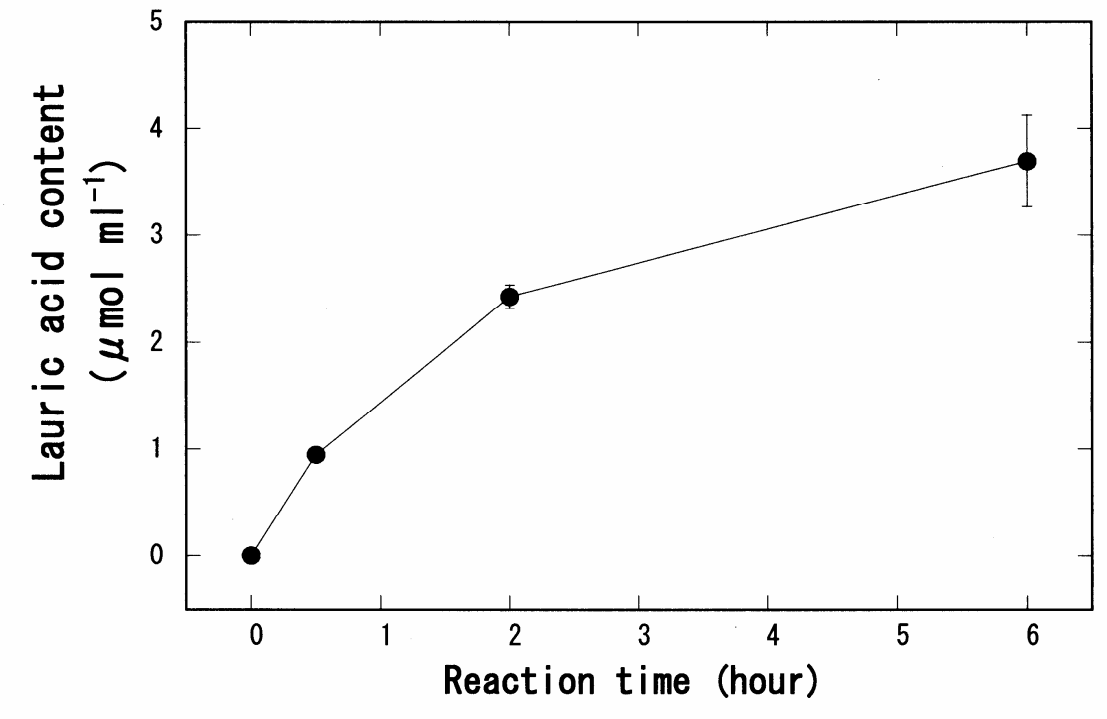
Lauric acid content
(μmol ml⁻¹)
0
1
2
3
4
5
0
1
2
3
4
5
6
Reaction time (hour)

Fig. 3.

