J. Japan. Soc. Hort. Sci. 76 (4): 338–344. 2007.
Available online at www.jstage.jst.go.jp/browse/jjshs

Effect of Heat Treatment on Catabolites Formation in Relation to Chlorophyll Degradation during Storage of Broccoli (*Brassica olearacea* L. Italica Group) Florets

Samak Kaewsuksaeng[1,2], Naoki Yamauchi[1,3]*, Yoshio Funamoto[3], Tomohiko Mori[1], Masayoshi Shigyo[1,3] and Sirichai Kanlayanarat[2]**

[1]*Faculty of Agriculture, Yamaguchi University, Yoshida, Yamaguchi 753–8515, Japan*
[2]*Division of Postharvest Technology, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand*
[3]*The United Graduate School of Agricultural Sciences, Tottori University, Koyama-Minami, Tottori 680–8553, Japan*

The effects of heat treatment on the formation of chlorophyll (Chl) derivatives and Chl-degrading enzyme activities in stored broccoli (*Brassica oleracea* L. Italica Group 'Ryokutei') florets were determined. The Chl *a* level barely changed during heat treatment, but each Chl *a* derivative level changed. Chlorophyllide (Chlide) *a*, pheophorbide (Pheide) *a*, C13^2-hydroxychlorophyll (C13^2-OHChl) *a*, Chl *a'*, an isomer of Chl *a*, and pheophytin (Phy) *a* were detected as a Chl derivative during heat treatment and during storage after treatment. Chlide *a*, Pheide *a*, and C13^2-OHChl *a* levels decreased during 2-h heat treatment (50°C), whereas Chl *a'* and Phy *a* levels increased. Chl-degrading enzyme activities, in particular, Mg-dechelation activity, were effectively suppressed after 2 h of heat treatment. The content of Chls *a* and *b* in control broccoli florets decreased greatly during storage at 15°C, while the content in heat-treated broccoli florets hardly changed at all. In Chl derivatives, the Pheide *a* level in broccoli florets treated with or without heat treatment, especially the former, increased appreciably during storage. The Chlide *a* level in control florets decreased markedly during storage. On the other hand, the Chlide *a* level in heat-treated broccoli florets did not change during storage. Mg-dechelation activity in control florets markedly increased after 4 days of storage at 15°C, but the enhancement of the activity was suppressed by heat treatment. These findings suggest that Chl derivatives, especially Pheide *a*, are accumulated as intermediates in heat-treated broccoli florets, and Mg-dechelating action, in conjunction with that of chlorophyllase and Chl-degrading peroxidase, could be involved in Chl degradation in stored broccoli florets.

Key Words: broccoli, chlorophyll degradation, chlorophyll derivatives, heat treatment.

Introduction

In general, the yellowing of leaves, florets and fruit pericarp is an important factor indicative of quality deterioration in stored horticultural products. Obviously, in broccoli, the most visible deterioration is the loss of sepal greenness that usually occurs with chlorophyll (Chl) breakdown (Costa et al., 2005; Yamauchi and Watada, 1991, 1993).

An early step in Chl *a* degradation seems to be removal of the side chain attached to the tetrapyrrole macrocycle to form chlorophyllide (Chlide) *a* by chlorophyllase. The newly formed Chlide *a* retains a green color (Amir-Shapira et al., 1987; Shimokawa et al., 1978). The elimination of Mg^{2+} from Chlide *a* to produce pheophorbide (Pheide) *a* is induced by a Mg-dechelatase (Langmeier et al., 1993) or Mg-dechelating substance (Shioi et al., 1996), and the Pheide *a* thus loses its green color. Finally, Pheide *a* is decomposed to fluorescent Chl catabolites, which are primary colorless catabolites, via a red Chl catabolite by both Pheide *a* oxygenase and red Chl catabolite reductase (Matile et al., 1999). In this pathway, the conversion of Chlide *a* to Pheide *a* could

Received; January 31, 2007. Accepted; June 6, 2007.
This work was supported in part by a Grant-in-Aid for Scientific Research (No. 17580029) from the Japanese Society for the Promotion Science and the Commission on Higher Education Staff Development Project, the Royal Thai Government.
* Corresponding author (E-mail address: yamauchi@yamaguchi-u.ac.jp).
** Present address: Kobeya Bakery Company, Higashiyodogawa-ku, Osaka, Japan.

be the first step in degreening, and Mg-dechelation activity related to this reaction seems to have an effect on the quality of horticultural produce.

