Water Soluble Fluorescent Substances in *Citrus unshiu* Fruit and their Possible Participation in Chlorophyll Catabolism

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Summary

Changes in levels of fluorescence substances (FS) in aqueous extracts of *Citrus unshiu* (C. unshiu) peels were measured by the 3-dimensional (3-D) fluorescence spectrophotometer. FS, extracted from C. unshiu peels, have characteristic fluorescence at excitation (Ex) 320–330 nm and emission (Em) 420–440 nm. Ex and Em of FS are very similar to those of lipofuscin-like compounds known to accumulate in tissues during aging and fluorescent chlorophyll-catabolites (FCC) appeared to be a product of oxidative cleavage of chlorophyll (Chl)–porphyrin. The amount of FS increased in yellow–green C. unshiu peels and decreased in yellow C. unshiu peels. In ethylene-treated C. unshiu peels, the amount of FS increased at the time of rapid disappearance of Chl, and decreased after that, whereas FS of non-ethylene-treated C. unshiu peels did not change during incubation. A possible participation of FS in Chl catabolism is discussed.

Key Words: chlorophyll catabolism, *Citrus unshiu*, ethylene, fluorescence substances.

Introduction

Senescence is normally studied by assessing parameters, such as Chl, lipid peroxidation (LPO), protein, RNA, and free amino acids. The pathway of Chl catabolism is comprised of several reactions, which lead to the formation of intermediary catabolites. After removal of phytol and Mg atom from Chl–porphyrin by chlorophyllase and Mg–dechelatase, respectively, the porphyrin macrocycle of phophorbide (Phed) a is cleaved. The porphyrin ring is cleaved by the enzyme Phed a oxygenase, forming red–Chl–catabolite (RCC). RCC is broken down by RCC deuctase (RCCR), eventually forming FCC. After hydroxylation and additional species-specific modifications, FCCs are tautomized nonenzymically to nonfluorescent–Chl–catabolites (NCCs) inside the vacuole (Matile et al., 1999).

In previous studies, we monitored changes in levels of Chl and their derivatives in ethylene-treated C. unshiu fruits in the dark and found that pyropheophorbide (Pyrophed) a was present in the green skin, but it gradually disappeared with ripening (Kurata et al., 1998; Maeda et al., 1998b). These results suggest that the catabolic pathway of Chl a to colorless catabolites exists in C. unshiu peels.

In this study, we investigated the FS levels in the peel of C. unshiu fruits that are similar to the fluorescence characteristic of lipofuscins–like compounds and FCCs.

Materials and Methods

Plant materials and ethylene treatment

The satsuma mandarin (*Citrus unshiu* Marc. cv. Nichinan No. 1) fruit were obtained from a local farm. Green fruit were harvested, and treated with 200 ppm ethylene within 6 hr of harvest. After the fruit were exposed for 16 hr in a 20-liter desiccator at 25 °C in darkness, the fruit were transferred to fresh air and held at 25 °C in the dark for 72 hr. For on-tree trial, green, yellowish green, and yellow fruit were harvested on the same day and analyzed.

Determination of chlorophyll content

Flavedo disks were incubated in N, N-dimethylformamide (DMF) at 4 °C, and total Chl content in DMF extract was determined spectrophotometrically by measuring absorbances at 664.5 and 647 nm under a dim,
green light at 25 °C. The amount of total Chl content was calculated, using the following equation; total Chl content (mg·liter⁻¹)= 17.90 × A647 + 8.08 × A664.5.

Extracts of Fluorescent compounds for 3-D spectrum measurement

Flavedo disks were frozen with liquid nitrogen, ground in a mortar and pestle, immediately homogenized with 0.1 M potassium phosphate buffer (pH 7.0) and the mixture was centrifuged and the supernatant mixed with −25 °C ethanol (final concentration was 80% [v/v]) to precipitate the proteins and then centrifuged.

Measurement of fluorescence compounds

FS extracted from *Citrus unshiu* fruits was monitored and analyzed using a 3-D spectrum measurement (HITACHI: F-4500 Fluorescence spectrophotometer) (Adachi et al., 1999).

