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Regulated Chlorophyll Degradation in Spinach Leaves during Storage

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Abstract. **Degradation of chlorophyll in spinach *(Spinacia olearacea* L. cv. Hybrid 612) appeared to be regulated through the peroxidase-hydrogen peroxide pathway, which opens the porphyrin ring, thus resulting in a colorless compound. This conclusion was arrived at from the analysis of chlorophylls (Chls) and their metabolizes by HPLC and of enzyme activities catalyzing the degradative reactions. Chls decreased at 25C but not at 1C. The chlorophyll oxidase pathway was not active, as noted by the lack of accumulation of a reaction product named Chl a-1. Lipid peroxidation increased with storage, but the products of the reaction. did not degrade chlorophyll, as noted by the lack of increase in Chl a-1. Chlorophyllase activity increased, but chlorophyllide, the expected product of the reaction, changed minimally during senescence. Ethylene at 10 ppm did not alter the pathway that degraded chlorophyll in spinach.**

One of the symptoms of senescence in harvested leafy vegetables is loss of greenness with the degradation of chlorophyll (Chl). Quantitative changes in Chl and degradation products of Chl and/or the hydrolyzing enzymes have been monitored in vegetables, but the mechanism or pathways of degradation are not clear and appear to differ among commodities.

Chlorophyllase activity, which catalyzes the release of the phytol chain from Chl to form chlorophyllide (Chd), has been reported to increase with yellowing in barley and oat leaves (Rodriguez et al., 1987; Sabater and Rodriguez, 1978), but in tobacco and radish leaves the activity decreases with senescence (Phillips et al., 1969; Shimizu and Tamaki, 1963). In ethylene-treated citrus fruit, chlorophyllase activity increased with concomitant decrease in Chl content (Amir-Shapira et al., 1987; Shimokawa et al., 1978). However, in regreening of Valencia oranges, chlorophyllase activity increased with the enhancement of-Chl, and the authors reported that the enzyme was involved in the biosynthesis of Chl (Aljuburi et al., 1979). Thus, chlorophyllase is involved in both biosynthetic and degradative reactions of Chl, but not necessarily in all commodities.

Chlorophyll oxidase, which requires linoleic acid to catalyze the reaction, is located in the thylakoid membranes of the chloroplast. It is operative in the oxidation of Chl to a metabolize identified as Chl a-1, which is suspected to be an intermediate in chlorophyll degradation rather than a final product (Luthy et al., 1984; Schoch et al., 1984).

Chloroplast lipids, such as monogalactosyldiglyceride, digalactosyldiglyceride, and phosphatidylglycerol, decreased markedly in senescing spinach leaves (Yamauchi et al., 1986, 1987), and the decrease was probably caused by lipolytic acyl hydrolase in the chloroplast, since its activity increased with senescence. The free fatty acids released by the hydrolase could be oxidized by lipoxygenase and form hydroperoxides. Free radicals, as one of the end products of unstable hydroperoxides, have the potential of degrading Chl.

In parsley, Chl is degraded by a peroxidase-hydrogen peroxide system containing apigenin, a major flavone required to catalyze this reaction (Yamauchi and Minamide, 1985).

We report here research on analyses of Chls and their degradation products and activities of enzymes known to catalyze the reactions that degrade Chl in spinach. We also determined if ethylene altered the degradation pathway.

Materials and Methods

About 1 kg of freshly detached mature leaves of spinach, free of defects or injuries, were stored at 25C in a covered container under a stream of humidified air with or without 10 ppm ethylene or at lC without ethylene. Air with or without ethylene was metered at a sufficient rate to keep the CO_2 level below 0.5%. Leaves (≈100 g) were removed at scheduled intervals during the 6 days and blades without midribs were used for the analyses. The study was repeated with spinach obtained at a later harvest date.