During the past few years, there has been increasing interest in heat-treatment methodologies for the control of insect pests, prevention of fungal rots, and modification of ripening or response to temperature extremes of commodities (Lurie, 1998). Heat treatment is frequently used to maintain the postharvest quality of many horticultural crops (Costa et al., 2005; Funamoto et al., 2002, 2003; Kazami et al., 1991; Lurie, 1998; Terai et al., 1999; Tian et al., 1996). The advantages of postharvest treatments that result in a reduction of the yellowing of broccoli florets and suppression of the activities of Chl-degrading enzymes, such as chlorophyllase and Chl-degrading peroxidase, have been reported (Funamoto et al., 2002, 2003).

In this paper, we evaluate the changes in Chl derivatives during storage in heat-treated broccoli florets. We also examine the changes of Chl derivatives and Chl-degrading enzyme activities during heat treatment. Finally, we discuss the physiological role of Chl-degrading enzymes, including their action in Mg-dechelation on Chl degradation in stored broccoli, and the effect of heat treatment on their regulation.

Materials and Methods

Plant material and preparations

Fresh broccoli (*Brassica oleracea* L. Italica Group 'Ryokutei') heads were harvested in Aio, Yamaguchi City and transported to the laboratory of Horticultural Science, Yamaguchi University. The broccoli heads were held in an incubator (MIR-153, Sanyo, Japan) in which hot air (50°C) was circulated for 2 h. During the treatment, heads were loosely covered with perforated polyethylene bags to reduce weight loss. The heads were kept in polyethylene film bags (0.03 mm in thickness) with the top folded over, placed in a corrugated cardboard box (290 mm × 400 mm × 170 mm), and stored at 15°C in the dark. Three replicates of three heads were removed at scheduled intervals during heat treatment and the 6-day period of storage. The floral tissues were analyzed.

Surface color

The surface color of broccoli florets was determined by measuring the hue angle with a colorimeter (NF 777, Nippon Denshoku, Japan).

Chlorophyll contents and chlorophyll-degrading enzyme assays

The Chl content in floral tissues was determined using *N,N*-dimethylformamide (Moran, 1982).

For enzyme assays, an acetone powder (500 mg) was suspended in 15 mL of a 10 mM phosphate buffer (pH 7.0) for Chl-degrading peroxidase and in 15 mL of a 50 mM phosphate buffer (pH 7.0) containing 50 mM KCl and 0.12% Triton X-100 for chlorophyllase and Mg-dechelation activities. A crude enzyme was stirred for 1 h at 0°C and then filtered through Miracloth (Calbiochem, USA). Subsequently, the filtrate was centrifuged at 16000 × *g* for 15 min at 4°C. The supernatant was used as the crude enzyme extract.

Chlorophyllase activity was determined by modification of the method of Amir-Shapira et al. (1987). The reaction mixture contained 0.5 mL enzyme solution, 0.1 mL 1% CHAPS, 0.2 mL Chl *a* acetone solution (Chl *a*-100 mg·L^{-1}), and 0.5 mL of a 0.1 M phosphate buffer (pH 7.5). The mixture was incubated in water at 25°C for 40 min, and the enzyme reaction was stopped by the addition of 4 mL of acetone. The remaining (non-degraded) Chl *a* was extracted with 4 mL of hexane and assayed by reading the absorbance at 663 nm (U-2001, Hitachi, Japan). The activity was based on the decrease in absorbance by Chl *a* at 663 nm.

