Hot air treatment decreases chlorophyll catabolism during postharvest senescence of broccoli (*Brassica oleracea* L. var. italica) heads

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Abstract: A hot air treatment was applied to broccoli (*Brassica oleracea* L. var. *italica*) florets and its effect on chlorophyll catabolism during postharvest senescence was analyzed. Florets were treated at 48 °C for 3 h and then placed in darkness at 20 °C. During storage, the yellowing of florets occurred simultaneously with a decrease in chlorophylls and an increase in pheophytins. Heat treatment delayed the appearance of yellowing by 2–3 days and a similar extension of shelf-life could be inferred. Also, the treatment delayed the onset of chlorophyll catabolism and slowed both the rate of chlorophyll *a* degradation and pheophytin accumulation. No effect on chlorophyll *b* degradation was found. Chlorophyllase and Mg-dechelatase activities increased from the first day of storage in untreated florets, whereas peroxidase-linked chlorophyll bleaching activity increased from day 3. In heat-treated florets, chlorophyllase activity did not increase until day 2 and then increased at lower rate than in controls. Mg-dechelatase and peroxidase-linked chlorophyll bleaching activities were similar in treated and control florets during the first 2 days of storage, but thereafter the activity of both enzymes was lower in heat-treated samples. In conclusion, a treatment at 48 °C for 3 h delayed chlorophyll *a* catabolism in broccoli during postharvest senescence and decreased the activities of chlorophyllase, Mg-dechelatase and peroxidase, three of the enzymes probably involved in chlorophyll degradation in plants.

Keywords: broccoli; heat treatment; postharvest, senescence, chlorophyll degradation

INTRODUCTION

Broccoli (*Brassica oleracea* L. var. *italica*) is a cole crop with floral heads composed of hundreds of florets arranged in whorls. The inflorescences are harvested while the floral heads, branchlets and florets are totally immature, with the sepals completely surrounding the flower. Harvesting causes severe stress conditions of nutrient and water deficiency and changes in hormonal status, determining an inability to maintain homeostasis and the appearance of senescence symptoms. This accelerated senescence leads to the degradation of chlorophyll (Chl), proteins, lipids and ascorbic acid.[1]

The broccoli quality is greatly reduced after harvesting because of the loss of chlorophylls and the consequent yellowing of sepals. Most postharvest methodologies such as refrigeration,[2] modified atmosphere[3,4] and different types of packaging[5] are directed to delaying senescence and the consequent degreening. The use of heat treatments as a methodology to extend postharvest life of different products has been studied and their beneficial effects are well documented.[6] In broccoli, it has been shown that hot water dips delay yellowing[7,8] and ethylene production.[8] In addition, hot air treatment has been useful in preventing broccoli tissue deterioration and chlorophyll degradation,[9] one of the contributing effects being the maintenance of membrane integrity and then avoiding contact among chlorophylls and low pH or H_2O_2.

Chlorophyll degradation starts with the elimination of the phytyl group from chlorophylls, a reaction catalyzed by chlorophyllase that generates chlorophyllides (Chld).[10] The second step would be the elimination of Mg^{2+} from Chld to produce pheophorbide (Pheo), in a reaction catalyzed by Mg-dechelatase (MDS). Some findings suggest that Mg-dechelatase should be considered as a heat-stable polypeptide catalyst with a low molecular mass rather than a typical enzyme.[11,12] Cleavage of Pheo *a* by the action of pheophorbide *a*-oxygenase leads to linearization of the tetrapyrrole ring, in a reaction that is considered to be the key regulatory step in the Chl catabolism pathway.[10,13,14] Other oxidative enzymes such as lypoxygenase, Chl oxidase and peroxidase could also cleave the tetrapyrrole in

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an alternative pathway. Studies performed in different systems support the possible participation of peroxidase in the catabolism of Chl.[15–17] Moreover, Funamoto *et al*.[18] suggested that a peroxidase isoenzyme is directly involved in chlorophyll catabolism in broccoli.

Although several studies have analyzed the effect of heating and other postharvest treatments on broccoli yellowing, the effect of those treatments on the underlying enzyme reactions involved in degreening of the produce remains largely unknown. Therefore, the objective of the present work was to study the effect of heat treatment on chlorophyll degradation during the postharvest senescence of broccoli heads. In particular, the effect on the activity of three enzymes related to chlorophyll degradation (chlorophyllase, Mg-dechelatase and peroxidase) was analyzed.

