Chlorophyll Degradation with Degreening of Kabosu

*(Citrus sphaerocarpa* Hort. ex Tanaka) Fruits

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Summary

Changes in concentrations of chlorophylls (Chls) a and b and their derivatives and peroxidase and chlorophyllase activities were determined to elucidate the pathway of Chl degradation in developing and stored kabosu (*Citrus sphaerocarpa* Hort. ex Tanaka) fruits.

Surface color of kabosu fruits gradually turned from green to yellow with fruit development. The level of phenolic compounds involving in Chl degradation and peroxidase activity increased concurrently.

The Chl contents sharply decreased concurrent with the degreening of stored kabosu fruits. The patterns of HPLC tracings derived from extracts of fresh kabosu fruit revealed the presence of pheophytin a, 10-hydroxychlorophylls a and b, and chlorophyllides a and b, as Chl derivatives. These Chl derivatives in rind extracts gradually disappeared as degreening progressed during storage at 20°C; some were barely detectable on day 50. Peroxidase activity in fruits held at 20°C increased appreciably during the first 20 days of storage and then decreased. Chlorophyllase activity in the rind of fruits stored at 20° and 4°C increased slightly on day 20 but subsequently decreased.

It is inferred from these results that most Chls in kabosu fruits are degraded into a colorless product through a peroxidase pathway.

Introduction

Kabosu (*Citrus sphaerocarpa* Hort. ex Tanaka) fruits are a kind of green sour orange similar to lime and sudachi fruits. One of the symptoms of deterioration of harvested kabosu fruits is the loss of green color in the rind (9, 17).

It is well known that in ethylene treated citrus fruits, chlorophyllase activity strongly increases concurrent with a sharp decline in chlorophyll (Chl) content (3, 5, 21). Amir-Shapira *et al.* (3) reported that in mature green tangerine fruits treated with ethylene, chlorophyllide content increased with the Chl degradation and the enhancement of chlorophyllase activity, suggesting that chlorophyllase is involved in Chl degradation. Moreover, Shimokawa *et al.* found that chlorophyllase activity in satsuma mandarin fruits treated with ethylene increased significantly with a sharp decrease in Chl content (21), but the enzyme activity of the fruits without ethylene treatment showed almost no change in spite of a gradual decrease of Chl content (20). Aljuburi *et al.* (1) also demonstrated that chlorophyllase activity increased with the rise of Chl in regreening of Valencia orange. These results indicate that chlorophyllase does not always participate in Chl degradation process.

It was reported in a preceding paper that chlorophyllase played a minor role in the degradation of Chls in spinach leaves stored with or without ethylene, and that a peroxidase system might be the major pathway for their degradation (26). Similarly, Chls in parsley leaves were degraded by the peroxidase-hydrogen peroxide system in the presence of apigenin, a major flavone in parsley leaves (24).

In the present study, changes in levels of Chls and their derivatives, and activities of enzymes known to catalyze Chl degrading reactions were monitored to determine the pathway of Chl degradation in stored green kabosu fruits. Concurrently, changes in concentration of phenolic compounds and enzyme activities related to Chl degradation in developing kabosu fruits were followed.
Materials and Methods

1. Materials

Green kabosu fruits were harvested at Oita Citrus Experimental Station and transported to the laboratories of Himeji College of Hyogo and Kagoshima University where they were kept at 20°C for 1 day. After that, 5 fruits per polyethylene film bag (16 × 20 cm in size, 0.03 mm in thickness, having 8 holes with a diameter of 6 mm), were stored at 20° or 4°C. The fruits were removed at scheduled intervals during the 60-day period and the flavedo tissues of rind were analyzed.