Mg-dechelation activity was determined by modification of the method of Suzuki and Shioi (2002). The reaction mixtures contained a crude enzyme solution (0.2 mL), 98 nM chlorophyllin *a* (0.3 mL), and a 50 mM Tris-HCl buffer (pH 8.0) (0.75 mL). Activity was measured at 35°C by following the change in OD at 686 nm. Chlorophyllin *a* was prepared according to the procedure of Vicentini et al. with slight modifications (1995). Thirty milliliters of a Chl *a* acetone solution was partitioned into 20 mL petroleum ether. The ether phase was separated, washed three times with 20 mL distilled water, and mixed with 1 μL of 30% (w/v) KOH in methanol per 30 μg Chl *a* to form chlorophyllin *a*. The solution in which chlorophyllin *a* was allowed to precipitate was centrifuged at 16000 × g at 4°C for 10 min. The precipitate was dissolved in distilled water and brought to pH 9 by adding 2 M Tricine.

Chl-degrading peroxidase was determined as described elsewhere (Yamauchi et al., 1997). The reaction mixture contained 0.5 mL enzyme solution, 0.1 mL 1.0% Triton X-100, 0.1 mL 5.0 mM *p*-coumaric acid, 0.1 mL 0.3% hydrogen peroxide, 0.2 mL Chl *a* acetone solution (Chl *a*-100 mg·L^{-1}), and 1.5 mL 0.1 M phosphate buffer (pH 5.5). Activity was determined spectrophotometrically by measuring the decrease of Chl *a* at 668 nm at 25°C.

One unit of chlorophyllase and Chl-degrading peroxidase was defined as a change of 1.0 μg Chl *a* degradation per min. Mg-dechelation activity was expressed as increased absorbance at 686 nm per min. The enzyme protein content was assayed by the method of Bradford (1976).

Analyses of chlorophyll and its derivatives
Pigment extraction

Broccoli florets (2 g) were homogenized in 9 mL of acetone-HEPES, which was prepared from 16 mL of cold acetone and 2 mL of a 50 mM HEPES buffer (pH 7.5), and the remaining solution (9 mL of acetone-HEPES) was then added. The extraction was continually

operated in the dark for 5 min. Subsequently, the aliquots were filtered through a DISMIC filter (0.45 μm, ADVANTEC, Japan) and used for HPLC analyses.

Preparation of chlorophyll and it derivatives

Chls were extracted from spinach (*Spinacia oleracea* L.), and Chls *a* and *b* were partially purified by adding 1,4 dioxane and distilled water to the acetone extract. The mixture was then allowed to stand until a precipitate formed. The mixture was then centrifuged at 15000 × g for 10 min, and the pellet was dissolved in 10 mL petroleum ether. Individual pigments were purified from the petroleum ether extract for use as a standard. Chls *a* and *b* were separated by sucrose column chromatography according to the method of Perkins and Roberts (1962).

Chlide *a* was prepared from Chl *a* by an enzymatic reaction using a chlorophyllase extracted from acetone powder of *Chenopodium album*, which has high chlorophyllase activity (Shioi et al., 1996). Pheophytin (Phy) *a* was prepared by adding one drop of 2N HCl to Chl *a* acetone solution. C13^2-hydroxychlorophyll (C13^2-OHChl) *a*, a derivative of Chl *a* oxidized at position 13^2, was prepared by adding peroxidase (horseradish, Sigma-Aldrich, USA) with the existence of H_2O_2 and *p*-coumaric acid to Chl *a* solution. Chl *a'*, an isomer of Chl *a*, was prepared by boiling the purified Chl acetone solution for 15 min.

Pheide *a* was purchased from Wako Pure Chemical Industries (Tokyo, Japan), and pyropheophorbide *a* was purchased from Tama Biochemical (Tokyo, Japan). The absorption spectrum and the retention time of each Chl and its derivative were measured and used as a standard.

HPLC analysis

Chl and its derivatives were 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 pigment was recorded at 665 nm. Pigments were separated on a LiChrospher C18 column (MERCK), 4 × 250 mm, using two solvents: A, methanol : water (80 : 20, v/v) and B, ethyl acetate in a gradient. Solvent B was added to solvent A at a linear rate until a 50 : 50 mixture was attained at the end of 20 min. The 50 : 50 mixture was then used isocratically for an additional 20 min, as described by Eskin and Harris (1981). The flow rate was 1 mL·min^{-1}, and the injection volume was 100 μL. The identification of Chl and its derivatives was based on the retention time and the visible absorption spectra.