Results and Discussion

A typical 3-D scan of water soluble FS, extracted from *Citrus unshiu* peel (Fig. 1), has characteristic fluorescence at Ex 320–330 nm and Em 420–440 nm. The fluorescence characteristics of FS extracted from *C. unshiu* was extremely similar to those of FCC and lipofucsin-like compounds. The FS level increased in yellow-green fruit and decreased in yellow fruit (Fig. 2). In ethylene-treated *C. unshiu* peels, the amount of FS increased concurrent with the rapid disappearance of Chl and then decreased. FS of non-treated *C. unshiu* peels remained almost constant during incubation (Fig. 3).

Senescence is normally studied by assessing parameters, such as LPO, protein, RNA and free amino acids. In addition, degradation of Chl has been widely considered to be a prominent feature of senescence in plant cells. Recent advances in the elucidation of Chl-porphyrin catabolism in senescent leaves suggest that the catabolism of Chl a to NCCs is catabolized via the following reaction: Chl a → chlorophyllide a → Phed a → FCC → FCC → NCC. FCC is a water-soluble compound that is generated by sequential reaction of Phed a → oxygenase and RCCR from Phed a. In addition, during periods of rapid Chl breakdown, trace amounts of FCCs are detectable in senescent leaves. FCCs have a spectrum (Ex: 320

![Fig. 1](image1.png)

![Fig. 2](image2.png)

![Fig. 3](image3.png)
nm/Em: 450 nm) typical for Schiff–base structures (–N=C–C–N–). The linear tetrapyrrole of FCCs suggests that the fluorescence is due to the unsaturated, γ methine bridge that links pyrroles C and D (Matile et al., 1999). A similar structure occurs in liposoluble, lipo­fuscin-like-compounds during natural or ethylene-induced ripening of fruit (Maguire and Haard, 1975) and senescent leaves (Wilhem and Wihelenova, 1981), indicating that they are products of LPO. Unlike the lipofuscin-like-compounds which accumulated in aging plant cells, the amount of FCC increased at the time of rapid disappearance of Chl, and subsequently decreased (Matile et al., 1999). In this experiment, quantitative change of FS in C. unshiu peels was similar to those of FCC. Furthermore, unlike liposoluble lipofuscin-like-compounds, FS is water-soluble. From these results, a possibility that water soluble FS was product of oxidative cleavage of Chl–porphyrin was suggested. In previous studies, FCC accumulated when Chl a, 2,4-dichlorophenol (DCP), and H2O2 were exposed to an enzymes prepared from ethylene-treated Raphanus sativus cotyledons (Adachi et al., 1999), Musa sapientum peels (Ma and Shimokawa, 1998), or C. unshiu peels (Takahashi et al., 2000). Contrarily, when DCP was changed into p-cumaric acid in the same enzyme reaction system of C. unshiu peels, FCC did not accumulate (Takahashi et al., 2000). Likewise, when Chl a was changed into Pyrophed, FCC did not accumulate (Maeda et al., 1998a). Kurata et al. (1998) also reported that Pyrophed a was present in the green C. unshiu peels but that it gradually disappeared with ripening which indicate that Pyrophed a is catabolized in C. unshiu peels. From these results, the enzyme which carries out oxidative-cleavage of the Chl–porphyrin, has been suggested existing beside Phed a oxygenase in the C. unshiu peel.

Literature Cited

ウンシュウミカン果実における水溶性蛍光色素とそのクロロフィル代謝への関与の可能性について

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摘要

ウンシュウミカン果皮に存在する水溶性蛍光物質の量的変化が、乾物重量三元素スペクトル法を使用し検討した。ウンシュウミカン果皮から抽出された水溶性蛍光物質は、Ex:320-330 nm/Em:420-440 nmの蛍光特性を示し、老化の間に蓄積することが知られているリポフシン様物質、およびクロロフィル・ポルフィリンが酸化分解した代謝産物であるFCCsの蛍光特性と極めて類似していた。樹皮での水溶性蛍光物質量は、葉緑色果実で増加し、黄色果実で減少した。エチレン処理果実の水溶性蛍光物質量は、クロロフィルが激激に消失する時期に上昇し、以後徐々に減少した。これに対し、無処理果実では、貯蔵の間ほとんど変化がみられなかった。本研究で得られた結果より、ウンシュウミカン果皮中に存在する水溶性蛍光物質のクロロフィル代謝への関与の可能性について考察した。

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