MATERIALS AND METHODS

Plant material and heat treatment

Broccoli (*Brassica oleracea* L. var. *italica*, cv. Cicco) heads were obtained from local producers and immediately sampled for analysis. Seventy-five broccoli heads were individually placed in trays and covered with PVC film to diminish water loss. The trays were placed in an air oven at 48 °C for 3 h. After treatment, trays were stored at 20 °C for 4 days in darkness and the superficial color was measured. Seventy-five heads without heat treatment were directly brought to 20 °C and used as controls. Fifteen heads were sampled immediately after the treatment and after 1, 2, 3 and 4 days of storage. For superficial color determinations, intact heads were used immediately. For analytical determinations (chlorophylls, pheophytins, chlorophyllase, Mg-dechelatase and peroxidase), stems of 15 heads were separated and only florets were analyzed. Florets were randomly grouped and frozen in liquid nitrogen and stored at −80 °C until analysis. The whole experiment was repeated three times.

Color measurement

Superficial color was evaluated by measuring the parameters L^*, a and b in nine positions of each broccoli head with a chromameter (Model CR-300, Minolta, Osaka, Japan). Hue angle (h°) was calculated as $h^\circ = \tan^{-1}(b/a)$ when $a > 0$ and $b > 0$ or as $h^\circ = 180^\circ - \tan^{-1}(b/a)$ when $a < 0$ and $b > 0$.

Extraction and dosage of chlorophylls and pheophytins

Approximately 60 g of frozen broccoli florets were crushed in a mill and 0.5 g of the powder obtained was poured into 5 mL of acetone at 0 °C, stirred and then centrifuged at 9000 × *g* for 15 min at 4 °C. The supernatant was used to measure the chlorophyll content.[19] To determine the pheophytin (Phe) content, all the chlorophylls present in the extract were transformed into Phe by adding one drop of 250 mL L^{-1} HCl to 5 mL of supernatant. The quantitative determination of Phe was performed by subtracting the amount of Chl in the original solution from the total Phe obtained after adding HCl.[19] Four replicates per condition were analyzed.

Enzyme extraction

Approximately 60 g of frozen broccoli florets were crushed in a mill and approximately 8 g were poured into 25 mL of the following extraction buffer: 0.1 mol L^{-1} Na_2HPO_4, 0.1 mol L^{-1} NaH_2PO_4, 2 mL L^{-1} Triton X-100, 30 g l^{-1} polyvinylpolypyrrolidone (PVPP), 1 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF), 5 mmol L^{-1} cysteine, pH 6.0. The mixture was stirred for 1 h at 4 °C and centrifuged at 9000 × *g* for 20 min at 4 °C. The supernatant was separated, vacuum-filtered and used to measure the enzyme activities.

Substrate preparation

Chlorophylls

Leaves of *Spinacia oleracea* (approximately 6 g) were homogenized in an Omnimixer with 60 mL of acetone–water (80:20) solution at 4 °C. The suspension was centrifuged at 9000 × *g* and 40 mL of petroleum ether were added to the supernatant to extract the chlorophylls. Subsequently, the ether was evaporated under N_2 and the chlorophylls were dissolved in 4–5 mL of acetone.

Chlorophyllin

Chlorophyllin was prepared according to Vicentini *et al*.[20] The Chl ethereal extract was prepared as described above, washed three times with 40 mL of water and then mixed with 1 mL of 300 mg kg^{-1} KOH in methanol per each milligram of chlorophyll present. The chlorophyllin was allowed to precipitate and then centrifuged at 5500 × *g* for 15 min. The precipitate was dissolved in distilled water and brought to pH 9 with 1 mol L^{-1} tricine.