Results

Changes in chlorophyll derivatives during heat treatment

The Chl *a* level barely changed during heat treatment, but each Chl *a* derivative level changed. The HPLC chromatogram of Chl derivatives extracted from broccoli florets demonstrated sequential elution of Chlide *a*, Pheide *a*, C13^2-OHChl *a*, Chl *a*', and Phy *a* during heat treatment (Fig. 1). Chlide *a*, Pheide *a*, C13^2-OHChl *a*, and Phy *a* were present in fresh broccoli florets. Chlide *a* level increased after 1 h of heat treatment at 50°C and then greatly decreased. Pheide *a* and C13^2-OHChl *a* levels decreased during heat treatment, whereas the Chl *a*' level increased during 2-h heat treatment. Phy *a* level also increased during heat treatment.

Changes in chlorophyll-degrading enzyme during heat treatment

As seen in Figures 2A and 2B, chlorophyllase and Mg-dechelation activities in broccoli florets increased after 1 h of heat treatment, and, later, those activities decreased by 9% and 20%, respectively. On the other hand, Chl-degrading peroxidase activity dropped gradually to 77% during heat treatment (Fig. 2C).

Changes in surface color and chlorophyll content during storage

Broccoli florets without heat treatment (control) remained green for 3 days of storage at 15°C and turned yellow on day 4. In comparison, heat-treated broccoli florets remained green through 6 days of storage. Hue angle values in the control declined gradually during storage at 15°C and then decreased significantly after day 6, in contrast to those of heat-treated broccoli florets, which changed little during storage (Fig. 3). Chls *a* and *b*

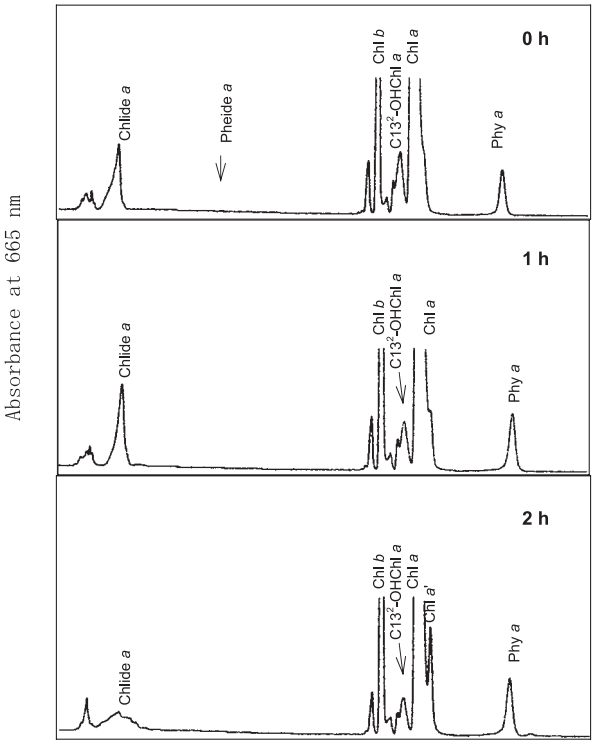

Fig. 1. Changes in HPLC chromatogram of chlorophyll and its derivatives of broccoli florets during heat treatment. Broccoli heads were held in hot air at 50°C for 2 h. Chl—Chlorophyll, Chlide—Chlorophyllide, Pheide—Pheophorbide, C13^2-OHChl—C13^2-hydroxychlorophyll, Phy—Pheophytin, Chl *a*'—an isomer of Chl *a*.