2. Extraction and purification of chlorophylls and their derivatives

Pigments were extracted by grinding 5 g of flavedo tissues in 20 ml of cold acetone adjusted to pH 7.0 with 1 ml of 1.2% Na2CO3 with a Polytron homogenizer. Upon filtering the homogenate and re-washing the residue with cold acetone until the residue became colorless, an aliquot of the combined filtrates was brought to 25 ml with additional acetone until the final concentration was 80%. The extract was re-filtered through a Millipore filter (0.45 µm) and read for photometric and high-performance liquid chromatography (HPLC) analyses. Purification and identification of Chl derivatives in the acetone extract were carried out by the method described in the preceding paper (26).

3. Determination of chlorophylls and their derivatives

Chl contents were measured spectrophotometrically according to the Arnon method (4). The Chl derivatives were determined by HPLC according to the method of Eskin and Harris (8) using the Shimazu Gradient-LC System. Pigments were separated by a Nucleosil 7C18 reversed phase column, 4.6 × 250 mm, using two solvents “A”, methanol: water (80:20), and “B”, ethyl acetate in a gradient. “B” was added to “A” at a linear rate for a 20 min period until a 50:50 mixture was attained at the end of a 20 min period and the 50:50 mixture was used isocratically for an additional 30 min. The flow rate was 1 ml/min and injection volume was 20 µl. Detection was at 435 nm by using TOSOH UV-8 model II.

4. Determination of chlorophyll degradation activity

The reaction mixture contained 0.2 ml of 70% ethanol extract of flavedo tissue (from which Chls and carotenoids were previously removed with hexane), 0.2 ml of Chls ethanol solution (Chl a 30 µg/0.2 ml), 0.04% Triton X-100, 20 µg of horseradish peroxidase (Sigma Chemical), 0.012% hydrogen peroxide, and 72 mM phosphate buffer (pH 6.0) in a final volume of 2.5 ml. The reaction was carried out for 5 min at 25°C after which the reaction was stopped by adding 5 ml of acetone. The remaining (non-degraded) Chls were extracted with hexane and determined spectrophotometrically by reading the absorbance at 663 nm. Degraded Chls were calculated by subtracting this value from the blank (distilled water instead of hydrogen peroxide) one. One unit of the Chl degradation activity was defined as the change of 0.01 in absorbance per min.

The Chl degradation activity shows the phenolic compound level of kabosu flavedo extract involving in Chl degradation by the peroxidase-hydrogen peroxide system.

5. Enzyme assay

Crude enzyme was dissolved by suspending acetone powder (50 mg) of kabosu flavedo in 10 ml of 5 mM phosphate buffer (pH 7.0), containing 50 mM KCl and 0.24% Triton X-100 for 1 hr at 8°C. The mixture was filtered through Miracloth and the filtrate was centrifuged at 16,000g for 15 min. The supernatant was used as the crude enzyme.

Peroxidase activity was determined by the method described elsewhere (23). Chlorophyllase activity was determined by a modification of the method of Amir-Shapira et al. (3). A reaction mixture contained 0.1 ml of enzyme, 0.1 ml of 2.64% Triton X-100, 0.12 ml of Chls acetone solution (Chls 80 µg/0.12 ml) and 1.0 ml of 100 mM phosphate buffer (pH 7.0). The reaction mixture was incubated in a water bath at 25°C for 20 min; the enzyme reaction was stopped by the addition of 4 ml of acetone. The remaining (non-degraded) Chls were extracted with 4 ml of hexane and assayed by reading the absorbance at 663 nm. Activity was based on the decrease in absorbance by Chl at 663 nm. One unit of the enzyme activities was defined as the change of 0.01 in absorbance per min.
Enzyme protein content was assayed by the method of Lowry et al. (11).

6. Determination of surface color and phenolic compounds

Surface color of kabosu fruits was measured by using a color difference meter (Nippon Denshoku ND-101D).

The kabosu extract for phenolic compounds was prepared by immersing 10 g of flavedo in 40 ml of 86% hot ethanol and heating the mixture on a boiling water bath for 15 min. The mixture was homogenized for 3 min in a Waring blender and the homogenate was filtered through Toyo #2 filter paper. The filtrate was used as kabosu extract. The total phenol content was determined by the method of Mizuno and Uno (16). Flavonoids content was determined spectrophotometrically by reading the absorbance at 412 nm after adding equal amounts of 70% ethanol solution containing 2% AlCl₃ to the kabosu extract (18).

