APPENDIX

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Effect of UV-B irradiation on chlorophyll-degrading enzyme activities and postharvest quality in stored lime (*Citrus latifolia* Tan.) fruit

Samak Kaewsuksaeng[a], Yoshiyuki Urano[b], Sukanya Aiamla-or[c], Masayoshi Shigyo[b,c], Naoki Yamauchi[b,c,*]

[a] Southern Tropical Plants Research Unit, Department of Agricultural Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung 93110, Thailand
[b] Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan
[c] The United Graduate School of Agricultural Sciences, Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan

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ABSTRACT

UV-B irradiation was applied to lime fruit to investigate its effect on chlorophyll (Chl)-degrading enzyme activities and postharvest quality during storage. Mature green lime fruit were irradiated with or without UV-B doses at 19.0 kJ m^{-2} and then stored at 25 °C in darkness. UV-B treatment efficiently delayed the decrease of hue angle values and Chl *a* contents. The activities of the Chl-degrading enzymes, chlorophyllase, Chl-degrading peroxidase and pheophytinase in the fruit with UV-B treatment were suppressed and Mg-dechelation activity was also retarded by the treatment. UV-B treatment induced a gradual increase in citric acid and suppressed the increase of sugar contents during storage. In addition, the ascorbic acid content with or without UV-B treatment decreased during storage, but the decrease in the control was faster than that with UV-B treatment. We concluded that UV-B treatment effectively suppressed chlorophyll degradation through the control of chlorophyll-degrading enzyme action and the changes in quality in mature green lime during storage.

1. Introduction

Quality in Tahitian lime (*Citrus latifolia* Tan.) deteriorates quickly after harvest. The most visible deterioration factor is the loss of peel greenness that usually occurs together with chlorophyll (Chl) degradation (Drazkiewice, 1994; Srilaong et al., 2011). Maintenance of the green color in the peel of lime is desirable during storage.

An early step of Chl *a* degradation seems to be the removal of the side chain attached to the tetrapyrrole macrocycle to form chlorophyllide (Chlide) *a* by chlorophyllase (Chlase). The Chlide *a* formed still retains a green color (Shimokawa et al., 1978; Amir-Shapira et al., 1987). The elimination of Mg^{2+} from Chlide *a* to produce pheophorbide (Pheide) *a* is induced by Mg-dechelatase (MD) (Langmeier et al., 1993; Kaewsuksaeng et al., 2006, 2007) or a Mg-dechelating substance (MDS) (Shioi et al., 1996; Aiamla-or et al., 2010; Kaewsuksaeng et al., 2010), and the Pheide *a* formed loses its green color. Finally, Pheide *a* is decomposed to fluorescent Chl catabolites, which are primarily colorless, via a red Chl catabolite by both Pheide *a* oxygenase and red Chl catabolite reductase (Matile et al., 1999). Chl-degrading peroxidase (Chl-POX) (Yamauchi et al., 2004; Kaewsuksaeng et al., 2007) is also suggested to be involved in Chl degradation as the first step enzyme oxidizing Chl *a* to form 13^2-hydroxychlorophyll *a*. In addition, a new Chl-degrading enzyme, pheophytinase (pheophytin pheophorbide hydrolase, PPH), which would dephytylate the Mg-free Chl pigment, pheophytin (Phein) *a* to give Pheide, has been recently reported (Schelbert et al., 2009).

UV treatments have been reported to maintain postharvest quality of many horticulture crops. UV-C seems to be especially effective in maintaining postharvest quality of strawberries (Erkan et al., 2008) and in inhibiting Chl degradation in stored broccoli florets (Costa et al., 2006a). Aiamla-or et al. (2010) found that UV-B treatment effectively delayed Chl degradation of broccoli during storage. Srilaong et al. (2011) also reported that UV-B treatment effectively suppressed Chl degradation and retarded the reduction of levels of Chl derivatives in mature green limes during storage. However, the effect of the UV-B on Chl-degrading enzyme activities and postharvest quality in lime fruit has not been determined.

* Corresponding author at: Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan. Tel.: +81 83 933 5843; fax: +81 83 933 5820.
E-mail address: yamauchi@yamaguchi-u.ac.jp (N. Yamauchi).

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In this paper, therefore, we examined the effects of UV-B irradiation on Chl-degrading enzyme activities, including PPH related to the new Chl degradation pathway and the resultant quality control.

2. Materials and methods

2.1. Plant materials and UV-B treatment

Mature green Tahitian lime (*C. latifolia* Tan.) fruit, imported by air from Mexico, were purchased from Tokio-Fukuoka Co., Ltd., Japan. After transportation to the laboratory, fruit were selected for uniformity in maturity, size, shape, peel color, and lack of defects. The fruit were irradiated with UV-B (spectral peak value: 312 nm, T-15M, VL). Each fruit was placed under UV-B lamps at a distance of 15 cm and irradiated for 20 min. A layer of aluminum film was placed under the fruit to ensure that UV-B would be irradiated to the bottom of the fruit. Unlike the dose reported previously (Aiamla-or et al., 2010), the UV-B energy doses measured in the region of 285–315 nm by a UV-B meter (UV 6.0, MK Scientific) were 19.0 kJ m^{-2}.

After irradiation, the fruit were kept in polyethylene film bags (0.03 mm in thickness) with the top folded over and stored at 25 °C in the dark. The samples (3 replications) were removed at 5 day intervals, and the peel tissues were sampled and used for analysis.

2.2. Surface color and chlorophyll assays

The surface color of lime fruit was determined by measuring the hue angle with a colorimeter (Nippon Denshoku NF 777). Chl *a* content was determined using *N,N*-dimethylformamide (Moran, 1982).

2.3. Preparation of chlorophyll a and resulting derivatives

2.3.1. Chlorophyll a

Spinach leaves were homogenized for 3 min in cold acetone. The homogenate was filtered through two layers of Miracloth (Calbiochem). The filtrates were treated with dioxane and distilled water and then kept for 1 h on ice. The filtrates were centrifuged at 10,000 × *g* for 15 min at 4 °C. After centrifugation, the pellets were treated again with acetone, dioxane, and distilled water, and then kept for 1 h on ice. Afterwards, the soluble pellets were centrifuged at 10,000 × *g* for 15 min at 4 °C and subsequently dissolved in petroleum ether. Soluble chlorophyll in petroleum ether was stored at 20 °C until the individual pigments were separated using sugar powder column chromatography (Perkins and Roberts, 1962). Finally, 500 μg mL^{-1} of Chl *a* was prepared in acetone.

2.3.2. Chlorophyllide a

Chlide *a* was prepared from a Chl *a* acetone solution (500 μg mL^{-1}) with 0.765 mg protein of partial purified Chlase (20–40% of $(NH_4)_2SO_4$) from green *Citrus unshiu* fruit. The reaction mixture was incubated at 25 °C for 40 min. The reaction was stopped using acetone and the remaining Chl *a* was separated by hexane. The lower part of the reaction mixture was used as the Chlide *a*.

2.3.3. Pheophytin a

Phein *a* was prepared by adding one drop of 0.1 N HCl into the Chl *a* acetone solution (500 μg mL^{-1}). After 2 min, 0.1 N NaOH was added to neutralize the solution, which was then employed as the substrate in the assays.

2.4. Analyses of chlorophyll-degrading enzyme activities

An acetone powder (500 mg) of peel tissues was suspended in 15 mL 10 mM phosphate buffer (pH 7.0) containing 0.6% CHAPS for Chlase. For MDS, an acetone powder (500 mg) of peel tissues was suspended in 15 mL 50 mM phosphate buffer (pH 7.0) containing 50 mM KCl and 0.24% Triton-X 100 or in 15 mL 10 mM phosphate buffer (pH 7.0) for Chl-POX. For PPH, an acetone powder (500 mg) was suspended in 15 mL 50 mM Tris–HCl buffer (pH 8.0). The crude enzyme was stirred for 1 h at 0 °C, and the mixture was filtered through two layers of Miracloth. The filtrate was then centrifuged at 16,000 × *g* at 4 °C for 15 min. The supernatant was used as the crude enzyme extract. The enzyme protein contents were determined on the basis of Bradford's method (Bradford, 1976).

2.4.1. Chlorophyllase activity

The reaction mixture contained 0.5 mL 0.1 mM phosphate buffer (pH 7.5), 0.2 mL 500 μg mL^{-1} Chl *a* acetone solution (100 μg mL^{-1}), and 0.5 mL enzyme solution. The reaction mixture was incubated in a water bath at 25 °C for 40 min, and the enzyme reaction was stopped by adding 4 mL of acetone. Chlide *a* was separated by adding 4 mL of hexane. The upper phase contained the remaining Chl *a* while the lower phase contained the Chlide *a*. The activity was spectrophotometrically detected by Chlide *a* formation at 667 nm per unit per mg protein.

2.4.2. Chlorophyll-degrading peroxidase activity

Chl-POX was determined as previously described (Yamauchi et al., 1997). The reaction mixture contained 50 μL enzyme solution, 100 μL 1.0% Triton-X 100, 100 μL 5 mM *p*-coumaric acid, 100 μL 500 μg mL^{-1} Chl *a* acetone solution, 500 μL 0.2 mM phosphate buffer (pH 5.5) and 50 μL 0.3% hydrogen peroxide. The activity was determined spectrophotometrically by measuring the decrease of 1.0 μg Chl *a* at 668 nm per min per mg protein at 25 °C.

2.4.3. Mg-dechelation activity

Mg-dechelation activity by MDS using Chlide *a* was determined by the method of Suzuki and Shioi (2002) with slight modifications. The reaction mixture contained 0.75 mL 10 mM phosphate buffer (pH 7.5), 0.25 mL Chlide *a* (8.80 μg) acetone solution and 0.2 mL enzyme solution. The activity of the MDS was measured on the basis of Pheide *a* formation by following the change in OD at 535 nm per min per mg protein.

2.4.4. Pheophytinase activity

PPH was modified by the method of Schelbert et al. (2009). The reaction mixture contained 0.5 mL 50 mM Tris–HCl buffer (pH 8.0), 0.2 mL Phein *a* (22.40 μg) acetone solution and 0.5 mL enzyme solution. The reaction mixture was incubated in a water bath at 25 °C for 40 min, and the enzyme reaction was stopped by adding 2 mL of acetone. After that, the sample was analyzed by HPLC using a Hitachi L-7100 pump with an automated gradient controller and a Hitachi L-2450 diode array detector or a Hitachi L-7420 UV–Visible spectrophotometer. The absorption spectrum of the product as Pheide *a* was recorded at 665 nm. The activity of PPH was calculated with the Pheide *a* formation (μg) based on the peak area per min per mg protein.

2.5. Postharvest quality parameters

The organic acids and sugars from the fruit juice (15 mL) were extracted with hot ethanol (final concentration 70% ethanol) for 15 min. A 1 mL aliquot of 70% ethanol extracts was vacuum-evaporated to dryness and dissolved in 1 mL of Milli-Q water again. The citric and malic contents were determined by HPLC using a Hitachi L-7420 UV–Visible spectrophotometer. The sample was

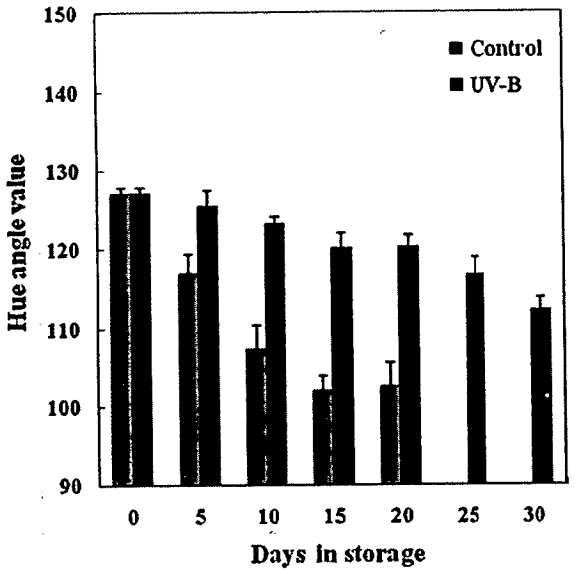

Fig. 1. Changes in the hue angle value in lime fruit treated with or without UV-B at 19.0 kJ m^{-2} during storage at 25 °C. Y-axis shows the value from 150 to 90. 90 shows yellow color and 150 shows blue green color. Vertical bars represent the average values with SE (n = 3).

separated on a Mightysil RP-18 column, 4 mm × 250 mm, using a solvent: water:methanol:50 mM phosphoric acid (69:1:30). The absorption spectrum of the citric and malic acid was recorded at 210 nm. The flow rate was 1 mL min^{-1}, and the injection volume was 100 μL. For sugar analysis, the ethanol extract was filtered by passing through Sep-pak C18 to remove the pigments and analyzed by HPLC (Hitachi L-7940 RI detector) using a LiChrospher NH_2 column, 4 mm × 250 mm, with a solvent: acetonitrile:water (80:20) according to the determined glucose, fructose, and sucrose contents. The ascorbic acid content was assayed by the method of Roe et al. (1948).

3. Results

3.1. Effect of UV-B irradiation on postharvest qualities of lime fruit

Surface color was evaluated by measuring the hue angle. As shown in Fig. 1, the hue angle value in the control declined significantly during storage at 25 °C with yellowing of the fruit peel. In contrast, that in UV-B-treated lime fruit changed only slightly during storage. The citric acid content, which is a major organic acid in lime fruit, decreased after 10 days of storage in the control, while that in UV-B-treated fruit increased constantly until day 20 and then decreased (Fig. 2A). On the other hand, the malic acid content decreased gradually in both UV-B-treated and control fruit (Fig. 2B). As a consequence, the fruit with UV-B treatment showed significantly higher contents of citric acid and malic acid. As shown in Fig. 3, the ascorbic acid content in limes with or without UV-B treatment decreased during storage at 25 °C, but that in the control declined faster than that in UV-B treatment. Fig. 4 shows the sugar level changes in lime fruit with or without UV-B treatment during storage. The glucose (Fig. 4A), fructose (Fig. 4B), and sucrose contents (Fig. 4C) in the control increased considerably on day 5 and then decreased during storage. With UV-B treatment, those contents also increased until day 10 and then showed a tendency to decline.

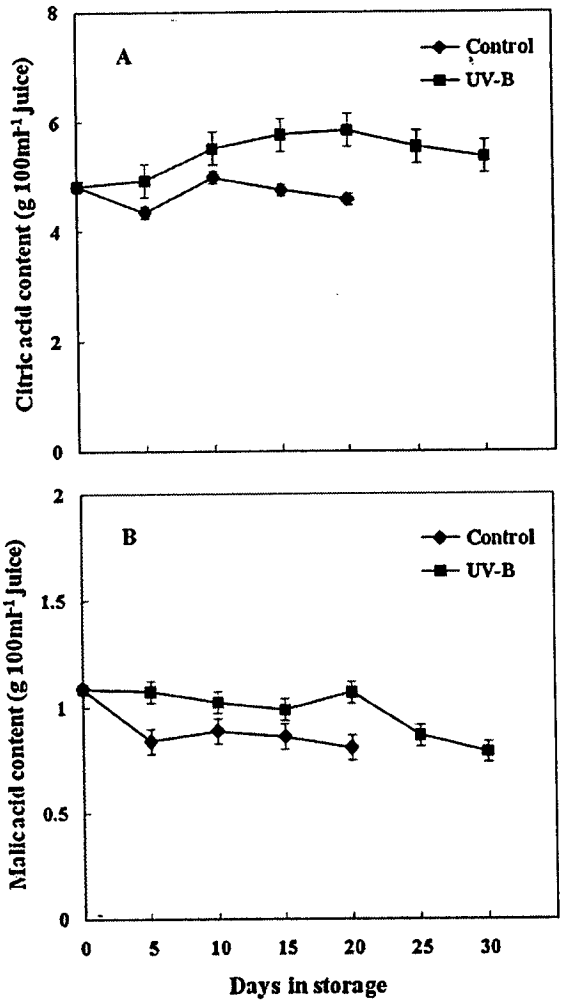

Fig. 2. Changes in citric acid (A) and malic acid (B) contents of the juice in lime fruit treated with or without UV-B at 19.0 kJ m^{-2} during storage at 25 °C. Vertical bars represent the average values with SE (n = 3).

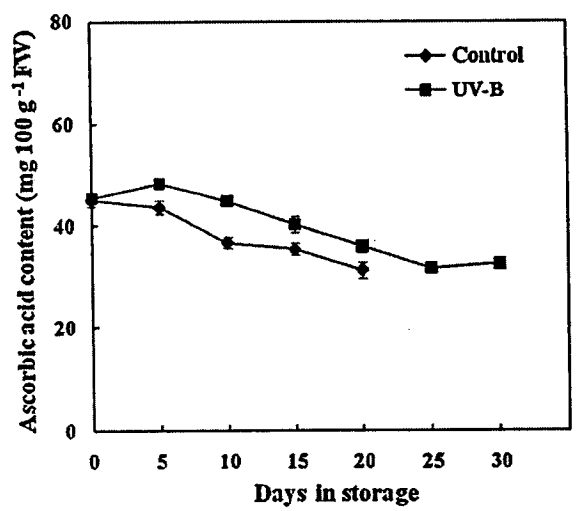

Fig. 3. Changes in ascorbic acid contents of the juice in lime fruit treated with or without UV-B at 19.0 kJ m^{-2} during storage at 25 °C. Vertical bars represent the average values with SE (n = 3).

