Chlorophyll Degradation by Peroxidase in Ethylene-treated Satsuma Mandarin Fruits[†]

Naoki YAMAUCHI* and Fumio HASHINAGA**

* *Himeji College of Hyogo,*

1-1-12 Shinzaike-honcho, Himeji 670

** *Faculty of Agriculture, Kagoshima University*

1-21-24 Korimoto, Kagoshima 890

Changes in chlorophylls (Chls) content, flavonoids level, and peroxidase and chlorophyllase activities were determined to clarify the pathway of Chl degradation in ethylene-treated satsuma mandarin (*Citrus unshiu* Marc.) fruits.

Chls content in the fruits decreased considerably even during ethylene treatment and then showed a sharp decline during storage at 20°C. On the other hand, Chls content in the fruits treated without ethylene decreased gradually after 1 day of storage. In the fruits treated with ethylene, Chl degradation activity, which shows the phenolic compound level involved in Chl degradation by a peroxidase-hydrogen peroxide system, increased markedly to a maximum on day 2, and then decreased to the original level. Chls were greatly degraded by the peroxidase-hydrogen peroxide system in the presence of naringenin and apigenin, which implies that flavonoids might relate to Chl degradation by the peroxidase system. The activities of peroxidase and chlorophyllase in ethylene-treated fruits increased significantly with the advance of degreening.

These results infer that Chls in ethylene-treated fruits might be degraded by the peroxidase pathway together with the chlorophyllase action.

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INTRODUCTION

The acceleration of chlorophyll (Chl) degradation is one of the physiological actions of ethylene¹⁾. In early ripening cultivar of satsuma mandarin fruits, the flesh attained to the harvesting stage while the peel still shows a green color. For this reason, ethylene is used for the enhancement of degreening in the peel²⁾.

It is well known that Chls in ethylene-treated citrus fruits decrease strongly with the enhancement of chlorophyllase activity^{1),3)}. Amir-shapira et al.⁴⁾ reported that chlorophyllide (chl) content in-

creased concurrent with the sharp decline of Chl content and with the increase of chlorophyllase activity in mature green tangerine treated with ethylene. These observations seem to indicate that chlorophyllase is involved in Chl degradation of ethylene treated citrus fruits.

We reported in a preceding paper⁵⁾ that Chls in green kabosu fruits without ethylene treatment decreased sharply with the degreening of fruits while Chl derivatives, such as 10-hydroxychlorophyll a (Chl a-1) and Chlide a, decreased gradually, and that peroxidase activity increased appreciably as degreening progressed during stor-

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age at 20°C, suggesting that Chls might be degraded through a peroxidase pathway.

In this paper, we deal with the changes of Chls, flavonoids, which are involved in Chl degradation by peroxidase, and peroxidase and chlorophyllase activities in ethylene-treated satsuma mandarin fruits to determine the involvement of the peroxidase pathway in Chl degradation.

MATERIALS AND METHODS

Materials

Green satsuma mandarin (*Citrus unshiu* Marc.) fruits were harvested at the Toso Orchard of Kagoshima University and transported to the laboratories of Himeji College of Hyogo and Kagoshima University where they were kept at 20°C for 1 day. The fruits were treated with or without 120 ppm ethylene for 12 hours in a desiccator (45 l) at 20°C. After the treatment, 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°C. The fruits were removed at scheduled intervals during the 9-day period and the flavedo tissues of rind were analyzed.

Extraction and determination of chlorophylls

Pigments were extracted by grinding 5 g of flavedo tissues in 20 ml of cold acetone adjusted to pH 7.0 with 0.5 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%. Chl contents were measured spectrophotometrically according to the Arnon method⁶⁾.

Determination of chlorophyll degradation activity

The reaction mixture contained 70% ethanol extract of flavedo tissue (0.08 g), from which Chls and carotenoids were previously removed with hexane, Chls ethanol solution (a-30 µg and b-11 µg/0.2 ml), 0.04% Triton X-100, 20 µg of horseradish peroxidase (Sigma chemical), 0.012% hydrogen peroxide and 80 mM phosphate buffer (pH 6.0) in a final

volume of 2.5 ml. This mixture contained 8% of ethanol. 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 flavedo extract involved in Chl degradation by a peroxidase-hydrogen peroxide system.

Enzyme assay

Crude enzyme was dissolved by suspending acetone powder (50 mg) of flavedo tissue 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 (Calbiochem.) 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 elsewhere⁷⁾. Chlorophyllase activity was determined by a modification of the method of Amir-shapira et al⁴⁾. The reaction mixture contained 0.3 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 0.8 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 at the change of 0.01 in absorbance per min.

Enzyme protein content was assayed by the method of Lowry et al⁸⁾.

