Completed Report

The Chlorophyll Degradation and Postharvest Quality Maintenance of Lime

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Abstract

Postharvest treatments such as UV-B irradiation and hot water treatments, were applied to lime (*Citrus latifolia* Tan. and *Citrus aurantifolia* Swingle cv. Paan) fruits to investigate their effect on chlorophyll degradation with chlorophyll-degrading enzyme activities and postharvest quality during storage at 25˚C. Mature green lime fruit were irradiated with UV-B doses at 19.0 kJm^{-2}, then stored at 25 ◦C in darkness. Green lime fruit were also treated with hot water at 50 ˚C for 3 and 5 min and then kept at 25 ˚C in darkness. UV-B treatment at 19.0 kJm^{-2} and hot water treatment at 50 ˚C for 5 min efficiently delayed the decrease of the hue angle value and the contents of chlorophylls *a* and *b*. Chlorophyllase, chlorophyll-degrading peroxidase and pheophytinase activities with UV-B treatment and hot water treatment were suppressed during storage, as well as the activity of Mg-dechelation activity was also retarded with these treatments. In postharvest quality, UV-B treatment induced a gradual increase in citric acid and malic 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. The weight loss and the opening of stomata were reduced by UV-B treatment. Moreover, hot water treatment at 50 ˚C for 5 min showed the highest maintenance of total acidity and the suppression of an increase of total soluble solid during storage.

Key words: lime, UV-B, hot water, chlorophyll degradation, chlorophyll-degrading enzyme, quality

บทคัดย่อ

การศึกษาการสลายตัวของคลอโรฟิลล์และการควบคุมคุณภาพหลังการเก็บเกี่ยวของมะนาว 2 พันธุ์ (*Citrus latifolia* Tan. and *Citrus aurantifolia* Swingle cv. Paan) โดยการฉายรังสียูวีบีและการจุ่มน้ำร้อน หลังจากนั้นชุดที่ฉายรังสียูวีบีนำมาเก็บรักษาในที่มืด ที่อุณหภูมิ 25 องศาเซลเซียส ขณะที่มะนาวชุดที่จุ่มน้ำร้อน เก็บรักษาที่อุณหภูมิ 13 องศาเซลเซียส การทดลองพบว่ามะนาวที่ฉายรังสียูวีบีที่ความเข้มข้น 19.0 กิโลจูลย์ต่อตารางเมตร และมะนาวที่จุ่มน้ำร้อนที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 3 และ 5 นาที มีประสิทธิภาพสูงในการชะลอการเปลี่ยนแปลงสีเปลือกจากสีเขียวไปเป็นสีเหลือง โดยชะลอการลดลงของค่า Hue angle ปริมาณคลอโรฟิลล์เอและบี และยับยั้งกิจกรรมเอนไซม์ที่เกี่ยวข้องกับการสลายตัวของคลอโรฟิลล์ 4 ชนิด ได้แก่ Chlorophyllase, chlorophyll-degrading peroxidase, pheophytinase และ Mg-dechelation ตลอดระยะเวลาการเก็บรักษาได้ดีกว่าชุดควบคุม นอกจากนี้มะนาวที่ผ่านการฉายรังสียูวีบีที่ความเข้มข้น 19.0 กิโลจูลย์ต่อตารางเมตร สามารถควบคุมคุณภาพภายในหลังการเก็บเกี่ยวได้ พบว่ามีชักนำให้เกิดการเพิ่มขึ้นของปริมาณกรดอินทรีย์ ได้แก่ กรดซิตริก กรดมาลิกและกรดแอสคอบิก และยับยั้งการเพิ่มขึ้นของปริมาณน้ำตาลตลอดระยะเวลาการเก็บรักษา เช่นเดียวกับที่มะนาวที่จุ่มน้ำร้อนที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 5 นาที มีประสิทธิภาพในการรักษาปริมาณกรดที่ไทเทรตได้และปริมาณของแข็งที่ละลายน้ำได้มากที่สุด ในทางการเปลี่ยนแปลงด้านสรีรวิทยาหลังการเก็บเกี่ยว มะนาวที่ผ่านการฉายรังสียูวีบีที่ความเข้มข้น 19.0 กิโลจูลย์ต่อตารางเมตร สามารถลดการสูญเสียน้ำและชักนำให้เกิดการปิดของปากใบที่เปลือกผลได้ดีกว่าชุดควบคุม และมะนาวที่จุ่มน้ำร้อนที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 5 นาที สามารถลดอัตราการหายใจและการผลิตเอทิลีนตลอดระยะเวลาการเก็บรักษาได้

คำสำคัญ; มะนาว ยูวีบี การจุ่มน้ำร้อน คลอโรฟิลล์ คุณภาพหลังการเก็บเกี่ยว

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PUBLICATIONS

1. **Kaewsuksaeng, S**., Urano, Y., Aiamla-or, S., Shigyo, M. and Yamauchi, N. 2011. Effect of UV-B irradiation on chlorophyll-degrading enzyme activities and postharvest quality in stored lime (*Citrus latifolia* Tan.) fruit. **Postharvest Biol. Technol.** 61: 124-130.
2. **Kaewsuksaeng**, S. 2011. Chlorophyll degradation in horticultural crops (Review). **Walailak J. Sci. & Technol.** 8(1): 9-19.
3. **Kaewsuksaeng, S.,** Tatmala, N., Srilaong, V. and Pongprasert, N., 2015. Postharvest heat treatment delays chlorophyll degradation and maintains quality in Thai lime (*Citrus aurantifolia* Swingle cv. Paan) fruit. **Postharvest Biol. Technol.** 100: 1-7.

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CHAPTER 1

INTRODUCTION

One of the symptoms of senescence in harvested horticultural crops is the loss of greenness that comes with the degradation of chlorophyll (Chl). With senescence, the chlorophyll-degrading enzyme activities such as chlorophyllase (Chlase) (Shimokawa et al., 1978; Amir-Shapira et al., 1987), Mg-chelatase (MD) or Mg-dechelation activity (Langmeier et al., 1993; Kaewsuksaeng et al., 2010), a new chlorophyll-degrading enzyme, pheophytinase (Schelbert et al., 2009), pheophorbidase and chlorophyll-degrading peroxidase (Yamauchi et al., 2004), which are involved in chlorophyll degradation, affected greatly in stored horticultural crops. The chlorophyll derivatives, especially chlorophyllide (Chlide), pheophytin (Phein), pheophorbide (Pheide) 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 envelop membrane of chloroplast and/ or the vacuole.

Qualities in limes (Citrus latifolia Tan. and Citrus aurantifolia Swingle cv. Paan) deteriorate quickly after harvest. The most visible deterioration factor is the loss of peel greenness that usually occurs together with Chl degradation (Drazkiewice, 1994; Srilaong et al., 2011). Maintenance of the green colour 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 Chlide *a* by chlorophyllase (Chlase). 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 Pheide *a* is induced by a Mg-dechelatase (MD) (Langmeier et al., 1993; Kaewsuksaeng et al., 2006, 2007) or 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 primary 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 with oxidizes 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, Phein *a* to give Pheide has been recently reported (Schelbert et al., 2009).

The UV treatment as an advance technique is required to maintain in postharvest quality of horticulture crop in order to delay the Chl degradation. UV-C, especially, seems to be 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 the Chl derivative levels in mature green lime during storage.

The 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 heat-treated citrus *nagato-yuzukichi* fruit (Yamaguchi et al., 2003), broccoli (Funamoto et al., 2002; Kaewsuksaeng et al., 2007), leek (Tsuvaltis et al., 2006) and celery (Viňa and Chaves, 2007).

However, the effect of the UV-B on Chl-degrading enzymes activities and postharvest quality in lime fruit has not been determined. In this paper, we therefore examined the effect of UV-B irradiation on Chl-degrading enzymes activities and the resultant quality control.

Moreover, the effect of the heat treatment on postharvest quality in lime fruit has not been yet applied. We, therefore, examined the effect of hot water treatment on Chl degradation with Chl-degrading enzyme activities changes. We also evaluate the physiological changes and the resultant quality control during storage in heat-treated lime.

Objectives

1 To study the mechanism of chlorophyll degradation in lime fruit.

2. To study the effect of UV-B irradiation on chlorophyll degradation and postharvest quality of lime fruit.

3. To study of effect of heat treatment on chlorophyll degradation and postharvest quality of lime fruit.

CHAPTER 2

LITERATURE REVIEWS

In general, the yellowing of leaves, florets and fruit pericarp is an important factor, indicating quality deterioration of stored horticultural products. Obviously, in spinach (Yamauchi and Watada, 1991), parsley (Yamauchi and Watada, 1993), broccoli (Costa et al., 2005), and lime (Win et al., 1991; Srilaong et al., 2011), 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 (Shimokawa et al., 1998). Thus, chlorophyll degradation is a characteristic symptom of leaf senescence and fruit ripening, and elucidating the mechanism of Chl degradation is important subject when considering the maintenance of the quality of harvested horticultural crops.

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 Chl degradation (Drazkiewice, 1994; Srilaong et al., 2011). Maintenance of the green color in the peel of lime is desirable during storage.

In Thailand lime (*Citrus aurantifolia* Swingle cv. Paan) is economically significant horticultural crop and the main production season is July to September (Pranamornkith et al., 2010). The lime fruits are harvest when the rind is still green. The price of lime fruit is strongly dependent on its availability and quality characteristics. Postharvest quality in lime deteriorates quickly after harvest. The most visible deterioration factor is the loss of peel greenness that usually occurs together with Chl degradation (Srilaong et al., 2011; Kaewsuksaeng et al., 2011). For the maintenance of postharvest quality, it is necessary to retain the green color in the peel of lime as long as possible.

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 (Shimokawa et al., 1978). The elimination of Mg^{2+} from Chlide *a* to produce pheophorbide (Pheide) *a* is induced by a Mg-dechelatase (MD) (Kaewsuksaeng et al., 2006, 2007) or Mg-dechelating substance (MDS) (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 primary colorless, via a red Chl catabolite by both Pheide *a* oxygenase and red Chl catabolite reductase (Matile et al., 1999). Chl-degrading peroxidase (POX) (Kaewsuksaeng et al., 2007) is also suggested to be involved in Chl degradation as the first 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 (Schelbert et al., 2009).

Chlorophyll structure

Chls are porphyrins containing basic tetrapyrrole rings, of which one is reduced. The four rings are coordinated with Mg^{2+} ion. A fifth isocyclic ring E, is found near the third pyrrole ring. At the fourth 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 2.1) (Hörtensteiner and Kräutler, 2000).

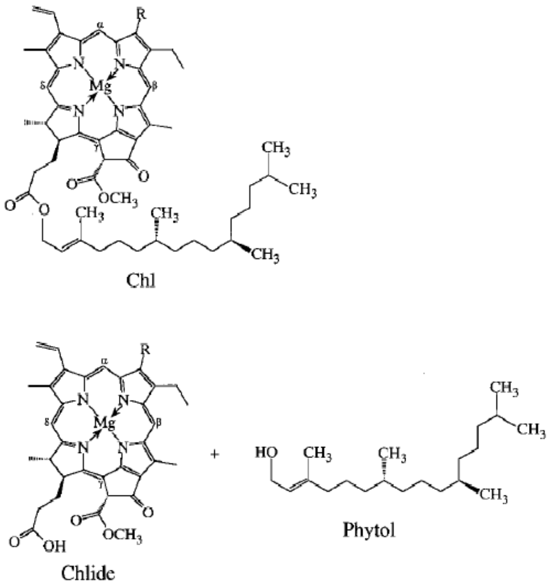

Figure 2.1 Structure formulae top: Chl *a* (R=CH_3), Chl *b* (R= CH=O); bottom: Chlide *a* (R=CH_3), Chlide *b* (R= CH=O) and phytol.

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 either by acid or alkali. Chlides *a* and *b* are prepared enzymatically, the hydrolysis being catalyzed by Chlase, enzyme commonly found in green plant tissues. Leaves that are especially rich in Chlase such as sugar beet (Holden, 1961), common cocklebur (*Xanthium pennsylvanicum*) (Pennington et al., 1964), goosefoot (*Chenopodium album)* (Shioi et al., 1996) and *Citrus unshiu* fruit (Yamauchi et al., 2003) 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% (Pennington et al., 1964; Hynninen et al., 1973).

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 (Pennington et al., 1964; Hynninen et al., 1771).

4. C13^2-hydroxychlorophyll a and b

Chl *a* are 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 (Maunders et al., 1983) and broccoli, which was prepared by adding peroxidase with the existence of H_2O_2 and *p*-coumaric acid to Chl *a* solution (Funamoto et al., 2002).

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 (Pennington et al., 1964).

The chlorophyll degradation pathway

The generally accepted pathway of chlorophyll degradation comprises two stages, before (early stage) and after (late stage) cleavage of 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 second 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 2.2) (Takamiya et al., 2000).