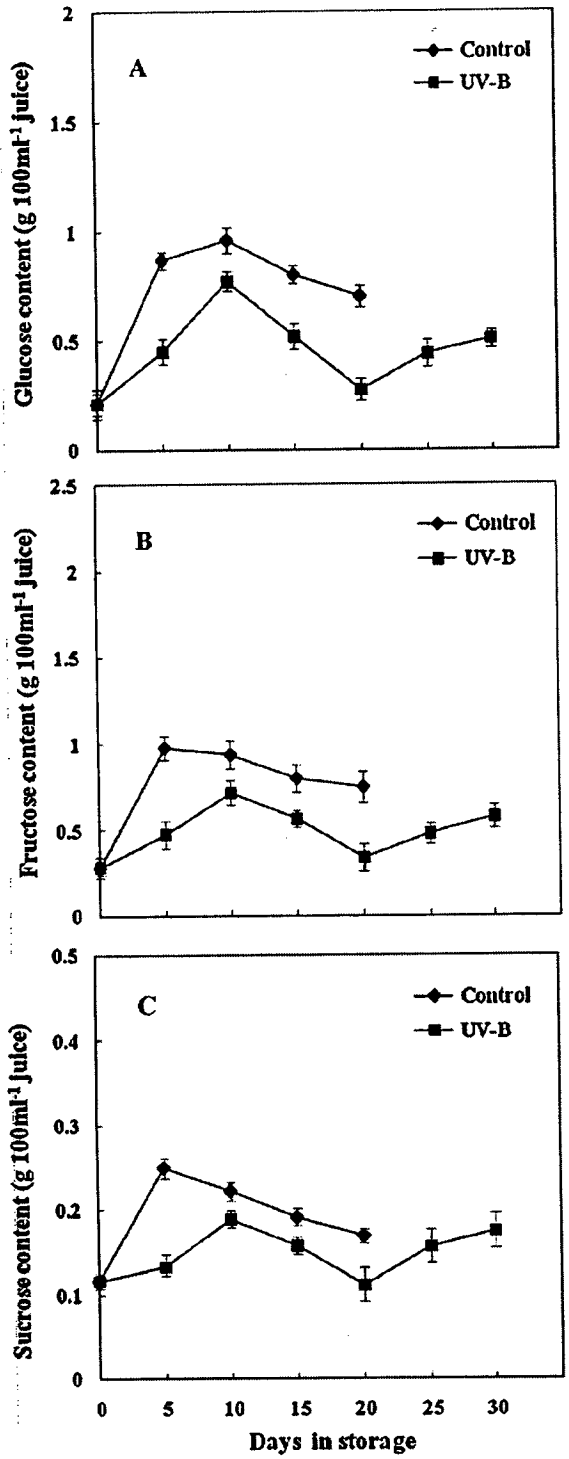

Fig. 4. Changes in glucose (A), fructose (B) and sucrose (C) contents of the juice in lime fruit treated with or without UV-B at 19.0 kJ m^{-2} during storage at 25 °C. Vertical bars represent the average values with SE (n = 3).

3.2. Effect of UV-B irradiation on chlorophyll degradation and chlorophyll-degrading enzyme activities in lime fruit

UV-B treatment delayed the reduction of Chl *a* content, as shown in Fig. 5. UV-B delayed the reduction in the peel during storage, whereas that in the control sharply decreased during storage. In Fig. 6A, Chlase activity in the control increased during storage at

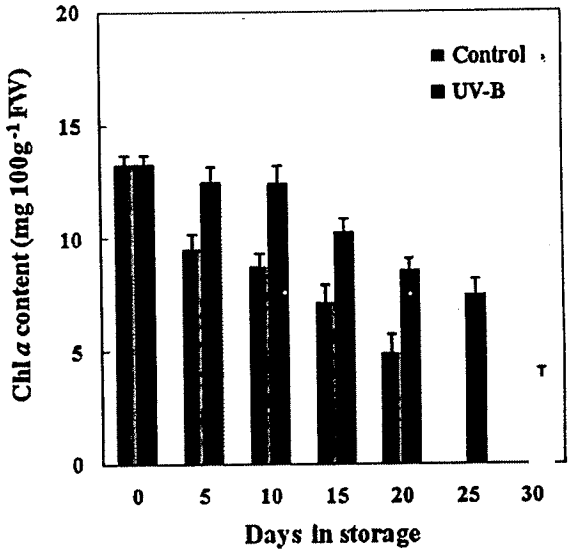

Fig. 5. Changes in Chl *a* content in lime fruit treated with or without UV-B at 19.0 kJ m^{-2} during storage at 25 °C. Vertical bars represent the average values with SE (n = 3). Chl, chlorophyll.

25 °C, whereas that with UV-B treatment decreased gradually during storage after a temporary increase on day 5. The activity with UV-B treatment was lower than that in the control. Chl-POX activity markedly increased in both the control and the UV-B-treated fruit during storage. However, the activity was lower in UV-B-treated fruit than in the control (Fig. 6B). Mg-dechelation activity by MDS was examined using Chlide *a* as a native substrate (Fig. 7A). In the control, Mg-dechelation activity showed an increase after 10 days of storage, whereas the activity with UV-B treatment also showed a temporary increase for the first 5 days of storage and then decreased. Moreover, PPH activity in the control started to increase until day 15 and then decreased slightly during storage. On the other hand, the activity in UV-B-treated fruit showed a decline during storage after a sharp increase on day 5 (Fig. 7B).

4. Discussion

The yellowing of leaves, florets, and fruit pericarp is an important factor indicative of quality deterioration in stored horticultural products. Obviously, in lime, the most visible deterioration is the loss of peel greenness that usually occurs with Chl degradation (Win et al., 2006; Srilaong et al., 2011). The maintenance of green color in the peel of lime during storage is required for fruit to maintain market prices (Pranmornkith et al., 2005). Many studies have demonstrated the efficacy of UV irradiation on fruit and vegetables for maintaining postharvest quality. Specifically, UV-C irradiation can reduce decay in grapefruit (D'hallewin et al., 2000), maintain postharvest quality of strawberry (Erkan et al., 2008), and suppress Chl degradation in stored broccoli florets (Costa et al., 2006a). In addition, UV-B is an alternative UV range that may maintain the postharvest quality of fresh produce. Aiamla-or et al. (2010) found that UV-B treatment effectively delayed Chl degradation of broccoli during storage. Previously, Srilaong et al. (2011) reported that UV-B treatment effectively suppressed Chl degradation in mature green limes during storage. In the present study, lime fruit were irradiated with UV-B at 19.0 kJ m^{-2}, and the results were compared with those of untreated fruit. Based on the results from the present study, we suggested that UV-B treatment effectively delayed chlorophyll breakdown in the lime peel.

Heat treatment is known to reduce Chl degradation through suppression of activities of Chl-degrading enzymes, including

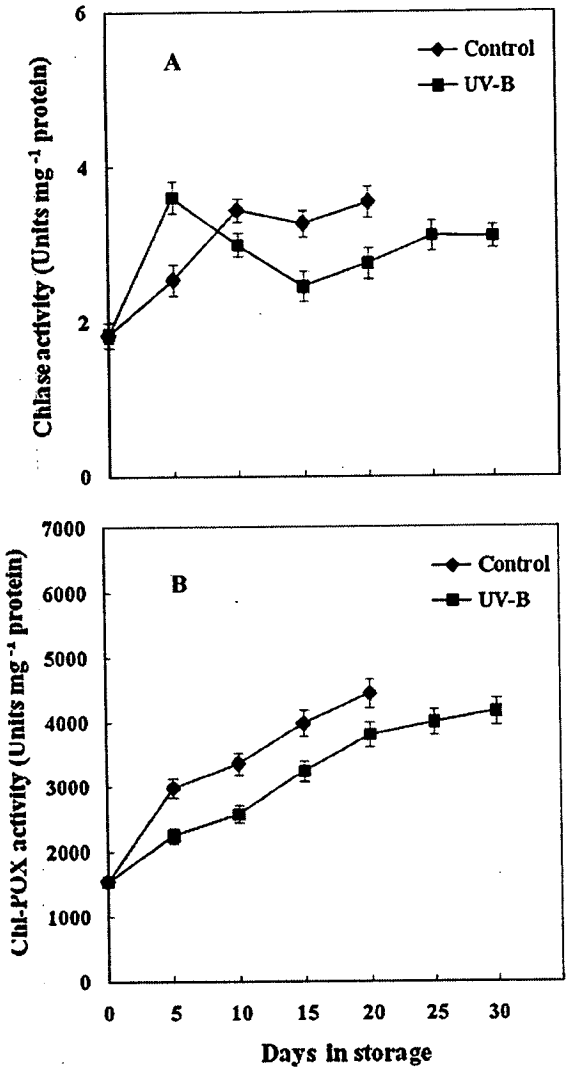

Fig. 6. Changes in Chlase (A) and Chl-POX (B) activities in lime fruit treated with or without UV-B at 19.0 kJ m^{-2} during storage at 25 °C. Vertical bars represent the average values with SE ($n = 3$). Chlase, chlorophyllase; Chl-POX, chlorophyll-degrading peroxidase.

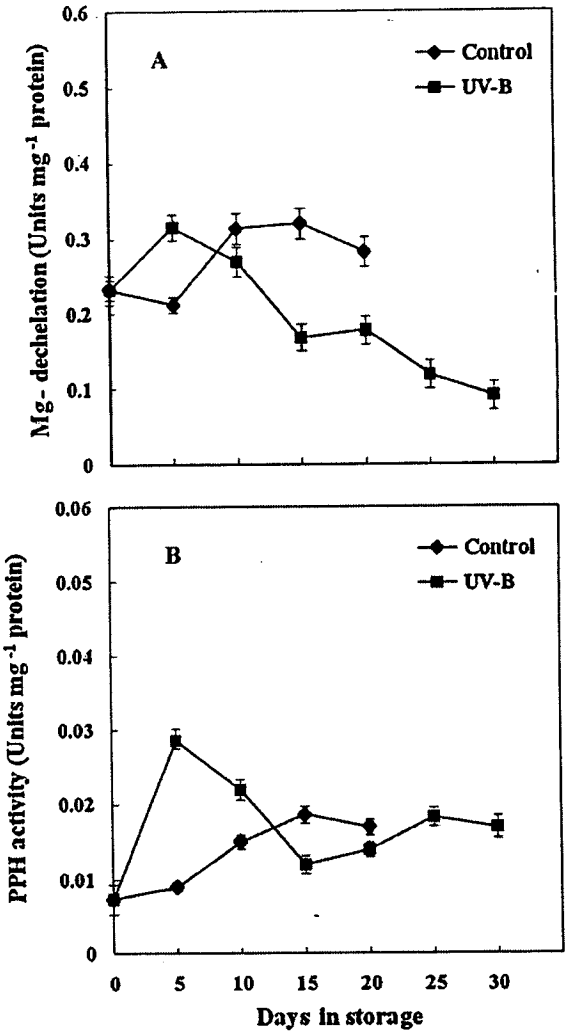

Fig. 7. Changes in Mg-dechelation (A) and PPH (B) activities in lime fruit treated with or without UV-B at 19.0 kJ m^{-2} during storage at 25 °C. Vertical bars represent the average values with SE ($n = 3$). PPH, pheophytinase.

Chlase, Mg-dechelation, Chl-POX, and Chl oxidase (Costa et al., 2006a; Funamoto et al., 2002; Kaewsuksaeng et al., 2007). UV-C (Costa et al., 2006b) and UV-B irradiation (Aiamla-or et al., 2010) also suppress the activities of Chl-degrading enzymes such as Chlase, Mg-dechelation and Chl-POX. In this study, Chl-degrading enzymes activities except Chl-POX in UV-B-treated lime fruit were reduced after a temporary increase during storage. UV-B treatment also suppressed effectively the enhancement of Chl-POX activity during storage in lime fruit. Chlase activity in the control gradually increased with senescence during storage. In contrast, it has been previously reported that Chlase activity decreased with degreening of *Citrus nagato-yuzukichi* (Yamauchi et al., 2003), *Citrus aurantifolia* Swingle cv. Paan (Win et al., 2006) and broccoli florets (Aiamla-or et al., 2010). We also found that Chlase activity was tentatively suppressed in lime fruit throughout storage by UV-B treatment. Mature green limes were shown to have high levels of Chlide *a* (Srilaong et al., 2011), which suggests that these high levels are due to increased Chlase action in the flavedo. This enzyme is involved in the first step of the chlorophyll catabolic pathway, which catalyzes the conversion of Chl *a* to Chlide *a* and phytol (Harpaz-Saad et al., 2007).

Our results indicate that Chl-POX activity markedly increased in lime fruit during storage, but its activity was clearly suppressed throughout the storage period in limes with UV-B treatment. In lime fruit, Chl *a* can be degraded by Chl-POX (Win et al., 2006) to form the oxidized Chl *a*, 13^2-hydroxychlorophyll *a*, which did not accumulate during storage by UV-B treatment (Aiamla-or et al., 2010). Mg-dechelation activity by MDS was also determined using Chlide *a* as native substrate in lime fruit. MDS, which is a small molecule and heat-stable substance, was required to remove the magnesium atom from Chlide *a* (Suzuki et al., 2005; Kaewsuksaeng et al., 2010). We found that Mg-dechelation activity increased slightly in the control and that UV-B treatment also effectively suppressed the enhancement of Mg-dechelation. These findings were similar to those by Aiamla-or et al. (2010). Srilaong et al. (2011) demonstrated that the Pheide *a* levels declined in UV-B-treated fruit, especially during the development of yellowing. This indicates that the degradation of Chlide *a* to Pheide *a* might be suppressed by UV-B, as was apparent in this study. Recently reported as a new Chl-degrading enzyme by Schelbert et al. (2009), PPH dephytylates Phein *a* to form Pheide *a*. Our results showed PPH activity gradually increased during storage in lime fruit, while the UV-B treatment effectively

suppressed PPH activity, which showed a temporary increase after treatment. Phein *a* was detected during storage of broccoli florets (Costa et al., 2006a; Kaewsuksaeng et al., 2006; Aiamla-or et al., 2010) and lime fruit (Srilaong et al., 2011), implying that MDS could play a role on the release of Mg^{2+} from Chl *a* to form Phein *a* as a substrate for PPH. However, Phein *a* accumulated at a higher rate in the UV-B-treated limes than in the control fruit (Srilaong et al., 2011). This finding seems to be due to the inhibition of PPH activity by UV-B treatment. Moreover, it is inferred that PPH activity measured in this study might include some activity of Chlase, since it can also dephytylate Phein *a* along with Chl *a* (McFeeters et al., 1971; Mínguez-Mosquera et al., 1994). Further study needs to be conducted to establish a correct measurement of PPH and clarify the role of PPH in Chl degradation of lime fruit.

Besides Chl degradation, composition changes in relation to quality occur during storage of lime fruit. The citric acid and malic acid contents in limes showed a slight change during storage. These contents were higher with UV-B treatment than in the controls during storage, implying that these acids might be maintained due to the suppression of respiration and senescence by the UV-B treatment. UV-B treatment also suppressed the changes in fructose, glucose and sucrose during storage. On the other hand, these sugar contents in the control increased with the advance of senescence. The results agreed with the effect of heat treatment on reducing sugar level changes (Lemoine et al., 2008). Thus, UV-B treatment seems to be a useful treatment for maintenance of internal quality in lime fruit. In addition, the ascorbic acid content was higher in the UV-B treated fruit than in the control. UV-B irradiation had a positive effect on antioxidants such as ascorbic acid, in lime fruit. In contrast, Liu et al. (2010) found that the content of ascorbic acid in tomato fruit was reduced after UV-B treatment.

In conclusion, the findings obtained in the present study show that a UV-B dosage of 19.0 $kJ\,m^{-2}$ effectively retarded degradation of Chl in lime fruit during storage. UV-B treatment also retarded the increase of activities of Chl-degrading enzymes, such as Chlase, Chl-POX, Mg-dechelation, and PPH, indicating that the suppression of those enzyme activities by UV-B treatment could be involved in the retardation of Chl degradation in stored lime fruit. Furthermore, UV-B treatment induced a gradual increase in citric acid and suppressed the increase of glucose, fructose, and sucrose contents during storage. UV-B treatment also maintained the content of antioxidants, such as ascorbic acid, during storage. We conclude that UV-B treatment could be a useful method to prolong the postharvest quality of lime fruit during storage.