Pigments were extracted by grinding 2.5 g leaves in 20 ml cold acetone and 2.5 ml distilled water with a mortar and pestle, filtering the homogenate, rewashing the residue with 80% cold acetone until the residue was colorless, and bringing the final volume to 50 ml in low light. Aliquots of the combined extracts (which were kept in darkness) were used for photometric analysis or passed through a millipore filter (0.22-µm pores) for HPLC analysis.

The apparatus for the HPLC consisted of Waters Model 6000 pumps (Waters, Milford, Mass.) with automated gradient controller and Model 712 WISP interfaced into a Hewlett-Packard 1040A rapid-scanning UV/visibIe photodiode array detector (Hewlett-Packard, Rockville, Md.) or Perkin-Elmer LC 95 spectrophotometer (Perkin-Elmer, Rockville, Md.). The absorption spectra of the pigments were recorded between 200 and 600 nm at the rate of 12 spectra/rein. Pigments were separated by a Beckman C_{18} ultrasphere column (Beckman, Columbia, Md.), 4.6 x 250 mm, using two solvents: A, 80 methanol : 20 water, and B, ethyl acetate in a gradient. Solvent B was added to A at a linear rate until a 50:50 mixture was attained at the end of 20 min. The 50:50 mixture then was used isocratically for an additional 25 rein, as described by Eskin and

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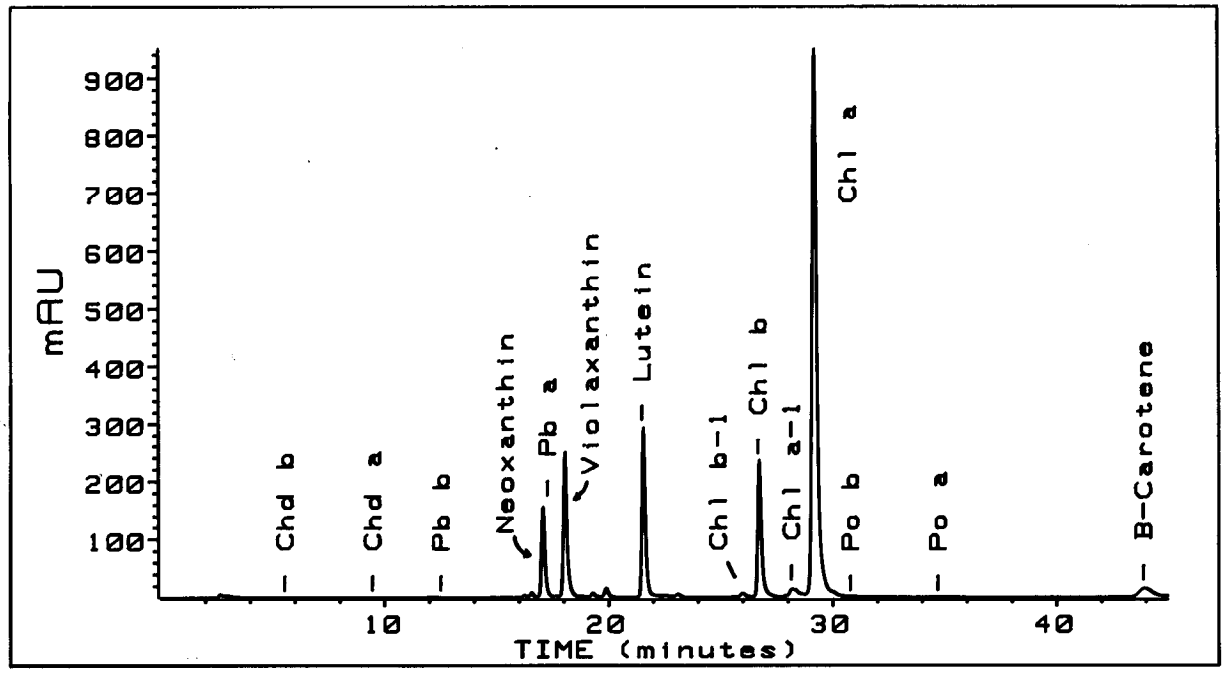


Fig. 1. Chromatogram of pigments extracted from spinach leaves and analyzed by HPLC with a photodiode array detector. Chd = chlorophyllide, Pb = pheophorbide, Chl = chlorophyll, Po = pheophytin.