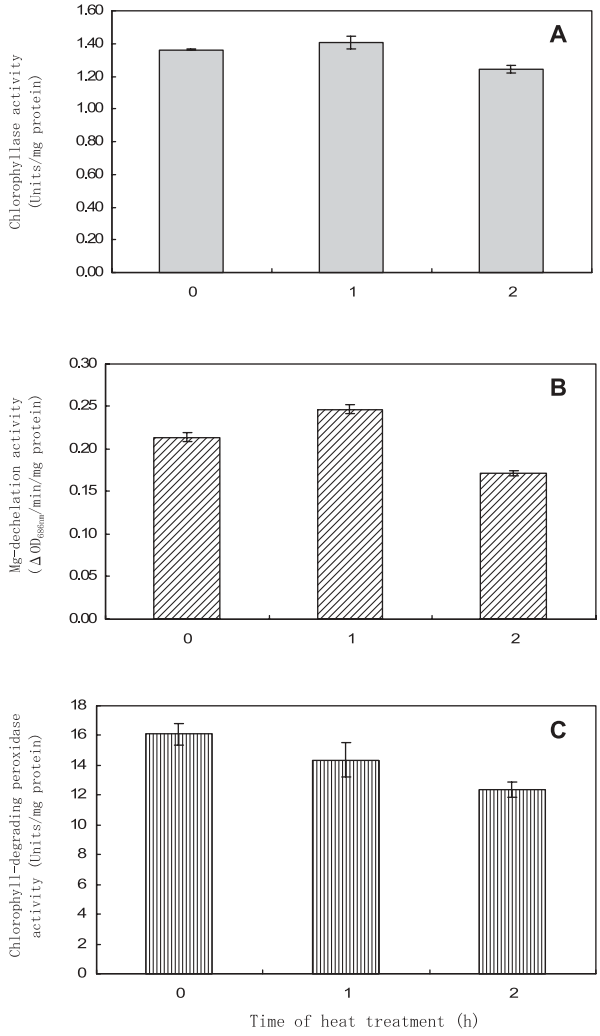

Fig. 2. Changes in chlorophyllase (A), Mg-dechelation activity (B) and chlorophyll-degrading peroxidase (C) of broccoli florets during heat treatment. Broccoli heads were held in hot air at 50°C for 2 h. Vertical bars represent average values with ± SD (n = 3).

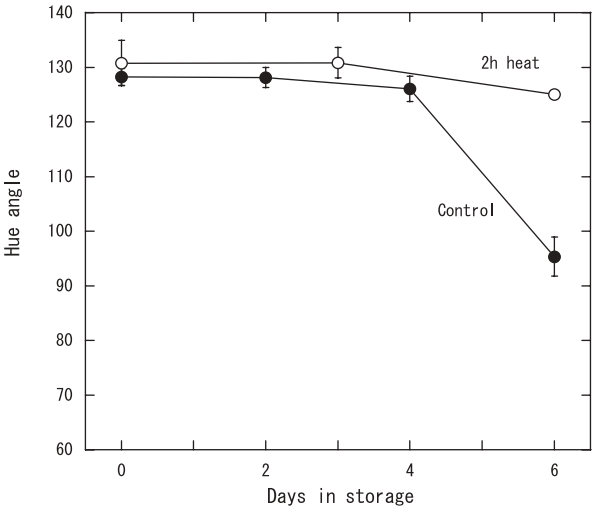

Fig. 3. Changes in hue angle of broccoli florets with or without heat treatment during storage at 15°C. Broccoli heads were held in hot air at 50°C for 2 h, followed by storage. Vertical bars represent average values with ± SD (n = 3).

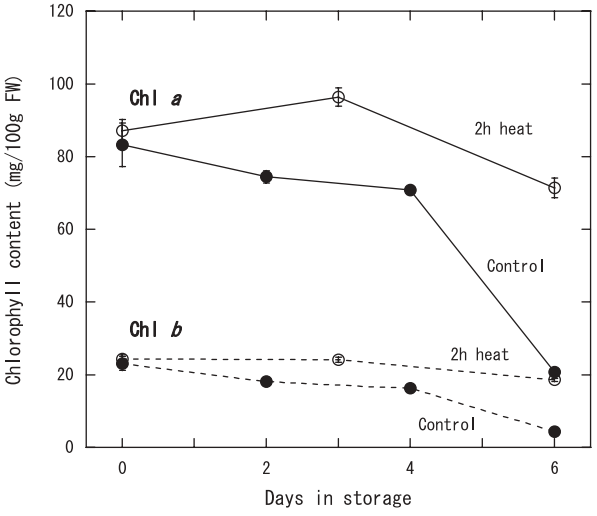

Fig. 4. Changes in chlorophylls contents of broccoli florets with or without heat treatment during storage at 15°C. Broccoli heads were held in hot air at 50°C for 2 h, followed by storage. Vertical bars represent average values with ± SD (n = 3). Chl—Chlorophyll.

contents in broccoli florets in the control decreased by 75% and 88% after 6 days of storage at 15°C, respectively, whereas the contents in heat-treated broccoli florets change little in the first 3 days of storage at 15°C and then decreased slightly (Fig. 4).

