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Effect of ethephon and 6-benzylaminopurine on chlorophyll degrading enzymes and a peroxidase-linked chlorophyll bleaching during post-harvest senescence of broccoli (*Brassica oleracea* L.) at 20 °C

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Abstract

Effects of ethephon and 6-benzylaminopurine (BAP) treatments on enzyme degrading enzymes and a peroxidase-linked chlorophyll bleaching were investigated in broccoli (*Brassica oleracea* L.) florets. The florets were dipped in solutions containing either BAP or ethephon and then incubated in darkness at 20 °C. The hue angle values and chlorophyll contents of ethephon-treated florets declined the most of the five-day experiment. In contrast, hue angle and chlorophyll content declined the least in BAP-treated florets. Pheophytin levels increased the most in ethephon-treated florets and the least in BAP-treated florets. Chlorophyllase, Mg-dechelatase, and peroxidase-linked chlorophyll bleaching increased over the five days in control (untreated) florets. Ethephon treatment enhanced chlorophyllase, Mg-dechelatase, and peroxidase-linked chlorophyll bleaching levels as compared with control. BAP treatment reduced chlorophyllase, Mg-dechelatase, and peroxidase-linked chlorophyll bleaching levels. It was concluded that the activities of chlorophyllase, Mg-dechelatase, and peroxidase-linked chlorophyll bleaching could be regulated by external application of either ethylene (applied as ethephon) or a cytokinin (applied as BAP). In the case of ethephon treatment, accelerated rates of chlorophyll degradation were found and in the case of BAP-treatment, reduced rates of chlorophyll degradation were found.

Keywords: Broccoli; Chlorophyll degradation; Ethephon; BAP

1. Introduction

Broccoli (*Brassica olaracea* L. var Italica) is a cole crop with floral heads composed of hundreds of florets arranged in whorls. The inflorescences are harvested

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while the floral heads, branchlets, and florets are totally immature, with the sepals completely surrounding the flower. This type of harvesting provokes a sudden disruption in nutrient, energy, and hormone supplies, which in turn induces a severe tissue stress that triggers the senescence process. Senescence leads to the degradation of chlorophyll (Chl), proteins, lipids, and ascorbic acid (Pogson et al., 1995). The phenotypic changes that occur during broccoli senescence are essentially similar to those seen in leaf senescence (Page et al., 2001). The process is delayed by cytokinins and promoted by ethylene. In intact plants, cytokinins are synthesized in the roots and transported to the flowers and their concentration diminishes abruptly after harvest, and this is generally considered inducing the organ senescence (Tian et al., 1994). The treatment with cytokinins delays the physiological changes that usually accompany the senescence of florets (Downs et al., 1997). On the contrary, ethylene seems to be the principal promoter of senescence and yellowing. The level of 1-amino-cyclopropane-1-carboxylic acid increases during broccoli post-harvest (Pogson et al., 1995) and the incubation of broccoli heads in ethylene-enriched atmospheres enhance the senescence (King and Morris, 1994).

The broccoli quality is highly reduced after harvesting because of the loss of green color and the consequent yellowing of its sepals. There are extensive works related to methodologies in delaying yellowing during post-harvest senescence of broccoli (Ballantyne et al., 1988; Barth et al., 1993). However, very few analyses of the enzymes involved in the chlorophyll degradation of broccoli have been performed.

In the last years, the chlorophyll degradation pathway has been outlined (Hörtensteiner, 1999). The activity of chlorophyllase, which removes the phytol and produces chlorophyllides (Chld), has been proposed to be the first step in Chl breakdown (Matile et al., 1999). The second step would be the elimination of Mg^{2+} from Chld to produce pheophorbide (Pheo), in a reaction catalyzed by Mg-dechelatase. Some findings suggest that Mg-dechelatase should be considered as a heat stable polypeptidic catalyst with a low molecular mass rather than a typical enzyme (Shioi et al., 1996; Costa et al., 2002). Further catabolism of Pheo involves the cleavage of Pheo *a* by pheophorbide *a*-oxygenase, which is considered as the key regulatory step in the Chl catabolism pathway (Matile and Schellemberg, 1996; Hortensteiner et al., 1998; Matile et al., 1999). Also, other oxidative enzymes such as lypoxygenase, Chl oxidase, and peroxidase could be involved in Chl catabolism, through alternative pathways. Studies performed in different systems support the possible participation of peroxidase in the catabolism of Chl (Matile, 1980; Huff, 1982; Maeda et al., 1998; Martínez et al., 2001). Moreover, Funamoto et al. (2002) have suggested that one isoenzyme of peroxidase is directly involved in chlorophyll catabolism of broccoli.