Harris (1981). Flow rate was 1 ml·min^{-1} and injection volume was 50 µl. Data from the photodiode array detector were stored and processed by means of a Hewlett-Packard 9000/series 300 computing system.

Individual pigments were purified from the acetone extract for use as standards. Chls a and b were purified by adding 3 ml 1,4-dioxane and 3 ml distilled water to 20 ml of the 80% acetone extract, allowing the mixture to stand until a precipitate formed, centrifuging the mixture at 12,000× g for 10 min, and dissolving the pellet in ?5 ml of acetone (Yoshiura and Iriyama, 1979). Part of this acetone mixture was used to prepare Chds a and b by treating it with chlorophyllase that was extracted from the acetone powder of spinach leaves, as described below. Pheophorbides a and b were prepared by adding one drop of 2 N HCl to the Chd solution. Another part of the acetone mixture was used to prepare pheophytins a and b by adding one drop of 2 N HCl. The individual pigments were separated by TLC using silica gel base with 60 n-hexane :20 acetone :5 t-butyl alcohol solvent, scraping the base from the plate, and extracting it with 80% acetone before analysis. Carotenoids were extracted from spinach leaves with cold 80% acetone and also separated by the same TLC method as for Chl pigments. Each carotenoid was identified by its absorption spectrum in ethyl alcohol (Davies, 1976).

For the crude enzyme extract, an acetone powder was prepared by macerating 5 g frozen spinach leaves (– 80C) with 50 ml cold acetone (– 18C) using a cold mortar and pestle, filtering the macerate with a Buchner funnel, washing the macerate with a small volume of cold acetone, remacerating the residue, refiltering, rewashing with a small volume of cold diethyl ether, and vacuum-drying the residue. Enzymes were extracted by suspending the acetone powder in 20 ml of 50 mM phosphate buffer (pH 7) containing 0.6% 3-[(3-Chlamido-propyl) dimothylammonio]-1-propanesulfonate (CHAPS) (a zwitterionic detergent) for 1 h at 4C, filtering the mixture through Miracloth, and centrifuging the filtrate at 24,000× g for 20 min. The crude extract was desalted by passing a 4-ml aliquot of the supenatant through 4 g of a 20-ml bed volume of Sephadex G-25 in a 1.6 × 18-cm column.

Peroxidase activity was determined as described by Yamauchi et al. (1985). Chlorophyllase activity was determined by a modification of the method by Shimokawa et al. (1978). The reaction mixture consisted of 1 ml crude enzyme extract, 1 ml acetone, and 100 kg chlorophyll in 10 mM phosphate buffer (pH 7) for a total volume of 2 ml. The reaction was carried out at 25C for 45 min and was stopped by adding 5 ml of a 3 acetone : 7 hexane mixture. Activity was based on a decrease in absorbance by chlorophyll at 663 nm. One unit of peroxidase or chlorophyllase activity was defined as a change of 0.01 absorbance unit per minute or hour, respectively.

Total Chl content was determined by the method of Amen (1949), that of protein by the method of Lowry et al. (1951). Thiobarbeturic acid (TBA) assay, as described by Stewart and Bewley (1980), was used to determine the concentration of malondialdehyde (MDA), an indicator of the degree of lipid peroxidation.