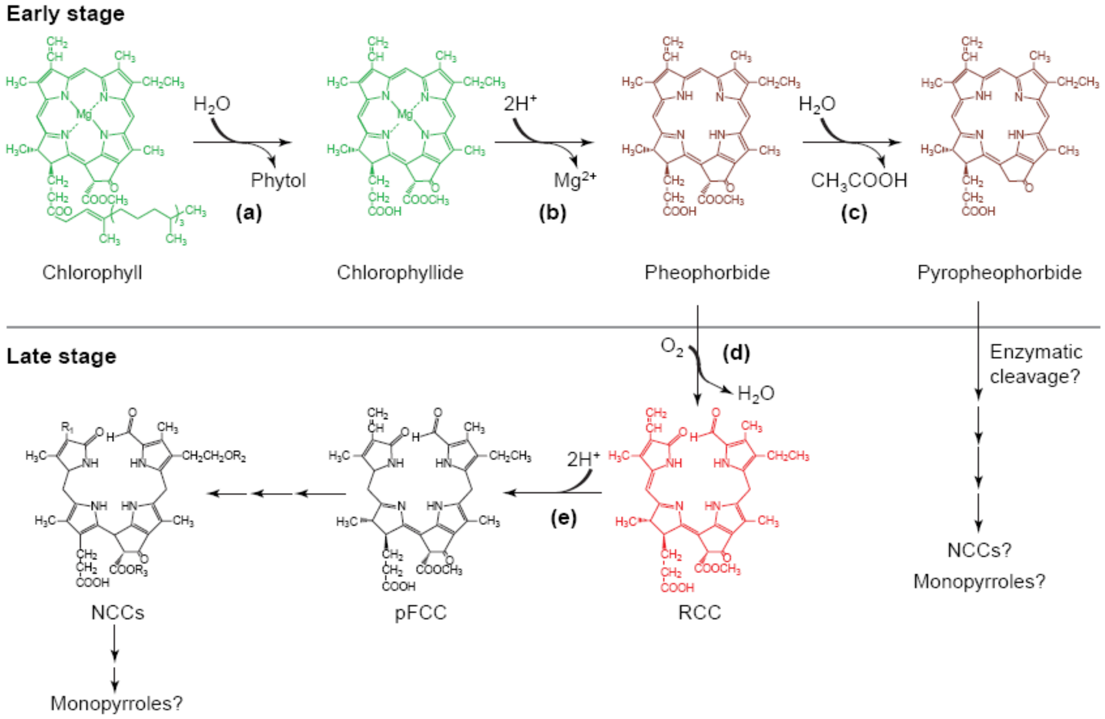

Figure 2.2 Chlorophyll degradation in higher plants (a) chlorophyllase (b) Mg-dechelatase (c) Pheophorbidase (d) Pheophorbide *a* oxygenase. (e) Red cholorphyll catabolite reductase. Abbreviations: NCCs, nonfluorescent chlorophyll catabolites; pFCC, primary fluorescent chlorophyll catabolite; RCC, red chlorophyll catabolite.

Characterization of Chlorophyll-degrading enzymes in relation to chlorophyll degradation

1. Chlorophyllase

The enzyme catalyzing the dephytylation, Chlase was one of the first plant enzymes to be studied (Willstätter and Stoll, 1913). 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 (Drazkiewice, 1994). In spite of repeated isolations of Chlase from various plants and algae (Trebitsh et al., 1993; Khalyfa et al., 1995; Tsuchiya et al., 1999), 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 first 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 (Matile et al., 1997). 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 (Matile et al., 1997; 1999). The Chl molecule or Chl–protein complex released from such plastoglobuli could be attacked by Chlase in the vacuole more than the chloroplast (Parthier, 1988; Satoh et al., 1998).

2. Mg-dechelation activity

Mg-dechelation takes place after dephytylation to yield pheophorbide *a* and pheophorbin *a* using Chlide *a* and Chlin *a* as substrates (Kanieda et al., 2005). Initially, the *in vivo* and *in vitro* accumulation of pheopigments during Chl degradation of algae and higher plants suggested the presence of MD enzymes

(Kaewsuksaeng et al., 2006, 2007). Furthermore, *in vitro* assays of the dechelatase activity revealed that it was associated with thylakoid membranes in a latent form of rape cotyledon (Langmeier et al., 1993). By contrast, in *Chenopodium album*, the activity could still be detected in a soluble with a low molecular mass (900 Da.) fraction after gel filtration of the enzyme, and it was heat stable. This activity was thus designated MDS (Shioi et al., 1996). Costa et al. (2002) reported that the Mg-dechelation activity was associated with a compound with a low molecular weight substance of 2180 ± 20 Da. Suzuki et al. (2005) and Kunieda et al. (2005) 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 be the molecular masses of 3.3 and 1.1 KDa (Kunieda et al., 2005). Lastly, the characterization of Mg-dechelation activity of stored broccoli florets was more investigated to clarify the mechanism of Chl degradation (Kaewsuksaeng et al., 2010). Only one peak of the activity was detected in fresh broccoli extract and no other isozyme with Mg-dechelating action was found in the yellow broccoli extract and molecular mass was about 70 KDa.

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 (Watanabe et al., 1999). This is not stable and therefore is nonenzymatically converted to pyropheophorbide (Pyropheide). Interestingly, pheophorbidase is located outside the chloroplast (Kunieda et al., 2005). If Pheide *a* is a true substrate of the enzyme, it suggests that there is another degradation pathway whose early steps occur outside 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 (Janave, 1997; Maeda et al., 1998) and might be an intermediate in the oxidative Chl bleaching pathway as well (Janave, 1997).

4. Chlorophyll degrading-peroxidase

The mechanism of *in vitro* Chl degradation by peroxidase can be summarized as shown in Figure 2.3 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 (Yamauchi et al., 2004) . Martinoia et al. (1982) demonstrated that peroxidative Chl bleaching activity was present in the thylakoid membrane of barley seedlings. Abeles et al. (1988) 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 (1998) 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 (Funamoto et al., 2002). By the method of native-PAGE, six anionic and two 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 (Funamoto et al., 2003).

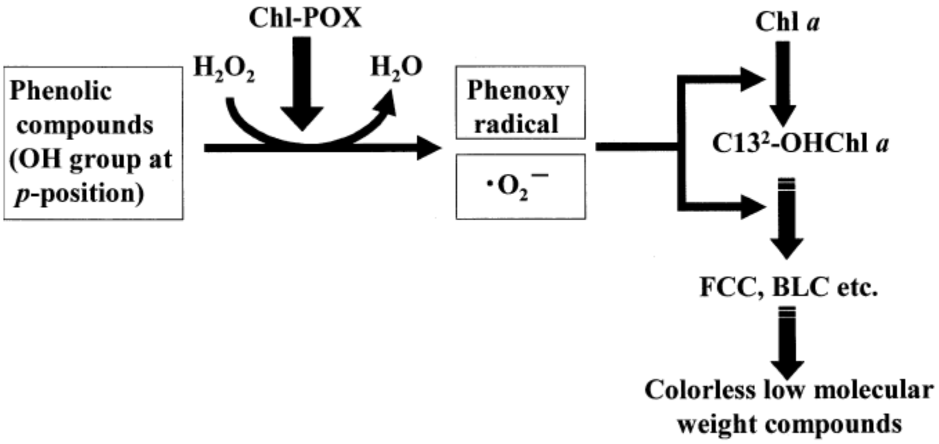

Figure 2.3 Pathway of peroxidase-mediated chlorophyll degradation. Chl-POX: Chlorophyll-degrading peroxidase, Chl: Chlorophyll, C13^2-OHChl: C13^2-Hydroxychlorophyll, FCC: Fluorescent chlorophyll catabolite, BLC: Bilirubin-like compounds

5. Pheophytinase

A new Chl degrading enzyme, PPH which would dephytylate the Mg-free Chl pigment, Phein *a* to give Pheide *a* has been recently reported (Schelbert et al., 2009). 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. 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.

Postharvest irradiation

The UV treatments have been reported to maintain the postharvest quality of horticulture crops. UV-C seems to be especially effective in maintaining the 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 the Chl derivative levels in mature green lime during storage.

Postharvest heat treatment

Heat treatment substitutes a non-damaging physical treatment for chemical prevention. There are three methods in use to heat commodities; hot water, vapor heat and hot air. Hot water is originally used for fungal control, but has been extended to disinfestation of insects. Vapor heat is developed specifically for insect control, and hot air has been used for both fungal and insect control and to study the response of commodities to high temperature. The last two methods (vapor heat and hot air) have subdivisions in that sometimes the air is relatively static, and sometimes air flow is quite high; additionally, hot air can have humidity control or not. All of these permutations may affect the response of the commodity to the heat treatment and affect the length of time of exposure needed to achieve a desired effect (Lurie, 1998).

Hot water dips and sprays

Hot water dips are effective for fungal pathogen control, because fungal spores and latent infections are either on the surface or in the first few cell layers under the peel of the fruit or vegetable. Postharvest dips to control decay are often applied for only a few minutes, at temperatures higher than heat treatments designed to kill insect pests located at the interior of a commodity, because only the surface of the commodity requires heating. Many fruits and vegetables tolerate exposure to water temperatures of 50–60˚C for up to 10 min, but shorter exposure at these temperatures can control many postharvest plant pathogens (Barkai-Golan and Phillips, 1991). In contrast, hot water dips for fruit require 90 min exposure at 46˚C. The extension of the hot water treatment has been the development of a hot water spray machine (Fallik et al., 1996a). This is a technique designed to be part of a sorting line, whereby the commodity is moved by means of brush rollers through a pressurized spray of hot water. By varying the speed of the brushes and the number of nozzles spraying the water, the commodity can be exposed to high temperatures for 10-60 s. The water is recycled, but because of the temperatures used (50–70˚C), organisms which are washed off the product into the water do not survive. This machine is in use both to clean and to reduce pathogen presence on a number of fruits and vegetables, such as mangos (Prusky et al., 1997) and peppers (Fallik et al., 1996b).

Vapor heat

Vapor heat is a method of heating fruit with air saturated with water vapor at temperatures of 40–50˚C to kill insect eggs and larvae as a quarantine treatment before fresh market shipment (Animal and Plant Health Inspection Service, 1985). Heat transfer is by condensation of water vapor on the cooler fruit surface. This procedure was first used to kill Mediterranean (*Ceratitis capitata* Wiedemann) and Mexican (*Anastrepha ludens* Loew) fruit fly (Hawkins, 1932; Baker, 1952) in a chamber without forced air. However, once ethylene dibromide and methyl bromide came into use as inexpensive chemical fumigants. In modern facilities the vapor heat includes forced air which circulates through the pallets and heats the commodity more quickly than vapor heat without forced air. Commercial facilities operate in many countries, mainly for use on subtropical fruits, particularly on mango and papaya (Paull and McDonald, 1994).

Hot air

Hot air can be applied by placing fruits or vegetables in a heated chamber with a ventilating fan, or by applying forced hot air where the speed of air circulation is precisely controlled. Hot air, whether forced or not, heats more slowly than hot water immersion or forced vapor heat, although forced hot air will heat produce faster than a regular heating chamber. The hot air chamber has been utilized to study physiological changes in fruits and vegetables in response to heat (Klein and Lurie, 1991, 1992). Forced hot air, however, has been used to develop quarantine procedures (Gaffney and Armstrong, 1990). One reason is that the high humidity in vapor heat can sometimes damage the fruit being treated, while the slower heating time and lower humidity of forced hot air can cause less damage. A high temperature forced air quarantine treatment to kill Mediterranean fruit fly, melon fly and oriental fruit fly on papayas has been developed (Armstrong et al., 1989; Hansen et al., 1990). This procedure may require rapid cooling after the heat treatment to prevent fruit injury, as may the forced hot air treatment for citrus (Sharp and Gould, 1994; Sharp and McGuire, 1996).

Effect of heat treatment on chlorophyll degradation

Heat treatment leads to an accelerated rate of degreening in apples (Liu, 1978; Klein et al., 1990). Chl content in apple peel, plantain peel and tomato pericarp decreased during a hot air treatment of 35–40˚C (Seymour et al., 1987; Lurie and Klein, 1991, 1992). Hot water immersion at 45˚C for 30–60 min can also induce yellowing of cucumbers (Chan and Linse, 1989), as does forced vapor heat for 30 min at 45˚C for zucchini (Jacobi et al., 1996) (zucchini are known as courgettes in some countries). Color changes in papaya skin or flesh were not affected by hot water immersion at 42˚C for 30 min followed by 49˚C for 90 min (Paull and Chen, 1990) and the same hot air treatment which stimulated degreening of plantains failed to degreen bananas (Seymour et al., 1987). Hot water dips at 43–55˚C for up to 10 min delayed yellowing of broccoli (Forney, 1995; Tian et al., 1996, 1997). The difference in responses of different commodities may be an indication of whether new enzymes must be synthesized to effect the color changes or not. In the case of apples chlorophyll degradation reveals the yellow of the underlying carotenoids already present, while other fruits may require synthesis of carotenoids. For example, it has been found that hot air at 38˚C or higher inhibits lycopene synthesis in tomatoes (Cheng et al. 1988). The inhibition of lycopene is due to the inhibition of transcription of mRNA for lycopene synthase, a key enzyme in the pathway, and this recovers after removal from heat (Lurie et al., 1996). In bananas the inhibition of degreening during the heat treatment appears to be due to the absence of the Chl oxidase enzyme resulting in the retention of Chl in the peel (Blackbourn et al., 1989). In broccoli hot water-treated at 45˚C for 14 min, floret yellowing was apparently retarded for 2 to 3 days at 20˚C storage (Kazami et al., 1991). Tian et al. (1996) observed that hot water treatment at 47˚C for 7.5 min was the best treatment for retarding floret yellowing. Terai et al. (1999) reported that hot air treatment at 50˚C for 2 or 3 h effectively inhibited floret yellowing, but the treatment for 3 h strongly retarded enzyme activity, such as that of ACC oxidase. Funamoto et al. (2005) showed that hot air treatment at 50˚C for 2 h. with broccoli could reduce Chl degradation due mainly to the suppression of Chl degrading enzyme activities such as Chlorophyllase, Chl-degrading peroxidase and Chl oxidase. Moreover, Costa et al. (2006a) also reported a treatment at 48˚C for 3 h delayed Chl *a* catabolism in broccoli during postharvest senescence and decreased the activities of chlorophyllase, Mg-dechelatase and peroxidase.

