ウンシュウミカン果実のエチレン誘導クロロフィル代謝
タイプI反応の二,三の酵素活性に対する阻害剤の影響

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Ethylene-enhanced Chlorophyll Catabolism in *Citrus unshiu* Fruit: Effects of Inhibitors on the Activity of Enzymes Involved in Type I Reactions

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Summary

The mechanism of ethylene (C_2H_4) action on type I reactions (the loss of phytol, the loss of magnesium, and the modification of the side chains of an isocyclic ring of the chlorophyll (Chl) structure) during Chl catabolism in *Citrus unshiu* (*C. unshiu*) fruit was investigated by using inhibitors of protein synthesis. C_2H_4 treatment promoted the degreening through increased chlorophyllase (Chlase) activity of *C. unshiu* fruit peel held in the dark. Cycloheximide (CH), an inhibitor of cytoplasmic protein synthesis, inhibited the C_2H_4-enhanced degreening. Chloramphenicol (CP), an inhibitor of plastid protein synthesis, did not inhibit the effects of C_2H_4. On the other hand, C_2H_4 treatment did not significantly increase chlorophyllide (Chlide)-degrading enzyme (CDE) activity nor $C13^2$-carboxyl-pyropheophorbide (Pyrophed) forming enzyme (CPFE) activity of *C. unshiu* fruit peels. Likewise, CDE and CPFE activities in C_2H_4-treated *C. unshiu* fruit were unaffected by CH- and CP-treatments. A high positive correlation between the degree of greenness of *C. unshiu* fruit peel and Chlase activity was obtained: Y = 1.368 X - 16.038, (r = 0.992), where X and Y are intensity of the green color of the peel and Chlase activity, respectively. However, correlations between the degree of greenness of the peel and 1) CDE activity (Y = 0.024 X +98.030, r = 0.460) and 2) CPFE activity (Y =0.015 X +103.581, r = 0.424) are low. These results indicate that C_2H_4 appears to enhance the degreening of the peel through *de novo* synthesis of Chlase, and that Chlase is the "key" enzyme in the degreening process.

Key Words: chlorophyllase, chlorophyllide-degrading enzyme, $C13^2$-carboxyl-pyropheophorbide forming enzyme, *Citrus unshiu*, ethylene.

Introduction

The Chl catabolism in C_2H_4-treated *Citrus* fruit is a very complex pathway (Amir-Shapira et al., 1987; Kurata et al., 1998a, b, 1999; Maeda et al., 1997, 1998; Shimokawa and Uchida, 1992; Shimokawa et al., 1978, 1990, 1992, 1994; Tanabe et al., 1996; Trebitsch et al., 1993; Yamauchi and Hashinaga, 1992; Yamauchi et al., 1991, 1997a, c; Yanagisako et al., 1994a, b). In previous studies, Shimokawa et al. (1990) and Yanagisako et al. (1994a, b), demonstrated that the degradation of Chl *a* in crude extracts of C_2H_4-treated *C. unshiu* fruit followed a complex enzymatic pathway: Chl *a* → Chlide *a* → Phed *a* → $C13^2$-carboxyl-Pyrophed *a* (Ito et al., 1998) → Pyrophed *a*. More recently, Kurata et al. (1998a, 1999) demonstrated that the crude extracts from the peel of non- and C_2H_4-treated *C. unshiu* fruit catalyzed the conversion of Chlide *a* into $C13^2$-carboxyl-Pyrophed *a*. This complex catabolic pathway involving Chlase, whose activity can be detected in green immature fruits, has been researched extensively. Its activity increases dramatically after C_2H_4 treatment as a result of *de novo* enzyme synthesis (Shimokawa et al., 1978; Trebitsch et al., 1993). However, participation of enzymes such as Chlide-degrading enzyme(CDE) and $C13^2$-carboxyl-Pyrophed forming enzyme (CPFE), has not been investigated during the C_2H_4-enhanced degreening of *C. unshiu* fruit.

In this study, the effects of the protein inhibitors, cycloheximide (CH) and chloramphenicol (CP) on the activities of CDE and CPFE were investigated

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Materials and Methods

Plant materials

The green satsuma mandarin (*Citrus unshiu* Marc. cv. Nichinan No. 1) fruit was obtained from a local farm at the end of September. The fruit was treated within 6 hr after harvest and held in the dark at 25 ℃.

