

PRESENCE OF PHEOPHYTIN AND ITS FORMATION AS A CHLOROPHYLL DERIVATIVE IN SELECTED CROP SPECIES

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ABSTRACT

Most of the green leaf vegetable and fruits lose their postharvest quality mainly due to yellowing as a consequence of chlorophyll (Chl) degradation. The study of Chl degradation of many plants have been revealed that the Chl degradation mainly progress through formation of chlorophyllide (Chlide) *a*. Recent studies have revealed that the Chl degradation could be progressing through formation of pheophytin (Phy) *a* as a Chl derivative. Present study was conducted to investigate the presence and formation of Phy *a* as Chl *a* derivative in selected stored plant species. Green tea leaves, bell pepper, parsley and Japanese bunching onion were used in the study. Presence of Chl *a* derivatives of 80% acetone extract of green tea leaves and Japanese bunching onion were detected using HPLC at the wave length of 663nm. Formation of Chl *a* derivatives were studied by incubating crude enzyme extract of bellpepper, parsley and bunching onion with Chl *a* and buffer solution. After one hour of incubation reaction was stopped by adding 80% acetone. Chl *a* derivatives were studied using HPLC at 663nm. Phy *a* level of processed green tea leaves was very high when compared to other plants. In parsley, bellpepper and Japanese bunching onion formation of Phy *a* was prominent. All these indicated that early studies of Chl degradation need further study to elucidate the mechanism of Chl degradation in green plants.

Keywords: Green tea leaves, Japanese bunching onion, Bellpepper, Parsley, Chlorophyll degradation, Chlorophyll derivatives, Pheophytin

INTRODUCTION

The economic significance of chlorophyll (Chl) breakdown is considered in the context of agriculture, especially when evaluating postharvest losses of stored green vegetables and ripening of fruits. Food losses are significant in horticultural crops by yellowing or off-colouring mainly as a consequence of Chl degradation. The application of different mechanisms to extend the shelf life of horticultural commodities can be effective if the process of Chl degradation is elucidated. Chl degradation, which is not extensively studied, is an essential and inevitable natural process in green plants. Study of Chl degradation mechanism of plants will give effective background to extend the keeping quality of green plants.

The studies of Chl degradation of many plants have revealed that the Chl degradation mainly

progress through formation of Chlide. The pathway for breakdown of Chl consists of several reaction steps. In the last few years, the degradation pathway of Chls has gradually become clear (Kräutler and Matile, 1999; Matile *et al.*, 1999, Hörtensteiner, 2006; Kräutler and Hörtensteiner, 2006). The hydrolysis of Chl *a* into Chlide *a* and phytol by the activity of chlorophyllase is thought to be the first step of Chl *a* degradation (Willstätter and Stoll, 1913; Holden, 1961; Shimokawa *et al.*, 1978; Amir-Shapira *et al.*, 1987). The second step would be the elimination of Mg⁺² from Chlide *a* to produce pheophorbide (Pheide) *a*, in a reaction catalyzed by Mg-dechelatease or metal chelating substances (Langmeier *et al.*, 1993; Vicentini *et al.*, 1995; Suzuki *et al.*, 2005). Finally, Pheide *a* was decomposed to fluorescent

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Chl catabolites, which are primary colourless catabolites, via red Chl catabolites, by both Pheide *a* oxygenase and red Chl catabolite reductase (Matile *et al.*, 1999). These reactions, which are thought to form the main pathway of Chl *a* degradation, occur in the chloroplast (Matile *et al.*, 1999) (Figure 01). However, the degradation pathway of Chls remains yet to be clarified, compared with the biosynthesis pathway of Chls.

On the other hand, peroxidase (Kato and Shimizu, 1985; Yamauchi and Minamide, 1985) or Chl oxidase (Schoch *et al.*, 1984) are also reported to be involved in *in vitro* Chl *a* oxidation to form C13²- hydroxychlorophyll

(OHChl) *a*. However, instead of removal of Mg⁺² from Chlide *a*, Tang *et al.* (2000) showed a direct removal of Mg⁺² from Chl *a* to form Phy *a* by Mg-dechelatease in *Ginkgo biloba* leaves (Figure 01). The degradation process of Chls to form Phy *a* is still not clearly clarified and demands further study to elucidate the pathway for Chl degradation.