CHAPTER 3

MATERIALS AND METHODS

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

Plant materials and UV-B treatment

Mature green Tahitian lime (*C. latifolia* Tan.) fruit 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. Fruit were irradiated with UV-B (spectral peak value: 280-315 nm, MK Scientific, Inc.). Each fruit was placed under UV-B lamps at a distance of 15 cm, resulting in UV-B energy doses of 0 (0min; control) and 19.0 (20 min) kJm^{-2}. A layer of aluminum film was placed under the fruit to ensure that UV-B would be irradiated to the bottom of the fruit. After irradiation, 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. The samples (3 replications) were removed at 5 d intervals, and the peel tissues were sampled and used for analysis. The fresh weight of each lime fruit was monitored every 5 d and data were expressed as percent of weight loss.

Surface colour and chlorophyll assays

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

Preparation of substrates

1. Chlorophyll a

Spinach leaves were homogenised for 3 min in cold acetone (-20 ˚C). The homogenate was filtrated through two layers of Miracloth (Calbiochem, USA). 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 where 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 µg/ml of Chl *a* was prepared in acetone.

2. Chlorophyllide a

Chlide *a* was prepared from a Chl *a* acetone solution (500 µg/ml) with 0.765 mg protein of partial purified Chlase (20–40% of $(NH_4)_2SO_4$) from green citrus fruits. 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*.

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). After 2 min, 0.1 N NaOH was added to neutralize the solution, which was then employed as substrate in the assays.

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 with two layers of Miracloth. The filtrate was then centrifuged at 16,000g at 4 ˚C for 15 min. The supernatant was used as the crude enzyme extract. The enzyme protein contents were determined based on Bradford’s method (1976).

1. Chlorophyllase activity

The reaction mixture contained 0.5 ml 0.1 mM phosphate buffer (pH 7.5), 0.2 ml 500 μg/ml Chl *a* acetone solution (100 μg/ml) 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. Chlorophyll-degrading peroxidase activity

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

3. Mg-dechelating substance activity

MDS using Chlide *a* substrate was determined by the method of Suzuki and Shioi (2002) with slight modification. The activity of Mg-dechelating substance was measured with Pheide *a* formation, the reaction mixture contained 0.75 ml 10 mM phosphate buffer (pH 7.5), 0.25 ml Chlide *a* (8.80 µg) and 0.2 ml of enzyme solution.

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* solution (22.40 µg) 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, 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.

5. Protein content

Protein content of broccoli floret were determined based on the Bradford method (Bradford, 1976) with bovine serum albumin as a standard by using 100 µl aliquots of crude extracts per 5 ml of a Coomassie protein assay reagent.

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 redissolved in 1 ml of Milli-Q water. The citric and malic contents were determined by HPLC using Hitachi L-7420 UV-Visible spectrophotometer. Sample were separated on a Mightysil RP-18 column, 4x250 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 $\text{ml}\cdot\text{min}^{-1}$, and the injection volume was 100 µl. The sugar extract was filtered by passing through Sep-pak C18 and analyzed by HPLC using Lichrocart column with a solvent: acetonitrile: water (80:20) according to determined glucose, fructose and sucrose contents.

Stomatal apertures

Stomatal of lime fruit was observed by Suzuki’s Universal Micro-Printing (SUMP) method using SUMP liquid and SUMP plate (SUMP Laboratory, Tokyo). The fruit peel was pressed onto 10 µl of SUMP liquid placed on a cover glass until liquid become solid. The copies SUMP resin images were then observed by light microscopy.

3.2 Experiment II Hot water treatment delays chlorophyll degradation and postharvest quality in lime (*Citrus aurantifolia* Swingle cv. Paan) fruit

Plant materials and heat treatment

The lime fruit were held in hot water (50˚C) was circulated for 3 or 5 min and dried at ambient temperature. After drying, five fruit per perfolated polyethylene-film bag (20 x 14 cm, 0.04 mm thick, with two 6 mm holes) and stored at 13˚C in the dark for 35 day. Three bags of each treatment were removed at scheduled intervals during the 35 d period could be analyzed.

Surface color

The surface color of broccoli florets was determined by measuring the hue angle with a colorimeter (Nippon Denshoku NF 777). Hue angle of 0, 90, 180 and 270 degree showed red-purple, yellow, bluish-green and blue color, respectively.

Chlorophyll contents

The Chl content was made on broccoli florets based on the method of Moran (1982). The broccoli florets (0.5 g) were extracted in 20 ml of *N*,*N*-dimethylformamide and keep overnight at 4˚C in the dark. An absorbance was read spectrophotometrically at 647 and 664 nm. Chls *a* and *b* were calculated with the following equations.

Chl a (µg/ml) = $12.64\ OD_{664} - 2.99\ OD_{647}$

Chl b (µg/ml) = $-5.6\ OD_{664} + 23.26\ OD_{647}$

The unit of µg/ml is converted into mg/100 FW of broccoli floret using the following equations:

$$\text{Chl } a \text{ (mg/100 gFW)} = (\text{Chl } a \text{ (µg/ml)}) \times 20.5 \times \frac{100}{0.5} \times \frac{1}{1000}$$

$$\text{Chl } b \text{ (mg/100 gFW)} = (\text{Chl } b \text{ (µg/ml)}) \times 20.5 \times \frac{100}{0.5} \times \frac{1}{1000}$$

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 with two layers of Miracloth. The filtrate was then centrifuged at 16,000g at 4 ˚C for 15 min. The supernatant was used as the crude enzyme extract. The enzyme protein contents were determined based on Bradford's method (1976).

1. Chlorophyllase activity

The reaction mixture contained 0.5 ml 0.1 mM phosphate buffer (pH 7.5), 0.2 ml 500 µg/ml Chl *a* acetone solution (100 µg/ml) 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. Chlorophyll-degrading peroxidase activity

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

3. Mg-dechelating substance activity

MDS using Chlide *a* substrate was determined by the method of Suzuki and Shioi (2002) with slight modification. The activity of Mg-dechelating substance was measured with Pheide *a* formation, the reaction mixture contained 0.75 ml 10 mM phosphate buffer (pH 7.5), 0.25 ml Chlide *a* (8.80 µg) and 0.2 ml of enzyme solution.

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* solution (22.40 µg) 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, 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.

5. Protein content

Protein content of broccoli floret were determined based on the Bradford method (Bradford, 1976) with bovine serum albumin as a standard by using 100 µl aliquots of crude extracts per 5 ml of a Coomassie protein assay reagent.

Postharvest quality parameters

Total titratable acidity (TA) was measured by titration 1 mL of extracted juice diluted with 9 mL of distilled water with 0.1 M NaOH to an endpoint of pH 8.1, using an automatic titrator (AUT-501, DKK-TOA Corporation, Tokyo, Japan). Total titratable acidity was expressed as percentage of citric acid. Total soluble solid (TSS) from fruit juice was measured by a digital refractometer (PAL-1, Atago, Tokyo, Japan). The units of TSS were expressed as the percentage.

Postharvest physiology parameters

Lime fruit from each treatment was weighed individually before and during storage period, and the percentage of weight loss was calculated using the equation below.

$$\text{Total weight loss (\%)} = \frac{(\text{initial weight of fruit} - \text{final weight of fruit})}{\text{initial weight of fruit}} \times 100$$

Respiration rates were determined by gas chromatography (GC) (Chromatopac, CR 8A, Shimadsu Co., Kyoto, Japan), with a thermal conductivity detector (TCD) fitted with a 80/100 mesh Porapack Q column using helium as the carrier gas. The ethylene production rates were measured by gas chromatography (Chromatopac, CR 14A, Shimadsu Co., Kyoto, Japan), with a frame ionization detector (FID) equipped with a 80/100 mesh Porapack Q column using nitrogen as the carrier gas. Five fruit were sealed in a plastic container (610 mL) then incubated at room temperature for 3 hours. One mL of gas sample was withdrawn from the headspace with a gas syringe type and then injected into the gas chromatography.

CHAPTER 4

RESULTS

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

1. Effect of UV-B irradiation on Postharvest qualities of lime fruit

Surface colour was evaluated through the hue angle. As shown in Fig. 4.1, hue angle value in the control declined significantly during storage 25 ˚C and enhancing the yellowing of peel fruit. In contrast to those of UV-B at 19.0 kJm^{-2} lime fruits which changed little during storage (Fig. 4.2). The citric acid content in the fruit juice in the control showed an important decreased after 10 d, while UV-B at 19.0 kJm^{-2} treated fruit increased constant until day 20 and then decreased (Fig. 4.3A). The malic acid content decreased markedly in both UV-B treatment and control (Fig. 4.3B). As a consequence, UV-B treatment showed significant higher contents of citric acid and malic acid. Immediately after treatment the fructose (Fig. 4.4A), glucose (Fig. 4.4B) and sucrose contents (Fig. 4.4C) were slightly higher in control of day 5 and then decreased during storage. UV-B treatment, the level of fructose, glucose and sucrose contents were also increased until day 10 and then slightly decreased with increased after day 20.

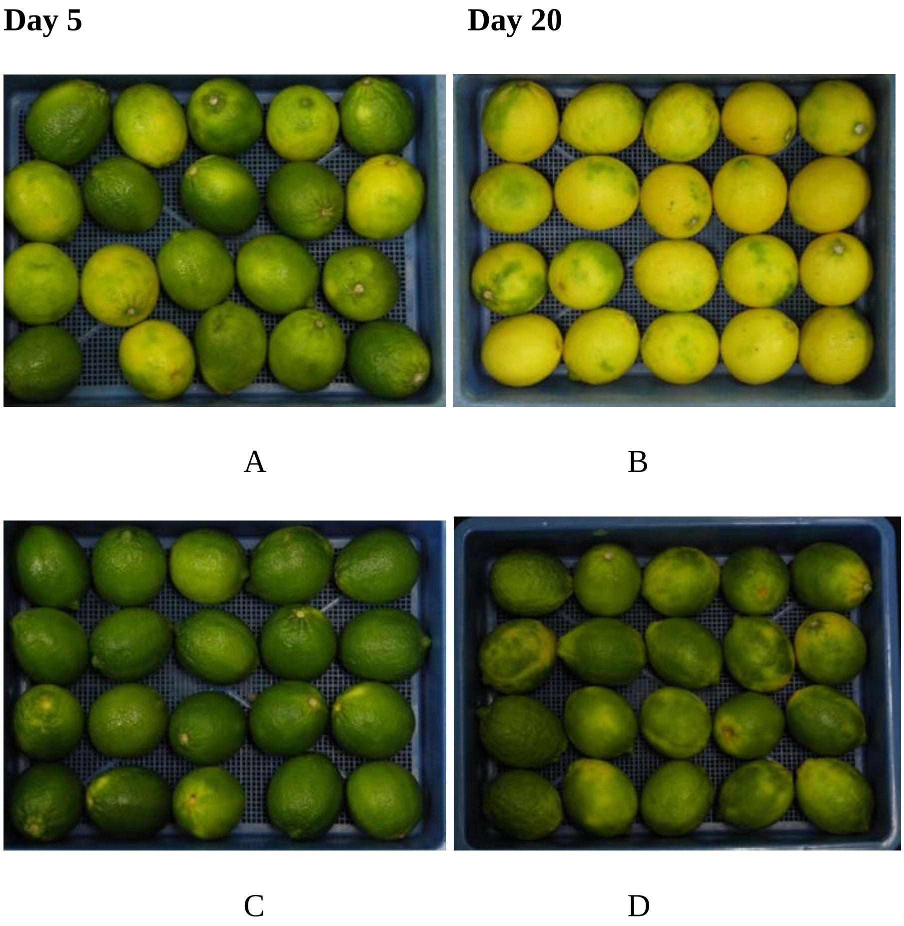

Fig. 4.1 Changes color of lime fruit treated with UV-B at 0 (Control) (A and B) and 19.0 kJm^{-2} (C and D) during storage at 25 ˚C of day 5 and 20.