Partial purification of chlorophylls

Chls used here were partially purified by adding

3 ml of 1,4-dioxane and 3 ml of distilled water to 20ml of the 80% acetone extract of fresh spinach leaves (5 g), allowing the mixture to stand until a precipitate formed, centrifuging the mixture at 12,000g for 10 min., and dissolving the pellet in ethanol or acetone⁹⁾.

Determination of surface color and flavonoids

Surface color of satsuma mandarin fruits was measured by using a color difference meter.

The flavedo extract (70% ethanol) for phenolic compounds was prepared by immersing 5 g of flavedo in 45 ml of 78% hot ethanol and heating the mixture on a boiling water bath for 15min. The mixture was homogenized for 5min in a Waring blender and the homogenate was filtered through Toyo #2 filter paper. The filtrate was used as flavedo extract. The flavonoid content was determined spectrophotometrically by reading the absorbance at 410 nm after adding equal amounts of 70% ethanol solution containing 2% AlCl_3 to the flavedo extract.

RESULTS

Satsuma mandarin fruits treated with ethylene kept their green color for the first day of storage at 20°C. After that, the green color of fruits turned markedly to yellow. On the contrary, fruits treated without ethylene retained the green color of the

fruits during the first 4 days of storage. The level of surface color ($L \cdot b / |a|$ value) of ethylene-treated fruits increased significantly with Chl degradation after ethylene treatment (Fig. 1). The level of surface color of the fruits treated without ethylene was nearly constant during the first 5 days of storage and then showed a slight increase. As shown in Fig. 2, the content of Chls a and b in the fruits decreased considerably even during ethylene treatment and then showed a sharp decline during storage at 20°C; by 6 days the Chl a content dropped to about 30% of the initial content. On the other hand, Chl contents in the fruits treated without ethylene decreased gradually after 1 day of

Fig. 1 Changes in surface color of ethylene-treated satsuma mandarin fruits during storage at 20°C.

Fig. 2 Changes in chlorophylls content of ethylene-treated satsuma mandarin fruits during storage at 20°C.

Fig. 3 Changes in flavonoids level of ethylene-treated satsuma mandarin fruits during storage at 20°C.

storage.

Fig. 3 shows the changes in flavonoid level of flavedo of satsuma mandarin fruits with or without ethylene treatment. The flavonoid level of flavedo of the fruits increased during ethylene treatment, which showed a peak value after 2 days of storage, and then decreased with degreening of fruits. Unlike the change in ethylene-treated fruits, the level of the fruits treated without ethylene showed almost no change during storage. The Chl degradation activity, which shows the level of phenolic compounds involved in Chl degradation by the peroxidase-hydrogen peroxide system, increased strongly during ethylene treatment, which showed a peak value after 2 days of storage, and then decreased significantly to the original level (Fig. 4). In the fruits without ethylene treatment, the activity increased slightly after 3 days of storage. As is apparent in Fig. 5, both peroxidase and chlorophyllase activities in the flavedo of ethylene-treated fruits increased markedly to a maximum on day 2, and then the peroxidase activity decreased while the chlorophyllase activity remained at a high level during storage.

Fig. 4 Changes in chlorophyll degradation activity of ethylene-treated satsuma mandarin fruits during storage at 20°C.

* Chlorophyll (Chl) degradation activity shows the level of phenolic compounds involved in Chl degradation by the peroxidase-hydrogen peroxide system.

** Unit = Δ O. D. 663 nm, 0.01/min.

Flavonoids such as narirutin, hesperidin, and apigenin glycoside, as reported by Nakabayashi¹⁰⁾, Nishiura et al.¹¹⁾ and Matsubara et al.¹²⁾, are contained in satsuma mandarin fruits. Table 1 shows the Chl degradation by the peroxidase-hydrogen peroxide system in the presence of naringenin and hesperetin as a aglycone of narirutin and

Fig. 5 Activities of peroxidase and chlorophyllase in ethylene-treated satsuma mandarin fruits.

* Unit = Δ O. D. 430 nm, 0.01/min.

** Unit = Δ O. D. 663 nm, 0.01/min.

The fruits were stored at 20°C.

Table 1 Chlorophyll degradation by peroxidase in the presence of several flavonoids.

Flavonoids		Chlorophyll degradation (%)
<u>Flavone</u>		
Apigenin	10 μ M	31.1
	20 μ M	46.9
Diosmetin	50 μ M	5.0
<u>Flavanone</u>		
Naringenin	10 μ M	30.6
	20 μ M	61.0
Hesperetin	50 μ M	4.8

Reaction mixture contained flavonoid ethanol solution (0.1 ml), chlorophylls ethanol solution (a—30 μ g and b—11 μ g/0.1 ml), 0.04% Triton X-100, 20 μ g of horseradish peroxidase (Sigma chemical), 0.012% hydrogen peroxide and 80 mM phosphate buffer (pH 6.0) in a final volume of 2.5ml. This mixture contained 8% of ethanol. 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) chlorophylls were extracted with hexane and determined spectrophotometrically by reading the absorbance at 663 nm.

hesperidin, and apigenin and diosmetin, which have the same structural form of B-ring of naringenin and hesperetin, respectively. Chls were degraded by the system in the presence of both flavanones or both flavones used. The level of Chl degradation, however, differed with the source of flavonoid as noted by the greater degradation with apigenin or naringenin than diosmetin or hesperetin.