Results

1. Chlorophyll degradation with fruit development of kabosu

The changes in surface color, levels of phenolic compounds, and enzyme activities involving Chl degradation in kabosu fruits harvested at three different stages of fruit development from August to September are shown in Table 1. Average weights of kabosu fruits harvested on Aug. 24, Sep. 11 and Sep. 27 were 58.9, 72.7 and 77.6 g, respectively. The surface green color of fruits gradually turned yellow with the fruit development. This agrees with the finding that the b value of the surface color increased with the fruit development. Both total phenol and flavonoid contents were highest in fruits harvested on Sep. 11 and decreased in fruits harvested on Sep. 27. However, Chl degradation activity increased with the fruit development, attaining the highest level in fruits harvested on Sep. 27. Simultaneous to the acceleration in Chl degradation activity, peroxidase activity in the rind increased strongly. On the other hand, chlorophyllase activity was lowest in those fruits harvested on Sep. 11 and subsequently

Table 1. Changes in surface color, contents of phenolic compounds, and enzyme activities involving in chlorophyll degradation with fruit development of kabosu.

<table>
<thead>
<tr>
<th>Item</th>
<th>Harvest date (1989)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aug 24</td>
</tr>
<tr>
<td>b value</td>
<td>9.8 ± 0.4</td>
</tr>
<tr>
<td>Total phenol (mg/100g flavedo)</td>
<td>126.5 ± 3.9</td>
</tr>
<tr>
<td>Flavonoid (O.D.412 nm)</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>Chl degradation activity²</td>
<td>105.5 ± 4.6</td>
</tr>
<tr>
<td>Peroxidase³</td>
<td>3941 ± 249</td>
</tr>
<tr>
<td>Chlorophyllase⁴</td>
<td>33.4 ± 3.8</td>
</tr>
</tbody>
</table>

The b value shows an index of the change of the surface color in fruits.

² Units/g flavedo, Unit = O.D.663 nm, 0.01/min.

Reaction mixture - Chl in ethanol solution, Chl a 30 µg + kabosu extract (70% ethanol) + Peroxidase (20 µg) + 0.04% Triton X-100 + 0.012% H₂O₂ + 72 mM phosphate buffer (pH 6.0).

Chlorophyll (Chl) degradation activity shows the level of phenolic compounds, which are included in the extract of kabosu flavedo, involving in Chl degradation by the peroxidase-hydrogen peroxide system.

³ Units/mg protein, Unit = O.D.430 nm, 0.01/min.

⁴ Units/mg protein, Unit = O.D.663 nm, 0.01/min.

⁵ Mean ± SE, n = 3.
increased in fruits harvested on Sep. 27.

2. Chlorophyll degradation in stored kabosu fruits

Kabosu fruits, which were harvested on Sep. 11, kept their harvest-freshness for the first 5 days of storage at 20°C. The quality and appearance of the stored fruits deteriorated gradually after becoming yellow; the yellow became more intense after day 50. On the contrary, fruits kept at 4°C remained fresh for nearly 50 days.

The b value of the surface color was nearly constant during the first 10 days at 20°C and then increased with the disappearance of Chls and appearance of the yellow pigments (Fig. 1). The value of b for fruits stored at 4°C remained unchanged. The contents of Chls a and b in kabosu fruits decreased slightly during the first 10 days of storage at 20°C, and then declined sharply; by day 50 Chl level decreased to one-third of the initial value (Fig. 2). Changes in levels of Chl derivatives in rinds of stored kabosu fruits at 20°C were determined by HPLC. The HPLC tracing of fresh kabosu fruit extracts showed chiefly the presence of pheophytin a, 10-hydroxychlorophylls a and b (Chls a-1 and b-1), and chlorophyllides a and b as Chl derivatives (Fig. 3). Chls a-1 and b-1 were detected as the oxidized forms of Chl a and b, respectively. Pheophorbide was hardly detectable in extracts of fresh kabosu fruit rinds. The contents of Chls a and b decreased greatly with the disappearance of green pigments in the rind. However, all Chl derivatives did not show an increase during the storage at 20°C. Finally, the derivatives were barely detectable on 50 days after storage.