The objective of the present work was to analyze the effect of growth regulators on chlorophyll degradation during the post-harvest senescence of broccoli heads, particularly focused on the chlorophyll degrading enzymes, such as chlorophyllase, Mg-dechelatase and peroxidase.

2. Materials and methods

2.1. Plant material and hormone treatment

Broccoli (*Brassica oleracea* L. var. Italica, c.v. Shogun) heads were obtained from local producers (La Plata, province of Buenos Aires, Argentina) and immediately processed for analysis. Individual branchlets were removed from the broccoli heads and dipped in solutions containing either 100 ppm 6-benzylaminopurine (BAP), 0.1% (v/v) dimethyl sulfoxide (DMSO) or 100 ppm ethephon, 0.1% (v/v) DMSO for 1 min. Control treatments were performed by dipping broccoli florets in 0.1% (v/v) DMSO for 1 min. After treatment, branchlets were placed in trays and covered (not sealed) with PVC film to diminish water loss. Eight branchlets were placed in each tray and 12 trays were used for each treatment. The trays were placed at 20 °C for 4 days; the weight of trays was taken every day and weight losses were calculated. Samples were also taken after 0, 1, 2, 3, and 4 days and processed immediately or frozen at −80 °C after removing the stems.

2.2. Color measurement

The superficial color was evaluated with a colorimeter (Minolta, Model CR-300, Osaka, Japan) by measuring the L^* and hue angle in nine positions of flower buds of each branchlet.

2.3. Extraction and dosage of chlorophylls and pheophytins

Frozen broccoli florets were crushed in a refrigerated mill (Tekmar, model A-10, Cole–Parmer Instrument Company, Chicago, IL, USA) and 0.5 g of the powder obtained were 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 determine the chlorophyll content (Lichtenthaler, 1987). To determine the pheophytin (Pheo) content, all the chlorophyll present in the extracts was transformed into Pheo by adding one drop of 25% (v/v) HCl to 5 ml of supernatant. The quantitative determination of Phes was performed by subtracting the amount of Chls in the original solution to the total Phes obtained after adding HCl according to the method of Lichtenthaler (1987). Four replicates per condition were analyzed.

2.4. Enzyme extraction

Frozen broccoli florets were crushed in a refrigerated mill (Tekmar, model A-10, Cole–Parmer Instrument Company, Chicago, IL, USA), and approximately 8 g were poured into 25 ml of the following extraction buffer: 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.2% (v/v) Triton X-100, 30 g/l polyvinylpolypyrrolidone (PVPP), 1 mM phenyl methyl sulfonyl fluoride (PMSF), 5 mM 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 determine enzyme activities.

2.5. Preparation of substrates

2.5.1. Chlorophylls

Spinach leaves (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 the supernatant was added with 40 ml of petroleum ether to extract the chlorophylls. After this, the ether was evaporated under N_2 and the chlorophylls dissolved in 4–5 ml of acetone.

2.5.2. Chlorophyllin

Preparation of chlorophyllin was done according to Vicentini et al. (1995). The Chl ethereal extract was prepared as described above, washed three times with 40 ml of water and then mixed with 1 ml 30% (w/v) KOH in methanol per each mg 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 M tricine.

2.6. Chlorophyllase activity

The following reaction mixture was used: 0.1 M sodium phosphate buffer pH 7.0 with 0.15 % (v/v) Triton X-100, 10 μM 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 min 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, and 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 absorbance at 663 nm in the lower phase. The enzyme activity was expressed as the increment of optical density at 663 nm per minute under the test conditions. Four replicates per condition were analyzed.

2.7. Mg-dechelatase activity

The following reaction mixture was used: 50 mM 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 reaction progress was evaluated by measuring the increase of OD at 686 nm. The Mg-dehelatase activity was expressed as the increment of OD at 686 nm per minute under the test conditions. Four replicates per condition were analyzed.