Chlorophyllase activity

The following reaction mixture was prepared to measure chlorophyllase activity: 0.1 mol L^{-1} sodium phosphate buffer, pH 7.0, 1.5 mL L^{-1} Triton X-100, 10 μmol L^{-1} Chls, 16% (v/v) acetone and 2 mL of enzymatic extract in a total volume of 13 mL. The mixture was incubated at 40 °C and duplicate samples of 2 mL were taken from 0 up to 60 min after the beginning of the reaction and poured into 5 mL of mixture of hexane–acetone (7:3) pre-cooled in ice–water. The mixtures were vigorously stirred until emulsion formation, then allowed to stand in the dark at 4 °C and centrifuged at 6000 × *g* for 5 min at 4 °C. The upper phase contained the remaining chlorophyll while the lower phase contained the chlorophyllide. The progress of the chlorophyllase activity was followed by measuring the optical density (OD) at 663 nm in the lower phase. The enzyme activity was expressed as the increment in OD at

663 nm per minute per gram of tissue. Four replicates per condition were analyzed.

Mg-dechelatase activity

The following reaction mixture was used: 50 mmol L^{-1} Tris–tricine buffer, pH 8.8, 50 μL chlorophyllin ($OD_{687\,nm} = 0.2$) and 150 μL of crude enzymatic extract in a total volume of 500 μL. The mixture was incubated at 37 °C and the increase in OD at 686 nm was followed. The Mg-dechelatase activity was expressed as the increment in OD at 686 nm per minute per gram of tissue. Four replicates per condition were analyzed.

Peroxidase chlorophyll-bleaching activity

The following reaction mixture was used: 0.02 mol L^{-1} sodium phosphate buffer, pH 5.0, 2 mL L^{-1} Triton X-100, 5 mmol L^{-1} *p*-coumaric acid, 60 mmol L^{-1} H_2O_2, 25 μL enzymatic extract, 10 μL chlorophyll, yielding an initial OD of 0.70 in a total volume of 1 mL. The mixture was incubated at 35 °C and the reaction was started by adding H_2O_2. Chlorophyll bleaching was evaluated by measuring the decrease in OD at 669 nm. The activity was expressed as the decrease in OD at 669 nm per minute per gram of tissue. Four replicates per condition were analyzed.

Statistical analysis

The entire experiment was carried out three times and performed according to a factorial design. Data were analyzed by means of ANOVA and the means were compared by the LSD test at a significance level of 0.05.

RESULTS AND DISCUSSION

In previous work, we analyzed the effect of hot air treatments on the senescence of broccoli heads during storage at 20 °C. The optimum combination of temperature and time was 48 °C for 3 h, which caused the greatest delay of senescence.[9] Therefore, these conditions were chosen to analyze the effect of heat treatment on chlorophyll degradation during induced senescence at 20 °C.

Superficial color

Control florets did not show appreciable color changes until day 2 and turned progressively yellow from day 3 on. The heat-treated samples showed a slight degreening only at day 4. Diminution of the hue angle value correlated with the progressive yellowing observed in the florets. Hue angle decreased from day 2 in control florets, whereas only a slight reduction was found in heat-treated samples from day 3 [Fig. 1a].

Pigments

The total chlorophyll content in untreated florets decreased continuously on keeping at 20 °C, showing