Peroxidase activity increased considerably during the first 20 days and then decreased between day 20 and day 50 in fruits held at 20°C (Fig. 4). But when stored at 4°C, peroxidase activity in the rind remained nearly constant for the first 10 days and then increased slightly. Unlike peroxidase activity, chlorophyllase activity in rinds of fruit kept at 20°C increased slightly on day 20 and then decreased. The chlorophyllase activity in the rind kept at 4°C paralleled that of fruits held at 20°C (Fig. 5).

Discussion

In the kabosu fruits, the levels of both Chl a-1 and chlorophyllide a were low during storage while the fruits turned yellow. Chl a-1 seems to be formed by Chl oxidase (12) or lipoxygenase (25). Maunders et al. (15) noted an accumulation of Chl a-1 with a decline of Chl a in senescing excised barley and bean leaves. Contrary to this finding,

![Fig. 2. Changes in chlorophyll content of flavedo of kabosu fruits stored at 20°C and 4°C.](image)

![Fig. 3. HPLC tracings of acetone extracts from flavedo of kabosu fruits after 0, 20, and 50 days of storage at 20°C. Chlide: Chlorophyllide, Chl: Chlorophyll, Phy: Pheophytin.](image)
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Chl a-1 content was very low in fresh spinach leaves and it decreased with yellowing in spinach leaves stored at 25°C with or without ethylene treatment (26). This finding that Chl a-1 content decreased in stored spinach leaves agrees well with that of stored kabosu fruits, inferring that both Chl oxidase and lipoxygenase pathways play an insignificant role in Chl degradation in kabosu fruits and spinach leaves. When fruits were treated with ethylene, Chl degradation was greatly accelerated as a result of enhanced chlorophyllase activity in satsuma mandarin (21), calamondin (5) and tangerine (3). The increase of chlorophyllide a content was determined with degreening of tangerine fruits treated with ethylene (3). These results indicate strongly that chlorophyllase is involved in the first step of Chl degradation of ethylene-treated citrus fruits. However, chlorophyllide formed by chlorophyllase shows a green color and the absorption maximum is similar to that of Chl (22), suggesting that Chl is not bleached by the chlorophyllase action alone. Chlorophyllide level was low in fresh and stored kabosu fruits and chlorophyllase activity did not increase significantly with yellowing during the storage at 20°C. Perhaps, these results indicate that chlorophyllase may play a minor role in the degradation of Chls in stored kabosu fruits.

Chls in parsley leaves are degraded by the peroxidase-hydrogen peroxide system in the presence of apigenin (24) and unlike Chl oxidase and lipoxygenase, Chl a-1 is not a major intermediate metabolite in Chl degradation in that system (unpublished). Hence, Chls are thought to be degraded directly into a colorless product. Our analyses revealed that peroxidase and Chl degradation activities increased with the development and concurrent degreening of kabosu fruits, suggesting that peroxidase might be involved in Chl degradation and Chls are degraded directly to a colorless product.

Huff (10) reported that chlorophyllides as well as Chls were degraded by the peroxidase-hydrogen peroxide system in the presence of phenolic compounds such as resorcinol and 2, 4-dichlorophenol. Moreover, Purvis (19) demonstrated that in the pathway of Chl degradation in calamondin fruits, chlorophyllase converts the lipid-soluble Chl into a more water-soluble chlorophyllide which is then catalytically bleached by hydrogen peroxide or enzymatically bleached by peroxidase. It is suggested that Chls in kabosu fruits may be degraded in part by both chlorophyllase and peroxidase actions; i.e. chlorophyllase hydrolyzes Chl to chlorophyllide and phytol. Peroxidase then degrades chlorophyllide to a colorless product. We determined that chlorophyllase activity in kabosu fruits is extremely high in comparison with that in spinach leaves (0.94 unit/mg protein). Amir-Shapira et al. (2) also found that Chl degradation by chlorophyllase in the chloroplast of citrus leaves and the chromoplast of citrus fruits was greater than that in parsley leaves. These findings also indicate that Chls in kabosu fruits may be degraded in part by both