2.8. Peroxidase-linked chlorophyll bleaching activity

The following reaction mixture was used: 0.02 M sodium phosphate buffer pH 5.0, 0.2% (v/v) Triton X-100, 5 mM *p*-coumaric acid, 1 mM H_2O_2, 25 μl enzymatic extract, 10 μl chlorophyll to yield 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. The bleaching of chlorophylls was evaluated by measuring the decrease of OD at 669 nm. The activity was expressed as the decrement of optical density at 669 nm per minute under the test conditions. Four replicates per condition were analyzed.

2.9. Statistical analysis

The entire experiment was done three times and performed according to a factorial design with treatments (three levels) and time (five levels) as factors. Data were analyzed using analysis of variance over time using the MGLH procedure of the Systat system. Differences among means were analyzed at a significance level of 0.05 by using the LSD test.

3. Results and discussion

3.1. Visual changes and surface color

Broccoli florets were treated with BAP or ethephon and stored at 20 °C for 4 days. After the storage, both the control and the treated florets showed a weight loss of 4–6% in relation to the initial value. The control florets did not show appreciable color changes until day 2, and turned progressively yellow from day 3 on. The florets treated with ethephon showed a premature and faster yellowing after 2 days at 20 °C, while the BAP-treated florets maintained their green color until day 4.

Changes in external color during broccoli senescence were evaluated through the hue angle and L* parameter (Fig. 1). Along the storage, the diminution of the hue angle value correlated with the progressive yellowing observed in the florets. Control florets reduced their hue angle from day 2 to day 3, while ethephon-treated florets started the reduction from day 1 to day 3 ($p < 0.05$). The rate of hue diminution in ethephon-treated florets was significantly faster than that of control florets; while the corresponding rate of BAP-treated florets was near to 0, detecting a slight decrease only after 4 days.

Broccoli lightness was evaluated through the L* parameter value (Fig. 1b). The L* value increased in all the samples during storage at 20 °C, but florets treated

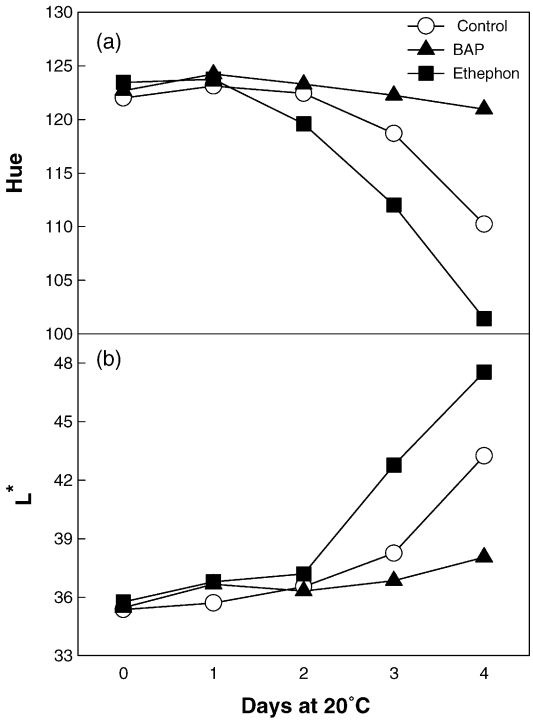

Fig. 1. Change of superficial color parameters during post-harvest senescence of broccoli incubated at 20 °C in darkness. Non-treated florets (◯); BAP-treated florets (▲); ethephon-treated florets (■) $LSD_{Hue} = 0.266$, $LSD_{L^*} = 0.176$.

with ethephon showed higher rate of increase of L* than the controls. Instead, BAP-treated florets maintained considerable lower rates of L* increment ($p < 0.05$).

