Involvement of Chlorophyll Degrading Enzymes with Chlorophyll Degradation in Stored Broccoli (*Brassica oleracea* L.) Florets

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Changes in chlorophyll (Chl) degrading enzyme activities were determined to clarify the pathway of Chl degradation in stored broccoli (*Brassica oleracea* L.) florets. Chl content in the florets decreased greatly after 2 days of storage at 15°C, whereas at 3°C they showed almost no change during storage. Chlorophyllase activities decreased gradually during storage at 15°C and 3°C. Chl degrading peroxidase activities showed a sharp increase and the hydrogen peroxide content a slight increase with the advance of floret yellowing at 15°C. Chl oxidase (linolenic or linoleic acid-dependent) activities decreased gradually during storage at 15°C. We suggest that Chl degrading peroxidase could be the main factor involved in Chl degradation in stored broccoli florets.

Keywords: chlorophyll degradation, chlorophyll degrading enzymes, senescence, broccoli

Yellowing, the loss of green color, is one of the main factors in the deterioration of quality in postharvest horticultural crops. In stored broccoli florets, yellowing is well known to occur dramatically with senescence. Therefore, much research has been performed on quality changes in stored broccoli florets (King & Morris, 1994; Tian *et al.*, 1997; Zhuang *et al.*, 1994).

The initial step of chlorophyll (Chl) degradation seems to be the formation of chlorophyllide (Chlide) by chlorophyllase (Shimokawa *et al.*, 1978; Amir-Shapira *et al.*, 1987) or that of 13²-hydroxychlorophyll a (Chl a-1) by oxidation related to Chl degrading peroxidase (Kato & Shimizu, 1985; Yamauchi *et al.*, 1997a) or Chl oxidase (Schoch *et al.*, 1984). Both Chlide a and Chl a-1 can be finally degraded to colorless low molecular weight compounds.

We reported that Chls content in broccoli florets treated with or without ethylene decreased strongly with yellowing during storage (Yamauchi & Watada, 1998). Unlike the changes in spinach (Yamauchi & Watada, 1991) and parsley (Yamauchi & Watada, 1993), Chlide a level in broccoli florets decreased with senescence during storage. The activity of Chlide a formation in the broccoli extracts also showed a decline with yellowing, but that of Chl a-1 formation showed almost no change (Yamauchi *et al.*, 1997b).

In this paper, we deal with the changes in Chl degrading enzymes (chlorophyllase, Chl degrading peroxidase and Chl oxidase) of stored broccoli florets to clarify the pathway of Chl degradation.

Materials and Methods

Plant material Broccoli (*Brassica oleracea* L., cv Haitzu) heads which were harvested at the Yamaguchi Agricultural Experiment Station farm were kept in polyethylene film bags (0.03 mm in thickness) with the top folded over,

placed in the corrugated cardboard box (290×400×170 mm) and stored at 3°C or 15°C in the dark. Three replicates of 3 broccoli heads were removed at scheduled intervals during the 7-day period and the florets analyzed.

Chlorophyll and hydrogen peroxide contents Chl content was determined using *N,N*-dimethylformamide (Moran, 1982). Hydrogen peroxide content was measured using peroxidase according to Kuroda and Sagisaka (1998).

Chlorophyll degrading enzymes assay An acetone powder (500 mg) of broccoli florets was suspended in 15 ml of 10 mM phosphate buffer (pH 7.0) for Chl degrading peroxidase and Chl oxidase or in 15 ml of 10 mM phosphate buffer (pH 7.0), containing 50 mM KCl and 0.24% Triton X-100, for chlorophyllase. The crude enzyme was stirred for 1 h at 0°C and the mixture was filtered through Miracloth (Calbiochem.). Afterwards, the filtrate was centrifuged at 16,000 *g* for 15 min at 4°C. The supernatant was used as the crude enzyme extract.