DISCUSSION

Degradation of Chl by the peroxidase-hydrogen peroxide system in the presense of phenolic compounds, such as 2, 4-dichlorophenol and resorcinol, has been reported by Huff¹³⁾ and Kato and Shimizu¹⁴⁾. In a previous paper¹⁵⁾, similarly, Chls in parsley leaves were degraded by the system in the presence of apigenin, a major flavone in parsley leaves. In this report, naringenin and apigenin showed a more effective degradation of Chls, as compared with hesperetin and diosmetin. The former flavonoids have a hydroxyl group at *p*-position of B-ring. This is in agreement with the finding by Kato and Shimizu¹⁴⁾ that each phenolic compound, which relates to Chl degradation by the poroxidase-hydrogen peroxide system, has an electron-attractive group at the *p*-position. Riov et al.¹⁶⁾ demonstrated that ethylene stimulated a phenylalanine ammonialyase (PAL) activity of peel of citrus fruits. The flavonoid content and Chl degradation activity increased with degreening of ethylene-treated satsuma mandarin fruits, inferring that PAL activity induced with ethylene treatment could enhance the synthesis of flavonoids involved in Chl degradation.

As reported by Amir-Shapira et al.⁴⁾, Chl degradation in ethylene-treated citrus fruits was greatly accelerated with the enhancement of Chlide level and chlorophyllase activity, which indicates that chlorophyllase is involved in the first step of Chl degradation in ethylene-treated citrus fruits. Chlide, however, still shows a green color and the spectral absorption is similar to that of Chl¹⁷⁾, which suggests that Chl is not degraded by the chlorophyllase action alone.

In stored kabosu fruits treated without ethylene, peroxidase activity increased appreciably with degreening of flavedo during storage at 20°C, whereas chlorophyllase activity increased slightly but Chlide a did not show an increase, which implies that most of Chls are degraded into a colorless products through the peroxidase⁵⁾. Out of accordance with this result, we found that the activities of peroxidase and chlorophyllase showed a sharp increase with the advance of Chl degradation in ethylene-treated satsuma mandarin fruits. Moreover, the degradation of Chlide by the peroxidase-hydrogen peroxide system has been reported by Matile¹⁸⁾ and Huff¹³⁾. These results suggest that in satsuma mandarin fruits treated with ethylene, most of Chls might be degraded by both chlorophyllase and peroxidase actions: chlorophyllase hydrolyzes Chl to Chlide and phytol, and then peroxidase degrades Chlide in the presence of flavonoids to colorless products.

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エチレン処理された温州ミカンのペルオキシダーゼによるクロロフィルの分解

山内直樹*・橋永文男**

*兵庫県立姫路短期大学(〒670姫路市新在家本町 1-1-12)

** 鹿児島大学農学部(〒890鹿児島市郡元 1-21-24)

エチレン処理された温州ミカン (*Citrus unshiu* Marc.) 果実のクロロフィル分解経路を明らかにするため、貯蔵に伴うクロロフィル含量、フラボノイド、クロロフィル分解活性、ペルオキシダーゼならびにクロロフィラーゼ活性の変化について検討を行った。

エチレン処理果は20℃貯蔵に伴い脱緑がみられたが、無処理果では貯蔵4日までほとんど変化がみられなかった。クロロフィル含量についてみたところ、エチレン処理中に減少し、処理後貯蔵に伴い急減した。処理果のフラボノイドは増大がみられ、貯蔵2

日で最大値を示し、その後処理前の値に減少した。クロロフィル分解活性(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量)は、フラボノイドの変化と同様の傾向を示し、その変化割合はより顕著であった。温州ミカンに含まれるフラボノイド・アグリコンであるナリンゲニン、ヘスペレチン、アピゲニンおよびヘスペレチンと同じB環構造を持つフラボンのジオスメチン存在下でのペルオキシダーゼによるクロロフィル分解を調べたところ、クロロフィルはすべてのフラボノイドにより分解され、特にナリンゲニンとアピゲニンの効果が顕著であった。ペルオキシダーゼならびにクロロフィラーゼ活性についてみたところ、両活性ともエチレン処理中に増大がみられ、ペルオキシダーゼ活性は貯蔵2日から減少したが、クロロフィラーゼは貯蔵中、高活性を維持した。

以上の結果より、エチレン処理された温州ミカンのクロロフィル分解は、クロロフィルがクロロフィラーゼにより分解され、その後生成したクロロフィリッドがフラボノイド存在下でペルオキシダーゼにより無色の物質に分解されるものと推察された。

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