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References

Aiamla-or, S., Kaewsuksaeng, S., Shigyo, M., Yamauchi, N., 2010. Impact of UV-B irradiation on chlorophyll degradation and chlorophyll-degrading enzyme activities in stored broccoli (*Brassica oleracea* L. Italica Group) florets. Food Chem. 120, 645–651.

Amir-Shapira, D., Goldschmidt, E.E., Altman, A., 1987. Chlorophyll catabolism in senescing plant tissues: in vivo breakdown intermediates suggest different degradative pathways for citrus fruit and parsley leaves. Proc. Natl. Acad. Sci. U.S.A. 84, 1901–1905.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Costa, L., Vicente, A.R., Civello, P.M., Chaves, A.R., Martinez, G.A., 2006a. UV-C treatment delays postharvest senescence in broccoli florets. Postharvest Biol. Technol. 39, 204–210.

Costa, M.L., Civello, P.M., Chaves, A.R., Martinez, G.A., 2006b. Hot air treatment decreases chlorophyll catabolism during postharvest senescence of broccoli (*Brassica oleracea* L. var. Italica) heads. J. Sci. Food Agric. 86, 1125–1131.

Drazkiewice, M., 1994. Chlorophyllase: occurrence, function, mechanism of action, effects of external factors. Photosynthetica 30, 301–309.

D'hallewin, G., Schirra, M., Pala, M., Ben-Yehoshua, S., 2000. Ultraviolet-C irradiation at 0.5 $kJ\,m^{-2}$ reduce decay without causing damage or effecting postharvest quality of star ruby grapefruit (*C. paradise* Macf.). J. Agric. Food Chem. 48, 4475–4571.

Erkan, M., Wang, S.Y., Wang, C.Y., 2008. Effect of UV treatment on antioxidant capacity, antioxidant enzyme activity and decay in strawberry fruit. Postharvest Biol. Technol. 48, 163–171.

Funamoto, Y., Yamauchi, N., Shigenaga, T., Shigyo, M., 2002. Effects of heat treatment on chlorophyll degradation enzymes in stored broccoli (*Brassica oleracea* L.). Postharvest Biol. Technol. 24, 163–170.

Harpaz-Saad, S., Azoulay, T., Arazi, A., Ben-Yaakov, E., Mett, A., Shiboleth, Y.M., Hörtensteiner, S., Gidoni, D., Gal-On, A., Goldschmidt, E.E., Eyal, Y., 2007. Chlorophyllase is a rate-limiting enzyme in chlorophyll catabolism and is post-translationally regulated. Plant Cell 19, 1007–1022.

Kaewsuksaeng, S., Yamauchi, N., Funamoto, Y., Shigyo, M., Kanlayanarat, S., 2006. Effect of Mg-dechelation activity on chlorophyll degradation in stored broccoli florets. Acta Hortic. 712, 705–709.

Kaewsuksaeng, S., Yamauchi, N., Funamoto, Y., Mori, T., Shigyo, M., Kanlayanarat, S., 2007. Effect of heat treatment on catabolites formation in relation to chlorophyll degradation during storage of broccoli (*Brassica oleracea* L. Italica group) florets. J. Jpn. Soc. Hortic. Sci. 76, 338–344.

Kaewsuksaeng, S., Yamauchi, N., Funamoto, Y., Aiamla-or, S., Mori, T., Shigyo, M., Kanlayanarat, S., 2010. Partially purification of Mg-dechelatase in relation to chlorophyll degradation in broccoli (*Brassica oleracea* L. Italica Group) florets. Acta Hortic. 875, 509–514.

Langmeier, M., Ginsburg, S., Matile, P., 1993. Chlorophyll breakdown in senescent leaves: demonstration of Mg-dechelatase activity. Physiol. Plantarum 89, 347–353.

Lemoine, M.L., Civello, P.M., Chaves, A.R., Martínez, G.A., 2008. Effect of combine treatment with hot air and UV-C on senescence and quality parameters of minimally processed broccoli (*Brassica oleracea* L. var. Italica). Postharvest Biol. Technol. 48, 15–21.

Liu, C., Han, X., Cai, L., Lu, X., Ying, T., Jiang, Z., 2010. Postharvest UV-B irradiation maintains sensory qualities and enhances antioxidant capacity in tomato fruit during storage. Postharvest Biol. Technol. 59, 232–237.

Matile, P., Hörtensteiner, S., Tomas, H., 1999. Chlorophyll degradation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 69–75.

McFeeters, R.F., Chichester, C.O., Whitaker, J.R., 1971. Purification and properties of chlorophyllase from *Ailanthus altissima* (tree-of-heaven). Plant Physiol. 47, 609–618.

Mínguez-Mosquera, M.I., Gandul-Rojas, B., Gallardo-Guerrero, L., 1994. Measurement of chlorophyllase activity in olive fruit (*Olea europaea*). J. Biochem. 116, 263–268.

Moran, R., 1982. Formulae for determination of chlorophyllous pigments extracted with *N,N*-dimethylformamide. Plant Physiol. 69, 1376–1381.

Perkins, H.J., Roberts, D.W., 1962. Purification of chlorophylls, pheophytins and pheophobides for specific activity determinations. Biochim. Biophys. Acta 58, 486–498.

Pranmornkith, T., Mawson, A.J., Heyes, J.A., 2005. Effect of CA and alternative postharvest treatments on quality of lime (*Citrus latifolia* Tanaka) fruit. In: Proceedings of 9th International Controlled Atmosphere Research Conference , 5–10 July. Michigan State University, ISHS, pp. 21–27.

Roe, J.H., Mary, B.M., Oesterling, M.J., Charlotle, M.D., 1948. The determination of diketo-gulonic acid, dehydro-L-ascorbic acid, and L-ascorbic acid in the same tissues extract by 2,4-dinitrophenyl hydrazine method. J. Biol. Chem. 174, 201–208.

Schelbert, S., Aubry, S., Burla, B., Agne, B., Kessler, F., Krupinska, K., Hörtensteiner, S., 2009. Pheophytin pheophorbide hydrolase (Pheophytinase) is involved in chlorophyll breakdown during leaf senescence in *Arabidopsis*. Plant Cell 21, 767–785.

Shimokawa, K., Shimada, S., Yaeo, K., 1978. Ethylene-enhanced chlorophyllase activity during degreening of *Citrus unshiu* Marc. Sci. Hortic. 8, 129–135.

Shioi, Y., Tomita, N., Tsuchiya, T., Takamiya, K., 1996. Conversion of chlorophyllide to pheophorbide by Mg-dechelating substance in extracts of *Chenopodium album*. Plant Physiol. Biochem. 34, 41–47.

Suzuki, T., Shioi, Y., 2002. Re-examination of Mg-dechelation reaction in the degradation of chlorophylls using chlorophyllin a as a substrate. Photosynth. Res. 74, 217–223.

Suzuki, T., Kunieda, T., Murai, F., Morioka, S., Shioi, Y., 2005. Mg-dechelation activity in radish cotyledons with artificial and native substrates, Mg-chlorophyllin *a* and chlorophyllide *a*. Plant Physiol. Biochem. 43, 459–464.

Srilaong, V., Aiamla-or, S., Soontornwat, A., Shigyo, M., Yamauchi, N., 2011. UV-B irradiation retards chlorophyll degradation in lime (*Citrus latifolia* Tan.) fruit. Postharvest Biol. Technol. 59, 110–112.

Win, T.O., Srilaong, V., Kyu, K.L., Poomputsa, K., Kanlayanarat, S., 2006. Biochemical and physiological changes during chlorophyll degradation in

lime (*Citrus aurantifolia* Swingle cv. 'Paan'). J. Hortic. Sci. Biotechnol. 81, 471–477.
Yamauchi, N., Harada, K., Watada, A.E., 1997. In vitro chlorophyll degradation in stored broccoli (*Brassica oleracea* L. var. Italica Plen) florets. Postharvest Biol. Technol. 12, 239–245.
Yamauchi, N., Eguchi, K., Tokuhara, Y., Yamashita, Y., Oshima, A., Shigyo, M., Sugimoto, K., 2003. Control of degreening by sucrose laurate ester and heat treatment of *Citrus nagato-yuzukichi* fruit. J. Hortic. Sci. Biotechnol. 78, 563–567.
Yamauchi, N., Funamoto, Y., Shigyo, M., 2004. Peroxidase-mediated chlorophyll degradation in horticultural crops. Phytochem. Rev. 3, 221–228.

WALAILAK JOURNAL

MiniReview

Chlorophyll Degradation in Horticultural Crops

Samak KAEWSUKSAENG

Southern Tropical Plants Research Unit, Department of Agricultural Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung 93110, Thailand

(Corresponding author; e-mail: samak@tsu.ac.th)

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Abstract

One of the symptoms of senescence in harvested horticultural crops is the loss of greenness that comes with the degradation of chlorophyll. With senescence, the chlorophyll-degrading enzyme activities such as chlorophyllase, Mg-dechelatase or Mg-dechelation activity, a new chlorophyll-degrading enzyme, pheophytinase, pheophorbidase and chlorophyll-degrading peroxidase, which are involved in chlorophyll degradation, affected greatly in stored horticultural crops. The chlorophyll derivatives, especially chlorophyllide, pheophytin, pheophorbide and C13^2-hydroxychlorophyll are accumulated as intermediates of chlorophyll degradation. In addition, chlorophyll degradation by the chlorophyll-degrading enzymes seems to occur in the thylakoid and envelope membrane of chloroplast and/ or the vacuole. The involvement of chlorophyll-degrading enzymes in senescing horticultural crops is also discussed.

Keywords: Chlorophyll, chlorophyll degradation, chlorophyll-degrading enzymes, horticultural crop

Introduction

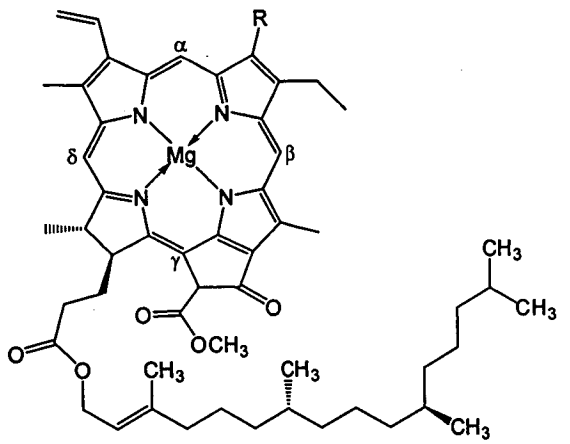
In general, the yellowing of leaves, florets and fruit pericarp is an important factor, indicating quality deterioration of stored horticultural products. Obviously, in spinach [1], parsley [2], broccoli [3] and lime [4,5], the most visible deterioration is the loss of sepal and peel greenness that usually occurs with chlorophyll (Chl) breakdown. On the other hand, in the fruit of an early-ripening cultivar, Wase Satsuma mandarin (*Citrus unshiu* Marc var. Tanaka), the peel is still green when the flesh matures and the fruit attains harvest maturity. To improve quality, ethylene is provided to the fruit to accelerate the degreening of the peel [6]. Thus, Chl degradation is a characteristic symptom of leaf senescence and fruit ripening, and elucidating the mechanism of Chl degradation is an important subject when considering the maintenance of the quality of harvested horticultural crops.

An early step of Chl *a* degradation seems to be the removal of the side chain attached to the tetrapyrrole macrocycle to form chlorophyllide (Chlide) *a* by chlorophyllase (Chlase). Chlide *a* formed still retains a green color [7,8]. The elimination of Mg^{2+} from Chlide *a* to produce pheophorbide (Pheide) *a* is induced by Mg-dechelatase (MD) [9-11] or a Mg-dechelating substance (MDS) [12-14], and the Pheide *a* formed then loses its green color. Finally, Pheide *a* is decomposed to fluorescent Chl catabolites, which are colorless, via a red Chl catabolite by both Pheide *a* oxygenase and red Chl catabolite reductase [15]. Chl-degrading peroxidase (POX) [11,16] is also suggested to be involved in Chl degradation as the 1st step enzyme with oxidizes Chl *a* to form 13^2-hydroxychlorophyll (C13^2-OHChl) *a*. In addition, a new Chl degrading enzyme, pheophytinase (pheophytin pheophorbide hydrolase, PPH) which would dephytylate the Mg-free Chl pigment, pheophytin (Phein) *a* to give Pheide has been recently reported [17].

In this review, we firstly deal with Chl structure and Chl derivatives, then the Chl degradation pathway by Chl-degrading enzymes, and, finally, with the characterization of Chl-degrading enzymes of postharvest horticultural crops.

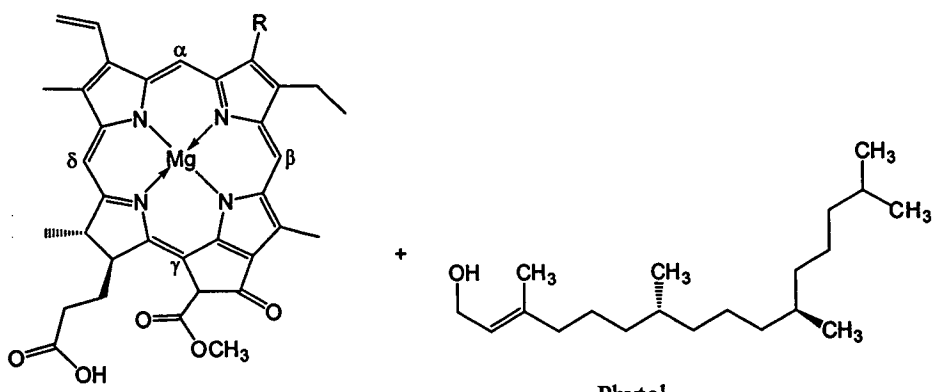
Chlorophyll structure

Chls are porphyrins containing basic tetrapyrrole rings, of which one is reduced. The 4 rings are coordinated to a Mg^{2+} ion. A 5th isocyclic ring E, is found near the 3rd pyrrole ring. At the 4th ring, the propionic acid substituent is esterified with diterpene alcohol phytol ($C_{20}H_{39}OH$), which is the hydrophobic side of the molecule, the rest of the molecule being hydrophilic. Chl *b* differs from Chl *a* only by having an aldehyde group (–CHO) in place of the methyl group at ring B position (**Figure 1**) [18].



Chl

(a)



Chlide (b) Phytol

Figure 1 Structure formulae (a) Chl *a* (R=CH_3), Chl *b* (R= CH=O); (b) Chlide *a* (R=CH_3), Chlide *b* (R= CH=O) and phytol.
Source: Hörtensteiner and Kräutler [18]

Chlorophyll derivatives

Chls can be readily transformed, both *in vivo* and *in vitro*, into a series of derivatives.

1. Chlorophyllides *a* and *b*

The phytyl ester can be easily hydrolyzed to give Chlide and phytol. The hydrolysis takes place under mild conditions in the presence of either an acid or alkali. Chlides *a* and *b* are prepared enzymatically, the hydrolysis being catalyzed by Chlase, an enzyme commonly found in green plant tissues. Leaves that are especially rich in Chlase such as sugar beet [19], common cocklebur (*Xanthium pennsylvanicum*) [20], goosefoot (*Chenopodium album)* [12] and *Citrus unshiu* fruit [21] are used as enzyme sources.

2. Pheophytins *a* and *b*

Pheins are the magnesium-free derivatives of Chls. Pheins *a* and *b* are easily obtained from Chlase by the action of dilute acids, which remove the magnesium. The reaction lasts 1 - 2 min, and the concentration of HCl used is 13 % [20,22].

3. Pheophorbides *a* and *b*

Pheides *a* and *b* are hydrolyzed Chl without phytol (Chlides) that have also lost the magnesium. The reaction may be prepared from Chls treated with concentrated acid (30 % HCl) or from acidified Chlides [20,22].

4. C13^2-hydroxychlorophyll *a* and *b*

Chl *a* is oxidized with the oxygen atom being located at the position C-13^2 and hydroxychlorophyll being formed. C13^2-OHChl *a* was indentified in senescing excised leaves [23] and broccoli, which was prepared by adding peroxidase with the addition of H_2O_2 and *p*-coumaric acid to a Chl *a* solution [24].

5. Pyrochlorophylls

Pyroderivatives of Chls or their derivatives are compounds that have lost the carbomethoxy group –$COOCH_3$ at C-10 of the isocyclic ring, the group being replaced by hydrogen. Chl *a*, methyl Chlide *a*, Phein *a*, or methyl Pheide *a* when heated in pyridine at 100 °C give rise to 'pyro' derivatives by decarbomethoxylation [20].

The chlorophyll degradation pathway

The generally accepted pathway of Chl degradation comprises 2 stages, before (early stage) and after (late stage) cleavage of the tetrapyrrole macrocyclic rings. The products of the early stage are greenish, whereas those of the late stage are essentially colorless. The early stage includes modification of the side chain of the tetrapyrrole macrocycle; hydrolysis of a phytol residue in ring IV (dephytylation), release of Mg^{2+} from the tetrapyrrole macrocycle by displacement with $2H^+$ (dechelation) and some modifications of the macrocycle that are probably specific for the plant species. The late stage includes the cleavage of the tetrapyrrole macrocycle by an oxygenase and subsequent reactions, such as reduction to yield colorless fluorescent and further nonfluorescent catabolites. The late stage is thus essential in the degreening of the Chl molecule and therefore it determines Chl degradation in leaf senescence and fruit ripening. In most cases of leaf senescence and fruit ripening, degradation intermediates do not accumulate to an appreciable extent, suggesting that there is a series of degradation reactions (**Figure 2**) [25].