Results

The HPLC of fresh spinach showed sequential elution of neoxanthin, violaxanthin, lutein, Chl b, Chl a-1, Chl a, and 13-carotene during the 45-rein run (Fig. 1). Chl b-1 eluted as a small peak. The elution time of Chd a and b, pheophorbide (Pb) a and b, and pheophytin (Po) a and b are also shown in Fig. 1 as reference points. Identity of each pigment was based on retention time, and some were confirmed by the absorption spectra of the eluting peak. The absorption spectra confirmed the identity of Chl a-1 and b-1, which eluted closely to Chl a and b, respectively. The absorption spectra of Chl a-1 and b-1 were similar in pattern to those of their respective sources, but the absorption below the maxima was stronger by Chl a-1 and b-1 than that by Chl a and b, respectively (Fig. 2 A and B).

Levels of Chl a and b in spinach held at lC did not change during 6 days of storage. At 25C, Chl decreased continually to ≈65% of the original level by day 4 (Fig. 3A, Table 1). Eth-

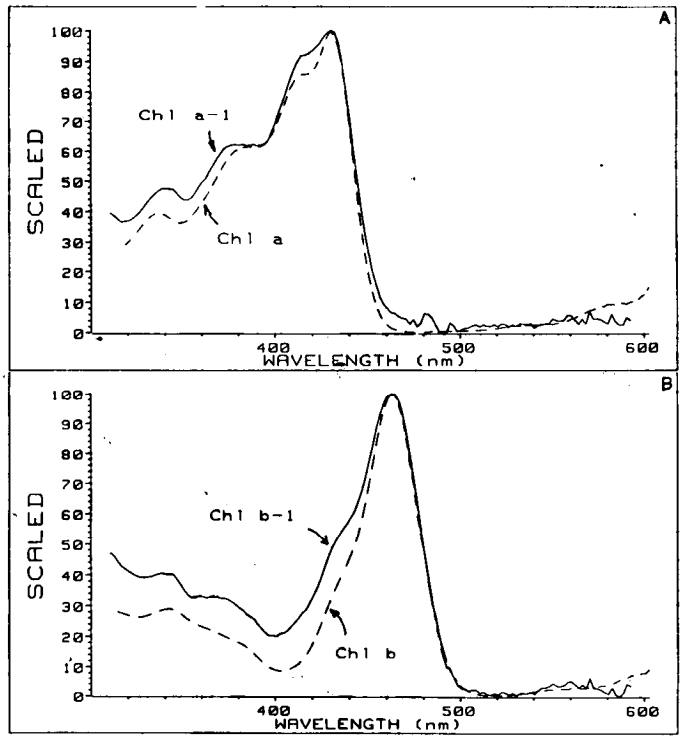


Fig.2. Spectrum of **(A)** chlorophyll a and a-1 and **(B)** chlorophyll b and b-1 in spinach leaves following separation by HPLC and spectra determined with a photodiode array detector.