Changes in chlorophyll derivatives during storage

Chlide *a*, Pheide *a*, C13^2-OHChl *a*, and Phy *a* were mainly detected as Chl derivatives during storage at 15°C. The Chlide *a* level in the control decreased markedly during storage at 15°C and was barely observable on day 6, whereas that in heat-treated broccoli florets did not change during storage (Fig. 5A). The Pheide *a* level in broccoli florets with or without heat treatment increased during storage at 15°C, and the level in heat-treated broccoli florets was higher than that in the control during storage (Fig. 5B).

As shown in Figure 6A, C13^2-OHChl *a* levels with or without treatment decreased greatly during storage. The Phy *a* level in broccoli florets with or without heat treatment increased slightly for the first 3 or 4 days of storage at 15°C and then decreased sharply (Fig. 6B). Furthermore, Chl *a*' levels with or without treatment did not show a constant pattern during storage.

Changes in Mg-dechelation activity during storage

Mg-dechelation activity in broccoli florets, as is apparent in Figure 7, was efficiently suppressed during heat treatment, and the activity in heat-treated broccoli increased slightly during storage. On the other hand, activity in the control increased 2 days after storage at

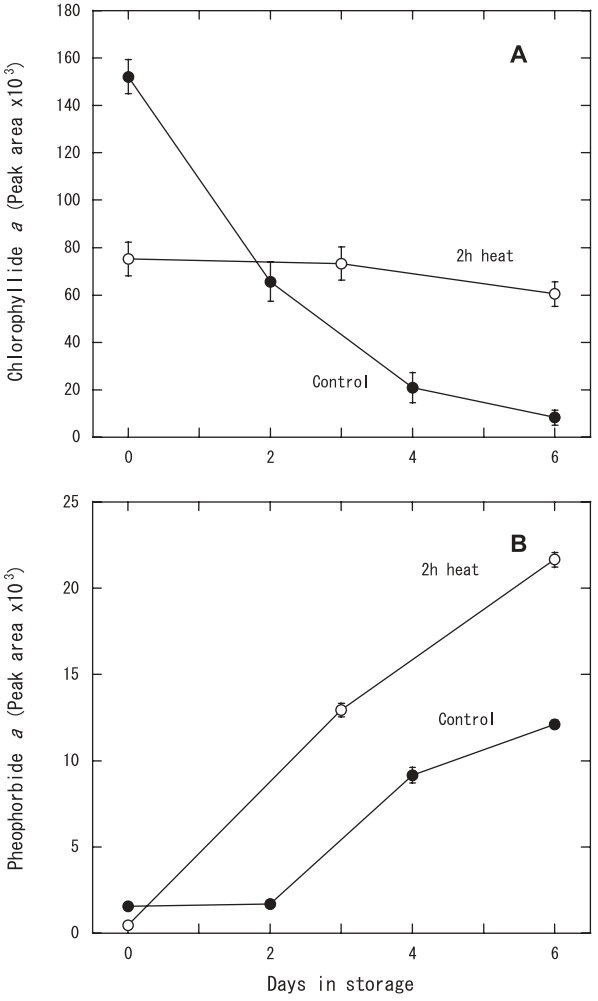

Fig. 5. Changes in chlorophyllide *a* (A) and pheophorbide *a* (B) levels of broccoli florets with or without heat treatment during storage at 15°C. Broccoli heads were held in hot air at 50°C for 2 h, followed by storage. Vertical bars represent average values with ± SD (n = 3).

