Degreening of *Citrus unshiu* Fruits Via Ethylene-induced Soluble Chlorophyllase

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Summary

The mechanism of ethylene action on degreening in satsuma mandarin (*Citrus unshiu* Marc. cv. Nichinan No. 1) fruits was investigated using inhibitors of protein synthesis and soluble chlorophyllase (chlorophyll-chlorophyllide hydrolase, Chlase; E.C. 3.1.1.14). This enzyme which catalyzes the dephytylation of chlorophyll (Chl) to chlorophyllide (Chlide), was partially purified.

Chlase catalyzes the first step in the Chl breakdown process. Ethylene treatments promoted the degreening and increased soluble Chlase activity of *C. unshiu* fruit peels held in the dark. Cycloheximide (CH), an inhibitor of cytoplasmic protein synthesis, inhibited the effects of ethylene, whereas chloramphenicol (CP), an inhibitor of plastid protein synthesis, also weakly inhibited the Chlase activity. These results suggest that ethylene appears to enhance the degreening of the peels through *de novo* synthesis of Chlase. Moreover, *de novo* cytoplasmic protein synthesis and chloroplast-dependent enzyme synthesis may regulate Chlase activity in the peel of ethylene-treated *C. unshiu* fruits.

Soluble Chlase was precipitated with acetone from *C. unshiu* fruit peels and purified by the following method: after ammonium sulfate fractionation, gel-filtration through Sephadex G-25 and Phenyl Sepharose CL-4B (hydrophobic chromatography) were used. The soluble Chlase was purified ca. 203.5-fold with a yield of 45.5%.

Key Words: chlorophyll, chlorophyllase, *Citrus unshiu*, degreening, ethylene.

Introduction

The plant hormone ethylene is produced and perceived in response to a wide variety of environmental and developmental cues. Ethylene regulates plant development and also coordinates the processes of fertilization, senescence, fruit ripening, healing of wounds and pathogen injuries. Thus, ethylene is used for degreening *Citrus* fruits.

Recent advances in the elucidation of Chl catabolism in senescent leaves suggest that the process comprises of three steps catalyzed by Chlase, Mg-dechelate, and pheophorbide *a* oxygenase, oxygenating pheophorbide *a* (Phed *a*)-cleavage enzyme (review: Matile et al., 1996). In *Citrus* fruit, however, the pathway of Chl catabolism is complex (Shimokawa et al., 1990; Yamauchi et al., 1991; Yamauchi and Hashinaga, 1992; Yamauchi et al., 1997a; b; Kurata et al., 1998; Maeda et al., 1998). To study fruit ripening of *Citrus* fruit, Chlase has been investigated extensively, even though its activity can be detected in green immature fruits. It is synthesized *de novo* upon exposure to ethylene (Shimokawa et al., 1978; Trebitsch et al., 1993).

We investigated the ethylene-enhanced Chl catabolism in *C. unshiu* fruit as an *in vivo/in vitro* Chl-

degrading system and found that Chlase is a key enzyme in the initial step of Chl breakdown (Shimokawa et al., 1978; Shimokawa, 1990; Yanagisako et al., 1994; Tanabe et al., 1996). Recently, Janave (1997) reported that soluble and Triton-solubilized enzyme extracts from Cavendish bananas (*Musa cavendishi*) degrade Chl by two distinct pathways, the Chlase and oxidative Chl degrading pathways. Shimokawa et al. (1990) using ^{14}C -Chl *a* found that in Triton-solubilized enzyme extracts from ethylene-treated *C. unshiu*, Chl *a* is degraded enzymatically as follows: Chl *a* → Chlide *a* → Phed *a* → pyropheophorbide *a*. More recently, soluble Chlase was prepared from peels of ethylene-treated fruits and its enzymatic properties reported by Azuma et al. (1998). However, little is known about the soluble enzymes responsible for the degreening of ethylene-treated *Citrus* fruit.

In the present study, the mechanism of ethylene-enhanced degreening in satsuma mandarin (*Citrus unshiu* Marc. cv. Nichinan No. 1) fruits was elucidated by investigating the effects of various inhibitors on the activity of partially purified soluble Chlase in the peels.

Materials and Methods

Plant materials

Mandarin fruits were obtained from a local farm at the end of September and treated within 6 hr of harvest.