3.2. Chlorophyll and pheophytin content

The chlorophyll and pheophytin levels were evaluated in control and treated florets during the holding at 20 °C. The total chlorophyll content decreased continuously along the incubation (Fig. 2a). BAP treated samples showed chlorophyll degradation also but the process was slower in relation to the control ($p < 0.05$). After 4 days, BAP florets retained approximately 65% of the initial chlorophyll while the control retained 50%. On the contrary, ethephon treated samples showed an enhanced chlorophyll catabolism and only 38% of the initial amount of chlorophylls were present after 4 days at 20 °C. It is worth to point out that after 2 days the chlorophyll content decreased, while no

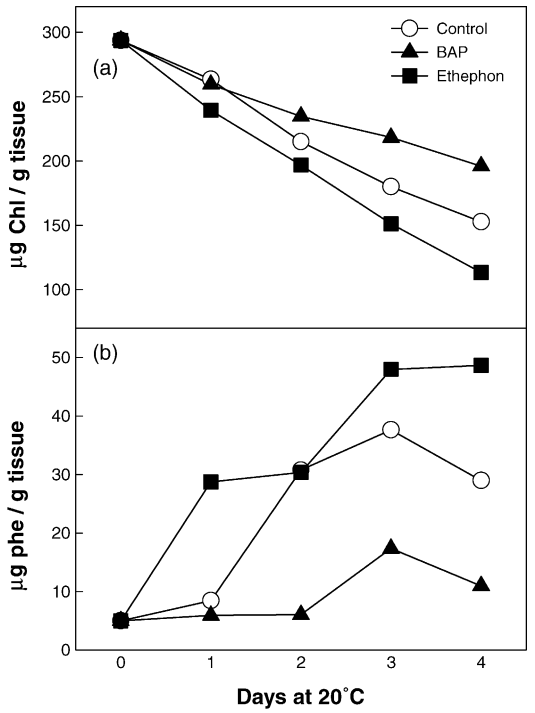

Fig. 2. Change of chlorophyll content (a) and pheophytin content, (b) expressed as μg pigment/g tissue, during post-harvest senescence of broccoli incubated at 20 °C in darkness. Non-treated florets (◯); BAP-treated florets (▲); ethephon-treated florets (■) LSD_{Chl} = 24, LSD_{Phe} = 8.

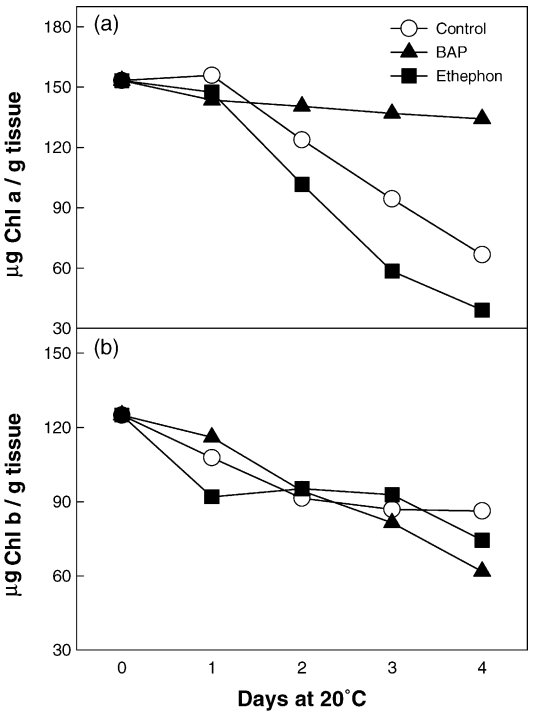

Fig. 3. Variation of chlorophyll *a* (a) and chlorophyll *b* (b) contents, expressed as μg pigment/g tissue, during post-harvest senescence of broccoli incubated at 20 °C in darkness. Non-treated florets (◯); BAP-treated florets (▲); ethephon-treated florets (■) $LSD_{Chl\ a}$ = 16, $LSD_{Chl\ b}$ = 10.

difference of hue angle was observed, indicating that the chlorophyll degradation begins before any color change is visible. These results agree with the reported effect of ethylene on chlorophyll degradation, as was showed by the application of propylene or anti-ethylene agents in broccoli (Tian et al., 1994; Wang, 1977). The effect of BAP on chlorophyll degradation coincides with other reports describing the delay in chlorophyll loss by exogenous application of cytokinins (Clarke et al., 1994; Downs et al., 1997). Moreover, transgenic broccoli with the isopentenyltransferase gene under the control of senescense-associated promoters showed an enhanced level of cytokinins when senescence begins, which caused a delay in yellowing (Chen et al., 2001).

