

ウンシュウミカン果実における水溶性蛍光物質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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著者名	倉田,裕文 東,理恵 中林,健一 下川,敬之 足立,勝
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Water Soluble Fluorescent Substances in *Citrus unshiu* Fruit and their Possible Participation in Chlorophyll Catabolism

Hirofumi Kurata^{1*}, Rie Azuma^{1**}, Kenichi Nakabayashi², Keishi Shimokawa^{1***}
and Masaru Adachi^{1****}

¹ Faculty of Agriculture, Miyazaki University, Miyazaki 889–2192

² Faculty of Education and Culture, Miyazaki University, Miyazaki 889–2192

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-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-dechelatease, respectively, the porphyrin macrocycle of pheophorbide (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 deductase (RCCR), eventually forming FCC. After hydroxylation and additional species-specific modifications, FCCs are tautomerized 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-dimethyl-formamide (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,

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*Present address: National Agricultural Research Center for Kyushu Okinawa Region, 2421 Nishigoshi, Kumamoto 861-1192 (Research Fellow of Cooperative System for Supporting Priority Research)

**Present address: Department of Food Science, Ehime Women's college, Ibuki, Uwajima, Ehime 798-0025

***Corresponding author.

****Present address: Department of Biochemistry, Miyazaki Medical College, Kihara, Kiyotake, Miyazaki 889-1692

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green light at 25 °C. The amount of total Chl content was calculated, using the following equation; total Chl content ($\text{mg} \cdot \text{liter}^{-1}$) = $17.90 \times A_{647} + 8.08 \times A_{664.5}$.

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 *C. unshiu* fruits was monitored and analyzed using a 3-D spectrum measurement (HITA-CHI: F-4500 Fluorescence spectrophotometer) (Adachi et al., 1999).

Results and Discussion

A typical 3-D scan of water soluble FS, extracted from *C. 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 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

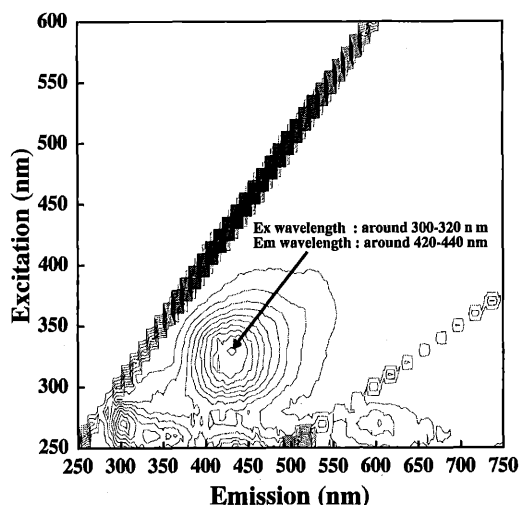


Fig. 1. 3D-fluorescence spectrum of fluorescence compounds extracted from *Citrus unshiu* fruit.

reaction: Chl *a* → chlorophyllide *a* → Phed *a* → RCC → 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

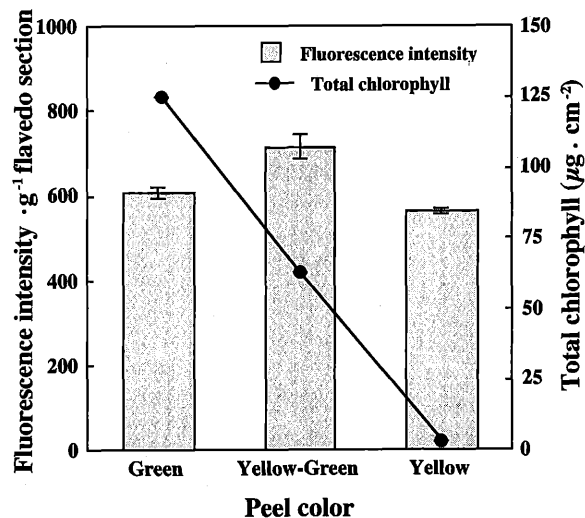


Fig. 2. Fluorescence compounds and chlorophyll content in *Citrus unshiu* fruit on the tree. The vertical bars represent SE of the means ($n=5$). Data are results from nine different experiments.

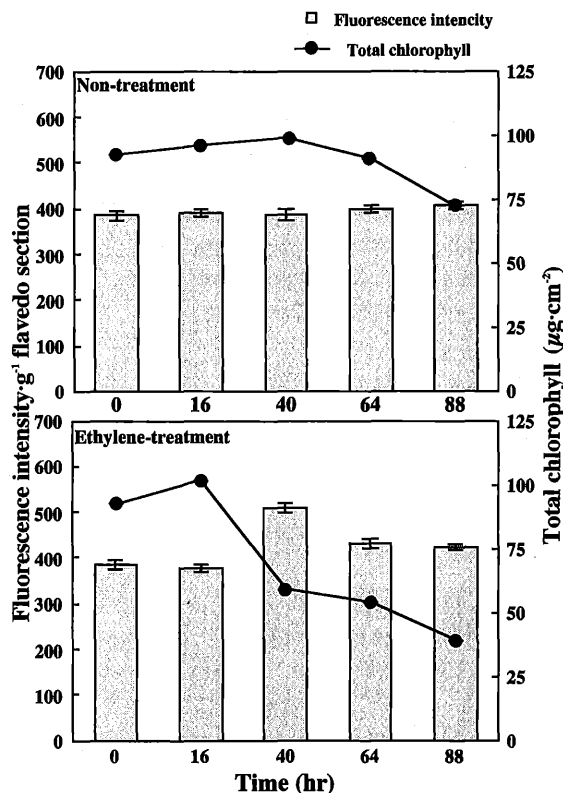


Fig. 3. Fluorescence compounds and chlorophyll content in non-treated and ethylene-treated *Citrus unshiu* fruit during incubation in the dark. The vertical bars represent SE of the means ($n=5$). Data are results from five different experiments.

nm/Em: 450 nm) typical for Schiff-base structures ($-N=C-C=C-N-$). The linear tetrapyrrolic 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 Wihelnova, 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 H_2O_2 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*-coumaric 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 Pyrophe *a*, FCC did not accumulate (Maeda et al., 1998a). Kurata et al., (1998) also reported that Pyrophe *a* was present in the green *C. unshiu* peels but that it gradually disappeared with ripening which indicate that Pyrophe *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 besides Phed *a* oxygenase in the *C. unshiu* peel.

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

倉田裕文^{1*}・東 理恵^{1***}・中林健一²・

下川敬之¹・足立 勝^{1***}

¹ 宮崎大学農学部 889-2192 宮崎市

学園木花台西1丁目1番地

² 宮崎大学教育文化学部 889-2192 宮崎市

学園木花台西1丁目1番地

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、蛍光分光三次元スペクトル法を使用し検討された。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スチン様物質、およびクロロフィル-ポルフィリンが酸化環裂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。樹上での水溶性蛍光物質量は、黄緑色果実で増加し、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、クロロフィルが急激に消失する時期に上昇し、以後徐々に減少した。それに対し、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。

* 現在：農業技術研究機構九州沖縄農業研究センター（重点研究支援協力員）熊本県菊池郡西合志町大字須屋2421

** 現在：愛媛女子短期大学 食物栄養科 愛媛県宇和島市伊吹町馬場421

*** 現在：宮崎医科大学 第二生化学 宮崎県宮崎郡清武町木原5200