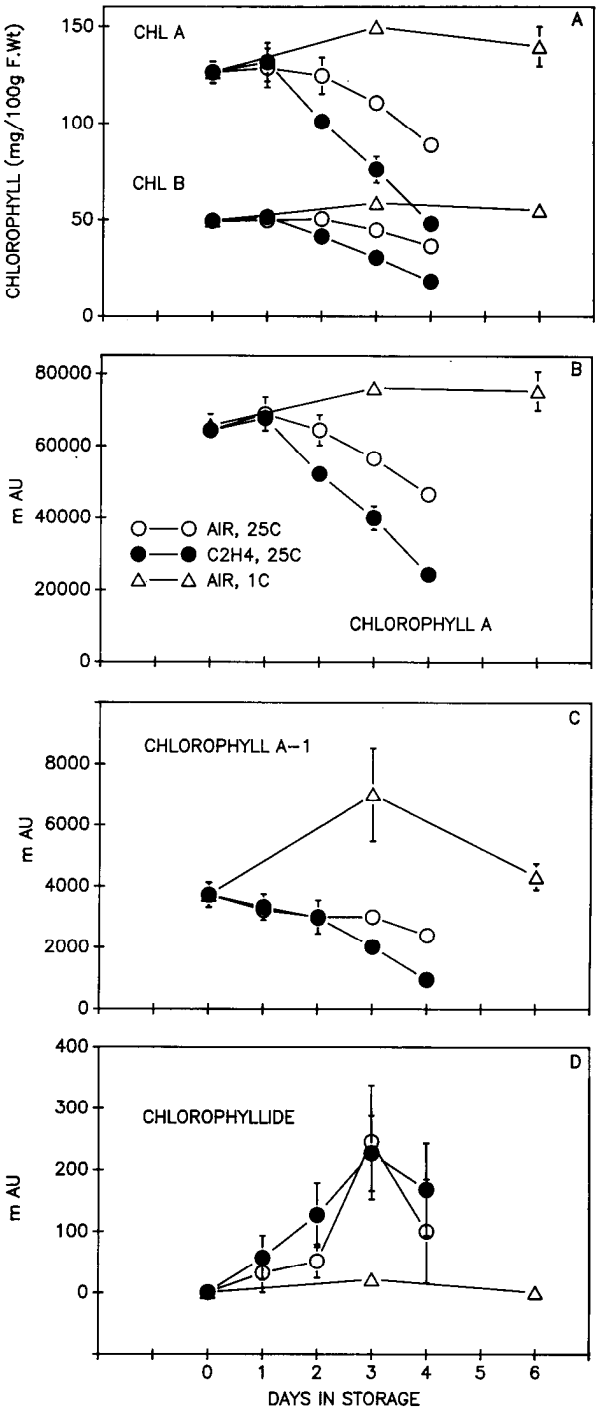
yleneat 10 ppm hastened this decrease at 25C, which was apparent on day 2; Chl content dropped to 40% of the original level by day 4. Chl b content in spinach at 25C, with or without ethylene, decreased similarly relative to Chl a, but the rate of decrease was not as great.

Chl a-1 decreased rather than increased at 25C, with the decrease slightly hastened by a 10-ppm ethylene treatment (Fig. 3C, Table 1). Chl a-1 content remained unchanged at 1C. The relative level of Chd a (Fig. 3D, Table 1), based on milliabsorbance units, was only ≈1% of that of Chl a (Fig. 3B) on day 0, and the level, overall, increased slightly at 25C. The increase was hastened slightly by ethylene. Pheophytin was detected in samples held at 1 or 25C, but the quantity in fresh spinach was very low (data not presented).

Chlorophyllase activity increased during the first 3 days in spinach held at 25C and then decreased sharply between days 3 and 4, with or without ethylene (Fig. 4A, Table 1). At 1C, there was a 50% reduction in chlorophyllase activity by day 3, and the level remained unchanged by day 6.

Peroxidase activity in spinach stored at 25C with or without ethylene increased by ≈30% after 1 day of storage and again increased sharply by day 4 (Fig. 4B, Table 1). It remained unchanged at 1C storage.

The TBA value, as indicated by the MDA concentration, increased in spinach stored at 25C, with the increase after day 3 having been greater in spinach treated with 10 ppm ethylene than in the control, but the difference probably is not significant (Fig. 4C, Table 1). At 1C, the activity remained unchanged during storage.



Quantitative changes of **(A)** chlorophyll a and b and relative changes of **(B)** chlorophyll a and **(C)** a-1 and **(D)** chlorophyllide in spinach leaves held in air or 10 ppm C_2H_4 in air at 25C or in air at 0C. Error bars not shown when masked by the symbol.

Discussion

In assessing the quantitative changes of pigments, only a slight increase in Chd was noted with the large decrease in Chl in spinach stored at 25C. One might expect a large increase in Chd with the noted increase in chlorophyllase activity during the first few days of storage, particularly since this enzyme catalyzes the removal of the phytol chain from Chl. The 10-ppm ethylene treatment, which hastened Chl degradation and yellowing of leaves, did not appear to affect this pathway of Chl degradation. In ethylene-treated citrus fruit peel, Arnir-Shapira et al. (1987) reported that Chl decreased and chlorophyllase activity increased, as noted here with spinach. However, the Chd content

Table 1. Statistical analysis by General Linear Models procedure of Chl a, Chl b, Chl a-1, Chd, chlorophyllase (C-ase), peroxidase (P-ase), and MDA changes in spinach stored in air or 10 ppm ethylene in air at 25C.