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Inhibitor and ethylene treatments

The fruits were administered aqueous solutions of 0.01% Triton X-100 containing $10 \mu\text{g} \cdot \text{ml}^{-1}$ cycloheximide, or $10 \mu\text{g} \cdot \text{ml}^{-1}$ chloramphenicol for 5min. They were blotted dry with paper towels and then exposed to 200 ppm ethylene, whereas the control was exposed to air. Both lots were incubated for 16 hr at 25 °C in the dark. After the treatment, the fruits were incubated for 24 hr at 25 °C in air before the peels were removed and stored at -20 °C. Peels of fruits dipped in 0.01% Triton X-100 solution were used as control.

Measurement of green density and determination of Chl content

Surface color of the satsuma mandarin fruits was determined by measuring L, a and b values at the same spots on each individual fruit with a Hunter's colorimeter (Minolta CR-200); the $(L+b)/2+a$ ratio was calculated (Tanabe et al., 1996). Chl content was measured according to Inskeep and Bloom (1985). Flavedo disks were incubated in N,N-dimethylformamide (DMF) at 4 °C and total Chl content in DMF extract determined spectrophotometrically 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 ($\text{mg} \cdot \text{l}^{-1}$)

$$= 17.90 \times A_{647} + 8.08 \times A_{664.5}.$$

Preparations of acetone powder and enzyme extract

Preparations of acetone powder and enzyme extract were carried out by the slightly modified procedure of Shimokawa et al. (1978).

Chlorophyllase activity

The reaction mixture contained 30 mM phosphate buffer (pH7.0) containing 0.2% Triton X-100 (P. buf.), 30 μM Chl *a* 1.5ml, and enzyme in a total vol. of 4.5ml. After incubation at 25 °C for 20min, the reaction was terminated by adding 3ml hexane, 2ml acetone, 1ml 2-butanone, and 0.2ml of 0.25M NaOH. The mixture was vigorously shaken and allowed to stand for 1min. Chlide *a* in the acetone layer was determined spectrophotometrically by using $74.9\text{mM}^{-1} \cdot \text{cm}^{-1}$ at 667nm. One unit of Chlase is defined as the amount of enzyme which catalyses the production of 1 μmol Chlide *a* $\cdot \text{min}^{-1}$.

Protein content

Protein content was determined according to the method of Lowry et al. (1951) using bovine plasma γ -globulin as the standard.

Purification of chlorophyllase

The acetone powder (100g) was ground further and extracted with 2 liters of 30 mM potassium phosphate buffer, pH 7.0 (P.P.buf.) at 25 °C for 10min. The extract

was filtered through miracloth and the filtrate centrifuged at $12,000 \times g$ for 20min. Ammonium sulfate (30% saturation) was added to the supernatant and the mixture re-centrifuged at $12,000 \times g$ for 30min. The supernatant was made to 50% saturation with ammonium sulfate and centrifuged at $12,000 \times g$ for 30min. The pellet was dissolved in P.P.buf. and the solution applied to a Sephadex G-25 column ($1.6 \times 45\text{cm}$) equilibrated with P.P.buf. The enzyme was eluted with the same buf. and the eluate collected in 3-ml fractions. The active fraction was applied to a small column of phenyl Sepharose CL-4B ($1 \times 10\text{cm}$) pre-equilibrated with 100 mM P.P.buf. (100ml). The column was eluted with a linear gradient of Triton X-100 (0–0.6%, total vol. 200ml). The fractions with the highest enzyme activities were collected and assayed.

Results and Discussion

1. Effect of inhibitors of protein synthesis on the soluble Chlorophyllase activity in *Citrus unshiu* fruits

A high negative correlation between the degree of greenness [$(L+b)/2+a$ ratio] of the satsuma mandarin fruits and total Chl content in the peel was obtained: $Y = -0.14X + 7.94$, ($r = -0.993$), where X = green density [$(L+b)/2+a$ ratio] of the fruits and Y = total Chl content in the fruits (Fig. 1). The changes in greenness of the fruits were determined using a Hunter's colorimeter (Fig. 2). Exposure to ethylene for 16 hr resulted in no distinct decrease in levels of green density. A greater change in green coloration in ethylene-treated fruits occurred after 88 hr, whereas greenness in the non-treated fruits remained constant. Ethylene-treatment

Fig. 1. Correlation between total Chl content and Hunter's color index $[(L+b)/2+a]$ in the *Citrus unshiu* fruits. Significant at 0.1% level. $r = -0.993$.