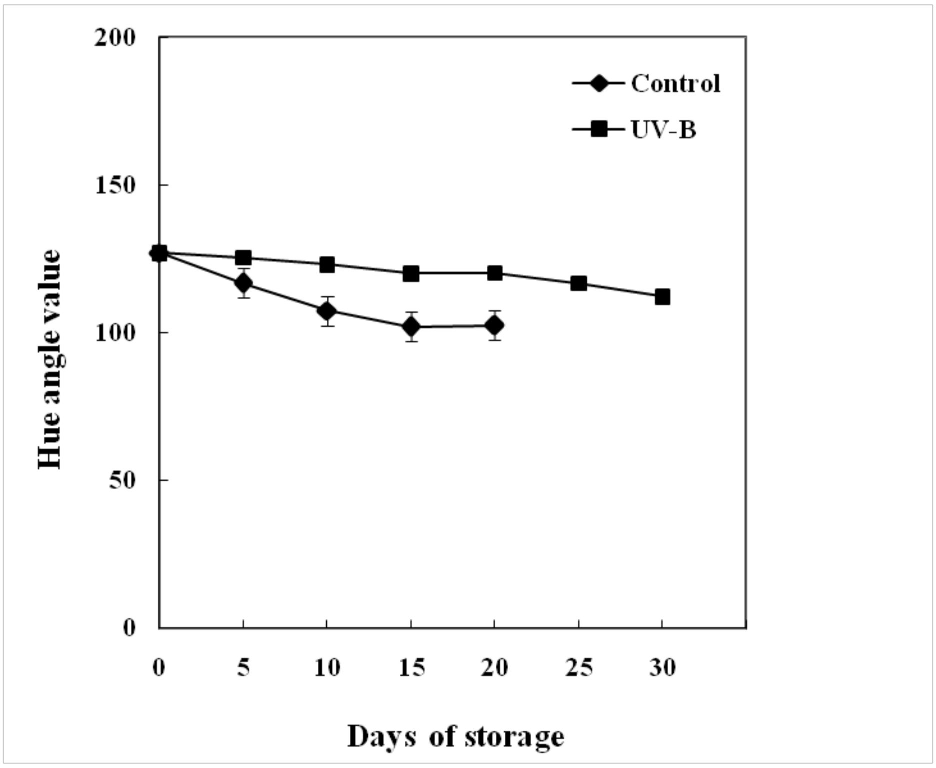

Figure 4.2 Changes in the hue angle value of the peel colour of lime fruits treated with UV-B at 0 (Control) and 19.0 kJm^{-2} during storage at 25 ˚C. Vertical bars represent the average values with SE (n = 3).

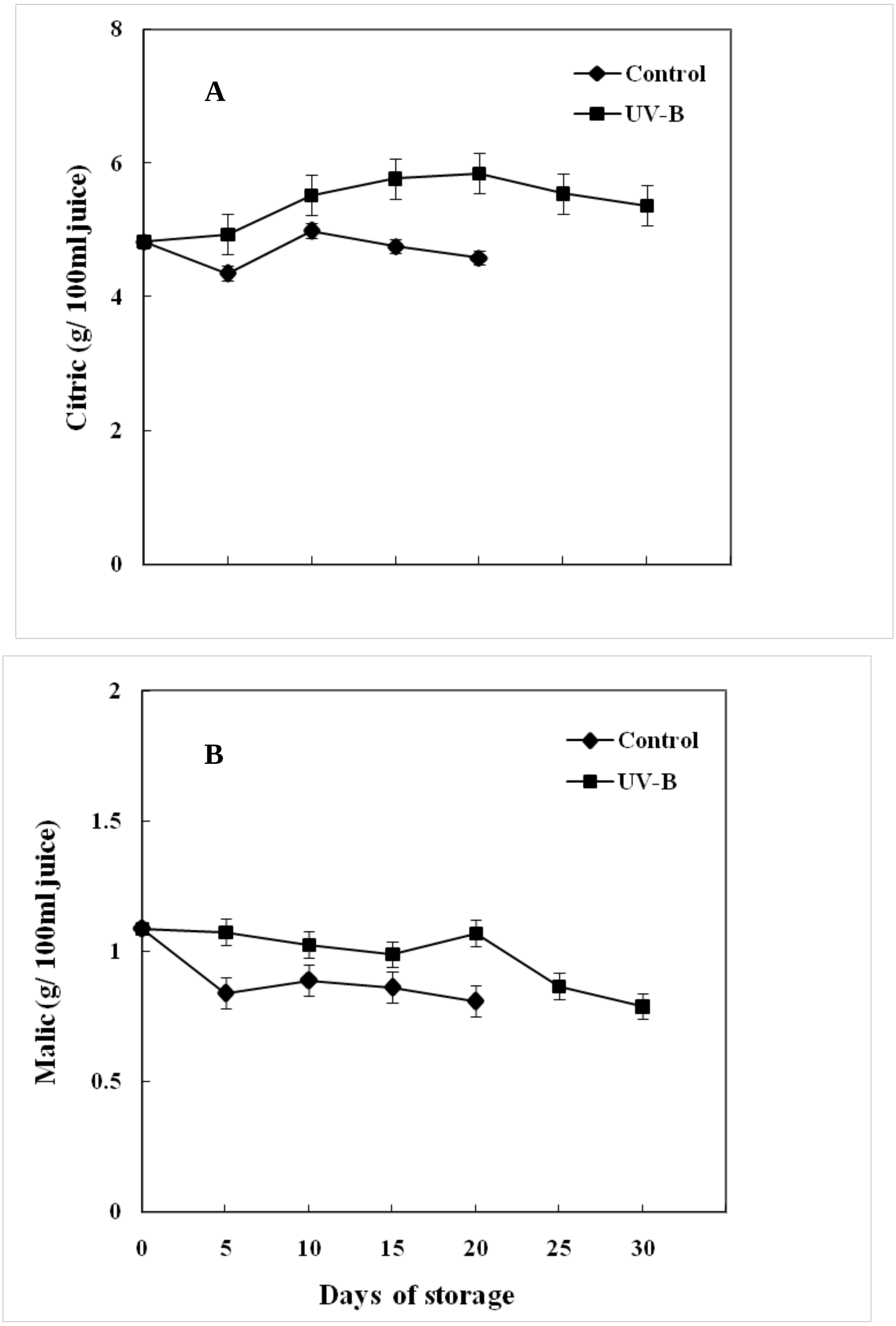

Figure 4.3 Changes in citric acid (A) and malic acid (B) contents of the juice in lime fruits treated with UV-B at 0 (Control) and 19.0 kJm^{-2} during storage at 25 ˚C. Vertical bars represent the average values with SE (n = 3).

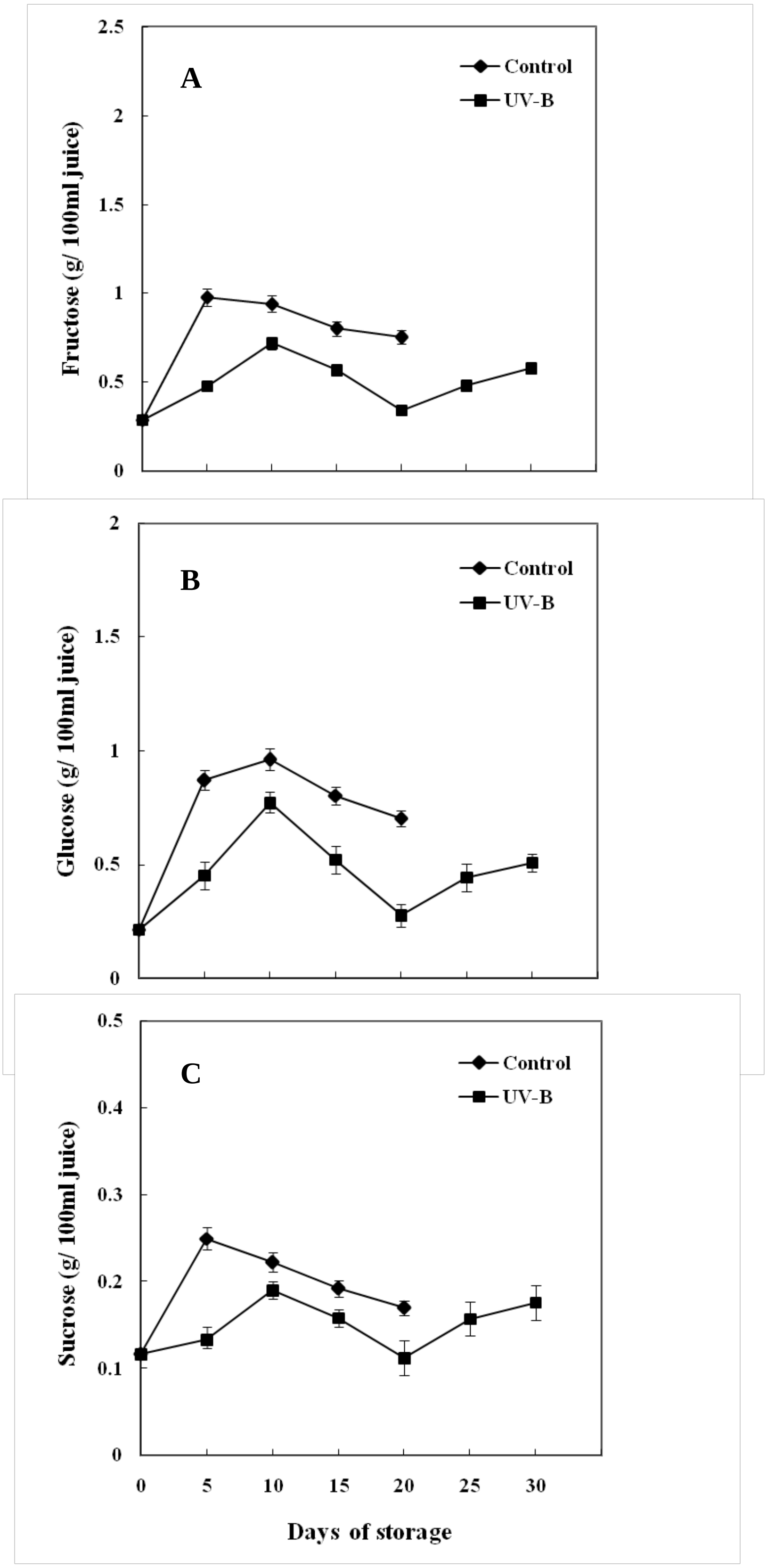

Figure 4.4 Changes in fructose (A), glucose (B) and sucrose (C) contents of the juice in lime fruits treated with UV-B at 0 (Control) and 19.0 kJm^{-2} during storage at 25 ˚C. Vertical bars represent the average values with SE (n = 3).

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

UV-B treatment delayed the reduction of Chl *a* and Chl *b* contents in limes, as shown in Fig. 4.5. The Chl *a* content in lime peel treated with UV-B was delayed of the reduction during storage, whereas that in the control sharply decreased after day 0 until the end of storage (Fig. 4.5A). In the case of Chl *b*, the change in content was similar to that of Chl *a* contents during storage (Fig. 4.5B). The lime fruit treated with UV-B had a higher content of Chl *b* than the control fruit.

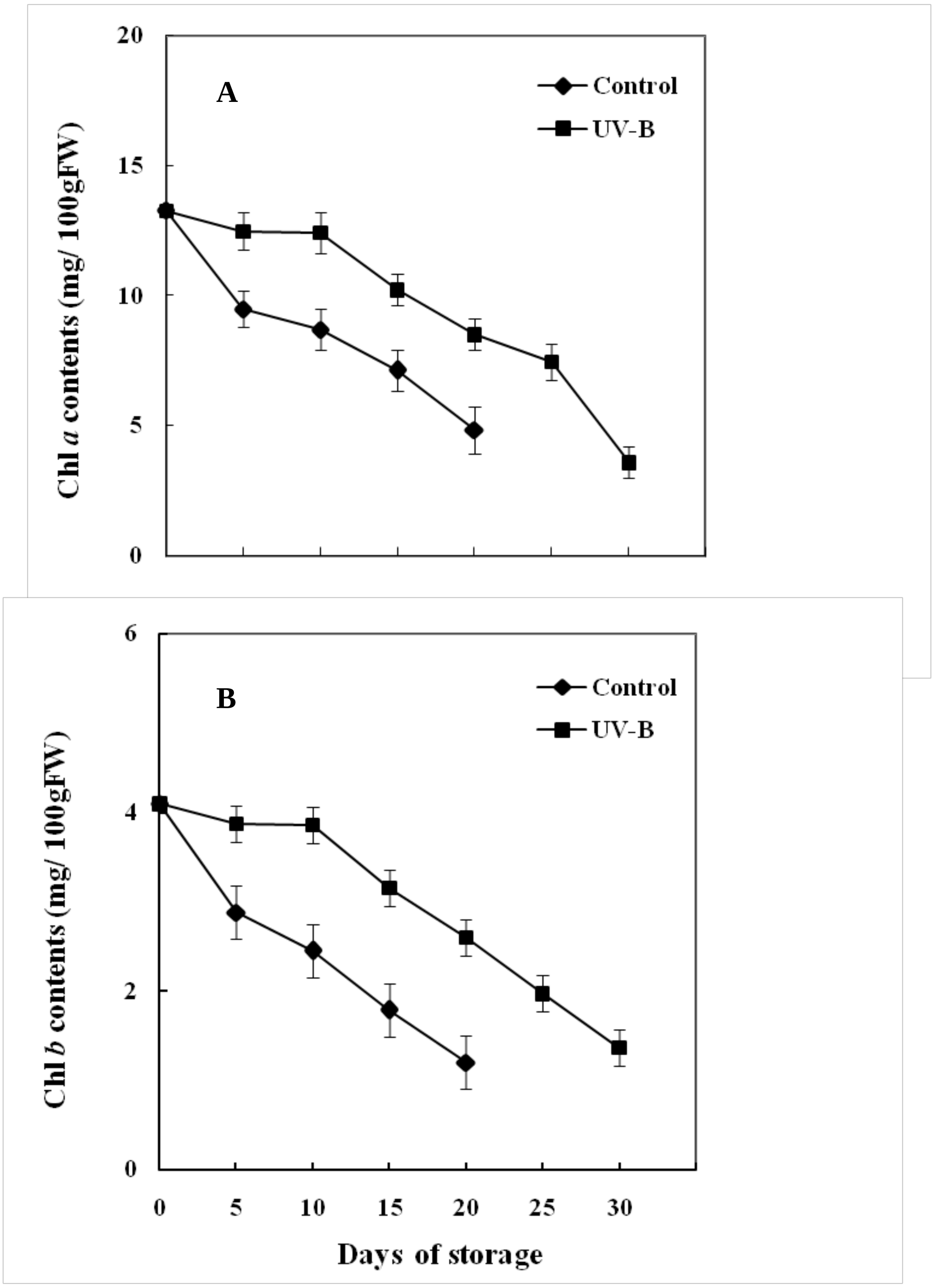

Figure 4.5 Changes in Chl *a* (A) and Chl *b* (B) contents in lime fruits treated with UV-B at 0 (Control) and 19.0 kJm^{-2} during storage at 25 ˚C. Vertical bars represent the average values with SE (n = 3).