Variables	df	Mean square				df	Mean square		
		Chl a	Chl b	Chl a-1	Chd		C-ase	P-ase	MDA
Treatment (T)	1	3705***	632***	221*	8850^{NS}	1	45^{NS}	10442^{NS}	806^{NS}
Rep (Treatment)	6	26^{NS}	6	53	36928***	2	808^{NS}	26880*	88^{NS}
Days									
Linear (D)	1	18758***	2622***	1891***	168177***	1	5040*	204919**	24750***
Quadratic (D^2)	1	2031**	401***	53	20628^{NS}	1	2987^{NS}	7110^{NS}	8
Cubic (D^3)	1	195^{NS}	12^{NS}	22	49551*	1	4687*	59213**	903*
T × D	1	2844***	551***	316*	1711^{NS}	1	1288^{NS}	7425^{NS}	697^{NS}
T × D^2	1	1^{NS}	5^{NS}	56	60^{NS}	1	2125^{NS}	1003^{NS}	168^{NS}
T × D^3	1	241^{NS}	38^{NS}	10	4727^{NS}	1	255^{NS}	5^{NS}	32^{NS}
Error	24	210	26	51	6155	8	393	4792	149

^{NS},*, * *,***Nonsignificant or significant at P = 0.05, 0.01, and 0.001, respectively.