The levels of chlorophyll *a* and *b* both decreased during the experiment (Fig. 3). In control florets, the chlorophyll *a* degradation rate was higher than that of chlorophyll *b*, as generally occurs during senescence (Kura-Hotta et al., 1987; Hidema et al., 1992). However, an interesting effect was found when the hormones were applied: both ethephon and BAP influenced the catabolism of chlorophyll *a* but not of chlorophyll *b*. In the case of chlorophyll *a*, the treatment with BAP delayed the loss of chlorophyll *a* and the treatment with ethephon increased its degradation. No clear effects on the content of chlorophyll *b* were found after treatments with ethephon or BAP over the experiment. Therefore, the differences found in total chlorophyll content were closely related to effect of BAP and ethephon on chlorophyll *a* rather chlorophyll *b*. 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 (Matile et al., 1999). The model is supported by the existence of a "Chl *b* reductase", which transforms chlorophyllide *b* to chlorophyllide *a* (Matile et al., 1999). Hence, this common degradation pathway of chlorophylls suggests that the differential effect of BAP and ethephon

on the degradation of the two chlorophylls might affect a step upstream the conversion of chlorophyll *b* in chlorophyll *a*.

Pheophytins accumulated after 2 and 3 days at 20 °C and decreased at day 4 in control samples (Fig. 2b). The treatment with ethephon caused an accelerated and continuous accumulation of pheophytins since 1 day ($p < 0.05$). In contrast, BAP inhibited this accumulation until 3 day, where a small increase was detected ($p < 0.05$). According to Matile et al. (1999), chlorophylls are degraded by first removing the phytol, and then the central Mg-ion, generating chlorophyllides and pheophorbides respectively, but not pheophytins. However, our results indicated that a transient accumulation of pheophytins can occur, which implies a release of Mg-ion directly from the chlorophylls. A similar fact was described during senescence of parsley leaves, where a gradual disappearance of chlorophyll was accompanied by an increase in pheophytins and other phytylated compounds but without appearance of dephytylated derivatives (Amir-Shapira et al., 1987). Moreover, the only chlorophyll derivative detected in yellow leaves of *Ginkgo biloba* was pheophytin *a* (Tang et al., 2000), suggesting that the removal of magnesium occurred before dephytylation.

3.3. Chlorophyll degrading enzyme activities

The activities of enzymes related to chlorophyll degradation (chlorophyllase, Mg-dechelatase, and peroxidase) were evaluated in control and treated florets during holding at 20 °C.

3.3.1. Chlorophyllase

In control samples, chlorophyllase activity increased during the experiment, reached the maximum at 3 day and decreased thereafter ($p < 0.05$). The samples treated with BAP showed a slight increase at 1 day and a posterior decrease in the chlorophyllase activity, remaining without changes until the end of the experiment. In contrast, the chlorophyllase activity increased continuously in ethephon-treated samples, and reached values three times higher than the control after 4 day ($p < 0.05$) (Fig. 4).

Chlorophyllase has been considered a hydrophobic protein of plastid membranes (Matile et al., 1999), but recently the cloning of several genes showed that some chlorophyllases contain a transit peptide typical of endoplasmic reticulum targeted proteins, suggesting the possibility of chlorophyllases with different localizations (Takamiya et al., 2000) and probably different regulations. In this sense, the chlorophyllase variation during senescence or ripening can show very different patterns according to the system analyzed. Chlorophyllase has a latent behavior and does not increase during leaf senescence of most species (Matile et al., 1999) and even decreases during senescence of *Phaseolus vulgaris* (Fang et al., 1998). However, activity can increase during senescence of barley (Sabater and Rodríguez, 1978) and spinach (Yamauchi and Watada, 1991), similarly with the data obtained from broccoli. The effect of ethylene can also be different according to the system. For example in spinach, the chlorophyllase activity increases during senescence and the treatment with ethylene does not produce an additional increment (Yamauchi and Watada, 1991). However, in Citrus fruit, chlorophyllase activity does not increase during ripening but the treatment with ethylene accelerates the chlorophyll breakdown and increases the chlorophyllase activity (Amir-Shapira et al., 1987; Jacob-Wilk et al., 1999). Our work indicates that the increment of chlorophyllase activity during broccoli senescence is up regulated by ethylene and down regulated by cytokinins.