In Fig. 4.6A, Chlase activity increased in the control lime during storage at 25 ˚C. While, Chlase activity in UV-B treatment was a little change during storage but remained below the activity in compare the control. Notably, Chlase activity was suppressed by UV-B treatment after 10 day of storage. Chl-POX activity markedly increased in both the control and the UV-B treatment along the storage period. Moreover, UV-B treatment showed lower Chl-POX activity than the control (Fig. 4.6B). In this study, MDS activity was examined by using Chlide *a* as a native substrate. MDS activity showed an increase until day 15 and then slightly decreases. UV-B treatment also showed an increase in MDS activity immediately after treatment and after 5 d. (Fig. 4.7A). However, after that MDS activity dropped to lower levels than those found in the control. In the case of a new enzyme, PPH activity was determined. PPH activity in the control started to increase until day 15 and then slightly decreased during storage. While, the UV-B treatment sharply reached in PPH activity after treatment through 5 d and then significant declined to lower than the control (Fig. 4.7B).

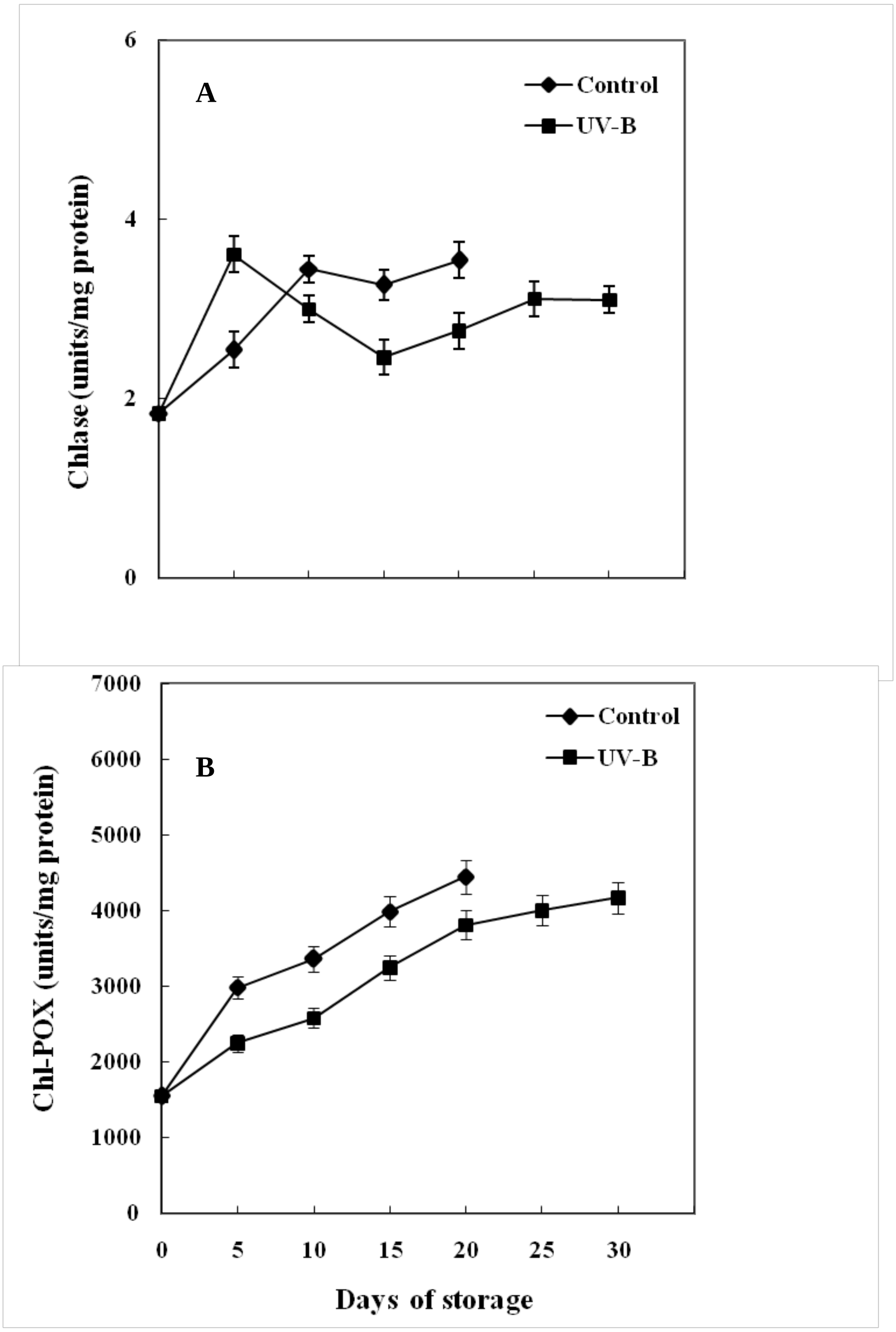

Figure 4.6 Changes in Chlase (A) and Chl-POX (B) activities in lime fruits treated with UV-B at 0 (Control) and 19.0 kJm^{-2} during storage at 25 ˚C. Vertical bars represent the average values with SE (n = 3).

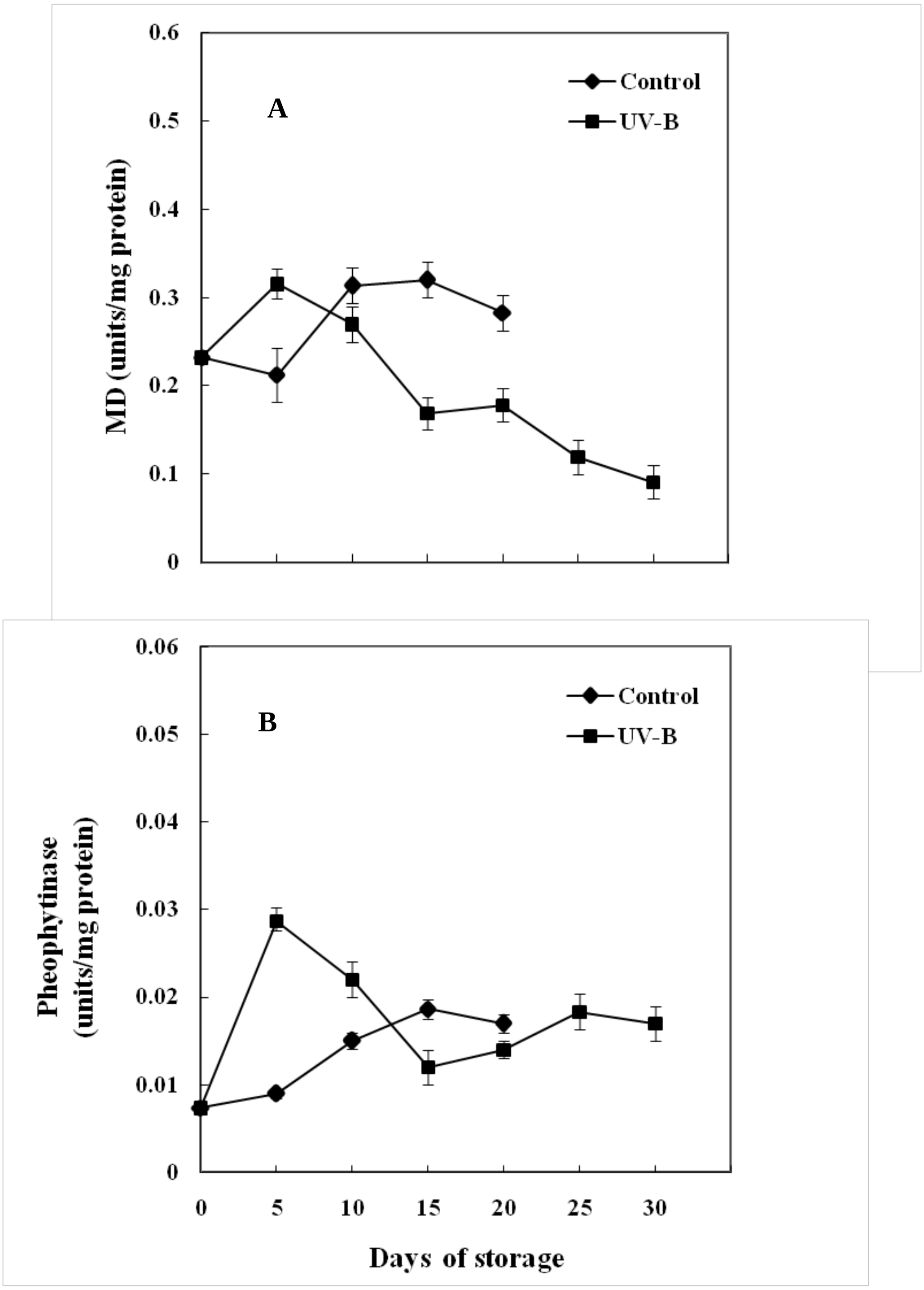

Figure 4.7 Changes in MDS (A) and PPH (B) activities in lime fruits treated with UV-B at 0 (Control) and 19.0 kJm^{-2} during storage at 25 ˚C. Vertical bars represent the average values with SE (n = 3).

3. Effect of UV-B irradiation on weight loss and stomatal apertures of lime fruit

Weight loss by the control and UV-B treated fruit in creased relative to length of the storage. Weight loss of the control was significantly greater than that in UV-B treated fruit (Fig. 4.8).

To evaluate the UV-B treatment that affects on stomatal using epidermal peels of lime fruit every 10 days until the end of storage. The stomatal in the control lime fruit showed still close from day 0 (Fig. 4.9A) and its started to little open on day 20 (Fig. 4.9B). However, stomatal was completely opened in last day of storage (day 30) of the control (Fig. 4.9C). On other hand, the stomatal in UV-B treatment was no significant opened throughout storage of lime fruit (Fig. 4.9 D, E and F).

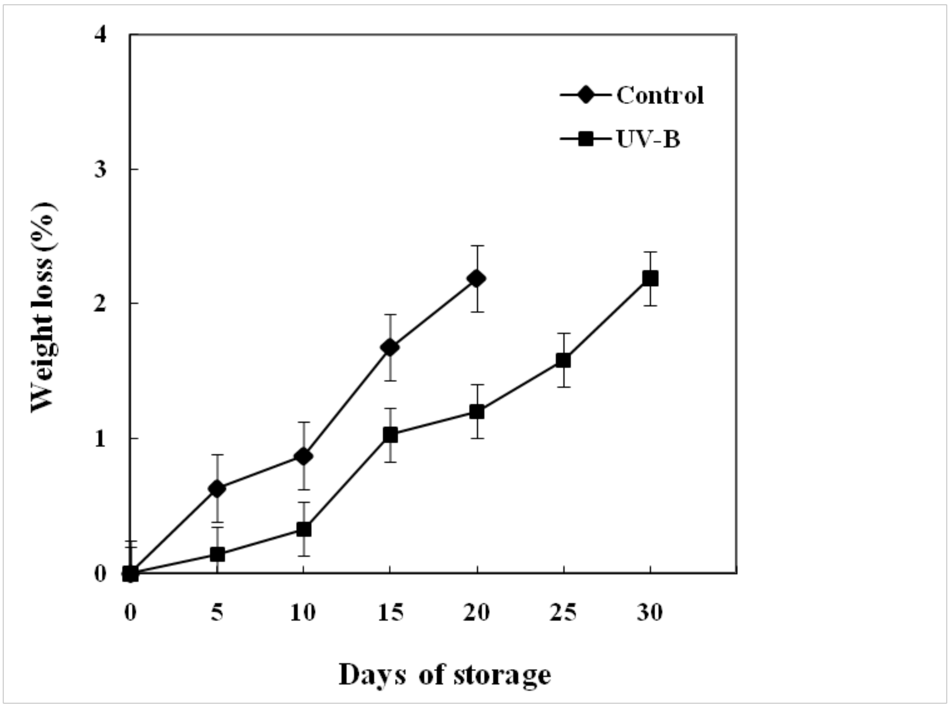

Figure 4.8 Changes in weight loss in lime fruits treated with or without UV-B at 19.0 kJm^{-2} during storage at 25 ˚C.