Recent studies have revealed that the chlorophyll degradation could progress through formation of pheophytin (Schelbert *et al.*, 2009). Therefore, this study was conducted to investigate the presence and formation of Phy as Chl derivative in selected plant species.

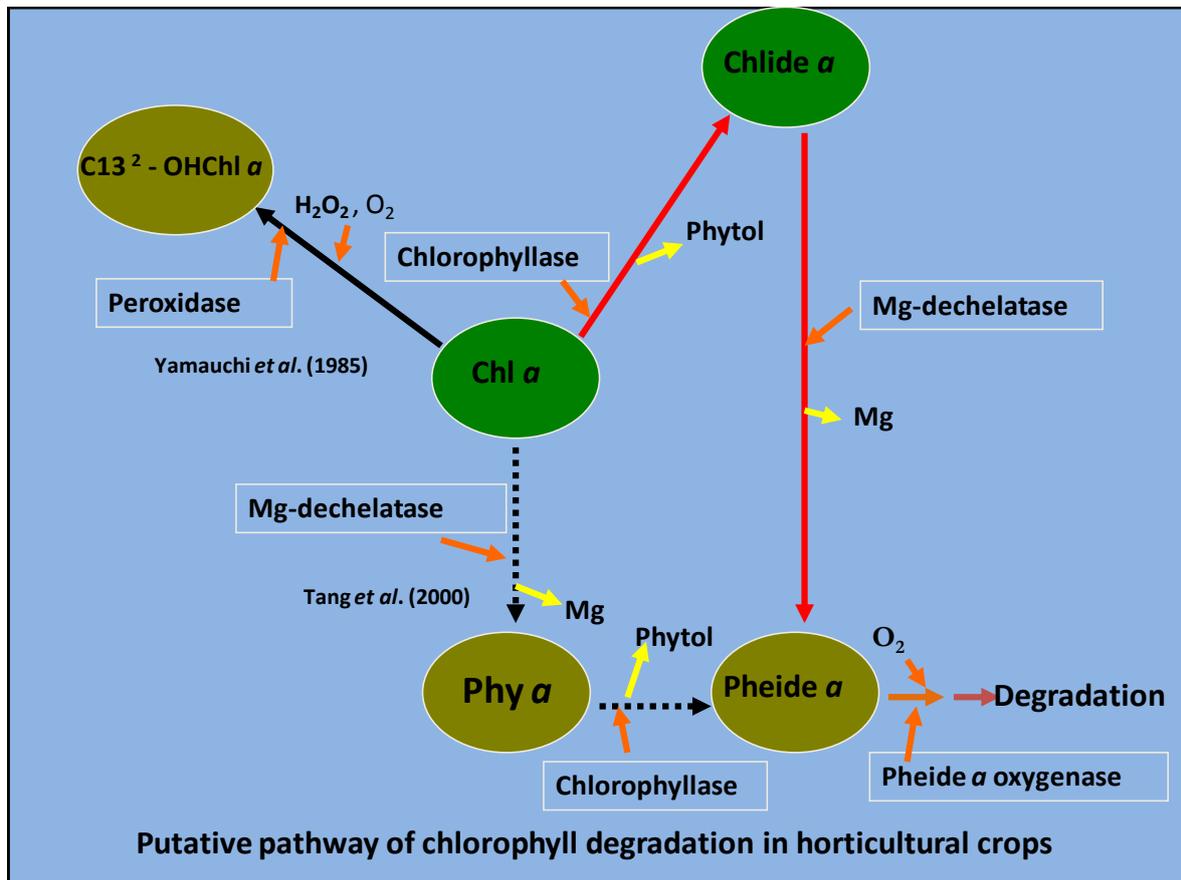


Figure 01: Putative pathway of Chlorophyll degradation in horticultural crops. Where; Chl *a* = Chlorophyll *a*, Chlide *a* = chlorophyllide *a*, Pheide *a* = pheophobide *a*, Phy *a* = Pheophytine *a*, C13²-OHChl *a* = C13²- hydroxychlorophyll *a*.