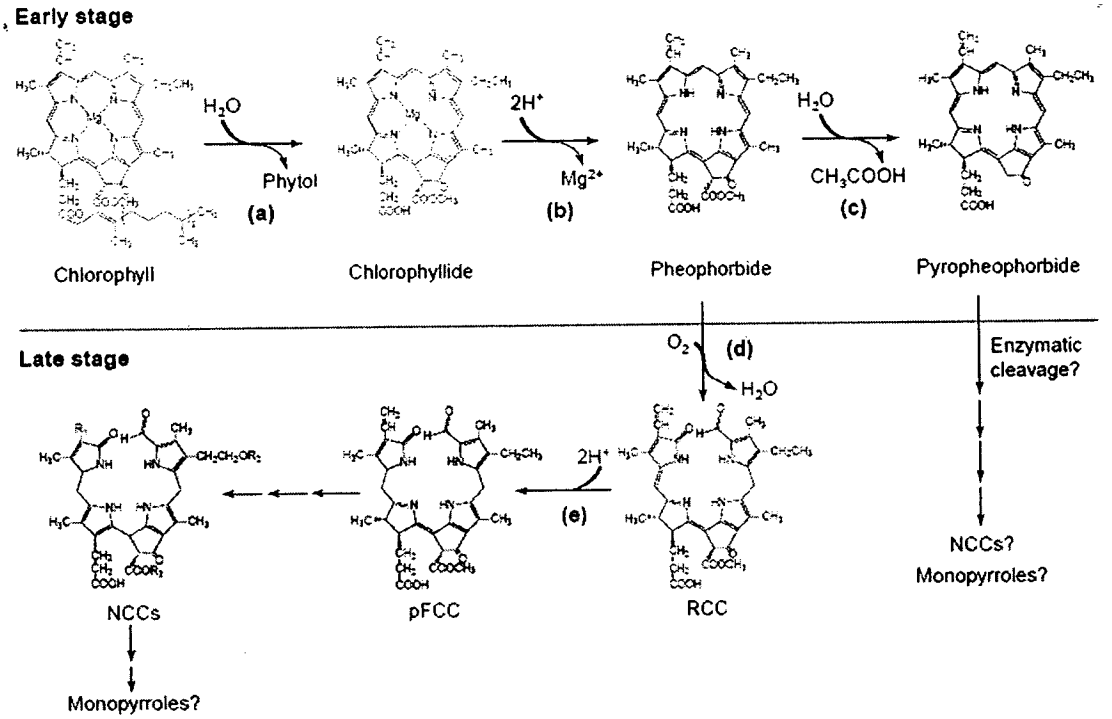


Figure 2 Chlorophyll degradation in higher plants (a) chlorophyllase (b) Mg-dechelatase (c) Pheophorbidase (d) Pheophorbide *a* oxygenase. (e) Red chlorophyll catabolite reductase. Abbreviations: NCCs, nonfluorescent chlorophyll catabolites; pFCC, primary fluorescent chlorophyll catabolite; RCC, red chlorophyll catabolite.
Source: Takamiya *et al* [25]

Characterization of Chlorophyll-degrading enzymes in relation to chlorophyll degradation

1. Chlorophyllase

The enzyme catalyzing the dephytylation, Chlase was one of the 1st plant enzymes to be studied [26]. There are many reports on Chlase activity, including the properties of crude enzymes and the effect of internal and external factors such as phytohormones and temperature stresses on the activity [27]. In spite of repeated isolations of Chlase from various plants and algae [28-30], molecular properties such as the entire amino acid sequence, functional domain, homology among Chlase and regulation of the expression of Chlase were unclear. The Chlase reaction is the 1st step of Chl degradation and therefore the location of Chlase is a factor in determining the site of Chl degradation. There is more than one compartment for Chlase localization. In most cases, the Chlase activity was latent in chloroplasts and *in vitro* which was considered to be thylakoid bound. Activity detection of Chlase using chloroplast subfractions localized Chlase activity to the envelope, probably in the inner membrane [31]. The latency appeared to be merely the result of the spatial inaccessibility of Chlase to Chl in the Chl–protein complex in thylakoid. Thus, based on the envelope location of Chlase, it was recently proposed that, *in vivo*, an as-yet unknown carrier protein for chlorophyll is synthesized in the senescent cell and transported to senescing chloroplasts and then it shuttles between the thylakoid and envelope membrane [15,31]. The Chl molecule or Chl–protein complex released from such plastoglobuli could be attacked by Chlase in the vacuole more than the chloroplast [32,33].

2. Mg-dechelation activity

Mg-dechelation takes place after dephytylation to yield Pheide *a* and pheophorbin *a* using Chlide *a* and Chlorophyllin (Chlin) *a* as substrates (**Figure 3**) [34]. Initially, the *in vivo* and *in vitro* accumulation of pheopigments during Chl degradation of algae and higher plants suggested the presence of MD enzymes [9,12,35,36]. Furthermore, *in vitro* assays of the dechelatase activity revealed that it was associated with thylakoid membranes in a latent form of rape cotyledon [9]. By contrast, in *Chenopodium album*, the activity could still be detected in a soluble low molecular mass (900 Da.) fraction after gel filtration of the enzyme, and it was heat stable. This activity was thus designated MDS [12]. Costa *et al* [37] reported that the Mg-dechelation activity was associated with a compound with a low molecular weight substance of 2,180 ± 20 Da. Suzuki *et al* [38] and Kunieda *et al* [34] demonstrated that the low molecular weight substances in radish cotyledons and mature leaves of *Chenopodium album* play a role in catalysis of the Mg-dechelation reaction using Chlide *a* as a substrate. Specifically, the low molecular weight substances in the mature leaves of *Chenopodium album* were found to have molecular masses of 3.3 and 1.1 kDa [34]. Lastly, the characterization of Mg-dechelation activity of stored broccoli florets was investigated to clarify the mechanism of Chl degradation [14]. Mg-dechelation activity in floret extracts was found in 2 different molecular weight fractions - a low molecular weight (< 5,000) fraction (LMWF) and a high molecular weight (> 5,000) fraction (HMWF), which seemed to be MD, using Chlin *a* or Chlide *a* as a substrate. The activity of the HMWF, which was partially purified by molecular exclusion chromatography (Sephacryl S-200) and using Chlin *a* as a substrate (**Figure 4a**), increased in yellowing broccoli florets after 6 days of storage (**Figure 4b**), whereas heat treatment reduced the enhancement of the activity concurrently with the inhibition of yellowing (**Figure 4c**). Only one peak of the activity was detected in fresh broccoli extracts and no other isozyme with Mg-dechelating action was found in the yellow broccoli extract which had a molecular mass of about 70 KDa. This high-molecular weight substance shows strong activity with the artificial substrate, Chlin *a*, but hardly an activity with native substrates, Chlide *a*. This means that the high-molecular weight substance (HMWS) does not have an activity with Chlide *a*. It is interpreted by the results obtained in this study. HMWS is not involved in the Chl degradation pathway of broccoli florets. It is necessary to purify the low molecular substance and clarify the characterization in the future.

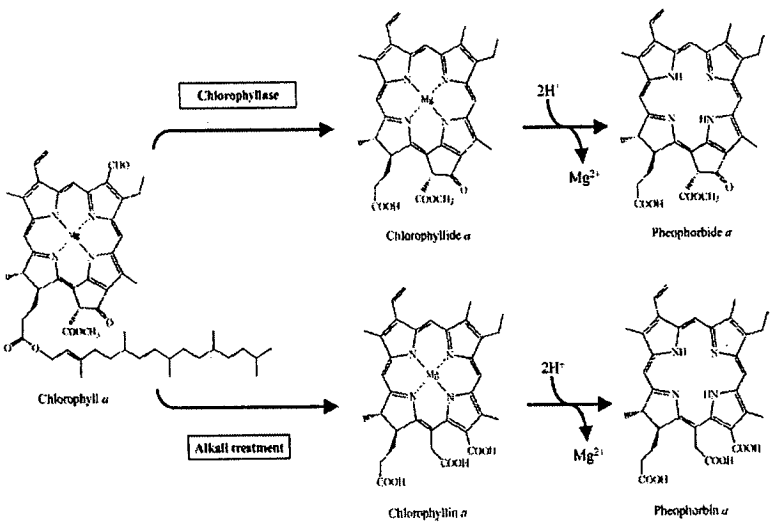


Figure 3 Structure of chlorophyll *a* and its derivative in relation to Mg-dechelation reaction. Chlorophyllide *a* is a native substrate, whereas chlorophyllin *a* is an artificial substrate in Mg-dechelation reaction.
Source: Kunieda *et al* [34]

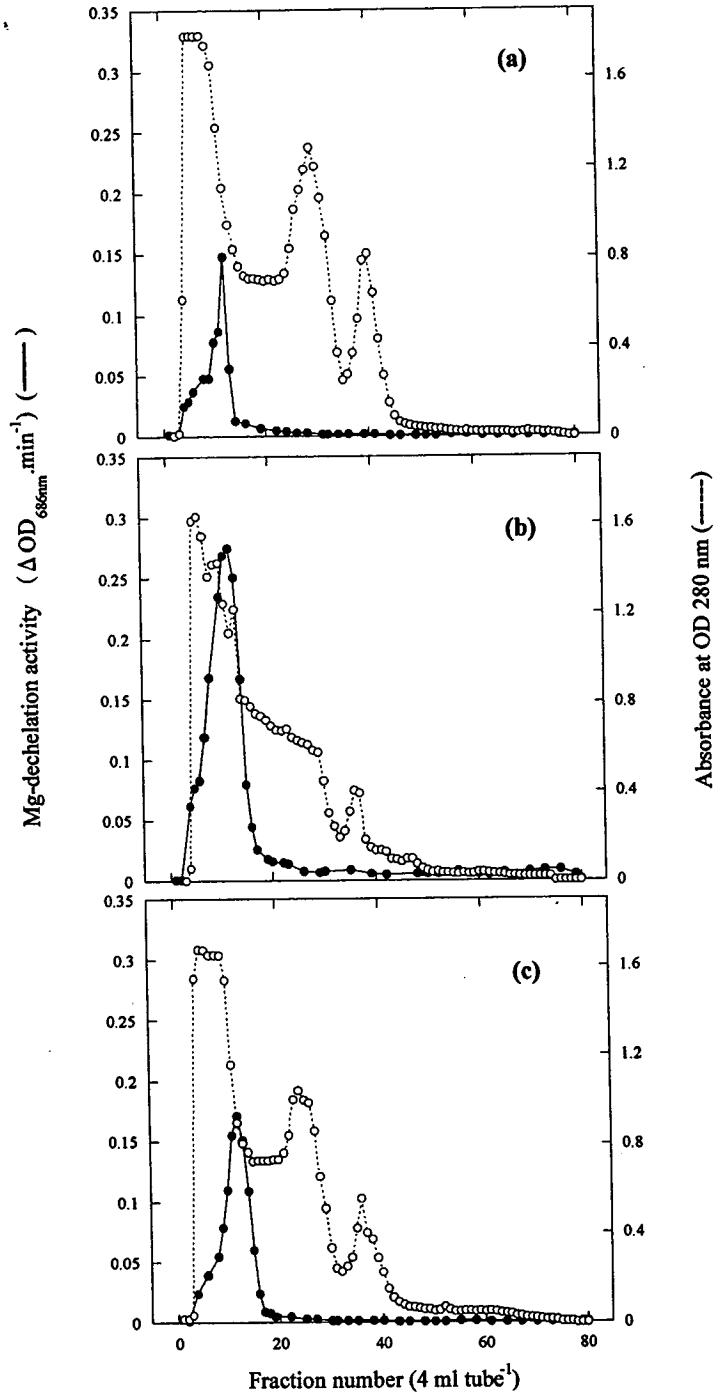


Figure 4 Elution profile on a Sephacryl S-200 column of Mg-dechelation activity in broccoli florets. (a) fresh broccoli florets, (b) broccoli florets stored for 6 days at 15 °C and (c) heat-treated broccoli florets stored for 6 days at 15 °C. a: Blue Dextran 2000.
Source: Kaewsuksaeng *et al* [14]

3. Pheophorbidase

Chl derivatives are found with modified side chains of a tetrapyrrole macrocycle. Recently, an enzyme named pheophorbidase (28 - 29 KDa) has been purified from *Chenopodium album*, which catalyzes the hydrolysis of the methyl ester bond of the isocyclic ring of Pheide to yield $C13^2$-carboxypyropheophorbide [39]. This is not stable and therefore is nonenzymatically converted to pyropheophorbide. Interestingly, pheophorbidase is located outside the chloroplast [34]. If Pheide *a* is a true substrate of the enzyme, it suggests that there is another degradation pathway whose early steps occur outside the chloroplast. Because the pheophorbidase activity is found in several, but not in all species of higher plants tested, this reaction might be specific for certain plants. $C13^2$-OHChl *a* is reported to be accumulated in ethylene-treated *Citrus* species and other plants [40,41] and might be an intermediate in the oxidative Chl bleaching pathway as well [41].

4. Chlorophyll-degrading peroxidase

The mechanism of *in vitro* Chl degradation by peroxidase can be summarized as shown in **Figure 5**. Peroxidase oxidizes the phenolic compounds, which have the hydroxyl group at the *p*-position, to form the phenoxy radical and superoxide anion; then, the radical and/or superoxide anion attacks Chl *a* to form $C13^2$-OHChl *a*. Chl *a* may be ultimately degraded in sequence to colorless low molecular weight compounds through Chl catabolites such as $C13^2$-OHChl *a* and bilirubin-like compounds [16]. Martinoia *et al* [42] demonstrated that peroxidative Chl bleaching activity was present in the thylakoid membrane of barley seedlings. Abeles *et al* [43] reported that in cucumber (*Cucumis sativus* L.) cotyledons treated with ethylene, cationic peroxidase (33KD, pI = 8.9), which degrades Chl *in vitro*, increased. Yamauchi and Watada [44] reported the involvement of peroxidase in Chl degradation of stored broccoli florets. The activity of peroxidase, which is involved in Chl degradation, showed a sharp increase concurrently with floret yellowing [24,45]. By the method of native-PAGE, 6 anionic and 2 cationic isoperoxidases were detected in fresh broccoli florets. In these isoperoxidases, only one cationic isoperoxidase (Rf 0.3) was related to Chl degradation. The cationic isoperoxidase was further purified by means of molecular exclusion chromatography and cationic exchange chromatography. Two Chl-degrading peroxidase isozymes (Type I and Type II) were contained in the cationic isoperoxidase [46]. This finding indicates that peroxidase might play a role in Chl oxidation. Further study is necessary to clarify the speculations about the action of peroxidase on Chl degradation and the mechanism of peroxidase-mediated Chl degradation in postharvest horticultural crops.

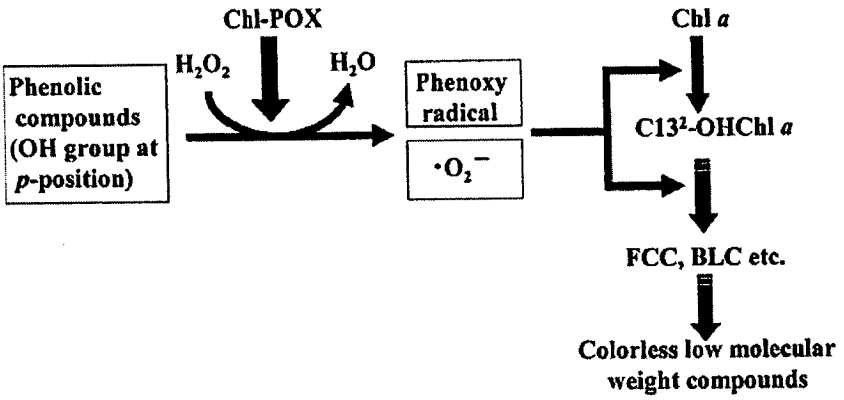


Figure 5 Pathway of peroxidase-mediated chlorophyll degradation. Chl-POX: Chlorophyll-degrading peroxidase, Chl: Chlorophyll, $C13^2$-OHChl: $C13^2$-Hydroxychlorophyll, FCC: Fluorescent chlorophyll catabolite, BLC: Bilirubin-like compounds.
Source: Yamauchi *et al* [16]

5. Pheophytinase

A new Chl degrading enzyme, PPH which would dephytylate the Mg-free Chl pigment, Phein *a* to give Pheide *a* has recently been reported [17]. They identified PPH, a chloroplast-located and senescence-induced hydrolase widely distributed in algae and land plants. *In vitro*, *Arabidopsis* PPH specifically dephytylates the Mg-free chlorophyll pigment, phein, yielding Pheide *a*. An *Arabidopsis* mutant deficient in PPH (*pph-1*) is unable to degrade chlorophyll during senescence and therefore exhibits a stay-green phenotype. Furthermore, *pph-1* accumulates Phein during senescence. Moreover, the *in vitro* activity of PPH in lime fruit was recently determined. The absorption spectrum of the product as Pheide *a* was recorded at 665 nm. The activity of PPH increased with Pheide *a* formation based on the total peak area (**Figure 6**). Therefore, PPH is an important component of the Chl breakdown machinery of senescent leaves, and we propose that the sequence of early Chl catabolic reactions be revised. Removal of Mg most likely precedes dephytylation, resulting in the following order of early breakdown intermediates: Chl → Phein → Pheide. Chlide, the last precursor of Chl biosynthesis, is most likely not an intermediate of breakdown. Thus, Chl anabolic and catabolic reactions are metabolically separated. Kaewsuksaeng *et al* [47] recently showed PPH activity gradually increased during storage in lime fruit, while the UV-B treatment effectively suppressed PPH activity. Further study needs to be conducted to establish a correct measurement of PPH and clarify the role of PPH in Chl degradation of horticultural crops.