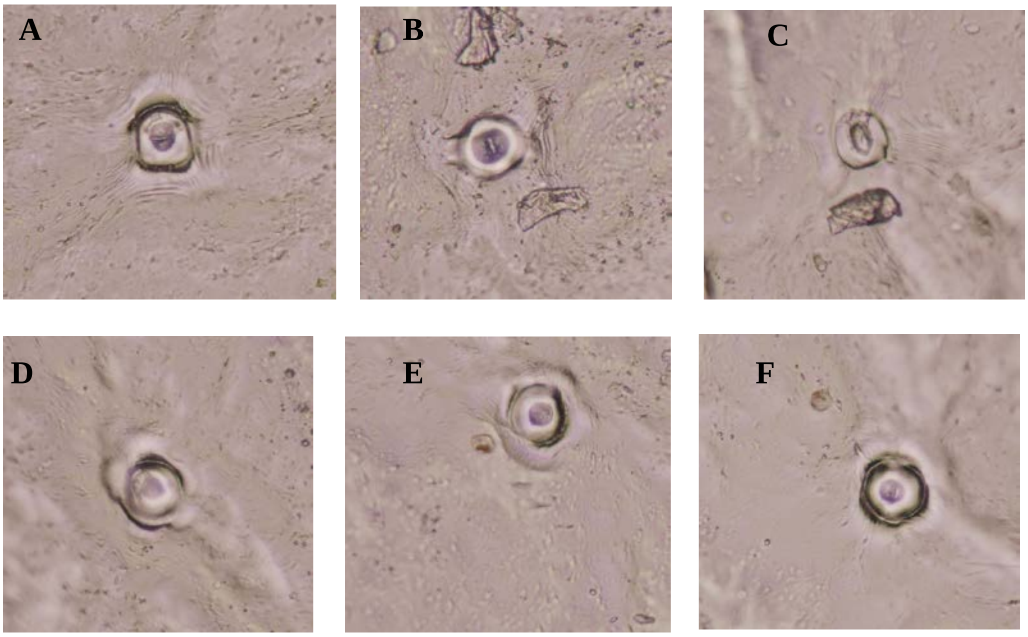

Figure 4.9 Changes in stomatal apertures in lime fruit at 25 ˚C. With UV-B at 0 kJm^{-2} (Control) at day 0 (A), day 20 (B) and day 30 (C). UV-B treatment at 19.0 kJm^{-2} at at day 0 (D), day 20 (E) and day 30 (F).

4.2 **Experiment II** Hot water treatment delays chlorophyll degradation and postharvest quality in lime (*Citrus aurantifolia* Swingle cv. Paan) fruit

1. Effect of hot water treatment on surface color and chlorophyll content during storage of lime fruit

Hue angle value in the control and hot water treatment at 50 ˚C for 3 min declined significantly during storage 13 ˚C and enhancing the yellowing of peel lime fruit. In contrast to those of hot water treatment at 50 ˚C for 5 min of lime fruits which changed little during storage (Fig. 4.10). Hot water treatment at 50 ˚C for 5 min efficiently delayed the decrease of the hue angle value.

Hot water treatment at 50 ˚C delayed the reduction of Chl *a*, *b* and total Chl contents in limes, as shown in Fig. 4.11. The Chl *a* and total Chl contents in lime peel treated with hot water treatment at 50 ˚C for 5 min was delayed of the reduction during storage, whereas that in the control sharply decreased after day 0 until the end of storage (Fig. 4.11A,C). In the case of Chl *b*, the change in content was similar to that of Chl *a* contents during storage (Fig. 4.11B). The lime fruit treated with hot water treatment at 50 ˚C for 5 min had a higher content of Chl *b* than the control and hot water treatment at 50 ˚C for 3 min.

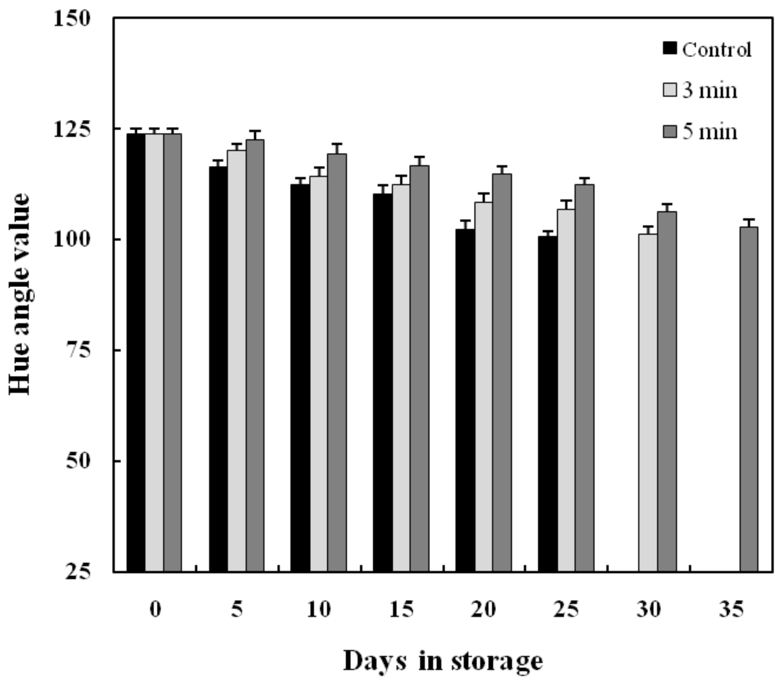

Figure 4.10 Changes in the hue angle value of the peel colour of lime fruits treated with hot water treatment at 0 (Control), 3 and 5 min during storage at 13 ˚C. Vertical bars represent the average values with SE (n = 3).

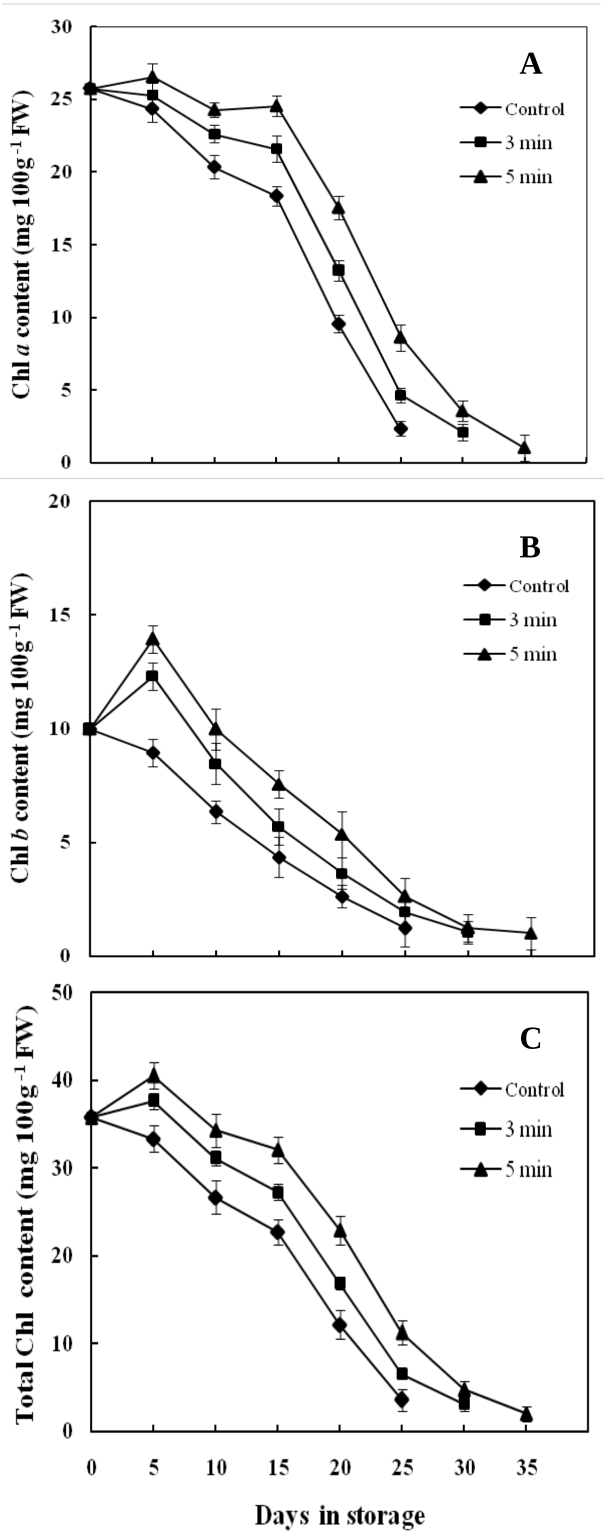

Figure 4.11 Changes in the Chl *a* (A) Chl *b* (B) and total Chl (C) contents of lime fruits treated with hot water treatment at 0 (Control), 3 and 5 min during storage at 13 ˚C. Vertical bars represent the average values with SE (n = 3).

2. Effect of hot water treatment on chlorophyll-degrading enzyme activities of lime fruit

In Fig. 4.12A, Chlase activity increased in the control lime during storage at 13 ˚C. While, Chlase activity in hot water treatment at 50 ˚C was a little change during storage but remained below the activity in compare the control. Notably, Chlase activity was most suppressed by hot water treatment at 50 ˚C for 5 min during storage. Chl-POX activity markedly increased in both the control and hot water treatment at 50 ˚C along the storage period. Moreover, hot water treatment at 50 ˚C for 5 min showed the lowest Chl-POX activity than the control (Fig. 4.12B). In this study, MDS activity was examined by using Chlide *a* as a native substrate. MDS activity showed an increase until day 5 and then slightly decreases. However, after that MDS activity of hot water treatment at 50 ˚C for 5 min dropped to lower levels than those found in the control (Fig. 4.13A). In the case of PPH activity was determined. PPH activity in the control started to increase until day 5 and then slightly decreased during storage. The hot water treatment at 50 ˚C for 5 min showed in PPH activity significant declined to lower than the control (Fig. 4.13B).

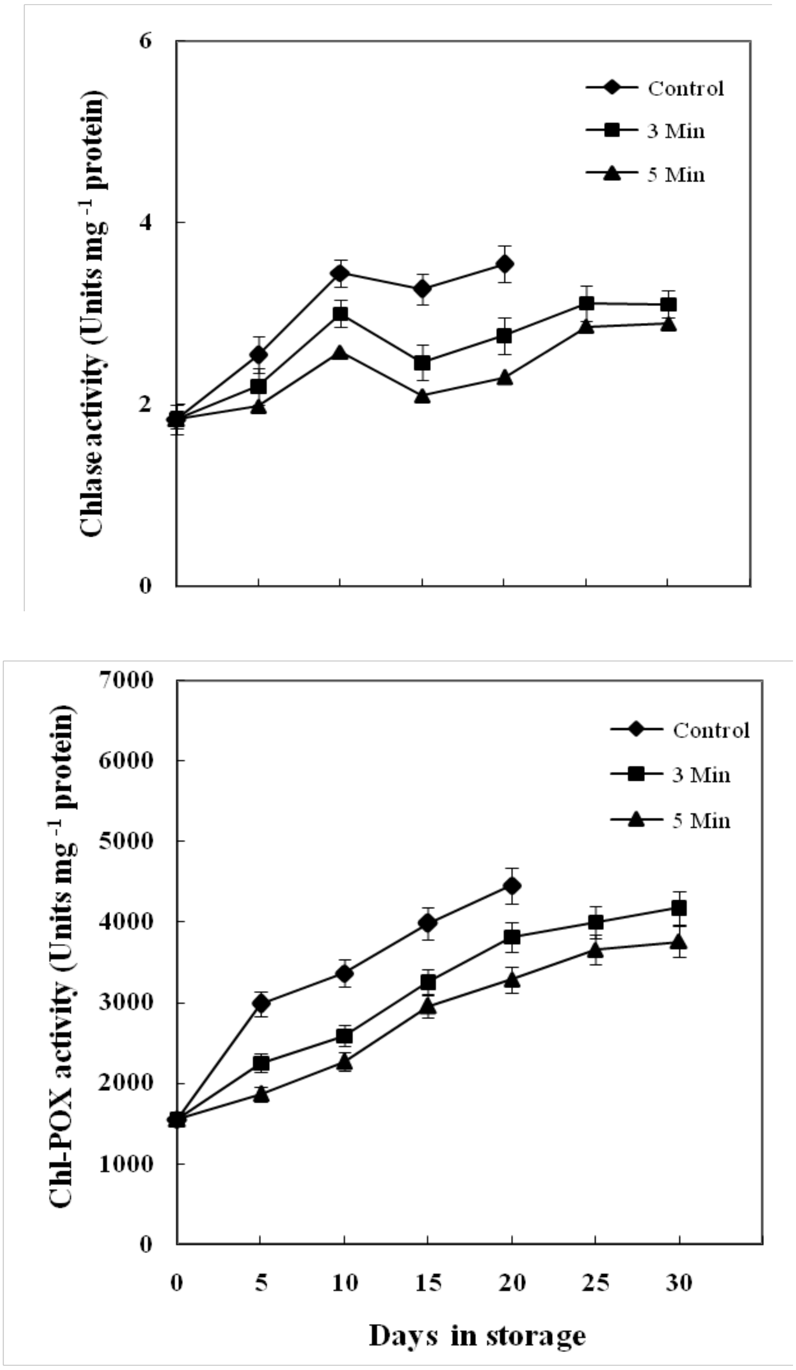

Figure 4.12 Changes in Chlase (A) and Chl-POX (B) activities of lime fruits treated with hot water treatment at 0 (Control), 3 and 5 min during storage at 13 ˚C. Vertical bars represent the average values with SE (n = 3).