METHODS AND MATERIALS

Plant material

Green tea leaves were obtained from a green tea factory in Japan. Parsley and bell pepper were obtained from farmers in Yamaguchi city, Japan. Leaves of Japanese bunching onion (FF) and its two alien monosomic addition line (AMAL)s (FF+3A and FF+4A) were obtained from experiment field at the experimental farm, Yamaguchi University, Japan. Eight AMALs of Japanese bunching onion (FF+1A, FF+2A, FF+3A, FF+4A, FF+5A, FF+6A, FF+7A and FF+8A) have been produced by Shigyo *et al.* (1996) inserting one extra chromosome from shallot (AA) (8 chromosome) to Japanese bunching onion.

Determination of presence of Chl derivatives

One g of leaf tissue of green tea leaves or Japanese bunching onion was homogenised on ice in an extraction mixture containing 8 mL of acetone and 1 mL of 50 mM HEPES (80% acetone), pH 7.5, using a mortar and pestle. After homogenisation, the mixture was kept on ice for 5 min to allow all pigments to partition into the extraction solution. The extracts were filtered using an ADVANTEC # 2 filter paper (Tokyo, Japan) and the filtrate was further purified through a 0.45 µm DISMIC filter (ADVANTEC) and used to analyse Chl *a* derivatives by HPLC. Pigments were separated on an HPLC (Hitachi, Tokyo, Japan) equipped with a Hitachi Model L-2130 pump with an automated gradient controller and a Model L-7420 UV-Visible spectrophotometer (Hitachi) using a LiChrospher 100 RP-18 column (250 mm x 4 mm; Merck, Tokyo, Japan) and two solvents. Solvent A was 80:20 (v/v) methanol: water, and solvent B was ethyl acetate. Solvent A was added to solvent B at a linear rate over 20 min, until a 50:50 (v/v) mixture was attained. The 50:50 (v/v) mixture was kept isocratic for an additional 20 min. The flow rate was 1 mL min⁻¹, and the sample injection volume was 100 µL. The absorption spectrum of the pigments was recorded at 665

nm. Identification of Chl *a* derivatives was based on their retention times and visible absorption spectra, using the standards (Yamauchi and Watada, 1991).

Determination of formation of Chl derivatives

Acetone powder of plant tissues of parsley (leaves), bell pepper (fruit), and Japanese bunching onion (leaves) were prepared and used for crude enzyme extraction. Five gram of leaf tissues were homogenized in 50mL of cold acetone in a homogenizer covered with dry ice pellets. The homogenate was vacuum filtered and remnant of leaf tissues on filter paper were used for further homogenization in cold acetone. After third homogenization and filtration, remnant on filter paper was washed using 100% chilled diethyl ether. The remains on filter paper were collected and vacuum dried and used as acetone powder. 500mg of acetone powder of plant tissues were suspended in 15mL of 10mM of phosphate buffer (pH 7) containing 50mM KCl and 0.24% CHAPS and stirred it for one h. The mixture was filtrated through Miracloth (Calbiochem, Japan) and filtrate was centrifuged at 16000xg for 15min at 4°C. The supernatant was used as crude enzyme. The reaction mixture containing 0.5mL crude enzyme, 0.2mL Chl *a*, 0.5mL Phosphate buffer (pH7.5) and 0.1mL 1% CHAPS (1g/100mL) was incubated for one hour and stopped the reaction by adding 80% acetone. Chl derivatives were analyzed using HPLC at 665nm.

RESULTS AND DISCUSSION

Presence of Chlorophyll derivatives

Presence of Chl derivatives in leaf extract of green tea leaves which are treated differently in the tea processing center is shown in Figure 02. It is evident that as the Chl derivatives OHChl *a*, Chlide *a*, Pheide *a*, and Phy *a* are present in leaf extract of Chl. Among them Chlide *a* and Phy *a* are prominent derivatives in extract. Presence of Phy *a* is the prominent Chl derivative and content is at least two times higher than the other compounds in all the

treatments.