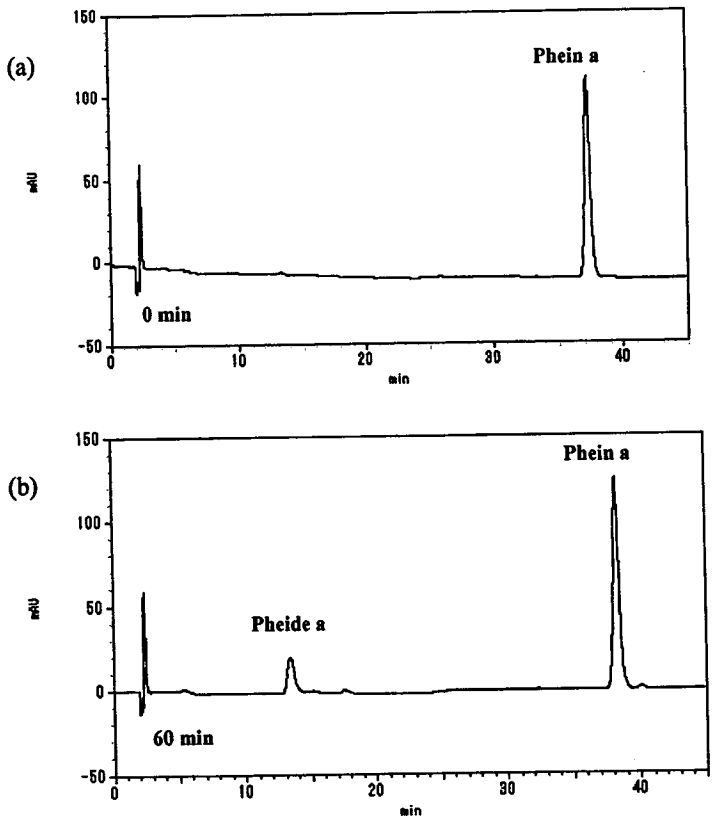


Figure 6 Analysis of Pheide formation of lime fruit. The *in vitro* pheophytinase reaction was held at 25 °C for 0 min (a) and 60 min (b), Pheide - Pheophorbide and Phein - Pheophytin.

Conclusions

A series of the reactions involved in Chl-degrading enzymes such as Chlase, MD or MDS, pheophytinase, pheophorbide *a* oxygenase and pheophorbidase, is thought to be the main pathway of Chl *a* degradation in horticultural crops and this occurs in the chloroplasts. On the other hand, Chl-degrading peroxidase is also reported to be involved in Chl oxidation to form $C13^2$-OHChl *a*. The initial step of Chl *a* degradation seems to be the formation of Chlide *a* by Chlase or Phein *a* by MD or that of $C13^2$-OHChl *a* by oxidation related to Chl-degrading peroxidase. Both Chlide *a*, Phein *a* and $C13^2$-OHChl *a* can be finally degraded to colorless, low molecular weight compounds.

References

[1] N Yamauchi and AE Watada. Regulated chlorophyll degradation in spinach leaves during storage. *J. of Amer. of Chem. Soc.* 1991; **116**, 58-62.

[2] N Yamauchi and AE Watada. Pigment changes in parsley leaves during storage in controlled or ethylene containing atmosphere. *Food Sci.* 1993; **58**, 104-8.

[3] ML Costa, PM Civello, AR Chaves and GA Martínez. Effect of hot air treatments on senescence and quality parameters of harvested broccoli (*Brassica oleracea* L var *Italica*) heads. *J. of the Sci. of Food and Agri.* 2005; **85**, 1154-60.

[4] TO Win, V Srilaong, KL Kyu, K Poomputsa and S Kanlayanarat. Biochemical and physiological changes during chlorophyll degradation in lime (*Citrus aurantifolia* Swingle cv. 'Paan'). *J. Hort. Sci. Biotech.* 2006; **81**, 471-7.

[5] V Srilaong, S Aiamla-or, A Soontornwat, M Shigyo and N Yamauchi. UV-B irradiation retards chlorophyll degradation in lime (*Citrus latifolia* Tan.) fruit. *Postharvest Biol. Technol.* 2011; **59**, 110-2.

[6] K Shimokawa. *Coloring. In*: Japanese Society for Horticultural Science (ed.). Shin-engeigaku zenpen, Youkendo, Tokyo, 1998, p. 585-8.

[7] K Shimokawa, S Shimada and K Yaeo. Ethylene-enhanced chlorophyllase activity during degreening of *Citrus unshiu* Marc. *Scientia Horti.* 1978; **8**, 129-35.

[8] D Amir-Shapira, EE Goldschmudt and A Altman. Chlorophyll catabolism in senescing plant tissues: *in vivo* breakdown intermediates suggest different degradative pathways for citrus fruit and parsley leaves. *In*: Proceedings of the National Academy of Sciences, USA, 1987, p. 1901-5.

[9] M Langmeier, S Ginsburg and P Matile. Chlorophyll breakdown in senescent leaves: demonstration of Mg-dechelatase activity. *Physiol. Plant.* 1993; **89**, 347-53.

[10] S Kaewsuksaeng, N Yamauchi, Y Funamoto, M Shigyo and S Kanlayanarat. Effect of Mg-dechelation activity on Chlorophyll Degradation in Stored Broccoli Florets. *Acta Hort.* 2006; **712**, 705-9.

[11] S Kaewsuksaeng, N Yamauchi, Y Funamoto, T Mori, M Shigyo and S Kanlayanarat. Effect of heat treatment on catabolites formation in relation to chlorophyll degradation during storage of broccoli (*Brassica oleracea* L. italica group) florets. *J. Jap. Soc. Hort. Sci.* 2007; **76**, 338-44.

[12] Y Shioi, N Tomita, T Tsuchiya and K Takamiya. Conversion of chlorophyllide to pheophorbide by Mg-dechelating substance in extracts of *Chenopodium album*. *Plant Physiol. Biochem.* 1996; **34**, 41-7.

[13] S Aiamla-or, S Kaewsuksaeng, M Shigyo and N Yamauchi. Impact of UV-B irradiation on chlorophyll degradation and chlorophyll-degrading enzyme activities in stored broccoli (*Brassica oleracea* L. Italica Group) florets. *Food Chem.* 2010; **120**, 645-51.

[14] S Kaewsuksaeng, N Yamauchi, Y Funamoto, S Aiamla-or, T Mori, M Shigyo and S Kanlayanarat. Partially purification of Mg-dechelatase in relation to chlorophyll degradation in broccoli (*Brassica oleracea* L. Italica Group) florets. *Acta Hort.* 2010; **875**, 509-14.

[15] P Matile, S Hörtensteiner and H Tomas. Chlorophyll degradation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1999; **50**, 69-75.

[16] N Yamauchi, Y Funamoto and M Shigyo. Peroxidase-mediated chlorophyll degradation in horticultural crops. *Phytochem. Reviews.* 2004; **3**, 221-8.

[17] S Schelbert, S Aubry, B Burla, B Agne, F Kessler, K Krupinska and S Hörtensteiner. Pheophytin pheophorbide hydrolase (Pheophytinase) is involved in chlorophyll

breakdown during leaf senescence in *Arabidopsis*. *Plant Cell.* 2009; **21**, 767-85.

[18] S Hörtensteiner and B Kräutler. Chlorophyll breakdown in oilseed rape. *Photosyn. Res.* 2000; **64**, 37-146.

[19] M Holden. The breakdown of chlorophyll by chlorophyllase. *J. of Biochem.* 1961; **78**, 359-64.

[20] FC Pennington, HH Strain, WA Svec, JJJ and Katz. Preparation and properties of pyrochlorophyll *a*, methyl pyrochlorophyllide *a*, pyropheophytin *a*, and methyl pyropheophorbide *a* derived from chlorophyll by decarbomethoxylation. *J. of Amer. of Chem. Soc.* 1964; **86**, 1418-26.

[21] N Yamauchi, K Eguchi, Y Tokuhara, Y Yamashita, A Oshima, M Shigyo and K Sugomota. Control of degreening by sucrose laurate ester and heat treatment of *Citrus nagato-yuzukichi* fruit. *J. of Hort. Sci. Biotech.* 2003; **78**, 563-7.

[22] PH Hynninen. Chlorophylls IV. Preparation and purification of some derivatives of chlorophylls *a* and *b*. *Acta Chem. Scand.* 1973; **27**, 1771-80.

[23] MJ Maunders, SB Brown and HW Woolhouse. The appearance of chlorophyll derivatives in senescing tissue. *Phytochem.* 1983; **22**, 2443-6.

[24] Y Funamoto, N Yamauchi, T Shigenara and M Shigyo. Effects of heat treatment on chlorophyll degrading enzymes in stored broccoli (*Brassica olearacea* L.). *Postharvest Biol. Technol.* 2002; **24**, 163-70.

[25] K Takamiya, T Tsuchiya and H Ohta. Degradation pathway(s) of chlorophyll: what has gene cloning revealed? *Trends in Plant Sci.* 2000; **5**, 1380-5.

[26] R Willstätter and A Stoll. *Die Wirkungen der Chlorophyllase*. *In*: Untersuchungen über Chlorophyll. Springer, Berlin, 1913, p. 172-87.

[27] M Drazkiewice. Chlorophyllase: occurrence, function, mechanism of action, effects of external factors. *Photosynt.* 1994; **30**, 301-9.

[28] T Trebitsh, EE Goldschmidt and J Riov. Ethylene induces de novo synthesis of chlorophyllase, a chlorophyll degrading enzyme, in *Citrus* fruit peel. *In:* Proceeding of National Academic Science, USA, 1993, p. 9441-5.

[29] A Khalyfa, S Kermasha, P Marsot, and M Goetghebeur. Purification and characterization of chlorophyllase from algae *Phaeodactylum tricornutum* by preparative native electrophoresis. *Apply Biochem. and Biotech.* 1995; **53**, 11-27.

[30] T Tsuchiya, H Ohta, K Okawa, A Iwamatsu, H Shimada and T Masuda, and K Takamiya. Cloning of chlorophyllase, the key enzyme in chlorophyll degradation: finding of a lipase motif and the induction by methyl jasmonate. *In*: Proceedings of the National Academy of Sciences, USA, 1999, p. 15362-7.

[31] P Matile, M Schellenberg and F Vicentini. Localization of chlorophyllase in the chloroplast envelope. *Planta.* 1997; **201**, 96-9.

[32] B Parthier. Gerontoplasts - the yellow end in the ontogenesis of chloroplasts. *Endo. Cell Resource.* 1988; **5**, 163-90.

[33] H Satoh, K Nakayama and M Okada. Molecular cloning and functional expression of a water-soluble chlorophyll protein, a putative carrier of chlorophyll molecules in cauliflower. *J. of Bio. and Chem.* 1998; **273**, 30568-75.

[34] T Kunieda, T Amano and Y Shioi. Search for chlorophyll degradation enzyme, Mg-dechelatase, from extracts of *Chenopodium album* with native and artificial substrates. *Plant Sci.* 2005; **169**, 177-83.

[35] TG Owensand and PG Falkowski. Enzymatic degradation of chlorophyll *a* by marine phytoplankton *in vitro*. *Phytochem.* 1982; **21**, 979-84.

[36] Y Shioi, Y Tatsumi and K Shimokawa. Enzymatic degradation of chlorophyll in *Chenopodium album*. *Plant Cell Physio.* 1991; **32**, 87-93.

[37] ML Costa, PM Civello, AR Chaves and GA Martínez. Characterization of Mg-dechelatase activity obtained from *Fragaria x ananassa* fruit. *Plant Physio. and Biochem.* 2002; **40**, 111-8.

[38] T Suzuki, T Kunieda, F Murai, S Morioka and Y Shioi. Mg-dechelation activity in radish cotyledons with artificial and native substrates, Mg-chlorophyllin *a* and chlorophyllide *a*. *Plant Physio. and Biochem.* 2005; **43**, p. 459-64.

[39] K Watanabe, H Ohta, T Tsuchiya, B Mikami, T Masuda, Y Shioi, and K Takamiya.

Purification and some properties of pheophorbidase in *Chenopodium album*. *Plant Cell and Physio.* 1999; **40**, 104-8.

[40] Y Maeda, H Kurata, M Adachi and K Shimokawa. Chlorophyll catabolism in ethylene-treated *Citrus unshiu* fruits". *J. Jap. Soc. Hort. Sci.* 1998; **67**, 497-502.

[41] MT Janave. Enzymic degradation of chlorophyll in Cavendish bananas: *in vitro* evidence for two independent degradative pathways. *Plant Physio. and Biochem.* 1997; **35**, 837-46.

[42] E Marutinoia, MJ Dalling and P Matile. Catabolism of chlorophyll: Demonstration of chloroplast-localized peroxidative and oxidative activities. *Z. Pflanzenphysiol.* 1982; **107**, 269-79.

[43] FB Abeles, LJ Dunn, P Morgens, A Callahan, RE Dinterman and J Schmidt. Induction of 33-KD and 60-KD peroxidases during ethylene-induced senescence of cucumber cotyledons. *Plant Physio.* 1988; **87**, 609-15.

[44] N Yamauchi and AE Watada. Chlorophyll and xanthophylls changes in broccoli florets stored under elevated CO_2 or ethylene-containing atmosphere. *Hort. Sci.* 1998; **33**, 114-7.

[45] N Yamauchi, Y Funamoto and Y Kanetsune. Involvement of chlorophyll degrading enzymes with chlorophyll degradation in stored broccoli (*Brassica oleracea* L.) florets. *Food Sci. Technol. Res.* 1999; **5**, 300-3.

[46] Y Funamoto, N Yamauchi and M Shigyo. Purification and characterization of chlorophyll-degrading peroxidase in broccoli florets. *J. Jap. Soc. Hort. Sci.* 2003; **72**, 137.

[47] S Kaewsuksaeng, Y Urano, S Aiamla-or, M Shigyo and N Yamauchi. Effect of UV-B irradiation on chlorophyll-degrading enzyme activities and postharvest quality in stored lime (*Citrus latifolia* Tan.) fruit. *Postharvest Biol. Technol.* 2011; **61**, 124-30.

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From: Ian Ferguson ian.ferguson@plantandfood.co.nz
Subject: Your Submission

Ms. Ref. No.: POSTEC-D-14-00385R1
Title: Postharvest heat treatment delays chlorophyll degradation and maintains quality in Thai lime (Citrus aurantifolia Swingle cv. Paan) fruit
Postharvest Biology and Technology

Dear Dr. Samak Kaewsuksaeng,

I am pleased to inform you that your paper "Postharvest heat treatment delays chlorophyll degradation and maintains quality in Thai lime (Citrus aurantifolia Swingle cv. Paan) fruit" has been accepted for publication in Postharvest Biology and Technology.

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Corresponding Author: Dr. Samak Kaewsuksaeng,

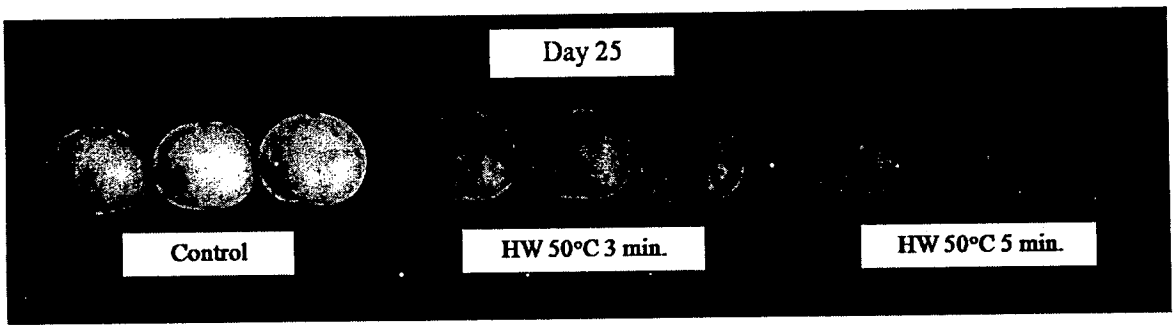
Corresponding Author's Institution:

First Author: Samak Kaewsuksaeng

Order of Authors: Samak Kaewsuksaeng; Nopparat Tatmala, Master; Varit Srilaong, Ph.D.; Nutthachai Pongprasert, Ph.D.