Fig. 2. Changes in level of greenness in ethylene-treated satsuma mandarin fruits held in the dark. Satsuma mandarin fruits were treated with (●) or without (○) 200 ppm ethylene for the first 16 hr, then left in CO₂-free air for an additional 72 hr. Vertical bars represent S.D. of the mean (n=7).

hastened degreening, the Chl levels decreasing ca. 60–67.5% after 40 hr (data not shown). Thus, ethylene treatments significantly decreased green values, compared with the control. When Chlase activity was followed spectrophotometrically at 667nm, absorbance decreased with time in freshly prepared crude extracts but not in the boiled preparations, indicating that the catabolism of Chl is catalyzed by heat labile enzyme. The catalysis was enhanced by ethylene.

Cycloheximide (CH), an inhibitor of protein synthesis in cytoplasmic 80S ribosomes of eukaryotes, inhibited both ethylene-enhanced degreening of mandarin peels and an increase in Chlase activity (Table 1), indicating that mRNA translation is involved in Chlase induction.

The inhibitory effect of CH on chloroplast senescence under various natural (Choe and Thimann, 1975) and artificial (Shimokawa et al., 1978; Azuma et al., 1998; Adachi and Shimokawa, 1998; Adachi et al., 1998) conditions has led to a postulation that chloroplast senescence requires *de novo* cytoplasmic protein synthesis. Moreover, chloramphenicol (CP), an inhibitor of plastid protein synthesis in the plastid of prokaryotes, also weakly inhibited degreening and chloroplast senescence (Table 1). These results suggested that chloroplast-dependent enzyme synthesis plays a role in the regulatory process of Chlase activity in ethylene-treated *C. unshiu* fruit peels. Thus, it is suggested that ethylene enhanced Chl loss in peels is through *de novo* synthesis of Chlase. The inhibitory effects of CH and CP indicate that *de novo* synthesis of ethylene-induced Chlase, a “key” enzyme, is essential in the degreening of the peels (Table 1). Ethylene is known to accelerate senescence in plants (Abeles et al., 1992), which is supported by our results (Fig. 2, Table 1).

2. Partial purification of the soluble Chlorophyllase from ethylene-treated *C. unshiu* fruits

Ethylene-induced soluble Chlase was purified from 100 g of acetone powder prepared from 1kg of *C. unshiu* peels. The enzyme was extracted with 30 mM P.P. buf. (pH 7.0) from acetone powder and purified by hydrophobic chromatography as summarized in Table 2. The enzyme was applied to Phenyl Sepharose CL-4B and eluted with a liner gradient of Triton X-100 (0–0.6%). This purification procedure is relatively rapid taking only 2 days. In addition, the activity of soluble Chlase recovered from the peels was 45.5% (Yield), a purification of about 203.5-fold. Shimokawa (1982) previously purified ethylene-enhanced, Triton X-100 solubilized Chlase from *C. unshiu* fruits only 16.6-fold, only a 13.7% recovery (Yield). Thus, the method is useful for the soluble Chlase purification.

The optimum pH for the Chlase extracted from acetone powder was 7.6 with both P. buf. and P.P. buf..

Table 1. Effect of cycloheximide and chloramphenicol on a soluble chlorophyllase activity in the peels of *Citrus unshiu* fruits.

Treatment	Concentration	Chlorophyllase activity	
		(n mol / hr / mg protein)	(% of control)
Ethylene	(200ppm, control)	14.1 ± 0.4 ^z	100.0 ± 2.8
Ethylene + Water		13.2 ± 0.6	93.6 ± 4.3
None		1.2 ± 0.3	8.5 ± 2.1
None + Water		0.6 ± 0.1	4.3 ± 0.7
Ethylene + Cycloheximide	(35.5 μM, 5min)	5.7 ± 0.2	40.4 ± 1.4
Ethylene + Chloramphenicol	(30.9 μM, 5min)	10.6 ± 0.8	75.2 ± 5.7
None + Cycloheximide	(35.5 μM, 5min)	1.0 ± 0.3	7.1 ± 2.1
None + Chloramphenicol	(30.9 μM, 5min)	0.9 ± 0.2	6.4 ± 1.4

^z Means ± S.E. (n=5)

Table 2. The purification of soluble chlorophyllase from *Citrus unshiu* fruits.