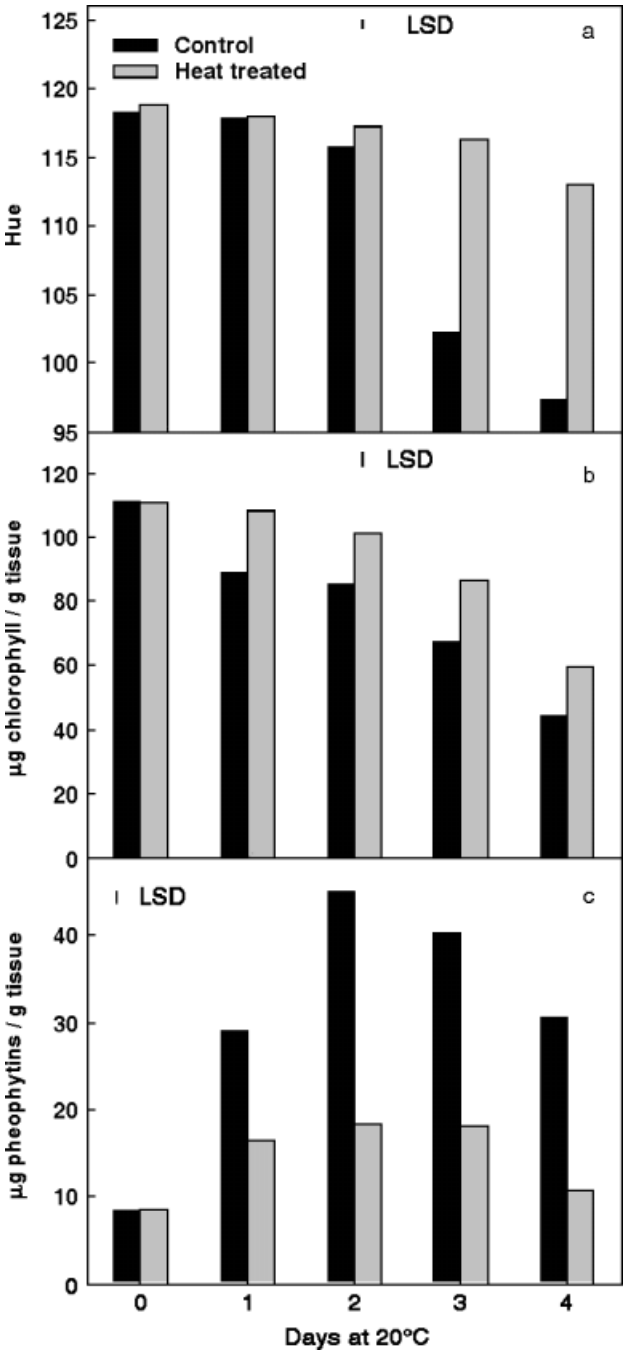

Figure 1. (a) Change in superficial color parameter (hue) during postharvest senescence of broccoli incubated at 20 °C in darkness. $LSD_{Hue} = 0.15$. (b) Variation in chlorophyll content, expressed as μg pigment g^{-1} tissue, during postharvest senescence of broccoli incubated at 20 °C in darkness. $LSD_{Chl} = 2.51$. (c) Change in pheophytin content, expressed as μg pigment g^{-1} tissue, during postharvest senescence of broccoli incubated at 20 °C in darkness. $LSD_{Phe} = 2.74$.

a decrease of more than 50% after 4 days [Fig. 1b]. In contrast, there was no chlorophyll degradation in heat-treated florets after 1 day. Heat treatment not only delayed the onset of chlorophyll catabolism by 1 day but also slowed the degradation rate. It is worth noting that after 2 days a decrease in chlorophyll was detected whereas no difference in hue angle was observed, indicating that the chlorophyll degradation begins before any color change is visible and that the

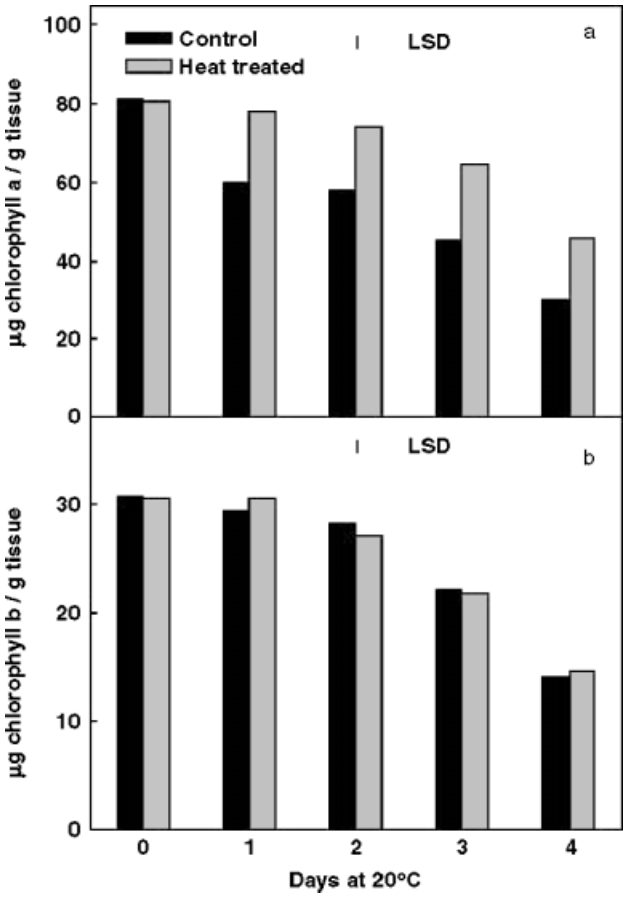

Figure 2. Variation of chlorophyll *a* (a) and chlorophyll *b* (b) contents, expressed as µg pigment g^{-1} tissue, during postharvest senescence of broccoli incubated at 20 °C in darkness. $LSD_{Chl\ a} = 2.31$; $LSD_{Chl\ b} = 2.23$.