Inhibitor and C_2H_4 treatments

The fruit of *C. unshiu* was administered aqueous solutions of 0.005%(w/v) Triton X-100 containing 50 μM CH, or 50 μM CP for 3min. They were then blotted dry with soft paper towels and then exposed to 200 ppm C_2H_4, whereas the control lot was dipped in 0.005% (w/v) Triton X-100 solution for 3min, and then exposed to air. Both lots were incubated for 16 hr at 25 ℃ in the

dark, which were then further incubated for 24 hr at 25 ℃ in air.

Measurement of color index

Surface color of the *C. unshiu* fruit peel was determined by measuring L, a and b values on one spot on individual fruits with a Hunter's colorimeter (Minolta CR-200); then the (L+b) / 2+a ratio was calculated (Tanabe et al., 1996). Using the data obtained at 0 and 40 hr of incubation, the changes in Hunter's color index were calculated from the expression, [(L+b)/2+a] at 40 hr - [(L+b)/2+a] at 0 hr.

Preparations of acetone powder and enzyme extract

Acetone powder was prepared as described previously (Kurata et al., 1999). The acetone powder (1g) was extracted by stirring with 40 ml of 30 mM potassium phosphate buffer (pH 7.0) with 0.2 %, w/v Triton X-100 for 30 min at 25℃. The mixture was filtered and the filtrate centrifuged at 12,000 × *g* for 20 min at 4 ℃. The supernatant was used as the crude enzyme extract. Its protein content was determined according to the method of Lowry et al. (1951), using bovine plasma γ-globulin as the standard.

Enzyme assay

The Chlase activity was determined according to the method of Shimokawa (1982). The reaction mixture for CDE and CPFE activities consisted of 0.15 ml crude extract, 35.9 μM Chlide *a* or 140.6 μM Phed *a*, mixed with 1.2 ml of 30 mM potassium phosphate buffer (pH 7.0). The reaction was allowed to proceed for 1hr or 2hr at 25 ℃ in the dark. Cold (-35 ℃) acetone (0.8 ml) was added to 0.2 ml of the reaction mixture to terminate the enzymatic reaction. Aliquots were used for HPLC analysis. The activity was based on the decrease in the peak area of Chlide *a* or Phed *a*.

Reagents for Analysis of Chl derivatives by HPLC

Reagents were prepared for HPLC analysis as described previously (Kurata et al., 1999).

Results and Discussion

Immediately after exposure to 200 ppm C_2H_4 for 16 hr, there was no significant difference in the color index in the peel between non-treated and C_2H_4-treated *C. unshiu* fruit (data not shown). After 40 hr, however, the color index increased significantly in the C_2H_4-treated *C. unshiu* fruit, compared with the controls (Table 1). CH, an inhibitor of protein synthesis on cytoplasmic ribosomes (80S), prevented C_2H_4-enhanced degreening, whereas, CP, an inhibitor of plastid protein synthesis, did not. Chlase activity was enhanced by C_2H_4 but CH countered it; CP had no effect (Table 1). CDE and CPFE activities were slightly influenced by all treatments which indicate that C_2H_4-treatment caused the degreening of the peel through *de novo* cytoplasmic Chlase synthesis.

A linear curve and a high significant positive coefficient of correlation (r = 0.992) express the relationship

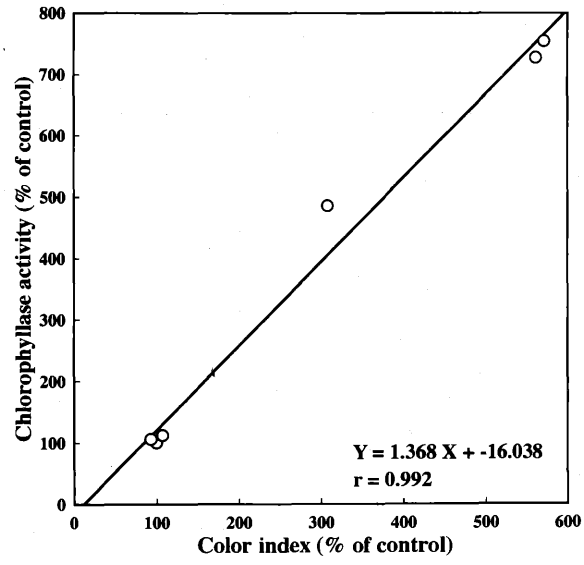


Fig. 1. A correlation between color index of *C. unshiu* fruit and Chlase activity. Data plotted from Table 1.