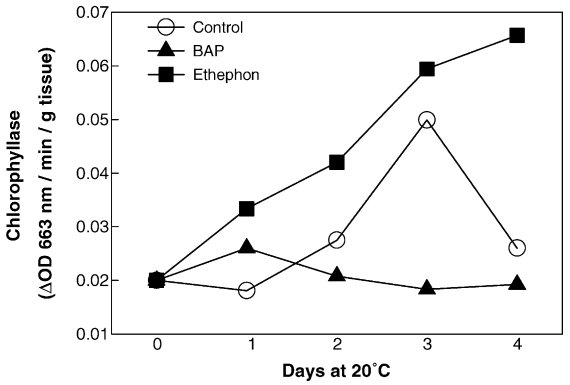

Fig. 4. Evolution of chlorophyllase activity during post-harvest senescence of broccoli incubated at 20 °C in darkness. Non-treated florets (◯); BAP-treated florets (▲); ethephon-treated florets (■) LSD = 0.003.

3.3.2. Mg-dechelatase

The activity of Mg-dechelatase increased along the course of senescence in control samples ($p < 0.05$),

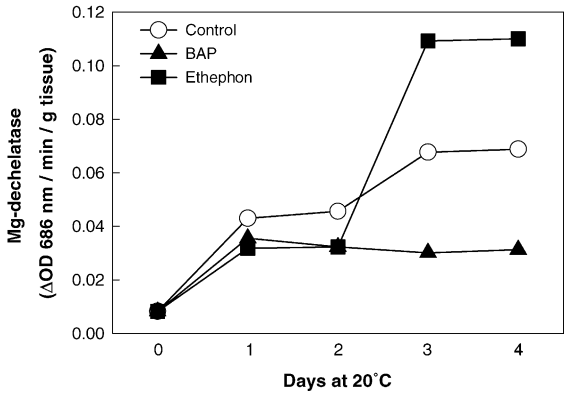

Fig. 5. Evolution of Mg-dechelatase activity during post-harvest senescence of broccoli incubated at 20 °C in darkness. Non-treated florets (◯); BAP-treated florets (▲); ethephon-treated florets (■) LSD = 0.003.

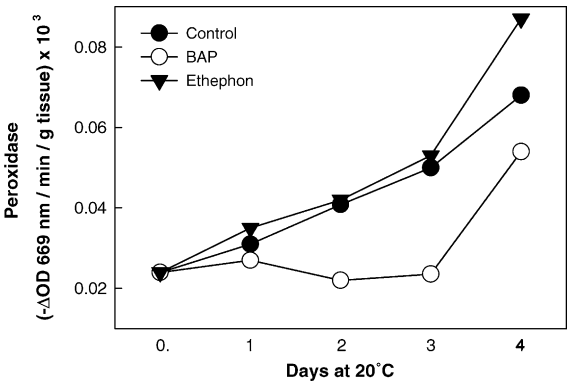

Fig. 6. Evolution of chlorophyll degrading peroxidase activity during post-harvest senescence of broccoli incubated at 20 °C in darkness. Non-treated florets (◯); BAP-treated florets (▲); ethephon-treated florets (■) LSD = 0.003.

and the activity after 4 day at 20 °C was seven times higher than the initial value (Fig. 5). Ethephon-treated samples showed a similar trend but the increase was near to eleven times after 4 days. The enzyme activity in florets that had been treated with BAP increased approximately four times ($p < 0.05$) at day one and remained constant thereafter ($p > 0.05$).

As other enzymes involved in chlorophyll catabolism, Mg-dechelatase has been little studied. Early studies done in oil-seed rape cotyledons indicated that the activity decreases during dark-induced senescence (Vicentini et al., 1995). However, Mg-dechelatase activity increases during ripening of strawberry fruit (Costa et al., 2002) and yellowing of *Ginkgo biloba* leaves (Tang et al., 2000). In the last case, the authors suggest that this could be the key step in chlorophyll degradation since yellow leaves showed a decrease in chlorophyllase activity and the only catabolite detected was pheophytin. In broccoli, the activity of Mg-dechelatase increases 1 day after harvest, while the chlorophyllase activity increases after 2 day, suggesting a possible accumulation of Mg-free derivatives (e.g. pheophytins) prior to phytol-free ones. Until now, "in vitro" experiments with Mg-dechelatase indicated that release of Mg occurs only from de-phytylated compounds (Shioi et al., 1996). However, Mg-elimination could be catalyzed by Mg-dechelatase in an "in vivo" reaction, by another non-described enzyme or by a non-enzymatic reaction. The fact that Ginkgo (Tang et al., 2000) and parsley (Amir-Shapira et al., 1987) leaves and broccoli florets accumulate pheophytins supports the possibility of releasing Mg from phytylated compounds.