Chlide *a*, Pheide *a*, OHChl *a* and Phy *a* are present in bunching onion leaves as Chl derivatives (Figure 03). Phy *a* is the prominent Chl *a* derivative and OHChl *a* also present in considerable amount in bunching onion leaves. However, level of Chlide *a*, which is

considered as main Chl derivatives in most of the plant studied is comparatively low in Japanese bunching onion. Level of Phy *a* is more than two times higher than that of OHChl *a* in FF+3A, where it was lower than . in FF and FF+4A.

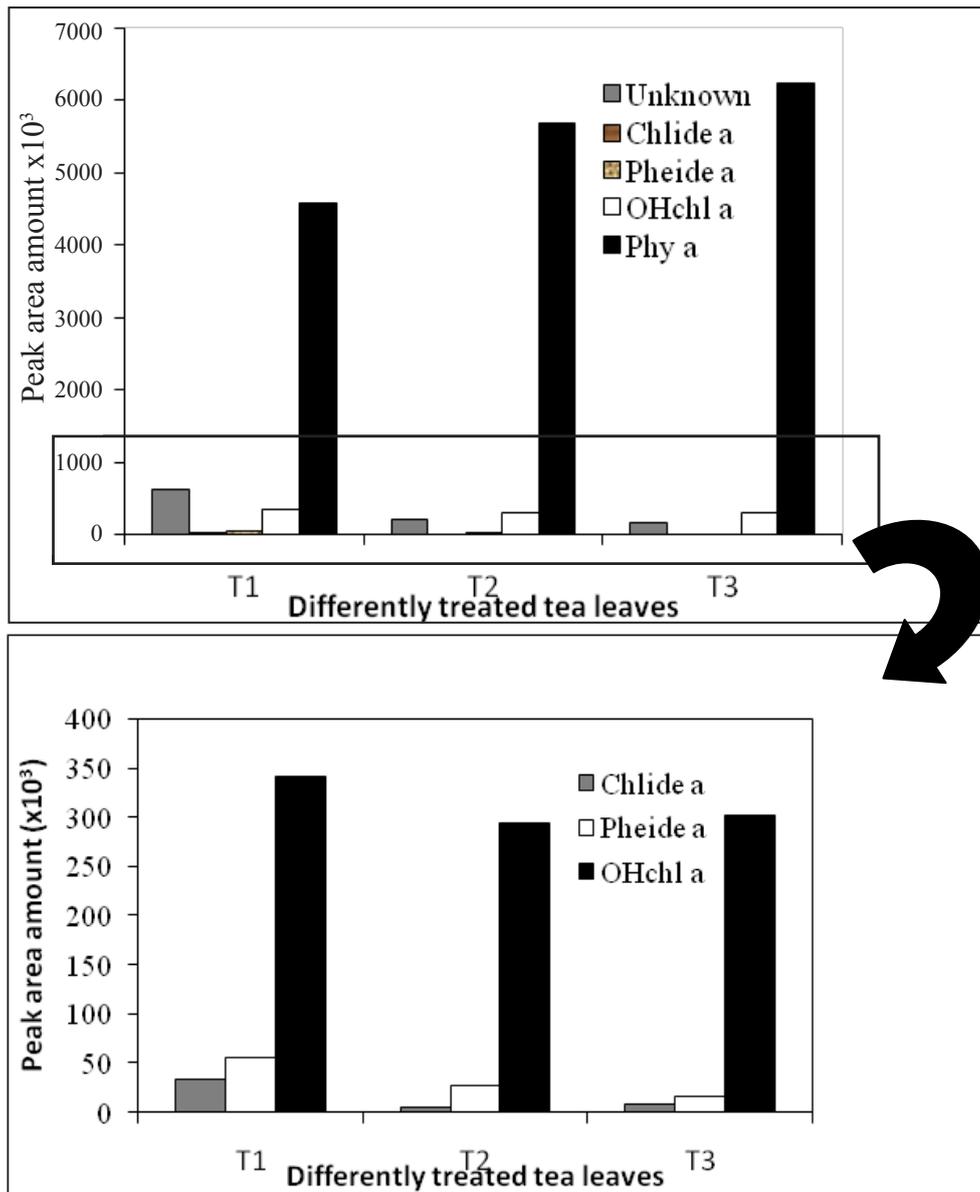


Figure 02: Presence of Chl (Chlorophyll) derivatives in differently treated (T1,T2,T3; treatments are not necessary as we are not checking treatment effect) green tea leaves detected by HPLC chromatogram. Where; Chlide a = chlorophyllide a, Phy a = pheophytin a, OHChl a = C13²⁻ hydroxychlorophyll a, Pheide a = pheophorbide.