Chlorophyllase activity was determined by a modification of the method of Amir-Shapira *et al.* (1987). The reaction mixture contained 0.5 ml enzyme solution, 0.1 ml 1.44% Triton X-100, 0.2 ml Chls acetone solution (Chl a-500 µg/ml) and 0.5 ml 0.1 M phosphate buffer (pH 7.5). The mixture was incubated in a water bath at 25°C for 40 min, and the enzyme reaction was stopped by the addition of 4 ml of acetone. The remaining (non-degraded) Chls were extracted with 4 ml of hexane and assayed by reading the absorbance at 663 nm. Activity was based on the decrease in absorbance by Chl a at 663 nm.

Chl degrading peroxidase was determined as described by Yamauchi *et al.* (1997a). 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 Chls acetone solution (Chl a-500 µg/ml) 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.

Chl oxidase was measured by a modification of the method of Blackburn *et al.* (1990). The reaction mixture contained 0.6 ml enzyme solution, 0.1 ml 3.0% Triton X-100, 0.1 ml 1.0 mM linoleic or linolenic acid, 0.2 ml Chls acetone solution (Chl a-500 µg/ml) and 2.0 ml 0.1 M phosphate buffer (pH 7.0). Activity was determined spectrophotometrically by measuring the decrease of Chl a at 668 nm at 25°C.

One unit of Chl degrading enzymes was defined as a change of 1.0 µg Chl a degradation per min.

Enzyme protein content was assayed by the method of Bradford (1976).

Partially purified Chls used for the enzyme analyses were prepared by the method of Yoshiura and Iriyama (1979).

Results

Chlorophyll changes The broccoli florets remained green for 2 days of storage at 15°C and turned yellow on day 3. In comparison, at 3°C they remained green through 7 days of storage. As shown in Fig. 1, Chls a and b content decreased greatly after 2 days of storage at 15°C, whereas the content showed almost no change during storage at 3°C.

Chlorophyll degrading enzyme changes Figure 2 shows the chlorophyllase activities in stored broccoli florets. The activity decreased gradually during storage at 15°C before the occurrence of Chl degradation with yellowing, while at 3°C the activity showed almost no change during storage.

As shown in Fig. 3, Chl degrading peroxidase activities increased slightly for the first 2 days of storage at 15°C; thereafter the activity showed a sharp increase and was 28 times higher on day 6 of storage than initially. The activity at 3°C increased slightly during storage. The specific activity of Chl degrading peroxidase also was higher than that of chlorophyllase or Chl oxidase during storage.

Figure 4 shows the Chl oxidase activities in stored broccoli florets. The linolenic acid-dependent Chl oxidase activities decreased gradually after 2 days of storage at 15°C, whereas the activity increased after 3 days of the storage at 3°C. The changes in the linoleic acid-dependent Chl oxidase activities were similar to those of the linolenic acid dependent-ones (data not shown).

Fig. 1. Changes in chlorophyll contents of broccoli florets during storage. The vertical bars represent average values with SE ($n=3$). Chl-Chlorophyll.

Hydrogen peroxide changes As apparent in Fig. 5, the hydrogen peroxide content increased slightly after 2 days of storage at 15°C, and also increased a little at 3°C.

Discussion

Chlorophyllase activity is known to increase with Chl degradation during storage in leaves of spinach (Yamauchi & Watada, 1991), barley (Rodriguez *et al.*, 1987; Sabater & Rodriguez, 1978) and oat (Sabater & Rodriguez, 1978). We also reported that Chlide a increased with senescence in stored spinach (Yamauchi & Watada, 1991) and parsley (Yamauchi & Watada, 1993) leaves. In broccoli florets, however, chlorophyllase activity at 15°C showed a slight decline in accordance with the decrease in Chlide a (Yamauchi & Watada, 1998).

Chlorophyllase, which catalyzes the release of the phytol chain from Chl a to form Chlide a, seems to be an enzyme related to an initial step of Chl degradation (Matile *et al.*, 1996). Chlide a is then degraded to pheophorbide (Pheide) a by Mg-dechelatase (Langmeier *et al.*, 1993) or the Mg-dechelating substance observed by Shioi *et al.* (1996). Finally, Pheide a is directly oxidized by Pheide a oxygenase (Matile *et al.*, 1996) or oxidized by monooxygenase (Curty *et al.*, 1995) after decarbomethoxylase removes a carbomethoxy group from Pheide a to form pyropheophorbide a (Shioi *et al.*, 1991). In stored broccoli florets, this pathway might not be the main one involved in Chl degradation during yellowing.