3.3.3. Peroxidase

The possible participation of peroxidase in the catabolism of Chl has been previously suggested (Matile, 1980; Maeda et al., 1998; Martínez et al., 2001). Peroxidase mediates chlorophyll degradation in the presence of a phenolic compound, through the following possible mechanism: the enzyme catalyzes the oxidation of a phenolic compound with hydrogen peroxide, generating a phenolic radical, which in turn degrades chlorophyll to a colorless compound (Kato and Shimizu, 1985; Yamauchi et al., 1997a,b). In our case the peroxidase-linked chlorophyll bleaching activity increased three times in control florets during the incubation period (Fig. 6). Ethephon-treated samples showed a similar behavior until day 3, but the increment of activity was higher in day 4 ($p < 0.05$). Otherwise, this peroxidase-linked chlorophyll bleaching activity in BAP treated florets remained constant until day 3 ($p > 0.05$) and increased in day 4 but in a lower extent than in the control ($p < 0.05$). Increment of guaiacol peroxidase activity (Toivonen and Sweeney, 1998) and peroxidase-linked chlorophyll bleaching activity (Funamoto et al., 2002) during broccoli senescence were previously reported. In the former paper, Toivonen and Sweeney (1998) compared two cultivars of broccoli with differences in chlorophyll loss and found that the c.v. that was resistant to losses of

chlorophyll during post-harvest had greater guaiacol-peroxidase activity. Authors suggested that higher superoxide dismutase and peroxidase levels enhanced the elimination of H_2O_2 and thus diminished the formation of hydroxyl radicals, preventing the damage of chlorophylls. It is interesting to point out that experiments performed by Toivonen and Sweeney (1998) were done at 13 °C, while senescence in our experiments was induced at 20 °C. Then, it is very probable that experiments at 13 °C focused in early events, while our experiments described relatively late senescence events. Before cell membranes become damaged, it is possible that only low level of phenolics would be in contact with peroxidase and chlorophyll, which would be insufficient for chlorophyll bleaching. In this case, the role of peroxidase would be more important in detoxification of H_2O_2. On the contrary, at late events of senescence, tonoplast, and chloroplast breakdown and phenolics can mix with chlorophyll and peroxidase, and thus peroxidase-linked chlorophyll bleaching would acquire relevance. The effect of peroxidase-linked chlorophyll bleaching was also studied by Funamoto et al. (2002), working with heat-treated broccoli florets. They found that the delay in chlorophyll degradation was closely related to a lower peroxidase activity in heat-treated samples. Chlorophyll *a*-1, an oxidative product of chlorophylls, was detected in an "in vitro" degradation system (Yamauchi et al., 1997a,b) and the authors proposed an important role of peroxidase in chlorophyll degradation of broccoli. The exact role of peroxidase in chlorophyll degradation remains to be elucidated (Matile et al., 1999); however, Guiamet et al. (1999) found that plastoglobuli of senescing soybean leaves including intact chlorophylls are eliminated from the chloroplast and carried to the vacuoles. In this location, highly active peroxidases and hydrogen peroxide could mediate degradation of chlorophylls.

4. Conclusions

According to our results, the loss of green color during broccoli senescence is associated to a reduction of chlorophyll *a* and *b* content, which occurs prior to the diminution of hue angle value. Also, a gradual activity increase of three chlorophyll-degrading enzymes (chlorophyllase, Mg-dechelatase and peroxidase-linked chlorophyll bleaching) was observed during senescence at 20 °C, indicating the activation of chlorophyll catabolism. Both, chlorophyll degradation and enhancement of enzymatic activities were stimulated by ethylene and delayed by BAP.

Acknowledgements

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