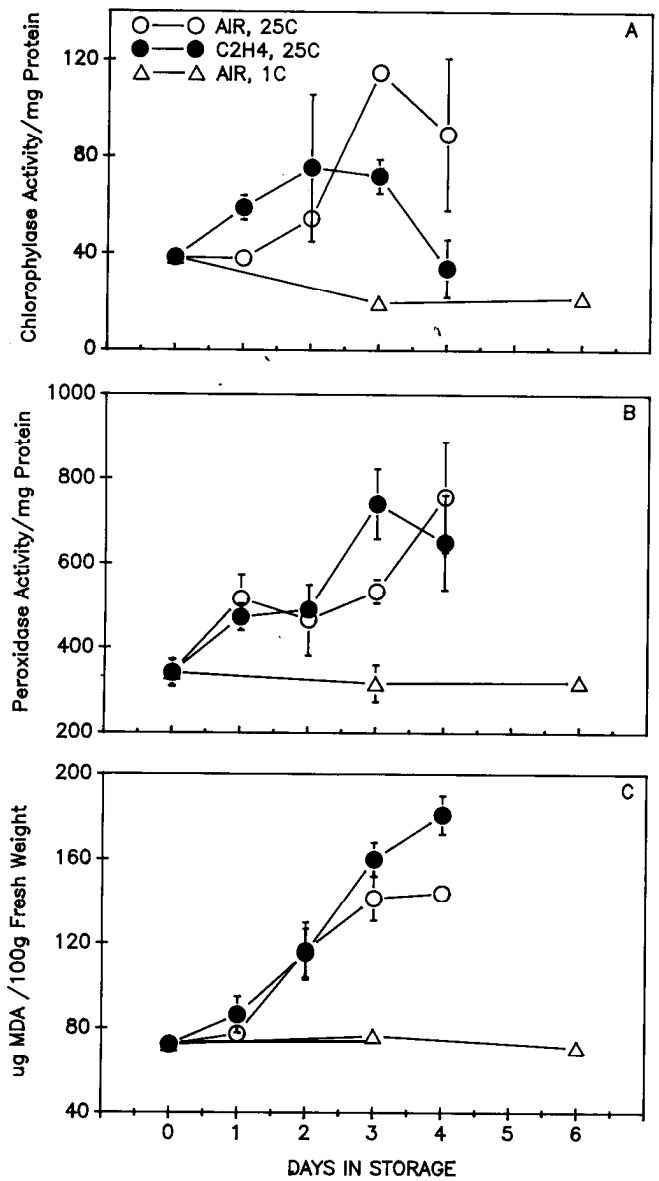


Fig. 4. Activities of **(A)** chlorophyllase and **(B)** peroxidase and changes in **(C)** malondialdehyde (MDA) content in spinach leaves held at 25C in air or 10 ppm C_2H_4 in air or at 0C in air. Error bars not shown when masked by the symbol.

was high and Chl a was low in the senescing fruit, which implies that degradation of Chl to Chd is a prominent pathway in citrus.

Chlorophyll degradation by chlorophyllase activity in the chloroplast of citrus leaves and the chromoplast of citrus fruit was reported to be greater than that noted in parsley leaves (Amir-Shapira et al., 1986). Perhaps the amount of chlorophyllase in spinach tissues is too low to degrade a significant amount of Chl, which may account for the very low levels of Chd.

Chlorophyll oxidase bleaches Chl in the presence of saturated fatty acids, such as stearic acid, and the enzyme is clearly different from lipoxygenase (Luthy et al., 1984). Schoch et al. (1984) reported Chl a-1 to be the only degradation product when Chl a was oxidized by chlorophyll oxidase in barley thylakoids. Maunders et al. (1983) noted an accumulation of Chl a-1 with a decline of Chl a in senescing excised barley and bean leaves. We have found the content of Chl a-1 to be very low in fresh spinach leaves, and it decreased with Chl a in spinach stored at 25C, with or without ethylene treatment, which implies that the chlorophyll oxidase pathway of degrading Chl is not significant in spinach leaves.

Degradation of Chl by peroxidase-hydrogen peroxide in the presence of phenolic compounds, such as 2,4-dichlorophenol and resorcinol, has been reported by Matile (1980), Huff (1982), and Kato and Shimizu (1985). Parsley leaves contain apigenin, a major flavone, which catalyzes the peroxidase-hydrogen peroxide reaction to degrade Chl (Yamauchi and Minamide, 1985). Spinach probably contains some form of phenolic compound to catalyze this reaction in degrading the Chl.

Lipid peroxidation can result in degradation of Chl as implied by Orthoefer and Dugan (1973) and Imamura and Shimizu (1974), where Chl was bleached in the presence of lipoxygenase and linoleic or linolenic acid. Imamura and Shimizu used leaf tissue extracts, while Orthoefer and Dugan used a model system consisting of Chl a, linoleic acid, and lipoxygenase. Similarly, chloroplast lipids, such as monogalactosyl-diglyceride, digalactosyldiglyceride, and phosphatidylglycerol, decreased during storage of spinach at 25C, and the decrease occurred before the beginning of chlorophyll degradation (Yamauchi et al., 1986). This decrease probably was due to the increasing amount of lipid peroxidation, as noted by the changing TBA values in spinach that we report. With the increasing amount of lipid peroxidation, which was hastened by the ethylene treatment, free radicals resulting from the unstable hydroperoxides have the potential of degrading Chl. However, if Chl a is oxidized to Chl a-1 in the lipoxygenase system, as it is in the chlorophyll

oxidase system, then it is questionable that the lipoxygenase system was very active due to the very low levels of Chl a-1 in stored spinach. Possibly, the free radicals formed by this system in spinach were scavenged by β– carotene or ascorbic acid, which are present in the chloroplast.

These results indicate that chlorophyllase plays a minor role in degradation of Chl in spinach leaves stored with or without ethylene and that most of the Chl appears to be degraded by the peroxidase pathway, where the pophyrin ring is opened and the resulting compound is colorless. Reactions of this pathway need to be examined further to identify enzyme(s) that can be manipulated at the molecular level for regulating color loss in spinach leaves during storage.

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