Chl degrading peroxidase is one of the enzymes involved in Chl oxidative degradation (Yamauchi & Minamide, 1985; Kato & Shimizu, 1985; Shimokawa & Uchida, 1992). Kuroda *et al.* (1990) reported that peroxidase activity increased during senescence of excised barley leaves and that one of the chloroplast isoperoxidases might function as a Chl-degrading enzyme. Moreover, Johnson-Flanagan and Spencer (1996) observed that Chlide and Pheide were detected in canola seeds but were absent in mustard seeds, and that Chl degradation was associated with high chlorophyllase activity and low Chl degrading peroxidase in canola, and low chlorophyllase and high Chl degrading peroxidase activity in mustard. In broccoli florets, Chl degrading peroxidase activity

Fig. 2. Chlorophyllase activity in stored broccoli florets. The vertical bars represent average values with SE ($n=3$). Unit: chlorophyll a degradation µg/min.

Fig. 3. Chlorophyll degrading peroxidase activity in stored broccoli florets. The vertical bars represent average values with SE ($n=3$). Unit: chlorophyll a degradation $\mu\text{g}/\text{min}$.

Fig. 4. Linolenic acid-dependent chlorophyll oxidase activity in stored broccoli florets. The vertical bars represent average values with SE ($n=3$). Unit: chlorophyll a degradation $\mu\text{g}/\text{min}$.

increased strongly with senescence during storage, suggesting that Chl degrading peroxidase could be the chief enzyme involved in Chl degradation. The hydrogen peroxide content did not decrease and it was maintained at a constant level with yellowing in stored broccoli florets. We infer that the hydrogen peroxide might be formed with floret yellowing, and that the Chl degrading peroxidase-flavonoid system might effectively act as a scavenging system of hydrogen peroxide. Chl degrading peroxidase degrades Chls in the presence of phenolic compounds. We reported in previous papers that Chl was degraded with the oxidation of hesperidin in satsuma mandarin fruit (Yamauchi *et al.*, 1997a) and apigenin in parsley leaves (Yamauchi & Minamide, 1985). Further study is needed regarding the detection of phenolic compounds related to Chl degradation in broccoli florets.

Chl oxidase bleaches Chl in the presence of fatty acids such as linoleic, linolenic and stearic acids, and the enzyme is clearly different from lipoxygenase (Lüthy *et al.*, 1984). As noted by Janave (1997), linoleic acid stimulated Chl degradation in the soluble enzyme extract from banana peel acetone powder, indicating that Chl oxidase is involved in Chl

Fig. 5. Changes in hydrogen peroxide content of broccoli florets during storage. The vertical bars represent average values with SE ($n=3$).

oxidation in banana peel. We found that Chl oxidase activity decreased gradually during storage at 15°C, which could suggest that this oxidase is not essential in Chl degradation in stored broccoli florets.

Chl a-1, the oxidized form of Chl a, seems to be an intermediate formed by Chl degrading peroxidase and/or Chl oxidase (Schoch *et al.*, 1984; Yamauchi & Watada, 1994). Adachi and Shimokawa (1995) demonstrated that a superoxide anion, a kind of active oxygen, was related to the reaction catalyzed by Chl bleaching enzyme extracted from radish cotyledons in the presence of hydrogen peroxide. One possibility based on these findings is that the superoxide anion, which is formed by the oxidation of phenolic compounds by Chl degrading peroxidase, could be involved in both Chl a-1 formation and oxidation to form the colorless low molecular weight compounds.

In conclusion, we suggest from the findings obtained here and reported earlier that, unlike the Chl degradation in stored spinach and parsley leaves, Chls in broccoli florets is chiefly degraded by the pathway involved in Chl degrading peroxidase.

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