Abstract: The influence of postharvest heat treatment by using hot water was applied to Thai lime fruit (Citrus aurantifolia Swingle cv. Paan) to investigate its effect on chlorophyll (Chl)-degradation and postharvest quality during storage. Mature green lime fruits were treated with water at ambient temperature (control) or with hot water at 50 °C for 3 and 5 min and then kept at 25 °C in darkness. The results showed that hot water treatment at 50 °C for 5 min efficiently delayed the decrease of hue angle value and the content of Chl a. Activities of Chl-degrading enzymes including chlorophyllase, Chl-degrading peroxidase and pheophytinase as well as Mg-dechelation activity in lime fruit were reduced by hot water treatments. This reduction was more pronounced in fruits treated by hot water for 5 min than in those treated for 3 min. Moreover, heat treatments prolonged storage life by 5 days in fruit treated for 3 min and by 10 days in fruit treated for 5 min. Hot water treatment at 50 °C for 5 min maintained the highest total acidity and delayed the increase of total soluble solids contents during storage. Respiration rate and ethylene production were also reduced by the heat treatments. In conclusion, these results suggest that hot water treatment at 50 °C for 5 min could be a useful method to delay Chl degradation and to maintain postharvest quality in mature green lime during storage at 25 °C.

Graphical Abstract



Research Highlights

1. Hot water treatment at 50 °C for 5 min could be a useful method to delay chlorophyll degradation in Thai lime fruit.
2. The activities of Chl-degrading enzymes in the lime fruit with hot water treatment at 50 °C for 5 min were suppressed.
3. Hot water treatment at 50 °C for 5 min caused the highest maintenance of postharvest quality.
4. The postharvest physiology effects were also the most reduced by the highest duration of hot water treatment at 50 °C for 5 min.

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Postharvest heat treatment delays chlorophyll degradation and maintains quality in Thai lime (*Citrus aurantifolia* Swingle cv. Paan) fruit

Samak Kaewsuksaeng[1,2*], Nopparat Tatmala[1,2] , Varit Srilaong[3], Nutthachai Pongprasert[3]

[1]Southern Tropical Plants Research Unit, Thaksin University, Phatthalung campus

[2]Department of Plant Science, Faculty of Technology and Community Development, Thaksin University, Phatthalung campus, Phatthalung 93110, Thailand

[3]Postharvest Technology Program, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, 126 Pracha-Utid Road, Bangmod, Thungkru, Bangkok 10140, Thailand

*Corresponding author at: Faculty of Technology and Community Development, Thaksin University, Phatthalung campus, Phatthalung 93110, Thailand Tel. & Fax: +66-74-693-996.
E-mail address: samak@tsu.ac.th (S. Kaewsuksaeng)

Key words: lime, heat treatment, chlorophyll degradation, chlorophyll-degrading enzyme, postharvest quality

Abstract

Postharvest heat treatments by using hot water were applied to Thai lime fruit (*Citrus aurantifolia* Swingle cv. Paan) to investigate their effect on chlorophyll (Chl)-degradation and postharvest quality during storage. Mature green lime fruit were treated with water at ambient temperature (control) or with hot water at 50 °C for 3 and 5 min and then kept at 25 °C in darkness. The results showed that hot water treatment at 50 °C for 5 min efficiently delayed the decrease of hue angle values and the contents of Chl *a*. Activities of Chl-degrading enzymes including chlorophyllase, Chl-degrading peroxidase and pheophytinase as well as Mg-dechelation activity in lime fruit were reduced by hot water treatments. This reduction was more pronounced in fruit treated by hot water for 5 min than in those treated for 3 min. Moreover, heat treatments prolonged storage life by 5 days in fruit treated for 3 min and by 10 days in fruit treated for 5 min. Hot water treatment at 50 °C for 5 min maintained the highest total acidity and delayed the increase of total soluble solids contents during storage. Respiration rate and ethylene production were also reduced by the heat treatments. In conclusion, these results suggest that hot water treatment at 50 °C for 5 min could be a useful method to delay Chl degradation and to maintain postharvest quality in mature green lime during storage at 25 °C.

1. Introduction

Lime (*Citrus aurantifolia* Swingle cv. Paan) is an economically significant horticultural crop in Thailand and the production season is from July to September (Pranamornkith et al., 2005). The lime fruit are generally harvested when the rind is still green, with highly aromatic compounds. The price of lime fruit strongly depends on its availability and quality characteristics. Postharvest quality of lime fruit deteriorates quickly after harvest. The most visible deterioration factor is the loss of peel greenness that usually occurs with chlorophyll (Chl) degradation (Kaewsuksaeng et al., 2011; Srilaong et al., 2011). For the maintenance of postharvest quality, it is necessary to retain the green color in the peel of lime fruit as long as possible.

An early step of Chl *a* degradation is the removal of the side chain attached to the tetrapyrrole macrocycle to form chlorophyllide (Chlide) *a* by chlorophyllase (Chlase). The Chlide *a* formed still retains a green color (Shimokawa et al., 1978; Amir-Shapira et al., 1987). The elimination of Mg^{2+} from Chlide *a* to produce pheophorbide (Pheide) *a* is induced by Mg-dechelatase (MD) (Langmeier et al., 1993; Kaewsuksaeng et al., 2006, 2007) or a Mg-dechelating substance (MDS) (Shioi et al., 1996; Aiamla-or et al., 2010; Kaewsuksaeng et al., 2010), and the Pheide *a* formed loses its green color. Finally, Pheide *a* is decomposed to fluorescent Chl catabolites, which are primarily colorless, via a red Chl catabolite by both Pheide *a* oxygenase and red Chl catabolite reductase (Matile et al., 1999). Chl-degrading peroxidase (POX) (Yamauchi et al., 2004; Kaewsuksaeng et al., 2007) is also suggested to be involved in Chl degradation as the first step enzyme, oxidizing Chl *a* to form 13^2-hydroxychlorophyll *a*. In addition, a new Chl-degrading enzyme, pheophytinase (pheophytin pheophorbide hydrolase, PPH), which would dephytylate the Mg-free Chl pigment, pheophytin (Phein) *a*, to give Pheide, has been recently reported (Schelbert et al., 2009).

Heat treatment is frequently used to maintain the postharvest quality of many horticulture crops. Much research has been performed on the maintenance of quality in broccoli (Funamoto et al., 2002; Kaewsuksaeng et al., 2007), celery (Viña et al., 2007), tomato (Lu et al., 2010) and peach (Spadoni et al., 2014). Yamauchi et al. (2003) reported that 2 % sucrose laurate ester with hot water treatment at 50 °C effectively reduces the degreening of green Nagato-yuzukichi fruit due to the suppression of Chl degradation. Recently, new physical technology such as UV-B irradiation to retard Chl degradation and maintain postharvest quality of Tahitian lime (*Citrus latifolia* Tan.) fruit has been used successfully (Kaewsuksaeng et al., 2011; Srilaong et al., 2011). However, the effect of the heat treatment on postharvest quality in lime fruit has not yet been invetsigated. In this study, we therefore examined the effect of hot water treatment on Chl degradation and changes in Chl-degrading enzyme activities. We also evaluated the physiological changes and resultant quality control during storage in heat-treated limes.

2. Materials and Methods

2.1. Plant materials and hot water treatment

Mature green Thai limes (*Citrus aurantifolia* Swingle cv. Paan) were harvested from a commercial orchard in Nakorn Sri Thammarat Province, Thailand. After transportation to the laboratory, fruit were selected for uniformity in maturity, size, shape, peel color, and lack of defects. The fruit were treated with hot water at 50 °C for 3 and 5 min or water at ambient temperature (control), and then dried for 1 h at ambient temperature.

After hot water treatment, the fruit were kept in polyethylene film bags (0.03mm in thickness) with the top folded over and stored at 25 °C in the dark. Samples (3 replications)

were removed at 5 day intervals, and the peel tissues were sampled and used for analysis. The entire experiment was performed in a completely randomized design with three replications. Each replication represented 20 samples of fruit for each parameter. The results show the average values with SE.

2.2. Surface color and chlorophyll assays

The surface color of lime fruit was determined by measuring the hue angle with a colorimeter (Nippon Denshoku NF 777). Chl *a* content was determined using *N,N*-dimethylformamide (Moran, 1982).

2.3. Preparation of chlorophyll a and resulting derivatives

2.3.1. Chlorophyll a

Spinach leaves were homogenized for 3 min in cold acetone. The homogenate was filtrated through two layers of Miracloth (Calbiochem). The filtrates were treated with dioxane and distilled water and then kept for 1 h on ice. The filtrates were centrifuged at 10,000g for 15 min at 4 °C. After centrifugation, the pellets were treated again with acetone, dioxane, and distilled water, and then kept for 1 h on ice. Afterwards, the soluble pellets were centrifuged at 10,000g for 15 min at 4 °C and subsequently dissolved in petroleum ether. Soluble chlorophyll in petroleum ether was stored at 20 °C until the individual pigments were separated using sugar powder column chromatography (Perkins and Roberts, 1962). Finally, five hundred $\mu g\ ml^{-1}$ of Chl *a* was prepared in acetone.

2.3.2. Chlorophyllide a

Chlide *a* was prepared from a Chl *a* acetone solution (500 $\mu g\ mL^{-1}$). The reaction mixture was incubated at 25 °C for 40 min. The reaction was stopped using acetone and the remaining Chl *a* was separated by hexane. The lower part of the reaction mixture was used as the Chlide *a*.

119

120

121 *2.3.3. Pheophytin a*

122 Phein *a* was prepared by adding one drop of 0.1 N HCl into the Chl *a* acetone solution
123 (500 μg ml^{-1}). After 2 min, 0.1 N NaOH was added to neutralize the solution, which was then
124 employed as the substrate in the assays.

125 *2.4. Analyses of chlorophyll-degrading enzyme activities*

126 An acetone powder (500 mg) of peel tissues was suspended in 15 mL of 10 mM
127 phosphate buffer (pH 7.0) containing 0.6% CHAPS for Chlase. For MDS, an acetone powder
128 (500 mg) of peel tissues was suspended in 15 mL of 50 mM phosphate buffer (pH 7.0)
129 containing 50 mM KCl and 0.24% Triton-X 100 or in 15 mL of 10 mM phosphate buffer (pH
130 7.0) for Chl-POX. For PPH, an acetone powder (500 mg) was suspended in 15 mL of 50 mM
131 Tris-HCl buffer (pH 8.0). The crude enzyme was stirred for 1 h at 0 °C, and the mixture was
132 filtered with two layers of Miracloth. The filtrate was then centrifuged at 16,000g at 4 °C for
133 15 min. The supernatant was used as the crude enzyme extract. Triplicates of supernatant
134 were utilized in each determination. The enzyme protein contents were determined on the
135 basis of Bradford's method (1976).

136 *2.4.1. Chlorophyllase activity*

137 The reaction mixture contained 0.5 mL of 0.1 mM phosphate buffer (pH 7.5), 0.2 mL of
138 500 μg mL^{-1} Chl *a* acetone solution (100 μg mL^{-1}), and 0.5 mL of enzyme solution. The
139 reaction mixture was incubated in a water bath at 25 °C for 40 min, and the enzyme reaction
140 was stopped by adding 4 mL of acetone. Chlide *a* was separated by adding 4 mL of hexane.
141 The upper phase contained the remaining Chl *a* while the lower phase contained the Chlide *a*.

142 The activity was spectrophotometrically detected by Chlide *a* formation at 667 nm per unit
143 per mg protein.

144

145 *2.4.2. Chlorophyll-degrading peroxidase activity*

146 Chlorophyll-degrading peroxidase (Chl-POX) was determined as previously described
147 (Yamauchi et al., 1997). The reaction mixture contained 50 µL of enzyme solution, 100 µL
148 of 1.0% Triton-X 100, 100 µL of 5 mM *p*-coumaric acid, 100 µL of 500 µg mL^{-1} Chl *a*
149 acetone solution, 500 µL of 0.2 mM phosphate buffer (pH 5.5) and 50 µL of 0.3% hydrogen
150 peroxide. The activity was determined spectrophotometrically by measuring the decrease of
151 1.0 µg of Chl *a* at 668 nm per min per mg protein at 25 °C.

152 *2.4.3. Mg-dechelation activity*

153 Mg-dechelation activity by MDS using Chlide *a* was determined by the method of
154 Kaewsuksaeng et al. (2006). The reaction mixture contained 0.75 mL of 10 mM phosphate
155 buffer (pH 7.5), 0.25 mL of Chlide *a* (8.80 µg) acetone solution and 0.2 mL of enzyme
156 solution. The activity of the Mg-dechelating substance was measured on the basis of Pheide *a*
157 formation by following the change in OD at 535 nm per min per mg protein.

158 *2.4.4. Pheophytinase activity*

159 PPH was determined by the method of Aiamla-or et al. (2012). The reaction mixture
160 contained 0.5 mL of 50 mM Tris-HCl buffer (pH 8.0), 0.2 mL of Phein *a* (22.40 µg) acetone
161 solution and 0.5 mL of enzyme solution. The reaction mixture was incubated in a water bath
162 at 25 °C for 40 min, and the enzyme reaction was stopped by adding 2 mL of acetone. After
163 that, the sample was recorded while performing the spectrophotometric measurement at 667

164 nm. The activity of PPH was calculated with the Pheide *a* formation (mole) per min per mg
165 protein.

166

167 *2.5. Determination of ethylene production and respiration rate*

168 The rate of ethylene production from lime fruit was measured by gas chromatography, with a
169 flame ionization detector (FID) equipped with an 80/100 mesh Porapack-Q column with
170 nitrogen as a carrier gas. Two fruit, in a 150 mL sealed plastic container, were incubated at
171 room temperature for 1 h. A 1 mL gas sample was withdrawn from the head-space and used
172 for ethylene determination. Respiration rates were also determined by gas chromatography
173 using an 80/100 mesh Porapack-Q column and a thermal conductivity detector (Chromatopac
174 C-R 8A; Shimadsu Co.)

175

176 *2.6. Postharvest quality parameters*

177 The titratable acidity (TA) of fruit juice was measured by titrating 5 mL juice with 0.1 M
178 NaOH, using 1-2 drops of 1% (v/v) phenolphthalein as an indicator until the end-point.
179 Titratable acidity was expressed as percentage of citric acid (meq. citric acid = 0.064). Total
180 soluble solid (TSS) contents were determined using a hand refractometer (Model N1; Atago
181 Co., Tokyo, Japan). Readings are shown as %.

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184

185

186 **3. Results**

187 *3.1. Effect of hot water treatment on postharvest qualities of lime fruit*

188 Lime fruit without hot water treatment (control) remained green for 4 days of storage at
189 25 °C and turned to yellow on day 5. In comparison, hot water-treated fruit remained green
190 through 10 days of storage. All treatments resulted in storage life of 25, 30 and 35 days for
191 control and hot water treatments at 50 °C for 3 and 5 min, respectively. Surface color was
192 also evaluated using hue angle. As shown in Fig. 1, the hue angle value in the control
193 declined significantly during storage associated with yellowing of fruit peel. In contrast, that
194 in hot water-treated lime fruit changed only slightly during storage. Titratable acidity was
195 expressed as a percentage of the citric acid content, which is a major organic acid in lime fruit.
196 In the control, acidity decreased after 5 days of storage, while that in hot water-treated fruit at
197 50 °C for 5 min increased constantly until day 35 (Fig. 2A). Figure 2B shows the total soluble
198 solids indicative of sugar level changes in fruit with or without hot water treatment during
199 storage. The total soluble solids in the control considerably increased on day 10 and then
200 decreased during storage. With hot water treatment, those contents were unchanged during
201 storage. However, the total soluble solids of hot water-treated fruit at 50 °C for 5 min were
202 higher than for 3 min.
203

204 *3.2. Effect of hot water treatment on chlorophyll degradation and chlorophyll-degrading*
205 *enzyme activities in lime fruit*

206 Hot water treatment at 50 °C for 5 min in lime fruit delayed the reduction of Chl *a* content
207 more than any other treatment, as shown in Fig. 3. The Chl *a* content in peel from fruit
208 treated with hot water delayed the reduction during storage, whereas that in the control
209 sharply decreased during storage after day 10 until the end of storage.