Table 1. Effect of inhibitors of protein synthesis on the color index and the activity of Chlase, CDE and CPFE in *Citrus unshiu* fruit.

Compounds	Concentration	Color index[z] (% of control)	Chlase activity (% of control)	CDE activity (% of control)	CPFE activity (% of control)
Control (Air, 40h)		100.0 ± 23.5[y]	100.0 ± 6.0[x]	100.0 ± 9.2[x]	100.0 ± 9.2[x]
Ethylene (200ppm, 40h)		561.3 ± 54.2	727.4 ± 3.6	97.0 ± 6.9	104.3 ± 3.5
Ethylene+CH	(50 μM)	307.9 ± 40.6	485.8 ± 14.9	120.6 ± 9.5	111.7 ± 4.9
Ethylene+CP	(50 μM)	571.4 ± 70.9	754.5 ± 16.2	119.0 ± 3.3	117.4 ± 3.5
+CH	(50 μM)	107.3 ± 10.4	112.4 ± 2.9	100.9 ± 5.6	114.7 ± 2.4
+CP	(50 μM)	93.2 ± 12.5	106.0 ± 4.0	92.3 ± 6.3	98.8 ± 5.1

[z] The color index was worked out as change of the coloring; eg. using the data obtained at 0 and 40 hr after C_2H_4 application, it was calculated from the expression, [(L+b)/2+a] at 40 hr - [(L+b)/2+a] at 0 hr.

[y] Values represent Means ± S.D. (n=6).

[x] Values represent Means ± S.E. (n=3).

Data are typical results from triplicate experiments.

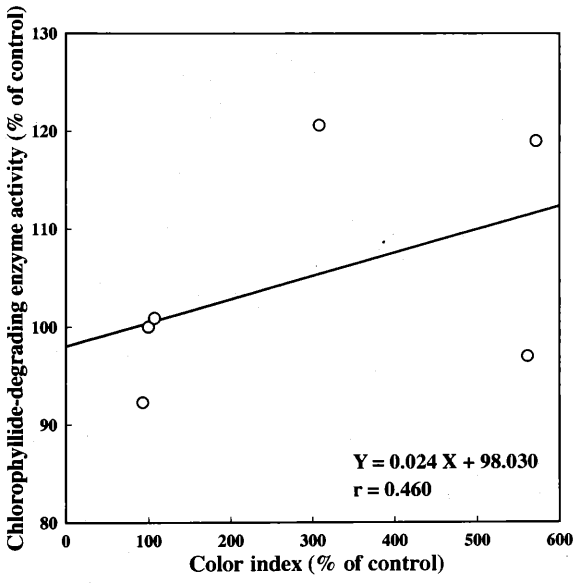


Fig. 2. A correlation between color index of *C. unshiu* fruit and CDE activity. Data plotted from Table 1.

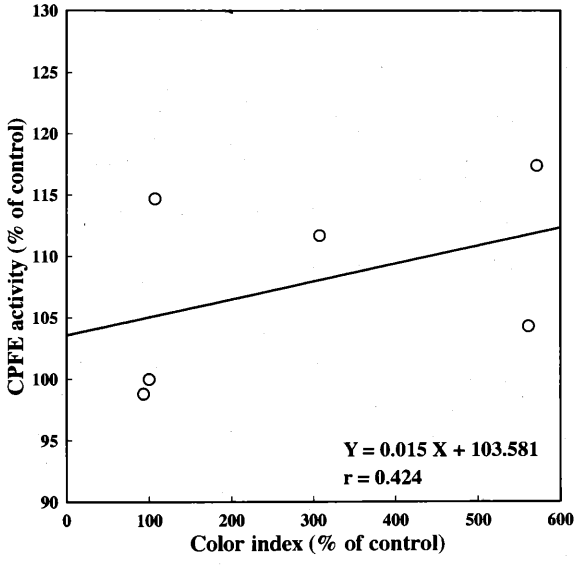


Fig. 3. A correlation between color index of *C. unshiu* fruit and CPFE activity. Data plotted from Table 1.

between the color index of the fruit peel and Chlase activity. The regression equation which is represented by: Y = 1.368 X - 16.038, (Fig. 1), where X and Y are color index of the fruit peel and Chlase activity, respectively. This relationship indicates that Chlase enzyme is the "Key" enzyme in the degreening process. On the other hand, correlations between the color index of the fruit peel and 1) CDE activity (Y = 0.024 X +98.030, r = 0.460, Fig.2) and 2) CPFE activity (Y =0.015 X +103.581, r = 0.424, Fig. 3) are low, indicating that CDE and CPFE activities are unaffected by the treatments.