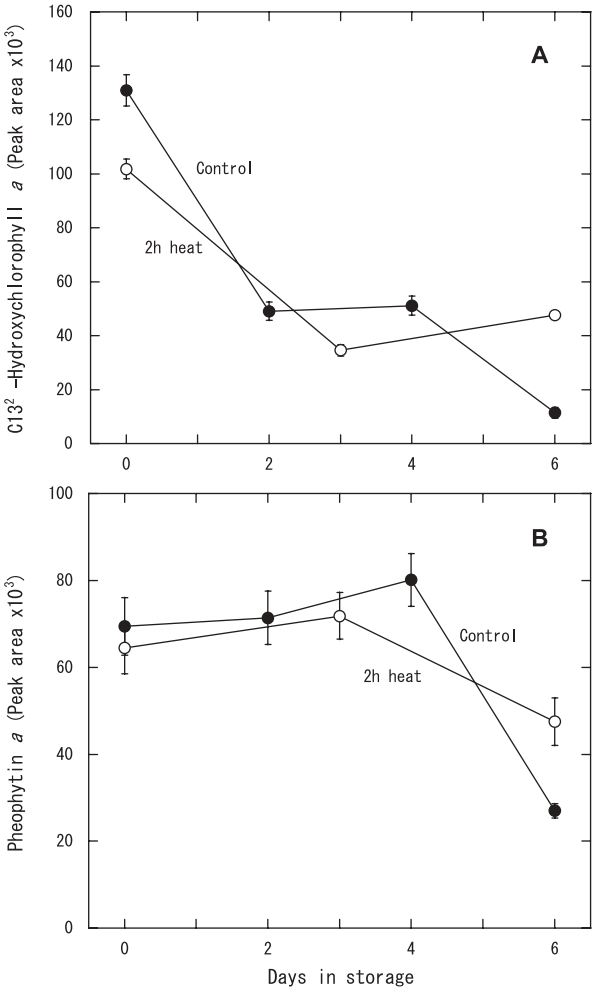

Fig. 6. Changes in C13^2-hydroxychlorophyll *a* (A) and pheophytin *a* (B) levels of broccoli florets with or without heat treatment during storage at 15°C. Broccoli heads were held in hot air at 50°C for 2 h, followed by storage. Vertical bars represent average values with ± SD (n = 3).

15°C. In heat-treated broccoli florets, Mg-dechelation activity was lower than that in the control during storage.

Discussion

With regard to the quantitative changes of Chl derivatives during heat treatment, the Chlide *a* level decreased significantly in heat-treated broccoli florets for 2 h following a temporary increase of Chlide *a*. The Pheide *a* level was only slightly observable in fresh broccoli florets and hardly detectable during heat treatment. Moreover, chlorophyllase and Mg-dechelation activities increased after 1 h of heat treatment. These findings suggest that the Chl degradation pathway, which is related to chlorophyllase and Mg-dechelation, might be temporally activated by heat treatment. The C13^2-OHChl *a* level declined during heat treatment with a concomitant decrease of Chl-degrading peroxidase activity. We reported in a previous paper (Funamoto et al., 2002) that heat treatment at 50°C for 2 h could reduce

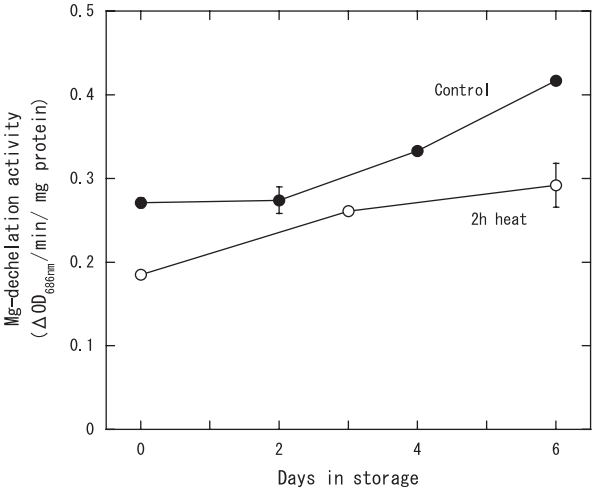

Fig. 7. Mg-dechelation activity of broccoli florets with or without heat treatment during storage at 15°C. Broccoli heads were held in hot air at 50°C for 2 h, followed by storage. Vertical bars represent average values with ± SD (n = 3).