Purification step	ml	Total activity (units)	Total protein (mg)	Specific activity (units / mg protein)	Yield (%)	Purification (– fold)
Acetone powder extract	1250.0	1.1	4647.5	0.0002	100.0	1.0
Gel filtration	13.0	0.8	113.9	0.0070	72.7	35.0
Hydrophobic	8.4	0.5	12.3	0.0407	45.5	203.5

The maximum enzyme activity with P. buf. at optimum pH was much higher than that of P.P.buf.. Purified Chlases are classified by their optimum pH into two groups; enzymes with an acidic pH optimum, such as those from *Ailanthus* (pH 4.52)(McFeeters et al., 1971) and tea leaves (pH 5.8)(Ogura, 1972); in the other group, the enzymes has a neutral pH optimum, such as those from ethylene-treated *C. unshiu* peel (pH 7.0, Triton X-100 solubilized Chlase)(Shimokawa, 1982), *Chlorella vulgaris* (pH 7.2–7.3)(Ichinose and Sasa, 1973), sugar beet (pH 7.1)(Bacon, 1970), and tobacco (pH 7.0–7.5)(Shimizu and Tamaki, 1962). Therefore, the ethylene-treated soluble Chlase belongs to the latter group. Moreover, it is proposed that soluble Chlase exists as an isozyme in ethylene-treated *C. unshiu* peel. The molecular weights of purified Chlases range from 27 kDa to 65 kDa, and their optimal pHs are mostly acidic. The differences may be due to differences in the plant species. The K_m for ours was 6.5 μM for Chl *a* which is similar or higher than those reported by other workers: 2.65 μM for mandarin peel (Shimokawa, 1982); 2.0 μM *Chlorella protothecoides* (Tamai et al, 1979), and lower than 106 μM for *Ailanthus* (McFeeters et al., 1971), 80 μM *phaseolus* (Moll and Stegwee, 1978) and 278 μM *C. limon* leaf (Fernandez-Lopez et al., 1992).

In our study, we found that ethylene-induced soluble Chlase which was partially purified, whereas CP and CH inhibited the degreening in *C. unshiu* peels. The study on the mechanism of ethylene-enhanced degreening in plants, such as fruits of *C. unshiu*, tomato and banana, and leafy vegetables provides useful knowledge on the preservation of freshness in fruits and vegetables.

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エチレン処理されたウンシュウミカン (*Citrus unshiu* Marc.) 果実の脱緑 — エチレン誘導可溶性クロロフィラーゼ —

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摘 要

ウンシュウミカン果実の脱緑に及ぼすエチレンの作用機構を明らかにするため、タンパク質合成阻害剤の影響を調査し、また、可溶性クロロフィラーゼ(クロロフィル *a* をクロロフィリド *a* に加水分解する酵素)を精製した。クロロフィラーゼはクロロフィル分解過程の最初のステップを触媒する。エチレン処理は、暗所下でのウンシュウミカン果実の脱緑を促進し、可溶性クロロフィラーゼ活性を増大させた。シクロヘキシミド(細胞質でのタンパク質合成阻害剤)はエチレン作用を阻害した。クロラムフェニコール(プラスチドタンパク質合成阻害剤)も弱く可溶性クロロフィラーゼ活性を阻害した。

これらの結果は、エチレンが可溶性クロロフィラーゼ酵素の合成を誘導することにより果皮の脱緑を促進することを示唆した。さらに、エチレン処理ウンシュウミカン果皮での可溶性クロロフィラーゼ活性の制御過程には、細胞質基質での *de novo* タンパク質合成とクロロプラスト依存のタンパク質合成が重要であることが示唆された。

可溶性クロロフィラーゼは、エチレン処理したウンシュウミカン果皮のアセトンパウダーより、ハイドロフォービッククロマトグラフィーで、収率45.5%, 203.5倍まで精製した。