The loss of Chl from leaves and fruits occurs during the senescence process and also the normal turnover of the pigment-protein complex. Type I reactions (Brown et al., 1991) of Chl catabolism includes loss of phytol, loss of magnesium, and the modification of the side chains of an isocyclic ring of the Chl structure, but not the degradation of the tetrapyrrole macrocycle. The accumulation of the Phed species, a part of type I reaction, has been reported in the breakdown of Chl *a* and Chl *b* in materials such as *Brassica napus* leaves (Langmeier et al., 1993), *Brassica oleracea* florets (Yamauchi et al., 1997b); *Chenopodium album* leaves (Shioi et al., 1991), *Chlorella fusca* (Ziegler et al., 1988), *Citrus* fruit peel (Amir-Shapira et al., 1987; Shimokawa et al., 1990; Ito et al., 1998; Kurata et al., 1998a, b, 1999; Maeda et al., 1997, 1998; Yamauchi et al., 1991, 1997a; Yanagisako et al., 1994a, b), *Melia azedarach* leaves (Amir-Shapira et al., 1987) and *Musa cavendishi* peels (Machhindra, 1997).

A large body of evidence has been accumulated on the degreening of the C_2H_4-treated *C. unshiu* fruit which suggests that Chlase plays an important role in this process: (1) a correlation between levels of Chlase activity and the Chl content (Shimokawa et al., 1992, 1994); (2) similarities between *in vivo* Chl degradation products during degreening and *in vitro* Chl products released by the action of Chlase; (Amir-Shapira et al., 1987) and (3) detection of endogeneous Chlase activity in green immature fruits and concurrent *de novo* synthesis during C_2H_4 induced degreening (Shimokawa et al., 1978; Trebitsch et al., 1993). The above evidence and our present results (Table 1 and Fig. 1) and those of Kurata et al. (1999) support the thesis that *de novo* synthesized Chlase is a "key" enzyme in the initial step of Chl *a* catabolism.

In contrast to Chlase, CDE and CPFE activities were hardly enhanced by C_2H_4 treatment (Table 1) which suggest that CDE and CPFE are present but inactive. Chlase is known as a carboxylesterase that specifically acts on Chls and their derivatives. The reaction mechanism of CPFE indicates that this enzyme is also a carboxylesterase but our data reveal that Chlase is clearly distinct from CPFE in the effects of C_2H_4, CH and CP treatments (Table 1).

Our contribution to the function and regulation of protein synthesis after C_2H_4-treatment should add to the biological basis for maintaining high quality of fruits, vegetables, and flowers.

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ウンシュウミカン果実のエチレン誘導クロロフィル代謝: タイプI反応の二，三の酵素活性に対する阻害剤の影響

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摘　要

ウンシュウミカン果実でのクロロフィル代謝のタイプI(フィトールの脱離，マグネシウムの脱離，クロロフィルのイソサイクリック環の側鎖の修飾)反応に対するエチレン作用のメカニズムが，タンパク質合成阻害剤を使用し調査された．エチレン処理は，暗黒条件下のウンシュウミカン果皮の脱緑を促進し，クロロフィラーゼ活性を増加した．シクロヘキシミド(細胞質でのタンパク質合成阻害剤)はエチレンが促進する脱緑を阻害したが，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)は阻害しなかった．一方，エチレン処理はクロロフィリッドを分解する酵素(CDE)活性と$C13^2$-カルボキシル-ピロフェオフォルビドを生成する酵素(CPFE)活性を増加しなかった．エチレン処理したウンシュウミカン果実でのCDE活性とCPFE活性は，シクロヘキシミドとクロラムフェニコール処理によってわずかに増加した．ウンシュウミカンの果皮色とクロロフィラーゼ活性との間には，高い正の相関が得られた：$Y = 1.368 X - 16.038$ ($r = 0.992$)(ただしXは果皮色，Yはクロロフィラーゼ活性)．しかしながら，ウンシュウミカンの果皮色と1) CDE活性 ($Y = 0.024 X - 98.030$ ($r = 0.460$)，2) CPFE活性 ($Y = 0.015 X - 103.581$ ($r = 0.424$)との相関は低かった．これらの結果は，エチレンがクロロフィラーゼ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，クロロフィラーゼが脱緑過程での鍵酵素であることを暗示した．