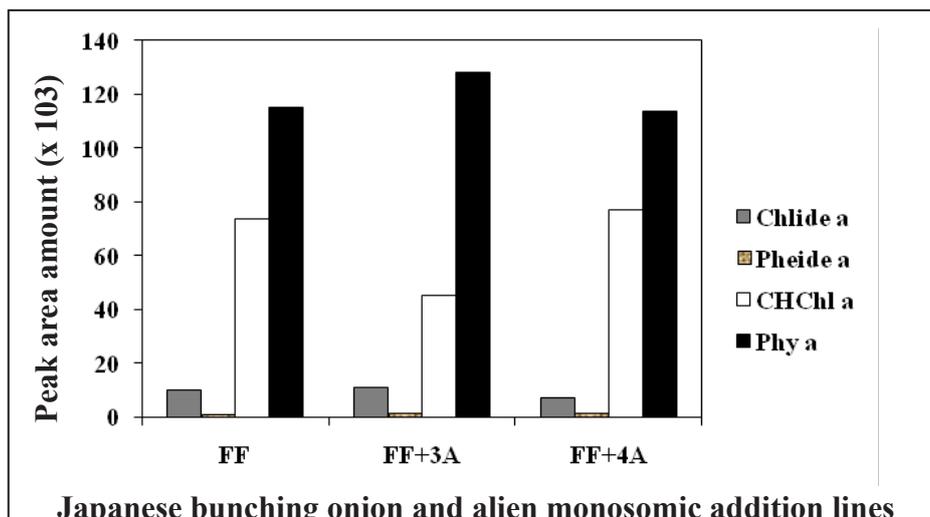


Figure 03: Presence of Chl (Chlorophyll) derivatives in Japanese bunching onion (FF) and two monosomic addition lines (FF+3A, FF+4A) detected by HPLC chromatogram. Where; Chlide a = chlorophyllide a, Phy a = pheophytin a, OHChl a = C13²- hydroxychlorophyll a, Pheide a = pheophorbide.

Formation of Chl Derivatives

Study of formation of Chl derivatives in Japanese bunching onion is present in Figure 04. It shows that Chlide *a* and Phy *a* are formed, to a great extent, during one hour of incubation of reaction mixture. The levels of formations of rest of the derivatives are minute. Formation of Phy *a* is relatively high in FF and in FF+3A. It is 10 times higher than that of Chlide *a* whereas in FF+4A level of formation of Phy *a* is just two times higher

than that of Chlide *a* formation.

Formation of Pheide *a* Chlide *a* and Phy *a* can be observed in bell pepper fruit peel extract (Figure 05). In contrast to Japanese Bunching onion, in here, formation of Chlide *a* is two times higher than the formation of Phy *a*. Formation of Pheid *a* is also more prominent compared to Japanese bunching onion. In Parsley formation of Phy *a* is nearly 10 times higher than formation of Chlide *a*.

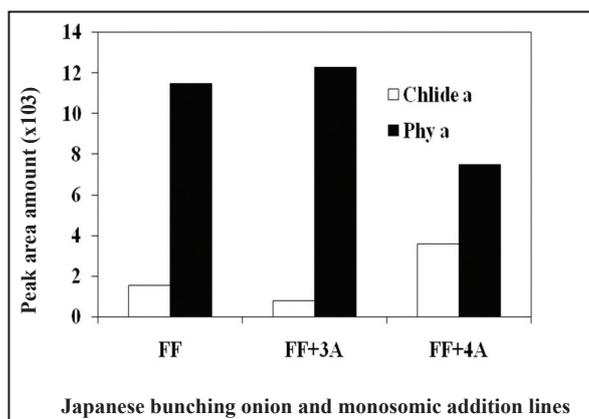


Figure 04: Formation of chlorophyll derivatives in leaf extract of Japanese bunching onion (FF) and two monosomic addition lines (FF+3A, FF+4A) after one hour of incubation with enzyme reaction mixture at 25°C. Where; Chlide a = chlorophyllide a, Phy a = pheophytin a.