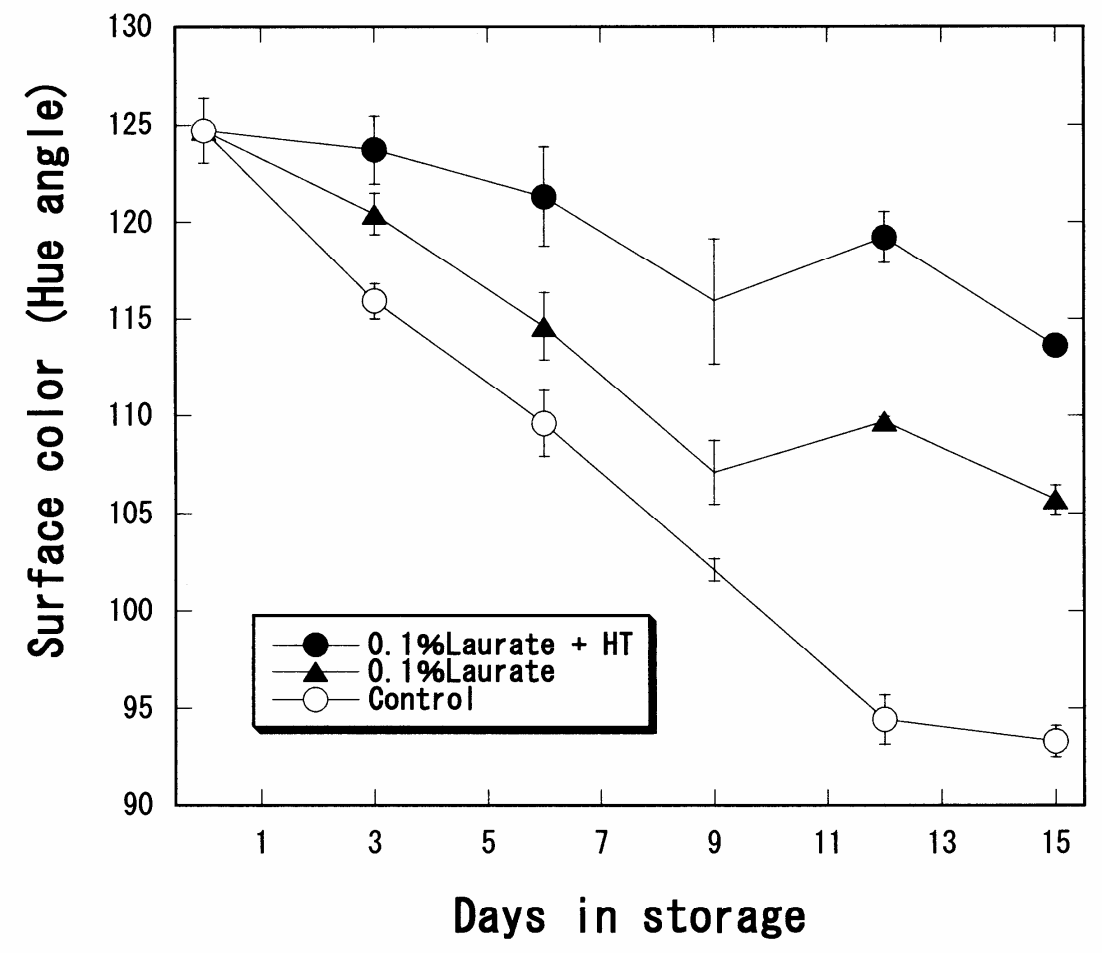
Surface color (Hue angle)
130
125
120
115
110
105
100
95
90
1
3
5
7
9
11
13
15
Days in storage
0.1%Laurate + HT
0.1%Laurate
Control

Fig. 4.

Table 1. Changes in surface color and chlorophyll content of sucrose fatty acid ester-treated Nagato-yuzukichi fruit during storage at 20℃.

Treatment	Surface color (Hue angle)	Chlorophyll *a* content (mg $100g^{-1}$flavedo)
Day 0		
Control	127±0.5*1 (100*2)	94.4±3.0 (100)
Day 20		
Control	104±2.8 (82)	20.3±9.6 (22)
SLE	114±1.6 (90)	36.0±6.3 (38)
SME	113±1.6 (89)	29.5±3.0 (31)
SPE	111±1.7 (87)	21.9±1.9 (23)
SSE	105±1.5 (83)	20.6±0.78 (22)

*1 mean±SE (n=9), *2 %
SLE: 2% sucrose laurate ester, SME: 2% sucrose myristate ester,
SPE: 2% sucrose palmitate ester, SSE: 2% sucrose stearate ester

Table 2. Effects of various fatty acids on the activities of chlorophyll-degrading enzymes.

	Activity (Units*1mg-1protein)	
	Chlorophyll-degrading Peroxidase	Chlorophyllase
Experiment 1		
Control	1334±85*2 (100*3)	2.49±0.41 (100)
1 mM Laurate (C_{12})	860±33 (64)	1.04±0.26 (56)
1 mM Myristate (C_{14})	1507±131 (113)	1.48±0.11 (60)
1 mM Palmitate (C_{16})	1453±128 (109)	1.77±0.11 (71)
1 mM Stearate (C_{18})	1435±129 (108)	2.63±0.04 (106)
Experiment 2		
Control	1072±109 (100)	3.44±1.07 (100)
1 mM Caprylate (C_8)	1331±206 (124)	2.44±0.18 (71)
1 mM Caprate (C_{10})	873±100 (81)	2.58±0.07 (75)
1 mM Laurate (C_{12})	434±46 (40)	1.63±0.23 (48)

*1 Unit = μ g chlorophyll *a* degradation min^{-1}

*2 mean±SE (n=3)

*3 %