Chl degradation due to suppression of the activities of Chl-degrading enzymes, such as chlorophyllase and Chl-degrading peroxidase, during storage. In this study, the inhibitory effect of Chl degradation seems to be related to the activities of Chl-degrading enzymes, chlorophyllase, Mg-dechelation, and Chl-degrading peroxidase, which decrease during heat treatment and continue to be suppressed during storage. In addition, as noted previously (Funamoto et al., 2002), 1 h heat treatment was insufficient to inhibit Chl degradation in stored broccoli florets, which suggests that Chl-degrading enzyme activities are not suppressed during heat treatment for 1 h.

Heat treatment of broccoli florets has been reported to effectively suppress Chl degradation during storage (Costa et al., 2005; Funamoto et al., 2002; Kazumi et al., 1991; Terai et al., 1999; Tian et al., 1996). In this study, the green color of heat-treated broccoli florets lasted longer than that in the control; however, the formation of Pheide *a* occurred continuously in heat-treated broccoli florets, suggesting that Pheide *a* might be accumulated as an intermediate of Chl degradation by the inhibition of Pheide *a* oxygenase and Mg-dechelation activities, especially the former. All Chl derivatives, except Pheide *a*, showed a slight decline in heat-treated broccoli florets during storage. In contrast, in the control, Chl *a* was promptly degraded without the accumulation of Chl *a* derivatives, except for Pheide *a*. Pheide *a* in the control also accumulated after 2 days of storage, but the accumulation was lower than that in heat-treated broccoli florets, as shown in Figure 5B. These results demonstrate that heat treatment could effectively suppress Chl degradation owing to inhibition of the action of Chl-degrading enzymes including Mg-dechelation activity.

Mg-dechelation activity was enhansed during the senescence of radish cotyledons (Suzuki et al., 2005), ripening of strawberry fruit (Costa et al., 2002), and yellowing of *Ginkgo biloba* leaves (Tang et al., 2000). In this study, Mg-dechelation activity in the control increased appreciably during storage at 15°C. The activity in heat-treated broccoli florets increased slightly during storage, but the increase of the activity was lower than that in the control. Also, we demonstrated that Mg-dechelation activity was more markedly inhibited during heat treatment than that of other Chl-degrading enzymes, which could suggest that Mg-dechelation activity is significantly involved in the Chl-degrading process. In addition, Chlide *a* levels in heat-treated broccoli florets were not accumulated during storage in spite of the inhibition of Mg-dechelation activity, inferring that Chl-degrading peroxidase might relate in part to the oxidation of Chlide *a* as well as Chl *a* (Huff, 1982).

Chlide *a*, which is formed from Chl *a* by chlorophyllase (Amir-Shapira et al., 1987; Shimokawa et al., 1978), is degraded to Pheide *a* by releasing Mg^{2+}. Mg-dechelatase (Langmeier et al., 1993) or Mg-dechelating substance, a heat-stable substance with a low-molecular mass (Shioi et al., 1996), is reported to be involved in this reaction. Costa et al. (2002) also indicated that Mg-dechelating activity in strawberry fruit was present in a low-molecular-mass compound sensitive to proteinase and Hg^{2+}, suggesting that the Mg-dechelating substance is a polypeptide containing the SH group. Further study is needed to clarify the characterization of the Mg-dechelating substance in broccoli florets.

In conclusion, Chl derivatives, especially Pheide *a*, are accumulated as intermediates of Chl degradation in heat-treat broccoli florets. In contrast, in control florets, Chl *a* was promptly degraded to colorless, low-molecular weight compounds without the accumulation of Chl derivatives except for Pheide *a*. Additionally, Mg-dechelating action, together with chlorophyllase and Chl-degrading peroxidase, is related to Chl degradation in stored broccoli florets. The inhibition of Chl degradation by heat treatment seems to be partly due to the suppression of these enzyme activities.

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