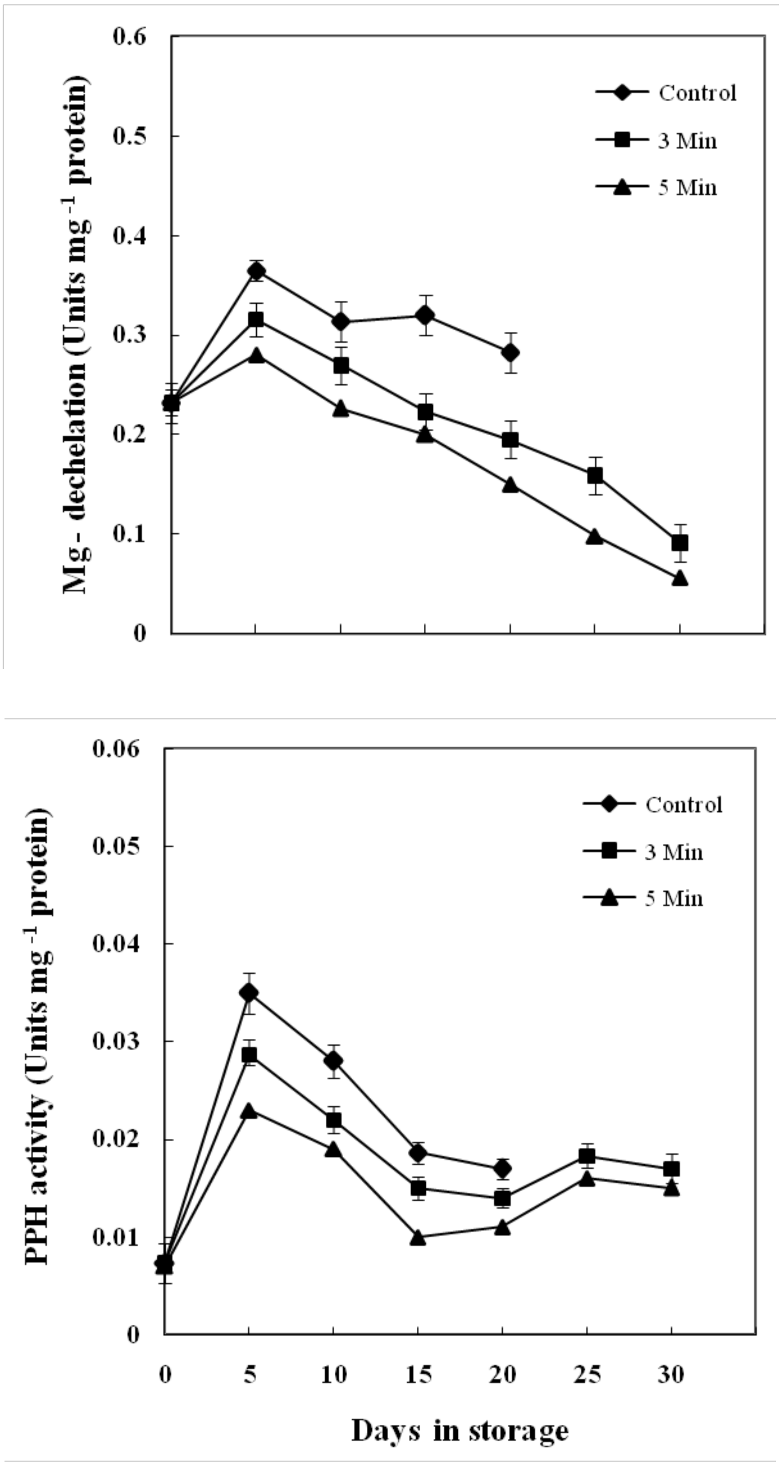

Figure 4.13 Changes in MDS (A) and PPH (B) activities of lime fruits treated with hot water treatment at 0 (Control), 3 and 5 min during storage at 13 ˚C. Vertical bars represent the average values with SE (n = 3).

3. Effect of hot water treatment on postharvest physiology of lime fruit

Weight loss by the control and hot water treatment at 50 ˚C increased relative to length of the storage. Weight loss of the control and hot water treatment at 50 ˚C for 5 min was significantly greater than that in hot water treatment at 50 ˚C for 3 min (Fig. 4.14).

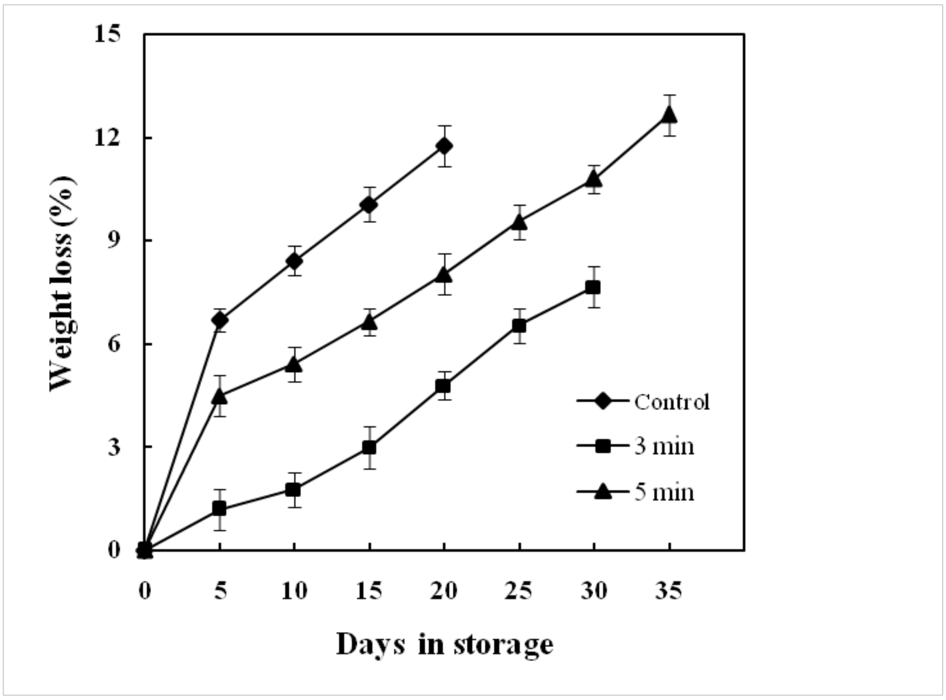

Figure 4.14 Changes in weight loss of lime fruits treated with hot water treatment at 0 (Control), 3 and 5 min during storage at 13 ˚C. Vertical bars represent the average values with SE (n = 3).

Respiration rate and ethylene production increased a little in both the control and hot water treatment at 50 ˚C along the storage period. Moreover, hot water treatment at 50 ˚C for 5 min showed the lowest respiration rate and ethylene production than the control (Fig. 4.15, 4.16).

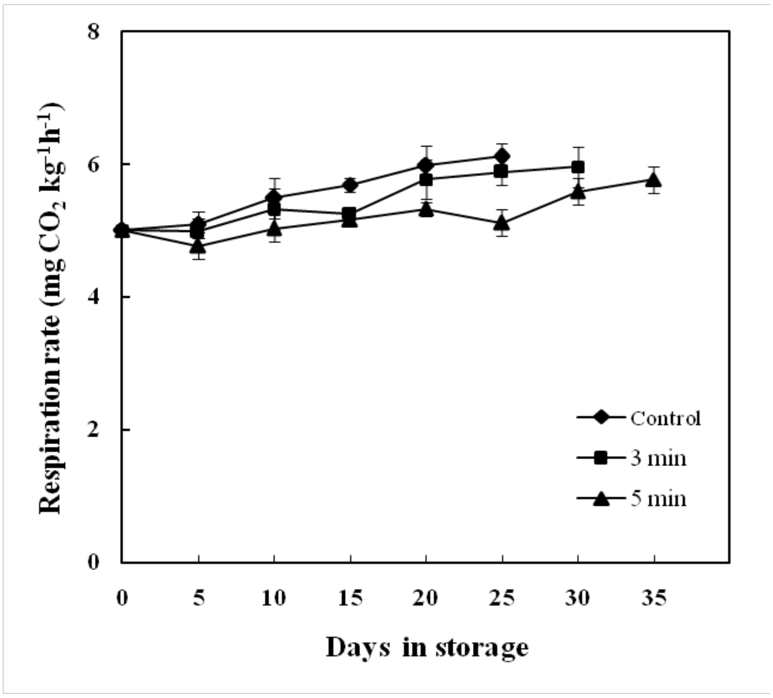

Figure 4.15 Changes in respiration rate of lime fruits treated with hot water treatment at 0 (Control), 3 and 5 min during storage at 13 ˚C. Vertical bars represent the average values with SE (n = 3).

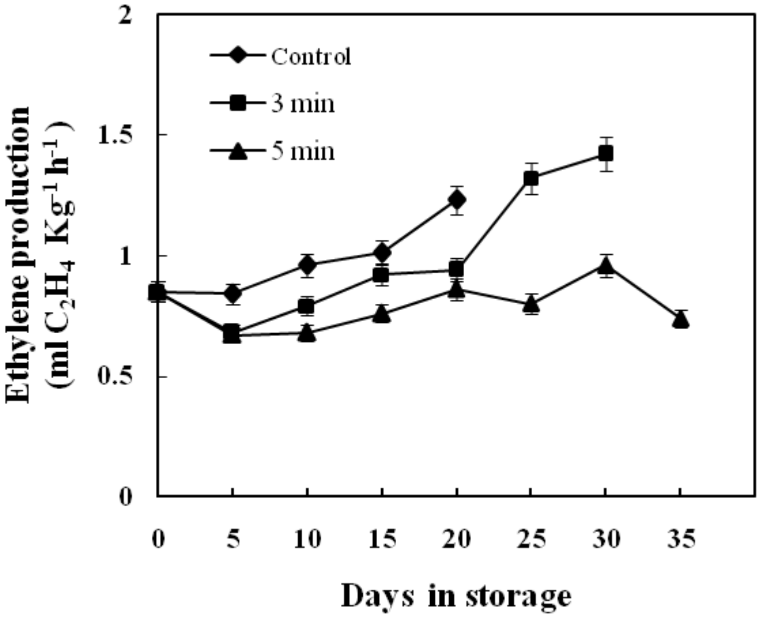

Figure 4.16 Changes in ethylene production of lime fruits treated with hot water treatment at 0 (Control), 3 and 5 min during storage at 13 ˚C. Vertical bars represent the average values with SE (n = 3).

4. Effect of hot water treatment on postharvest qualities of lime fruit

In relation to postharvest quality, all treatment resulted in storage life of 25, 30 and 35 days at control and hot water treatment at 50 ˚C for 3 and 5 min, respectively. Hot water treatment at 50 ˚C for 5 min caused the highest maintenance of total acidity (Fig. 4.17) and suppression the increase of total soluble solid during storage (Fig. 4.18).

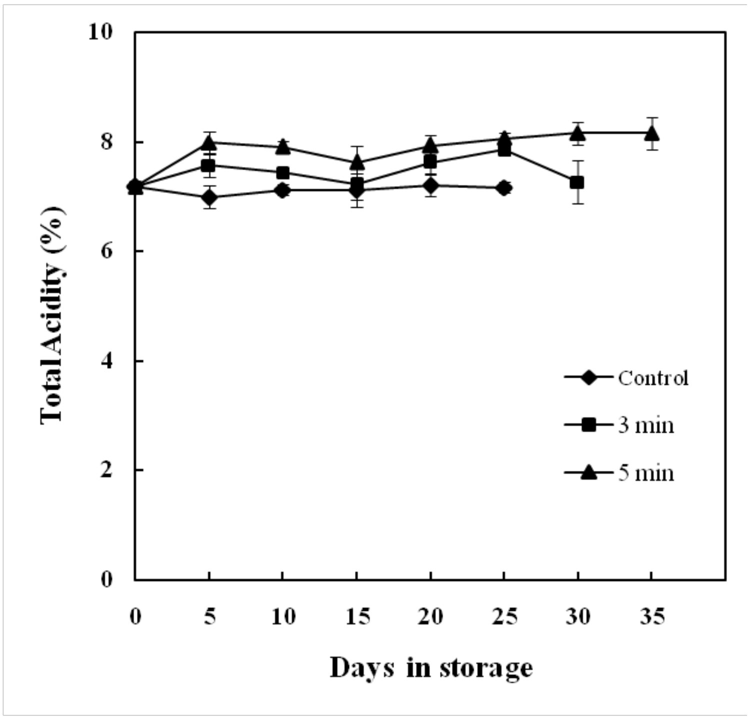

Figure 4.17 Changes in titratable acidity of lime fruits treated with hot water treatment at 0 (Control), 3 and 5 min during storage at 13 ˚C. Vertical bars represent the average values with SE (n = 3).