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\text{Fig. 4. Peroxidase activities in flavedo of kabosu fruits stored at 20°C and 4°C.}
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One unit of the enzyme activity was defined as a change of 0.01 in absorbance at 430 nm per min.

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\text{Fig. 5. Chlorophyllase activities in flavedo of kabosu fruits stored at 20°C and 4°C.}
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One unit of the enzyme activity was defined as a change of 0.01 in absorbance at 663 nm per min.
chlorophyllase and peroxidase actions.

Most Chls appear to be finally degraded through a pathway in which the porphyrin ring is opened, resulting in a colorless compound. Duggelin et al. (6) reported that lipofuscin-like compounds (fluorescent compounds) accumulate with senescence of fescue leaves and guessed that the compounds represent catabolites of Chl. Matile et al. (13) also found pink pigments as Chl catabolites, which are attributed to the opening of the porphyrin ring. The pigments are acidic and the pink pigment has a conspicuous absorption maximum at 526 nm. Furthermore, both lipofuscin-like compounds (7) and pink pigments (14) are mainly localized in vacuole. Additionally, the study on Chl catabolites formed by the peroxidase-hydrogen peroxide system and cell localization of the catabolites in kabosu fruits throws further light on the mechanism of Chl degradation in kabosu fruits.

Acknowledgment

The authors kindly thank M.S. Hiroaki Mine and Mr. Takashi Satoh, Research Scientists of Oita Citrus Experimental Station, for supplying with kabosu fruits. The authors also thank Dr. Seki Shimizu, Professor of Ochanomizu University, for his suggestion. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 01560039) from the Ministry of Education, Science and Culture of Japan.

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カボス果实の脱緑に伴うクロロフィルの分解

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摘 要

カボス (Citrus sphaerocarpa Hort. ex Tanaka) 果実のクロロフィル分解経路を明らかにするため、果実の発育（8月～9月）・貯蔵に伴うクロロフィルおよびクロロフィル分解物、ペルオキシダーゼならびにクロフィラーゼ活性の変化について検討を行った。

カボス果実の果皮色は、発育に伴い徐々に黄化した。ペルオキシダーゼによるクロロフィル分解に関与するフェノール化合物含量は、果実の発育に伴い増加し、またペルオキシダーゼ活性も同様に増大した。

カボス果実を20℃に貯蔵すると、果皮の脱緑に伴いクロロフィル含量の急減が認められた。高速液体クロマトグラフィーによりクロロフィル分解物を調べたところ、貯蔵日数の果実において、フェオフィチン a，10-ハイドロオキシクロロフィル a および b，クロフィリッド a および bが検出された。しかしながら、これらの分解物は20℃貯蔵に伴い増加はみられず、貯蔵50日後の黄化した果実ではほとんど検出されなかった。

クロロフィルの酸化分解に関与していると考えられるペルオキシダーゼ活性について調べたところ、20℃貯蔵に伴い活性が増大し、貯蔵20日で最高の活性を示し、その後減少した。一方、クロフィラーゼ活性は、4ならびに20℃貯蔵に伴い、わずかに活性の増大が認められ、その後減少を示した。このように、20℃貯蔵におけるクロフィラーゼ活性の変化は、ペルオキシダーゼとは異なり、顕著な活性の増大は認められなかった。

以上の結果から、カボスの脱緑に伴い、クロロフィルは、ポルフィリン構造の開環に基づく無色の物質にそのほとんどが直接分解されるものと思われ、その分解ペルオキシダーゼを含む系が関与しているものと推察された。