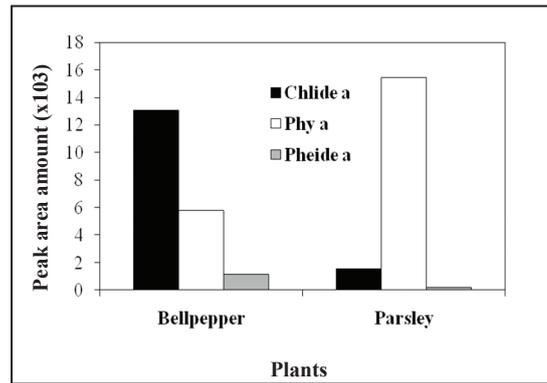


Figure 05: Formation of chlorophyll derivatives in leaf extract of Parsley and Bellpepper after one hour of incubation with enzyme reaction mixture at 25°C. Where; Chlide a = chlorophyllide a, Phy a = pheophytin a, Pheide a = pheophorbide.

In present study the presence of Phy *a* in tea leaf extract is recorded very high. This is an interesting finding because it gives clues to think the pathway of Chl degradation in green tea leaves, and accordingly in green tea leaves the Chl degradation could progress through formation of Phy *a*. Even though the levels of Phy *a* is different to each other in differently treated green tea, the Phy *a* is the prominent Chl derivative in green tea leaves. The pathway of Chl breakdown consists of several reaction steps (Hörtensteiner, 2006; Kräutler and Matile, 1999; Matile *et al.*, 1999). The presence of Phy as Chl derivatives were not found significant and thus the pathway of Chl degradation could be progressing through Chlide. However, resent studies have been reveling the presence of Phy *a* as a Chl derivatives in *Ginkgo biloba* leaves (Tang *et al.*, 2000). On the other hand, in plant processing the tendency to form Phy is high. Therefore, further study is demanding in this regard.

Japanese Bunching onion and two AMALs (FF+3A and FF+4A) also showed Phy *a* as Chl derivatives. This inevitably indicated that the Phy *a* is the prominent Chl *a* derivative in Japanese bunching onion leaves. The ex-

periments conducted to investigate the formation of Chl *a* derivatives during one hour of incubation of enzymatic reaction mixture also clearly showed that the Phy *a* formation has taken place due to enzymatic reaction. In previous studies FF+3A was identified as a relatively fast Chl degrading line among 8 different AMALs of JBO, whereas the FF+4A is the slowest Chl degrading line among the lot. The FF also showed fast Chl degradation (Disanayake, 2008). The results revealed a relationship between rate of Chl degradation and rate of formation of Phy *a* (Figure 04). With the increase of rate of Chl degradation formation of Phy *a* was also increased. Formation of Chlide *a* also showed low compared to Phy *a* formation. This indicated that the Chl degradation could possibly be taking place through formation of Phy *a*. It is worthy to investigate the reason behind formation of Phy *a* in fast Chl degrading lines of Japanese bunching onion. Despite the fact that there were differences in the pattern of formation of Phy *a* between fast and slow Chl-degrading lines, from the present findings it was shown that Phy *a* was formed as a catabolite of Chl *a* degradation in JBO leaves.

The study on formation of Phy *a* in bellpepper and parsley leaves also showed that Phy

a formed during Chl degradation. Moreover, the findings of Amir-Shapira *et al.* (1987) on the formation of Phy *a* in parsley leaves during storage supports the present results. However, Yamauchi and Watada (1993) did not observe increasing Phy *a* in stored parsley leaves. In bellpepper, the formation of Chlide *a* is higher than that of Phy *a*. All these results bring our attention to the pathway of Chl degradation, which needs further clarification. The question in current context is whether Chl degradation taken place through the formation of Chlide *a* or Phy *a* or through both ways.

CONCLUSIONS

The results lead to the conclusion, the Chl degradation pathway is not necessarily go through Chlide *a* but Phy *a* too. This emphasises that the elucidation of Chl degradation needs further attention in future experiments.

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