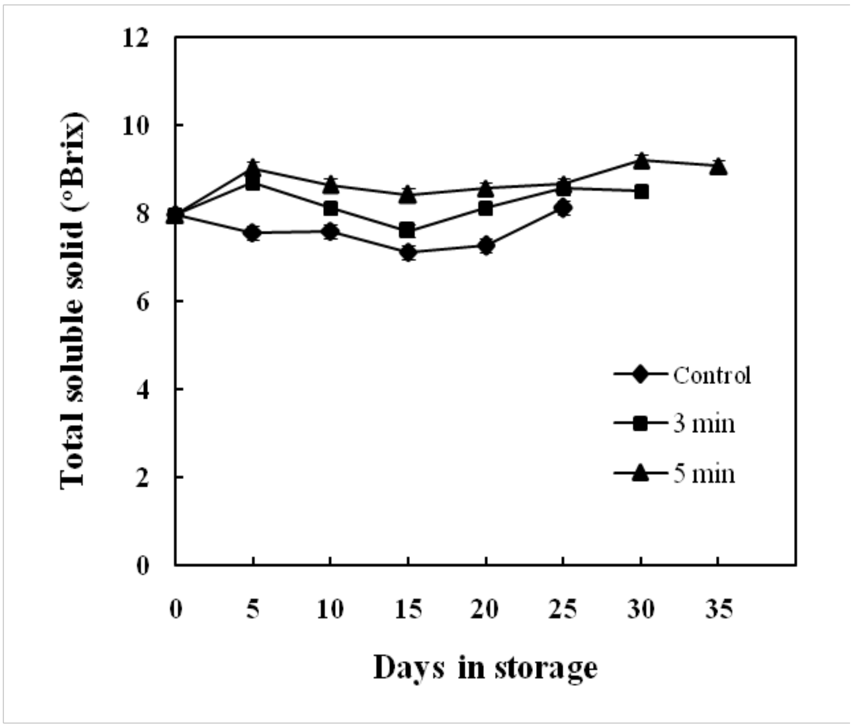

Figure 4.18 Changes in total soluble solid of lime fruits treated with hot water treatment at 0 (Control), 3 and 5 min during storage at 13 ˚C. Vertical bars represent the average values with SE (n = 3).

CHAPTER 5

DISCUSSION

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

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 if fruit are to add value prices (Pranmornkith et al., 2005). Many studies have shown the deleterious effect of UV irradiation on fruits and vegetables to maintain the postharvest quality. Specifically, UV-C irradiation can reduce decay in grapefruit (D'hallewin et al., 2000) or maintain the postharvest quality of strawberry (Erkan et al., 2008) and in suppressing Chl degradation in stored broccoli florets (Costa et al., 2006a). Also, UV-B is an alternative UV range that may maintain postharvest quality of fresh produce. Aimala-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 lime during storage. In the present study, lime fruit were irradiated with UV-B at 19.0 kJm^{-2} and the results were compared with those of untreated fruit. Base on the results of hue angle and Chl *a* and Chl *b* contents, we suggested that UV-B treatment effectively delayed the chlorophyll breakdown in the lime peel. The delay of Chl degradation with UV-B treatment may have the same effect in broccoli florets as 1-MCP induced reduction in Chl-degrading peroxidase and Chlase activities (Gong and Mattheis, 2003). Heat treatment reduce the Chl degradation through the suppression of activities of Chl-degrading enzymes, including Chlase, MD, Chl-degrading peroxidase 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 (Aimala-or et al., 2010) which also suppressed Chl-degrading enzyme activities as Chlase, MD, MDS and Chl-degrading peroxidase. In this study, a UV-B treatment at 19.0 kJm^{-2} effectively suppressed the activities of Chl-degrading enzymes such as Chlase, Chl-POX, MDS and PPH in lime fruits. During storage, Chlase activity gradually increased in lime fruit with or without UV-B treatment. In contrast, it was 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 (Aimala-or et al., 2010). In the study, we found that Chlase activity was tentatively suppressed in stored lime fruit throughout storage by UV-B treatment. The mature green lime was shown to have a high level of Chlide *a* which suggests that the high level of Chlide *a* is caused by 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). This result agrees with a report on lime fruits that the level of Chlide *a* was retained at high levels in limes with UV-B treatment (Srilaong et al., 2011). This outcome could be attributed to the UV-B treatment, which effectively inhibits the activity of Chlase, resulting in the retention of the Chlide *a* levels. Our results showed that Chl-POX activity was markedly increased in lime fruit during storage, but its activity was clearly suppressed throughout the storage life of lime treated with UV-B. In lime fruits, Chl *a* can be degraded by Chl-POX (Win et al., 2006) with 13^2-hydroxychlorophyll *a* is formed as an intermediate and does not accumulate by UV-B treatment (Aimala-or et al., 2010). MDS activity was also determined by using Chlide *a* as native substrate in lime fruits. MDS, which is 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 MDS activity was a little increased in control stored lime fruits and UV-B treatment also effectively suppressed MDS activity. These findings were similar to the finding by Aimala-or et al. (2010). Our result also correlates with Srilaong et al., (2011) found that the levels of Pheide *a* were lower in UV-B-treated limes than in the control fruit. This indicates that the degradation of Chlide *a* to Pheide *a* may be suppressed by UV-B treatment. Accordingly, we suggest that MDS could be involved in Mg-dechelation from Chlide *a* in lime fruits. In addition, a new enzyme in relation Chl degradation as PPH activity was determined in lime fruit. PPH specially dephytilates Phein *a* to produce Pheide *a* (Schelbert et al., 2009). Our result showed the PPH activity gradually increased during storage of lime fruits. While, the UV-B treatment effectively suppressed PPH activity even though activation after treatment. Sequential degradation of Chl by PPH activity implies formation of Pheide *a* in lime fruit is possibly included. Several studies have detected Phein *a* accumulation 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), which suggest the presence of enzymatic that release Mg^{2+} from Chl *a* as Phein *a* and thus provides substrates for PPH. However, Phein *a* was accumulated at a higher rate in the UV-B-treated limes than in the control fruit (Srilaong et al., 2011). Suggesting that the high accumulation of Phein *a* in UV-B treatment initiate also non-enzymatic formation to produce substrate for PPH. Further study needs to clarify the role of PPH in Chl degradation of lime fruit.

Besides Chl degradation, the composition changes in relation to the internal quality occur during storage of lime fruits. The citric acid and malic acid contents in lime fruit almost no changed during storage. However, theirs were higher in UV-B treatment than in the control. UV-B treatment may suppress the respiration rate, which are necessary to maintain organic acids in lime fruit. As a consequence, the level of fructose, glucose and sucrose contents showed a decrease during storage, but the decrement was higher in UV-B treatment of lime fruit. In this case, UV-B may have inhibited enzymes that inter-convert different carbohydrates such as invertase or enzymes that are normolly enhance during postharvest senescence. The results agreed with effect of heat treatment on reducing sugar (Lemoine et al., 2008). Thus, UV-B treatment seems to be a useful treatment for maintenance of internal quality in lime fruit.

The UV-B treatment that also affects on stomatal closure of epidermal peels of lime fruit during storage. We agreed with Dai et al. (1995) reported UV-B irradiation reduced the opening of stomata on the surface of rice leaves. In addition, Nogués et al. (1999) also found that UV-B irradiation can cause stomatal closure in plants. Our result supporting with Srilaong et al. (2011) showed UV-B treatment was markedly reduced weight loss and shriveling in the lime fruit.

5.2 Experiment II Hot water treatment delays chlorophyll degradation and postharvest quality in lime (*Citrus aurantifolia* Swingle cv. Paan) fruit

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; Srilaong et al., 2011; Kaewsuksaeng et al., 2011). The maintenance of green color in the peel of Thai limes during storage and shelf life are required for fruit to maintain their value prices (Pranmornkith et al., 2005). Due to consumers' 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 the quality. Heat treatment has also been demonstrated to show physiological effects on the control of ripening and senescence and the tolerance to chilling injury in postharvest fruits 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 the heat treatment at 50 ˚C for 3 min with the solution of 2% sucrose laurate ester delayed the 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 fruits 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 hot water treatment at 50 ˚C for 5 min retarded the Chl breakdown with delayed decline of hue angle value and Chl *a* and *b* contents in the lime fruit peel.

Heat treatment has been known to reduce the Chl degradation through the suppression of activities of Chl-degrading enzymes, including Chlase, Mg-dechelation activity, Chl-degrading peroxidase, and Chl oxidase (Costa et al., 2006; Funamoto et al., 2002; Kaewsuksaeng et al., 2007). UV-B irradiation in Tahitian lime (*Citrus latifolia* Tan.) (Kaewsuksaeng et al., 2011) also suppressed the activities of Chl-degrading enzymes such as Chlase, Mg-dechelation, Chl-degrading peroxidase and PPH. In this study, hot water treatment at 50 ˚C for 3 and 5 min also effectively suppressed the enhancement of Chlase and Chl-POX activity during storage in lime fruit, especially the latter. Mg-dechelation and PPH activities in hot water-treated lime fruit at 50 ˚C for 5 min reduced after a temporary increase during storage. Chlase activity in the control gradually increased with senescence during storage in Thai lime (*Citrus aurantifolia* Swingle cv. Paan), the same as in Tahitain lime fruits (Kaewsuksaeng et al., 2011). In contrast, it was previously reported that Chlase activity decreased with degreening of *Citrus nagato-yuzukichi* (Yamauchi et al., 2003) and broccoli florets (Aiamla-or et al., 2010). We also found that Chlase activity was tentatively suppressed in lime fruit throughout storage by hot water treatment at 50 ˚C for 5 min. The mature green lime was shown to have a high accumulation level of Chlide *a* during storage (Srilaong et al., 2011), which suggests that the high level of Chlide *a* is due to the 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 indicated that Chl-POX activity markedly increased in lime fruit during storage, but its activity was clearly suppressed throughout the storage period with hot water treatment at 50 ˚C for 5 min. In lime fruit, Chl *a* can be degraded by Chl-POX (Win et al., 2006a) to form the oxidized Chl *a*, 13^2-hydroxychlorophyll *a*, which did not accumulate during storage by UV-B treatment (Aimala-or et al., 2010). Mg-dechelation activity by MDS was also determined by 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 sharply in day 5 after hot water treatment due to the stress condition from high temperature and after that hot water treatment at 50 ˚C for 5 min also effectively inhibited Mg-dechelation. These results were similar to those by Kaewsuksaeng et al. (2011) that Mg-dechelation activity in Tahitian lime increased slightly in the control and that UV-B treatment also effectively suppressed the enhancement of Mg-dechelation. Srilaong et al. (2011) demonstrated that the Pheide *a* level 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. Further study needs to clarify the Chl derivatives and Chi-degrading enzymes in heat-treated lime fruit. These findings show that Mg-dechelation activity is significantly involved in the Chl-degrading process in lime fruit peel.

Recently reported as a new Chl-degrading enzyme, PPH dephytylates Phein *a* to form Pheide *a*. It is inferred that PPH activity measured in this study might include some activity of chlorophyllase, since it can also dephytylate Phein *a* along with Chl *a* (McFeeters et al., 1971; Mínguez-Mosquera et al., 1994). Kaewsuksaeng et al. (2011) showed that PPH activity gradually increased during storage in Tahitian lime fruit, while the UV-B treatment effectively suppressed PPH activity. For broccoli florets, it was found that PPH activity with or without UV-B treatment also increased during storage at 15 ˚C, but UV-B did not significantly affect PPH activity (Aiamla-or et al., 2012). In contrast, our results found a temporary increase in PPH activity in Thai lime after heat treatment, after that a decline during storage of lime fruits. However, hot water treatment at 50 ˚C for 5 min could greatly inhibit PPH activity in lime fruit. These findings seems to be due to the suppression of Chl-degrading enzymes such as Chlase, Mg-dechelation, Chl-degrading peroxidase and PPH activities by stress treatment such as hot water in Thai lime fruit. Further study needs to be carried out to clarify the characterization of Chl-degrading enzymes in relation to Chl degradation of lime fruit.

Besides Chl degradation, the physiological effects were determined in ethylene production and respiration rate. The results showed that the endogenous ethylene production slightly increased from mature green to full yellow in control of lime fruit, while the hot water-treated fruit at 50 ˚C for 5 min did not change during storage. Application of heat treatment such as hot water at 50 ˚C for 5 min significantly reduced ethylene production in lime fruit. This might be because of the suppression of ethylene production by hot water treatment due to the decrease of ACC (1-aminocylocpropane-1-carboxylic acid) oxidase action in the ethylene pathway in lime fruit (Win et al., 2006b). The composition changes in relation to quality occur during the storage of lime fruit. The citric acid content, determined to be titratable acidity in lime fruit, showed a slight change during storage. This content was higher in hot water- treated fruit at 50 ˚C for 5 min than any other during storage, suggesting that this acid might be maintained due to the suppression of respiration rate by hot water treatment. Hot water treatment was similar to that UV-B treatment in maintaining the acid content in lime fruit (Kaewsuksaeng et al., 2011). Hot water treatment at 50 ˚C for 5 min also suppressed the changes in total soluble solid during storage. On the other hand, total soluble solids 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, hot water treatment seems to be a useful treatment for the maintenance of postharvest internal quality in lime fruit.

CHAPTER 6

CONCLUSION

1. The findings obtained in the present study show that UV-B dosage of at 19.0 kJm^{-2} effectively retarded the degradation of Chl in lime fruits during storage. The reduction of Chl-degrading enzyme activities such as Chlase, Chl-POX, MDS and PPH were also suppressed by UV-B treatment, indicating that the suppression of those enzyme activities by UV-B treatment could be involved in retardation of Ch degradation in stored lime fruits. Furthermore, UV-B treatment maintained the internal quality as citric acid and malic acid contents and greatly reduced the level of glucose, fructose and sucrose contents. Finally, UV-B treatment has an inductive effect on stomatal closure in lime fruit peel. We suggest that UV-B treatment could be a useful method to prolong postharvest senescence and quality of lime fruit during storage at 25 ˚C.
2. These results suggest that heat treatment such as hot water at 50 ˚C for 5 min effectively retarded the Chl degradation in Thai lime fruit during 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 the 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 solid 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.

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APPENDIX