In Fig. 4A, Chlase activity in all the treatments increased during storage at 25 °C. The activity with hot water treatment at 50 °C for 5 min was lower than that in other treatments. Chl-POX activity markedly increased in both the control and the hot water-treated fruit during storage. However, the activity was lower in hot water-treated fruit at 50 °C for 5 min than for 3 min and the control (Fig. 4B). Mg-dechelation activity was examined using Chlide *a* as a substrate (Fig. 5A). In 3 treatments, Mg-dechelation activity showed an increase in the first 5 days of storage, and after that showed a sharp decrease until the end of storage. The activity was lowest in hot water-treated fruit at 50 °C for 5 min. Moreover, PPH activity also started to increase on the first 5 days and then decreased slightly during storage in all treatments. The activity in hot water-treated fruit at 50 °C for 5 min was the most effective for suppression during storage (Fig. 5B).

3.3. Effect of hot water treatment on postharvest physiology in lime fruit

A climacteric pattern of ethylene production was observed in the fruit. Ethylene production rates of the control fruit slightly increased during storage, whereas in the hot water treatment they declined in the first 5 days and increased until the end of storage, especially in hot water-treated fruit at 50 °C for 3 min. Nevertheless, hot water treatment at 50 °C for 5 min effectively suppressed ethylene production during storage compared to the other treatments (Fig. 6A). The respiration of lime fruit was unchanged throughout storage in the treatment at 50 °C for 3 min and in untreated fruit (Fig. 6B). The respiration rate was slightly reduced by hot water treatment at 50 °C for 5 min in lime fruit.

4. Discussion

For postharvest horticultural crops such as leafy vegetables, broccoli florets, and limes, one of the main factors related to quality deterioration is the loss of green color with Chl degradation (Win et al. 2006a; Kaewsuksaeng et al., 2011; Srilaong et al., 2011). The maintenance of green color in the peel of Thai limes during storage and shelf-life is required for fruit to maintain their value prices (Pranmornkith et al., 2005). Due to consumer interest in decreasing the postharvest use of chemicals, heat treatments such as hot water, hot air, and vapor heat treatment have gained interest for the control of fruit quality. Heat treatment has also been demonstrated to show physiological effects on the control of ripening and senescence and tolerance to postharvest chilling injury in fruit and vegetables (Fallik, 2004; Lurie and Mitcham, 2007).

The effects of postharvest stress treatments such as heat and UV treatments on yellowing or degreening were determined in stored horticultural produce (Yamauchi, 2013). Broccoli floret yellowing was effectively retarded during storage treated with hot air at 50 °C for 2 h (Funamoto et al., 2002). Additionally, heat treatment at 45 °C for 2.5 and 3 h had an inhibitory effect on Chl degradation, but the effect was less than that of 50 °C for 2 h (Funamoto et al., 2003). Yamauchi et al. (2003) reported that heat treatment at 50 °C for 3 min with a solution of 2% sucrose laurate ester delayed degreening in Nagato-yuzukichi (*Citrus nagato-yuzukichi* hort. Ex Y. Tanaka). Green yuzu (*Citrus junos* Siebold ex Tanaka) and Nagato-yuzukichi hot water treatments at 40 and 45 °C efficiently suppressed the decline of hue angle values during storage at 25 °C. Yuzu fruit treated with hot water at 40 °C for 5 and 10 min and Nagato-yuzukichi fruit treated at 45 °C for 5 min kept their green peel color (Ogo et al., 2011). In the present study, Thai lime fruit were treated with hot water at 50 °C for 3 and 5 min, and the results were compared with those of untreated fruit. We found that

258 hot water treatment at 50 °C for 5 min retarded Chl breakdown with delayed decline of hue
259 angle value and Chl *a* and *b* contents in the lime fruit peel.

260 Heat treatment has been known to reduce the Chl degradation through the suppression of
261 activities of Chl-degrading enzymes, including Chlase, Mg-dechelation activity, Chl-
262 degrading peroxidase, and Chl oxidase (Funamoto et al., 2002; Costa et al., 2006;
263 Kaewsuksaeng et al., 2007). UV-B irradiation in Tahitian lime (*Citrus latifolia* Tan.)
264 (Kaewsuksaeng et al., 2011) also suppressed the activities of Chl-degrading enzymes such as
265 Chlase, Mg-dechelation, Chl-degrading peroxidase and PPH. In this study, hot water
266 treatment at 50 °C for 3 and 5 min also effectively suppressed the enhancement of Chlase and
267 Chl-POX activity during storage in lime fruit, especially the latter. Mg-dechelation and PPH
268 activities in hot water-treated lime fruit at 50 °C for 5 min reduced after a temporary increase
269 during storage. Chlase activity in the control gradually increased with senescence during
270 storage in Thai lime (*Citrus aurantifolia* Swingle cv. Paan), the same as in Tahitian lime fruit
271 (Kaewsuksaeng et al., 2011). In contrast, it was previously reported that Chlase activity
272 decreased with degreening of *Citrus nagato-yuzukichi* (Yamauchi et al., 2003) and broccoli
273 florets (Aiamla-or et al., 2010). We also found that Chlase activity was tentatively suppressed
274 in lime fruit throughout storage by hot water treatment at 50 °C for 5 min. The mature green
275 lime was shown to have a high accumulation level of Chlide *a* during storage (Srilaong et al.,
276 2011), which suggests that the high level of Chlide *a* is due to the increased Chlase action in
277 the flavedo. This enzyme is involved in the first step of the chlorophyll catabolic pathway,
278 which catalyzes the conversion of Chl *a* to Chlide *a* and phytol (Harpaz-Saad et al., 2007).

279 Our results indicated that Chl-POX activity markedly increased in lime fruit during
280 storage, but its activity was clearly suppressed throughout the storage period with hot water
281 treatment at 50 °C for 5 min. In lime fruit, Chl *a* can be degraded by Chl-POX (Win et al.,
282 2006a) to form the oxidized Chl *a*, 13^2-hydroxychlorophyll *a*, which did not accumulate

283 during storage by UV-B treatment (Aimala-or et al., 2010). Mg-dechelation activity by MDS
284 was also determined by using Chlide *a* as a native substrate in lime fruit. MDS, which is a
285 small molecule and heat-stable substance, was required to remove the magnesium atom from
286 Chlide *a* (Suzuki et al., 2005; Kaewsuksaeng et al., 2010). We found that Mg-dechelation
287 activity increased sharply in day 5 after hot water treatment due to the stress from high
288 temperature and after that hot water treatment at 50 °C for 5 min also effectively inhibited
289 Mg-dechelation. These results were similar to those by Kaewsuksaeng et al. (2011) that Mg-
290 dechelation activity in Tahitian lime increased slightly in the control and that UV-B treatment
291 also effectively suppressed the enhancement of Mg-dechelation. Srilaong et al. (2011)
292 demonstrated that the Pheide *a* level declined in UV-B-treated fruit, especially during the
293 development of yellowing. This indicates that the degradation of Chlide *a* to Pheide *a* might
294 be suppressed by UV-B. Further study needs to clarify the Chl derivatives and Chi-degrading
295 enzymes in heat-treated lime fruit. These findings show that Mg-dechelation activity is
296 significantly involved in the Chl-degrading process in lime fruit peel.

297 Recently reported as a new Chl-degrading enzyme, PPH dephytylates Phein *a* to form
298 Pheide *a*. It is inferred that PPH activity measured in this study might include some activity
299 of chlorophyllase, since it can also dephytylate Phein *a* along with Chl *a* (McFeeters et al.,
300 1971; Mínguez-Mosquera et al., 1994). Kaewsuksaeng et al. (2011) showed that PPH activity
301 gradually increased during storage in Tahitian lime fruit, while the UV-B treatment
302 effectively suppressed PPH activity. For broccoli florets, it was found that PPH activity with
303 or without UV-B treatment also increased during storage at 15 °C, but UV-B did not
304 significantly affect PPH activity (Aiamla-or et al., 2012). In contrast, our results found a
305 temporary increase in PPH activity in Thai lime after heat treatment, after that a decline
306 during storage of lime fruit. However, hot water treatment at 50 °C for 5 min could greatly
307 inhibit PPH activity in lime fruit. These findings seems to be due to the suppression of Chl-

308 degrading enzymes such as Chlase, Mg-dechelation, Chl-degrading peroxidase and PPH
309 activities by stress treatment such as hot water in Thai lime fruit. Further study needs to be
310 carried out to clarify the characterization of Chl-degrading enzymes in relation to Chl
311 degradation of lime fruit.

312 Besides Chl degradation, ethylene production and respiration rate were also determined.
313 The results showed that the endogenous ethylene production slightly increased from mature
314 green to full yellow in the control, while that in the hot water-treated fruit at 50 °C for 5 min
315 did not change during storage. Application of heat treatment such as hot water at 50 °C for 5
316 min significantly reduced ethylene production. This might be because of the suppression of
317 ethylene production by hot water treatment due to the decrease of ACC (1-
318 aminocylocpropane-1-carboxylic acid) oxidase action in the ethylene pathway in lime fruit
319 (Win et al., 2006b). The composition changes in relation to quality occur during the storage
320 of lime fruit. The citric acid content, determined as titratable acidity in lime fruit, showed a
321 slight change during storage. This content was higher in hot water- treated fruit at 50 °C for 5
322 min than any other during storage, suggesting that this acid might be maintained due to the
323 suppression of respiration rate by hot water treatment. Hot water treatment was similar to a
324 UV-B treatment in maintaining the acid content in lime fruit (Kaewsuksaeng et al., 2011).
325 Hot water treatment at 50 °C for 5 min also suppressed the changes in total soluble solids
326 during storage. On the other hand, total soluble solids in the control increased with the
327 advance of senescence. The results agree with the effect of heat treatment on reducing sugar
328 level changes (Lemoine et al., 2008). Thus, hot water treatment seems to be a useful
329 treatment for the maintenance of postharvest internal quality in lime fruit.

330 In conclusion, the findings obtained in the present study show that heat treatment such as
331 hot water at 50 °C for 5 min effectively retarded the Chl degradation in Thai lime fruit during
332 storage. Hot water treatment at 50 °C for 5 min also suppressed activities of Chl-degrading

enzymes, such as Chlase, Chl-POX, Mg-dechelation, and PPH, indicating that the inhibition of those enzyme activities by heat treatment could be involved in the retardation of Chl degradation in stored lime fruit. Furthermore, hot water treatment at 50 °C for 5 min reduced physiological changes such as ethylene production and respiration rate during storage. Hot water treatment at 50 °C for 5 min induced a gradual increase in titratable acidity and suppressed the increase of total soluble solids during storage. We conclude that hot water treatment at 50 °C for 5 min could be a useful method to prolong the postharvest quality of Thai lime fruit during storage.

Acknowledgement

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References

Aiamla-or, S., Kaewsuksaeng, S., Shigyo, M., Yamauchi, N., 2010. Impact of UV-B irradiation on chlorophyll degradation and chlorophyll-degrading enzyme activities in stored broccoli (*Brassica oleracea* L. Italica Group) florets. Food Chem. 120, 645–651.

Aiamla-or, S., Nakajima, T., Shigyo, M., Yamauchi, N., 2012. Pheophytinase activity and gene expression of chlorophyll-degrading enzymes relating to UV-B treatment in postharvest broccoli (*Brassica oleracea* L. Italica Group) florets. Postharvest Biol. Technol. 63, 60–66.

Amir-Shapira, D., Goldschmidt, E. E., Altman, A., 1987. Chlorophyll catabolism in senescing plant tissues: in vivo breakdown intermediates suggest different degradative

357 pathways for citrus fruit and parsley leaves. Proc. Natl. Acad. Sci. USA 84, 1901 –
358 1905.

359 Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram
360 quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem.72,
361 248–254.

362 Costa, M. L., Civello, P. M., Chaves, A. R., Martinez, G. A., 2006. Hot air treatment
363 decreases chlorophyll catabolism during postharvest senescence of broccoli (*Brassica*
364 *oleracea* L. var. italica) heads. J. Sci. Food Agric. 86, 1125–1131.

365 Falik, E., 2004. Prestorage hot water treatments (immersion, rinsing and brushing).
366 Postharvest Biol. Technol. 32, 125–134.

367 Funamoto, Y., Yamauchi, N., Shigenaga, T., Shigyo, M., 2002. Effects of heat treatment on
368 chlorophyll degradation enzymes in stored broccoli (*Brassica oleracea* L.). Postharvest
369 Biol. Technol. 24, 163–170.

370 Funamoto, Y., 2003. Chlorophyll degradation in stored broccoli (*Brassica oleracea* L.)
371 florets and its control by heat treatment. Ph.D. Thesis. The United Graduate School of
372 Agriculture Sciences, Tottori Univ., Tottori (In Japanese with English summary).

373 Harpaz-Saad, S., Azoulay, T., Arazi, A., Ben-Yaakov, E., Mett, A., Shiboleth, Y.M.,
374 Hörtensteiner, S., Gidoni, D., Gal-On, A., Goldschmidt, E.E., Eyal, Y., 2007.
375 Chlorophyllase is a rate-limiting enzyme in chlorophyll catabolism and is
376 posttranslationally regulated. Plant Cell. 19, 1007–1022.

377 Kaewsuksaeng, S., Yamauchi, N., Funamoto, Y., Shigyo, M., Kanlayanarat, S., 2006. Effect
378 of Mg-dechelation activity on chlorophyll degradation in stored broccoli florets. Acta
379 Hort. 712, 705-709.

380 Kaewsuksaeng, S., Yamauchi, N., Funamoto, Y., Mori, T., Shigyo, M., Kanlayanarat, S.,
381 2007. Effect of heat treatment on catabolites formation in relation to chlorophyll
382 degradation during storage of broccoli (*Brassica oleracea* L. italica group) florets.
383 J. Jap. Soc. Hort. Sci. 76, 338–344.
384 Kaewsuksaeng, S., Yamauchi, N., Funamoto, Y., Aiamla-or, S., Mori, T., Shigyo, M.,
385 Kanlayanarat, S., 2010. Partially purification of Mg-dechelatase in relation to
386 chlorophyll degradation in broccoli (*Brassica oleracea* L. Italica Group) florets. Acta
387 Hort. 875, 509 – 514.
388 Kaewsuksaeng, S., Urano, Y., Aiamla-or, S., Shigyo, M., Yamauchi, N., 2011. Effect of UV-
389 B irradiation on chlorophyll-degrading enzyme activities and postharvest quality in
390 stored lime (*Citrus latifolia* Tan.) fruit. Postharvest Biol. Technol. 61, 124–130.

391 Langmeier, M., Ginsburg, S., Matile, P., 1993. Chlorophyll breakdown in senescent leaves:
392 demonstration of Mg-dechelatase activity. Physiol. Plant. 89, 347 – 353.

393 Lemoine, M. L., Civello, P. M., Chaves, A. R., Martínez G. A., 2008. Effect of combine
394 treatment with hot air and UV-C on senescence and quality parameters of minimally
395 processed broccoli (*Brassica oleracea* L. var. *Italica*). Postharvest Biol. Technol. 48,
396 15–21.

397 Lu, J., Charles, M. T., Vigneault, C., Goyette, B., Raghavan, G.S.V., 2010. Effect of heat
398 treatment uniformity on tomato ripening and chilling injury. Postharvest Biol. Technol.
399 56, 155–162.
400 Lurie, S., Mitcham, E. J., 2007. Physiological response of agriculture commodities to heat
401 treatment. P. 79-104. In: J. Tang et al. (eds.) Heat treatments for postharvest pest
402 control: theory and practice. CABI, Oxon.

403 Matile, P., Hörtensteiner, S., Tomas, H., 1999. Chlorophyll degradation, Annu. Rev. Plant
404 Physiol. Plant Mol. Biol. 50, 69 – 75.

405 McFeeters, R.F., Chichester, C.O., Whitaker, J.R., 1971. Purufication and properties of
406 chlorophyllase from *Ailanthus altissima* (tree-of-heaven). Plant Physiol. 47, 609-618.

407 Mínguez-Mosquera, M.I., Gandul-Rojas, B., Gallardo-Guerrero, L., 1994. Measurement of
408 chlorophyllase activity in olive fruit (*Olea europaea*). J. Biochem. 116, 263-268.

409 Moran, R., 1982. Formulae for determination of chlorophyllous pigments extracted with *N,N*-
410 dimethylformamide. Plant Physiol. 69, 1376–1381.

411 Ogo, Y., Umeno, C., Shigyo, M., Yamauchi, N., 2011. Control of degreening during storage
412 by heat treatment in green sour citrus fruit. Hort. Res. (Japan) 10 (Suppl. 1): 473 (In
413 Japanese).

414 Perkins, H. J., Roberts, D. W., 1962. Purification of chlorophylls, pheophytins and
415 pheophobides for specific activity determinations. Biochim. Biophys. Acta,
416 58, 486–498.

417 Pranmornkith, T., Mawson, A. J., Heyes, J. A., 2005. Effect of CA and alternative
418 postharvest treatments on quality of lime (*Citrus latifolia* Tanaka) fruit. In: Proceedings
419 of 9th International Controlled Atmosphere Research Conference, July 5–10, 2005,
420 Michigan State University, ISHS, pp. 21–27.

