(Format No. 3)

# SUMMARY OF DOCTORAL THESIS

Name: Sukanya Aiamla-or

Title: Postharvest changes in activities and gene expression of enzymes relating to chlorophyll degradation in broccoli florets and their control by UV-B treatment

ブロッコリー花蕾のクロロフィル分解に関与する酵素の活性および遺伝子発現における収穫後変化と UV-B 処理によるその制御

---

Broccoli (Brassica oleracea L. Italica Group) contains high amounts of antioxidants, vitamins and anti-carcinogenic compounds, which are health-promoting phytochemicals. Green coloration of florets, which indicates freshness or good quality, is preferred by consumers. However, postharvest broccoli rapidly undergoes floret yellowing. The shelf life of broccoli florets transported and stored at ambient temperature will only be one or two days at market, then the florets begin to turn yellow, resulting in chlorophyll (Chl) degradation. Therefore, suitable postharvest treatments are required to maintain broccoli quality. In this study, postharvest changes in activity and gene expression of Chl-degrading enzymes relating to Chl degradation in broccoli florets were determined and the control of Chl degradation by UV treatment was also examined to maintain quality during storage.

Broccoli florets were irradiated with UV-A and UV-B and then kept in darkness at 15 ºC. UV-B irradiation was more effective in retaining the green color of broccoli florets as compared to UV-A irradiation. UV-B doses of at least 19 kJ m–2 to broccoli florets resulted in surface color with a higher hue angle, as compared to those treated with 9.5 kJ m–2 UV-B or without UV-B. Therefore, a UV-B dose of 19 kJ m–2 was selected and applied to broccoli cultivars ('Pixel' and 'Sawayutaka'), harvested during the winter and early summer seasons and kept at different storage temperatures. Broccoli florets stored at 4 and 15 °C showed slow floret yellowing, while broccoli florets stored at 25 °C turned yellow more quickly during storage. In contrast, UV-B treatment delayed floret yellowing and the decrease of hue angle value at 4 ºC and 15 ºC. The 'Sawayutaka' cultivar exhibited a slower decrease in green coloration of florets during storage, when compared to the 'Pixel' cultivar. UV-B treatment delayed floret yellowing and Chl degradation. Broccoli harvested in winter or early summer and irradiated with UV-B during storage at 15 ºC displayed higher Chl content and hue angle value than broccoli without UV-B treatment, suggesting that UV-B irradiation is effective in retaining the green color of florets during storage.

The formation of Chl derivatives with Chl degradation during storage was examined in broccoli florets with a UV-B treatment dose of 19 kJ m–2 or without UV-B treatment. Chlorophyllide a and 132-hydroxychlorohyll a gradually decreased with senescing broccoli florets. Pheophorbide (Pheide) a and pyropheophorbide a levels were significantly higher in broccoli florets without UV-B treatment. Moreover, changes in the activities of Chl-degrading enzymes, such as chlorophyllase (Chlase), Chl-degrading peroxidase (POX), Mg-dechelation enzyme (Mg-dechelatase; MD) and Mg-dechelating substance (MDS), were determined in broccoli florets during storage. Chlase and Chl-degrading POX activities with UV-B treatment were suppressed, as well as the activity of MD. MDS activity was also suppressed with this treatment. Recently, pheophytinase (PPH) has been reported to be an important enzyme involved in Chl degradation, such that Arabidopsis mutants deficient in PPH accumulated pheophytin (Phy) a during senescence (Schelbert et al., 2009). However, the accurate measurement of Pheide a formation by PPH during plant senescence could be difficult because Chlase also uses Phy a as a substrate. Hence, a methodology for measuring PPH activity in

broccoli florets using Phy a as a substrate was defined. The PPH protein fraction was separated from Chlase protein by ammonium sulfate precipitation. The protein precipitated by 45-60% saturated ammonium sulfate included a little bit of Chlase activity and was suitable for PPH determination. PPH activity in broccoli florets treated with a UV-B dose of 19 kJ m–2 was repressed for the first 2 days of storage at 15 °C, whereas it increased gradually with senescence of control broccoli florets.

Considering that in Chl-degrading enzymes, especially Chl-degrading POX activity was markedly increased with floret senescence, purification and characterization of Chl-degrading POX were made in an attempt to clarify its physiological role on Chl degradation in broccoli florets. Chl-degrading POX isozymes, Type 1, Type 2 and Type 3, were separated by CM-Sepharose ion exchange chromatography. Type 1 was detected in fresh broccoli florets and the activity gradually increased with floret yellowing. On the other hand, the activity of Type 1 was not clearly inhibited by UV-B treatment. The activities of Type 2 and Type 3, especially the latter, were detected in broccoli florets on day 4 and those were suppressed by UV-B treatment. Conversely, these two types were not found in fresh broccoli florets, indicating that Type 2 and Type 3, especially the latter, might be appreciably involved in Chl degradation. Furthermore, Type 1 and Type 3 were purified by successive chromatographic techniques and afterward, the characterization of them was also determined. The results showed that only Type 1 was a glycoprotein, and absorbed by Concanavalin A (ConA) resins and stained by the periodic acid-Schiff (PAS) reaction method. Molecular sizes of Type 1 and Type 3 were 43 and 34 kDa, respectively. In addition, the possibility of localization of Type 3 in chloroplasts was examined since it hardly had a glycoside chain. Intact chloroplasts from green and green-yellow broccoli florets were separated by Percoll gradient centrifugation. Protein immunoblot using antibodies obtained from purified Chl-degrading POX (Type 1 and Type 3) showed that only Type 3 was found in the intact chloroplast fraction that was isolated from green-yellow florets. Besides, both Type 1 and Type 3 were not localized in broken chloroplast fraction containing mainly thylakoid membranes. These results demonstrated that Type 3 Chl-degrading POX could be in the stroma and/or envelope of chloroplasts and relate to Chl degradation in the chloroplast. Furthermore, N-terminal amino acid sequences of Type 1 and 3 proteins were revealed as Ala-Arg-Ala-Asp-Ala-Asp-Ala-Met-Ala-Trp and Cys-Met-Pro-Gln-Leu-Pro-Ala-Pro-Ala, respectively. The pattern of amino acid sequences in both Types differed considerably with each other.

The gene expressions of Chl-degrading enzymes in broccoli florets with or without UV-B treatment dose of 19 kJ m–2 were determined. The expression level of BoCLH1 was reduced in broccoli florets on day 4 of storage, while BoCLH2 and BoCLH3 were up-regulated with UV-B treatment. A high BoPAO expression level was found in senescent broccoli florets, and the up-regulation of this gene was delayed by UV-B treatment. The highest expression level of BoPPH was found in the control, and its expression was clearly repressed by UV-B treatment on day 2 of storage. These results suggest that the up-regulation of Chl-degrading enzyme genes could be delayed by UV-B treatment, resulting in the suppression of floret yellowing in stored broccoli.

In conclusion, the enhancement of Chl-degrading enzymes, especially Chl-degrading POX, could be related to Chl degradation in stored broccoli florets. Two cationic POX isozymes, especially Type 3 POX, were found in appreciable amounts in senescing broccoli florets, and Type 3 was localized in the chloroplast, suggesting that Type 3 could be involved in Chl degradation with senescence in broccoli florets. Gene expressions of BoPAO and BoPPH increased with floret yellowing during storage. The activities and gene expressions of Chl-degrading enzymes were effectively suppressed not by UV-A but by UV-B treatment, which could suggest that UV-B treatment is useful as a postharvest treatment for maintaining quality of broccoli florets.