measure of chlorophyll is a more sensitive parameter than hue for following broccoli senescence.

The levels of both chlorophyll *a* and *b* decreased during senescence at 20 °C (Fig. 2). As occurs in other senescent vegetables, the degradation rate of chlorophyll *a* was higher than that of chlorophyll *b*.[21,22] Heat treatment delayed the degradation of chlorophyll *a* but did not affect chlorophyll *b*. A similar effect had been found with hormone treatments; applications of benzylaminopurine (BAP) or ethephon delayed or accelerated, respectively, the degradation of chlorophyll *a* but did not affect the catabolism of chlorophyll *b*.[23] Therefore, the differences found in total chlorophyll content were closely related to the effect of heat treatment on chlorophyll *a* rather than on chlorophyll *b* catabolism. It has been proposed that all the final catabolites of chlorophylls are derived from chlorophyll *a* and that chlorophyll *b* must transform into chlorophyll *a* derivatives before being degraded.[10] The model is supported by the existence of a 'Chl *b* reductase', which transforms chlorophyllide *b* to chlorophyllide *a*.[10] Hence this common chlorophyll degradation pathway suggests that the differential effect of heat treatment on the degradation of both chlorophyll *a* and *b* might affect a step downstream of the conversion of chlorophyll *b* into chlorophyll *a*.

Pheophytins accumulated after 2 days at 20 °C and decreased thereafter in control samples [Fig. 1c]. Heat-treated samples accumulated less than 50% of the amount found in controls after 2 days. According to Matile *et al.*,[10] the degradation of chlorophylls begins by the sequential elimination of the phytol and then the central Mg^{2+} ion. These reactions generate chlorophyllides and pheophorbides, respectively, but the pheophytin formation is not predicted in this model. The results obtained in the present and in a previous study[23] showed that a transient pheophytin accumulation occurs during broccoli senescence, which indicates the release of Mg^{2+} ion directly from the chlorophylls. Senescent leaves of parsley[24] and *Ginkgo biloba*[25] also showed accumulation of this catabolite, indicating that in these systems the magnesium could be also removed before dephytylation. Release of Mg^{2+} from chlorophylls or their derivatives could be catalyzed by Mg-dechelatase (see below) or another unknown enzyme. However, chlorophylls may also lose the Mg^{2+} ion by acidification. The possibility that plastoglobuli containing intact chlorophylls are exported from chloroplast to vacuoles during senescence of soy leaves has been suggested.[26] The acidic pH present in the vacuole could enhance the production of pheophytins by a non-enzymatic reaction.

Chlorophyll degrading enzyme activities

The activity of three enzymes related to chlorophyll degradation (chlorophyllase, Mg-dechelatase and peroxidase) was evaluated in control and heat-treated florets during storage at 20 °C.

Chlorophyllase

The chlorophyllase activity of control samples increased during the storage, reaching a maximum after 3 days and decreasing thereafter (Fig. 3). Instead, the activity in heat-treated samples remained constant for 2 days and then increased, but to a lesser extent than that observed in controls. Data on chlorophyllase localization and properties are contradictory. For years, this enzyme has been considered a hydrophobic protein localized in the chloroplast membranes, but the cloning of several chlorophyllase genes showed that other localizations and even different regulations should not be ignored.[27] Chlorophyllase activity can show very different patterns depending on the system analyzed. In many species, the enzyme shows functional latency, even during Chl breakdown in senescent leaves,[10] but in others the activity increases during senescence.[28,29] In the case of broccoli, we have found that both Cicco and Shogun cultivars (this work and Costa *et al.*,[23] respectively) showed an increment of chlorophyllase activity during postharvest senescence. In the latter cultivar, the activity is up-regulated by ethylene and down-regulated by cytokinins. In contrast, Funamoto *et al.*[18] did not detect appreciable changes in activity in Haitsu cultivar during senescence, but they reported the inhibition of

chlorophyllase activity on application of heat treatment. It could be that different broccoli cultivars show differences in their chlorophyll catabolism, as proposed by Toivonen and Sweeney.[30]