421 Schelbert, S., Aubry, S., Burla, B., Agne, B., Kessler, F., Krupinska, K., Hörtensteiner, S.,
422 2009. Pheophytin pheophorbide hydrolase (Pheophytinase) is involved in chlorophyll
423 breakdown during leaf senescence in *Arabidopsis*. Plant Cell. 21, 767 – 785.

424 Shimokawa, K., Shimada, S., Yaeo, K., 1978. Ethylene-enhanced chlorophyllase activity
425 during degreening of *Citrus unshiu* Marc. Sci. Hort. 8, 129 – 135.

426 Shioi, Y., Tomita, N., Tsuchiya, T., Takamiya, K., 1996. Conversion of chlorophyllide to
427 pheophorbide by Mg-dechelating substance in extracts of *Chenopodium album*. Plant
428 Physiol. Biochem. 34, 41 – 47.

429 Spadoni, A., Guidarelli, M., Sanzani, M.S., Ipplito, A., Mari, M., 2014. Influence of hot
430 water treatment on brown rot of peach and rapid fruit response to heat stress.
431 Postharvest Biol. Technol. 94, 66–73.

432 Srilaong, V., Aiamla-or, S., Soontornwat, A., Shigyo, M., Yamauchi, N., 2011. UV-B
433 irradiation retards chlorophyll degradation in lime (*Citrus latifolia* Tan.) fruit.
434 Postharvest Biol. Technol. 59, 110–112.

435 Suzuki, T., Kunieda, T., Murai, F., Morioka, S., Shioi, Y., 2005. Mg-dechelation activity in
436 radish cotyledons with artificial and native substrates, Mg-chlorophyllin *a* and
437 chlorophyllide *a*. Plant Physiol. Biochem. 43, 459 – 464.

438 Viña, S. Z., Osornio, M. M. L., Chaves, A. R., 2007. Quality changes in fresh-cut celery as
439 affected by heat treatment and storage. J. the Sci. of Food and Agri. 87 (7), 1400–1407.

440 Win, T. O., Srilaong, V., Kyu, K. L., Poomputsa, K., Kanlayanarat, S., 2006a. Biochemical
441 and physiological changes during chlorophyll degradation in lime (*Citrus aurantifolia*
442 Swingle cv. 'Paan'). J. Hort. Sci. Biotech. 81, 471 – 477.

443 Win, T. O., Srilaong, V., Heyes, J., Kyu, K. L., Kanlayanarat, S., 2006b. Effect of different
444 concentrations of 1-MCP on yellowing of West Indian lime (*Citrus aurantifolia*,
445 Swingle) fruit. Postharvest Biol. Technol. 42, 23–30.

446 Yamauchi, N., Harada, K., Watada, A. E., 1997. In vitro chlorophyll degradation in stored
447 broccoli (*Brassica oleracea* L var. italica Plen) florets. Postharvest Biol. Technol., 12,
448 239–245.

449 Yamauchi, N., Eguchi, K., Tokuhara, Y., Yamashita, Y., Oshima, A., Shigyo, M., Sugimoto,
450 K., 2003. Control of degreening by sucrose laurate ester and heat treatment of *Citrus*
451 *nagato-yuzukichi* fruit. J. Hort. Sci. Biotechnol. 78, 563 – 567.
452 Yamauchi, N., Funamoto, Y., Shigyo, M., 2004. Peroxidase-mediated chlorophyll
453 degradation in horticultural crops. Phytochem. Rev. 3, 221–228.
454 Yamauchi, N., 2013. Quality maintenance of postharvest horticultural crops by stress
455 treatment and approach for elucidation of its mechanism. J. Jap. Soc. Hort. Sci. 82, 1–
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477 **Legends of Figures**

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479 Figure 1. Changes in the hue angle value in lime fruit treated with hot water at 50 °C for 3 or
480 5 min during storage at 25 °C. Vertical bars represent the average values with SE (n
481 = 3).
482
483 Figure 2. Changes in titratable acidity (A) and total soluble solids (B) of the juice in lime fruit
484 treated with hot water at 50 °C for 3 or 5 min during storage at 25 °C. Vertical bars
485 represent the average values with SE (n = 3).
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487 Figure 3. Changes in Chl *a* content in lime fruit treated with hot water at 50 °C for 3 or 5 min
488 during storage at 25 °C. Vertical bars represent the average values with SE (n = 3).
489 Chl: chlorophyll
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492 Figure 4. Changes in Chlase (A) and Chl-POX (B) activities in lime fruit treated with hot
493 water at 50 °C for 3 or 5 min during storage at 25 °C. Vertical bars represent the
494 average values with SE (n = 3).
495 Chlase: chlorophyllase , Chl-POX: chlorophyll-degrading peroxidase
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498 Figure 5. Changes in Mg-dechelation (A) and PPH (B) activities in lime fruit treated with hot
499 water at 50 °C for 3 or 5 min during storage at 25 °C. Vertical bars represent the
500 average values with SE (n = 3).
501 PPH: pheophytinase
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504 Figure 6. Changes in ethylene production (A) and respiration rate (B) in lime fruit treated
505 with hot water at 50 °C for 3 or 5 min during storage at 25 °C. Vertical bars
506 represent the average values with SE (n = 3).
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523 **Figure 1.**
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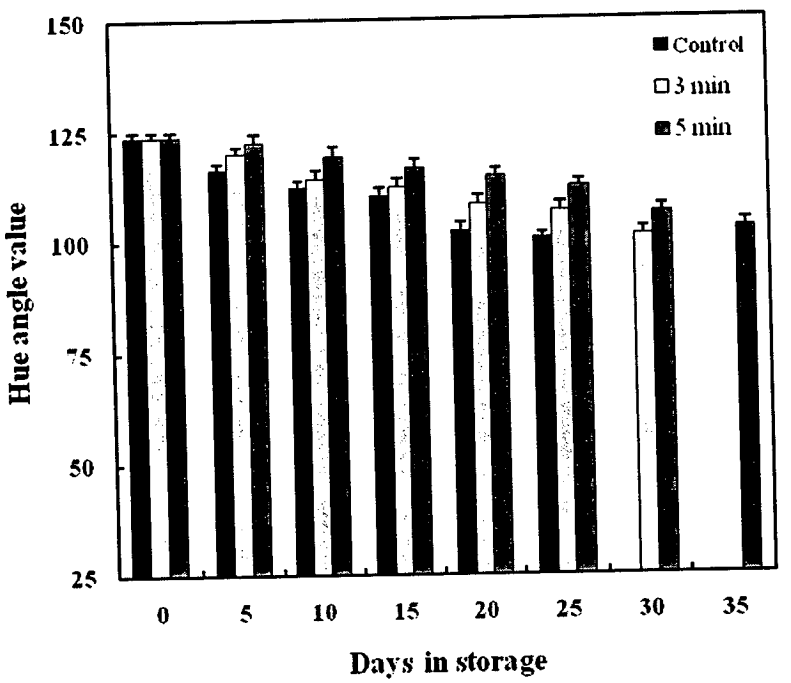

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550 **Figure 2.**

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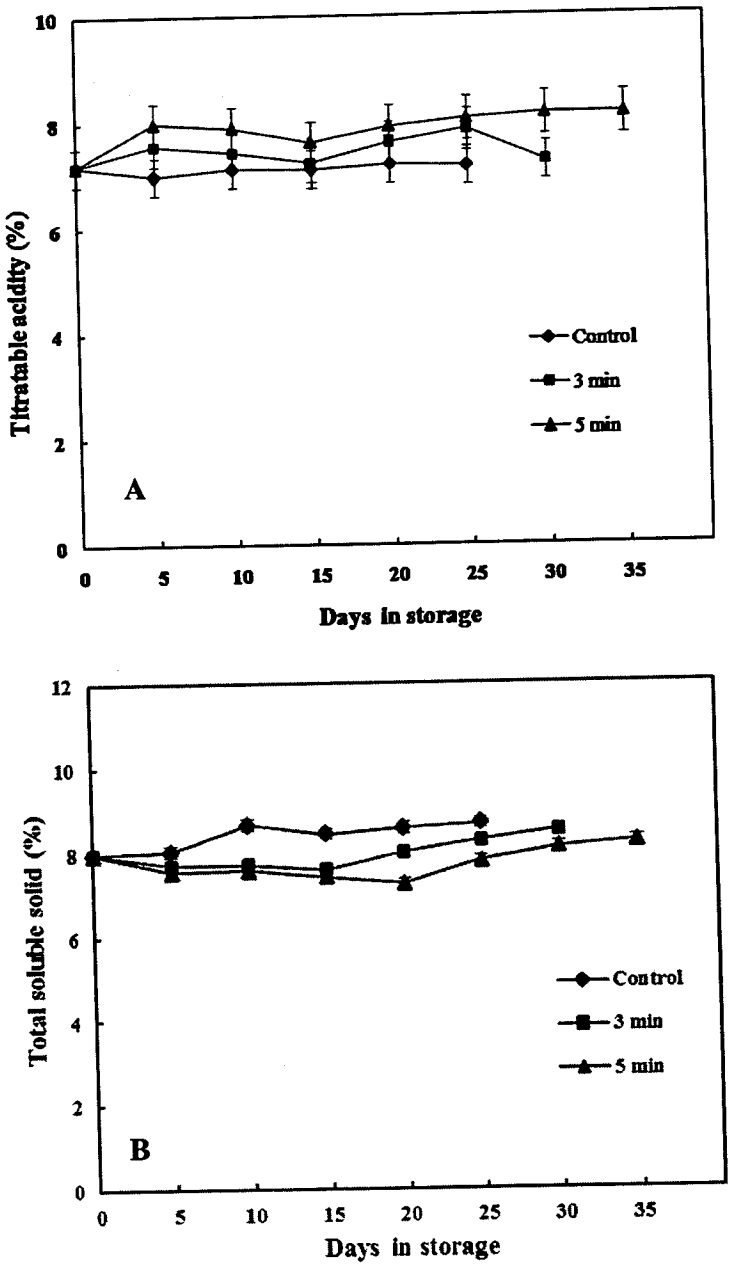

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577 **Figure 3.**

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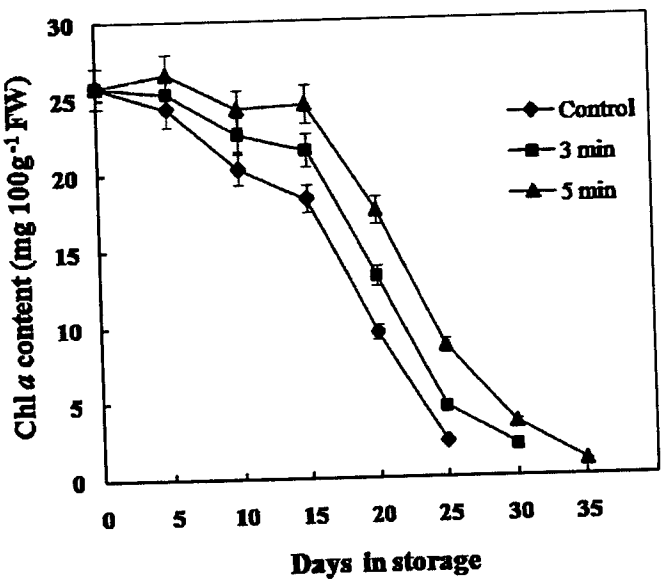

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605 **Figure 4.**

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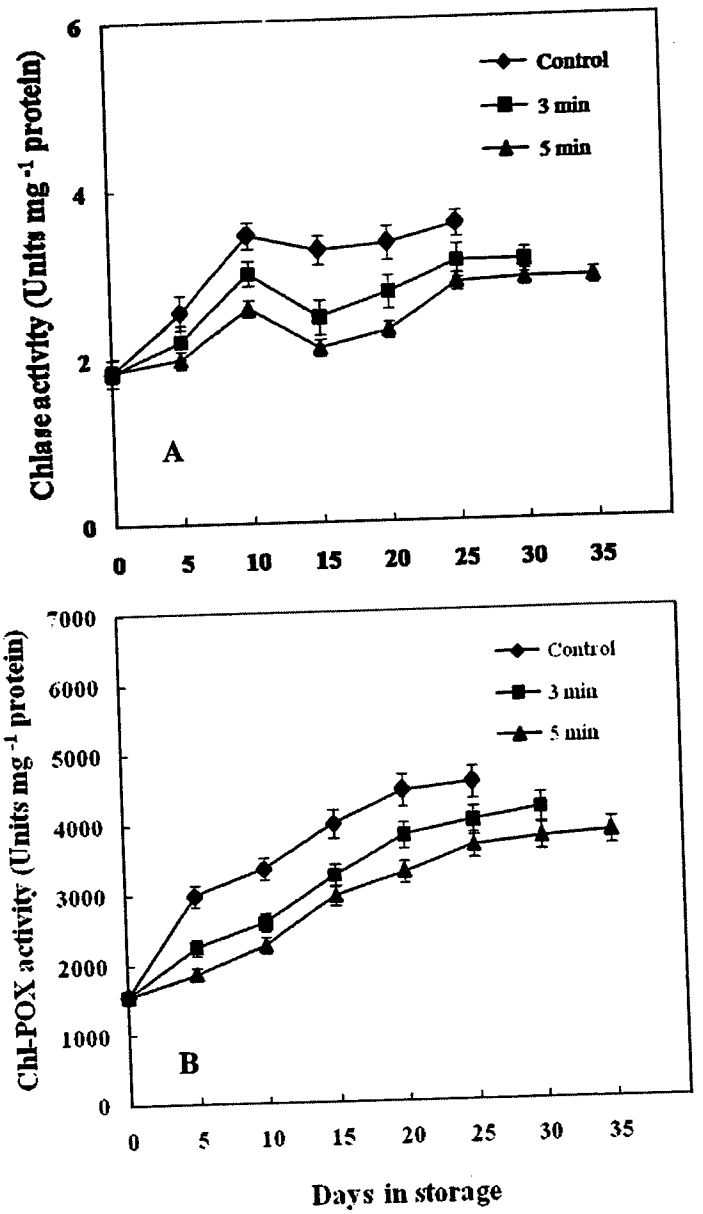

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631 **Figure 5.**

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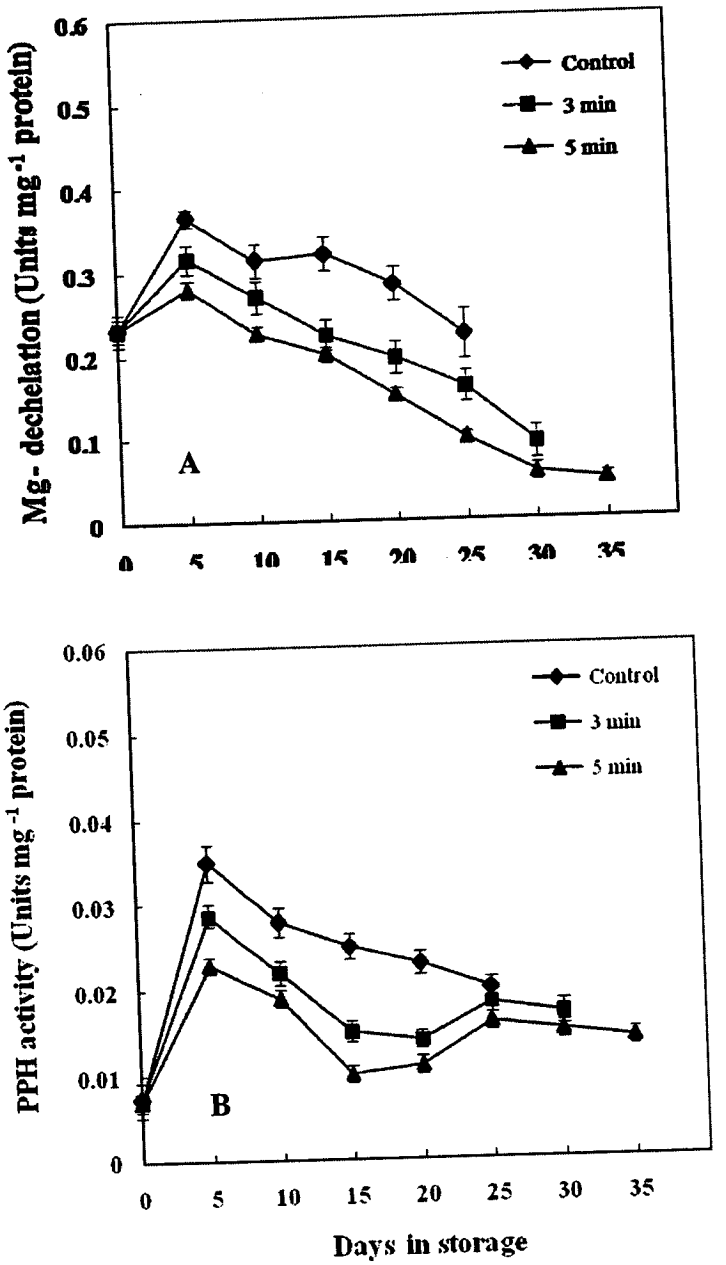

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