Mg-dechelatase

Mg-dechelatase activity increased during senescence in control samples, reaching values 10-fold higher than the initial level after 4 days at 20 °C (Fig. 4). The activity in heat-treated samples increased also at the beginning, but the rate of increase slowed after 3 days at 20 °C and at the end of the experiment the MDS activity was significantly lower. Release of Mg^{2+} from chlorophyllide is a necessary step to produce pheophorbide, the substrate of Pheo *a* oxygenase, which catalyzes the cleavage of the tetrapyrrole ring.[10] However, few studies on Mg-dechelatase have been performed. It has been reported that MDS activity increased during dark-induced senescence of oilseed rape cotyledons,[20] ripening of strawberry fruit[12] and yellowing of *Ginkgo biloba* leaves.[25] In the last case, the authors suggested that MDS could catalyze the key step in chlorophyll degradation since yellow leaves showed low chlorophyllase activity and the only catabolite detected was pheophytin. In broccoli, the activity of Mg-dechelatase, chlorophyllase and chlorophyll-bleaching peroxidase increases during senescence and this increment is up- and down-regulated by ethylene and cytokinins, respectively.[23] It has been reported that Mg-dechelatase releases the Mg^{2+} ion only from dephytylated compounds.[11] The fact that *Ginkgo*[25] and parsley[24] leaves and broccoli florets accumulate pheophytins supports the possibility that Mg^{2+} ions were released directly from phytylated compounds. Heat treatment inhibited the accumulation of pheophytins from day 1 and later the increase in Mg-dechelatase activity from day 3, suggesting that another mechanism of Mg^{2+} release could be also operating.

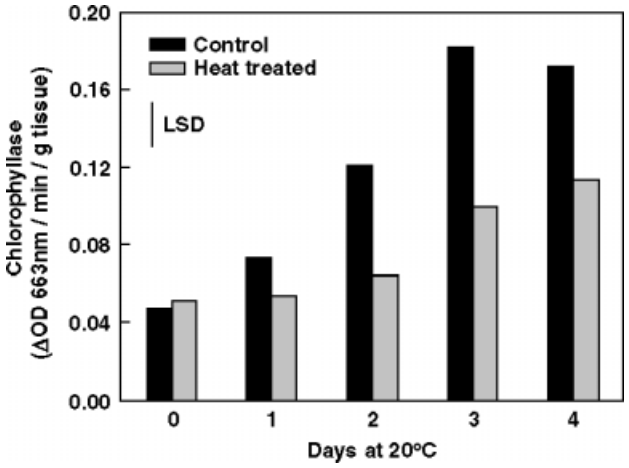

Figure 3. Chlorophyllase activity during postharvest senescence of broccoli incubated at 20 °C in darkness. Activity is expressed as the increase in OD at 663 nm per minute per gram of tissue. LSD = 0.018.

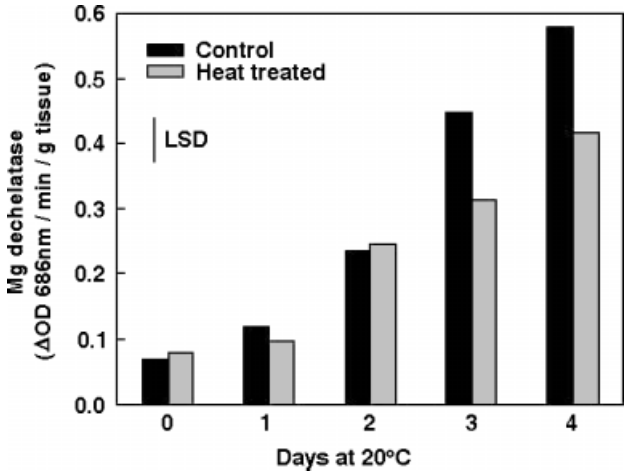

Figure 4. Mg-dechelatase activity during postharvest senescence of broccoli incubated at 20 °C in darkness. Activity is expressed as the increase in OD at 686 nm per minute per gram of tissue. LSD = 0.022.

Peroxidase

It has been suggested that peroxidase could mediate chlorophyll degradation in the presence of a phenolic compound. The proposed mechanism is that the enzyme catalyzes the oxidation of a phenolic compound by hydrogen peroxide, generating a phenolic radical, which in turn degrades chlorophyll to a colorless compound.[16,31,32] Some evidence of the hypothetical role of peroxidase in chlorophyll degradation has been obtained in broccoli; chlorophyll *a*-1, an oxidative product of chlorophylls, was detected in an '*in vitro*' degradation system[32] and the authors proposed an important role for peroxidase in chlorophyll degradation of broccoli. In control florets, we found that peroxidase-linked chlorophyll bleaching activity remained approximately constant during the first 2 days at 20 °C and then increased around 2-fold after 4 days (Fig. 5). In the case of heat-treated samples, the activity also increased after 2 days but at a lower rate and at the end of the experiment the peroxidase chlorophyll bleaching activity was significantly

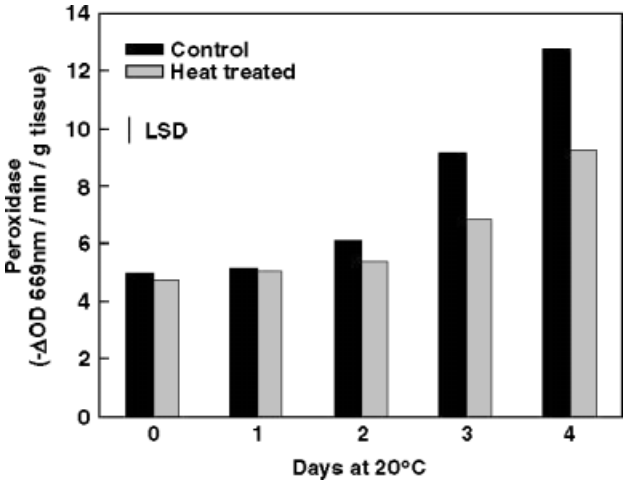

Figure 5. Chlorophyll degrading peroxidase activity during postharvest senescence of broccoli incubated at 20 °C in darkness. Activity is expressed as the decrease in OD at 669 nm per minute per gram of tissue. LSD = 0.84.

lower than in controls. A similar effect was described by Funamoto *et al.*[18] in florets of Haitsu cultivar and the authors suggested that the delay in chlorophyll degradation was closely related to a lower peroxidase activity in heat-treated samples. It is worth pointing out that peroxidase-linked chlorophyll bleaching activity increased in relatively late events of senescence, and after that tonoplast and chloroplast breakdown allows the mixture among phenolics with chlorophyll and peroxidase. Moreover, if plastoglobuli of senescing tissues including intact chlorophylls are eliminated from the chloroplast and carried to the vacuoles,[26] then, in this location, highly active peroxidases and hydrogen peroxide could mediate the degradation of chlorophylls.

CONCLUSIONS

During induced senescence in broccoli, a decrease in the hue angle value associated with a reduction in the amount of chlorophyll *a* and *b* and an increase in pheophytins was observed. Also, an increase in chlorophyllase and Mg-dechelatase activity was detected from the beginning of induced senescence, whereas peroxidase-linked chlorophyll bleaching activity increased after 3 days. The treatment of broccoli heads at 48 °C for 3 h in air delayed chlorophyll degradation at 20 °C. Chlorophyll *a* loss and pheophytin accumulation were reduced in heat-treated florets and the activities of enzymes related to chlorophyll catabolism were also decreased. Heat treatments, when applied in moderate doses, cause a temporary stress in the tissue that inhibits the normal metabolism and, in this case, the senescence. After stress, the tissue continues its normal metabolism but a residual effect can remain and be detected after a few days. In this work, a delay in chlorophyll loss and an increase in pheophytin content and chlorophyllase activity were detected after 1 day of induced senescence, but the effect on Mg-dechelatase activity and peroxidase-linked chlorophyll bleaching activity was observed only after 3 days at 20 °C.

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