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Formation of pyrochlorophylls and their derivatives in spinach leaves during heating

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Abstract

The changes of chlorophylls and their derivatives during heating of spinach leaves were studied. Chlorophylls and their derivatives were analyzed by high performance liquid chromatography (HPLC) with photodiode-array detection or positive ion fast atom bombardment mass spectrometry (FAB–MS). No chlorophyll derivatives were detected in fresh spinach leaves. The degradations of both chlorophylls, a and b, fit the first-order model, and the rate constant (min^{-1}) by microwave cooking or blanching was greater than that by steaming or baking. The major chlorophyll derivatives formed during baking and blanching included chlorophyll epimers and pheophytins. Pyrochlorophylls a and b were detected in spinach leaves after steaming for 30 min or microwave cooking for 1 min. Pyropheophytins a and b were not formed until steaming or microwave cooking time reached 30 or 5 min, respectively. Steaming favored the formation of pheophytins a and b while microwave cooking favored that of pyrochlorophylls a and b.

1. Introduction

Chlorophylls a and b are the major pigments of green vegetables. It has been well established that chlorophylls are susceptible to chemical and physical changes during processing of vegetables (Bacon & Holden, 1967; Schwartz & Lorenzo, 1991; Chen & Chen, 1993). For instance, the bright green appearance of vegetables can be converted to dull olive color as a result of heat treatment. Since color is one of the most important quality attributes of vegetable products, numerous studies have been conducted to investigate the color changes or degradation of chlorophylls during heating (Schwartz et al., 1981; Khachik et al., 1986; Chen & Chen, 1993). The chlorophyll derivatives, including chlorophyll epimers, chlorophyllides, pheophytins and pyropheophytins were reported to occur during cooking of vegetables (Bacon & Holden, 1967; Schwartz et al., 1981; Strain, 1954; von Elbe et al., 1986). Of the various cooking methods, blanching vegetables at low temperature (70°C) was found to result in formation of chlorophyll epimers, pheophytins, chlorophyllides and pheophorbides (Bacon & Holden, 1967; Chen & Chen, 1993; Khachik et al., 1986). In addition, severe heat treatments such as canning is necessary for decarbomethoxylation of pheophytins to form pyropheophytins (Khachik et al., 1986; Schwartz & Lorenzo, 1991; Schwartz et al., 1981).

The stability of chlorophyll standards during heating was also studied by several researchers. Pennington et al. (1963) heated purified chlorophyll a and its derivatives in pyridine at 100°C and observed that a series of pyro-derivatives, such as pyrochlorophyll a, methylpyrochlorophyllide a, pyropheophytin a and methylpyropheophorbide a, were present. However, the formation of pyrochlorophylls during heating of vegetables remains questionable.

The quantity of chlorophylls retained during processing of vegetables depends upon temperature and length of heat treatment (Schwartz & Lorenzo, 1991). Canjura et al. (1991) studied the thermal degradation of chlorophylls in spinach puree and found that the degradation of chlorophylls followed a first-order model, and chlorophyll a was degraded at a rate of two to six times higher than chlorophyll b. Chen and Chen (1993) cooked sweet potato leaves in a microwave oven for 8 min and reported that the amounts of most chlorophyll derivatives increased with increasing heating time. Since no information is available as to the formation of pyrochlorophylls during cooking of

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vegetables, the effects of various heat treatments on the chemical changes of chlorophyll derivatives have to be studied. The objectives of this study were to investigate the degradation of chlorophylls and formation of pyrochlorophylls and their derivatives in spinach leaves during baking, blanching, steaming, and microwave cooking.

2. Materials and methods

2.1. Materials

Both chlorophylls, a and b standards, were purchased from Sigma Co. (St. Louis, MO, USA). Pheophytins a and b were prepared by adding a few drops of 0.1 N HCl to chlorophylls a and b, respectively. All the solvents used were HPLC grade and were from Mallinckrodt Inc. (Paris, KY, USA). A total of about 10 kg fresh spinach was obtained from a local supermarket in Taipei. The spinach was washed and dried with paper towels, and the leaves were collected and cut into small pieces of about 2×5 cm^2.

2.2. Instrumentation

The HPLC instrument consisted of a Phenomenex DG-440 degassing system (Torrance, CA, USA), a Jasco PU-980 pump (Tokyo, Japan), a Rheodyne 7161 injector fitted with a 20-μl loop (Cotati, CA, USA), a Showa Denko K.K. stainless-steel C_{18} column (250×4.6 mm i.d.) packed with Ultremex 5-μm particle size (Tokyo, Japan). A Jasco UV-970 UV/VIS detector (Tokyo, Japan) was used to monitor the separation and a Jasco MD-915 photodiode-array detector (Tokyo, Japan) was used to determine the absorption spectra of chlorophylls and their derivatives. Borwin software version 1.2 (Le Fontanil, France) was used for processing data. Positive ion fast atom bombardment (FAB) mass spectra of pyrochlorophylls were determined using a Joel SX102A double-focusing mass spectrometer (Tokyo, Japan) equipped with a MS-MT 7000 data system. The microwave oven (Model ER 761 M) with an output power of 700 W was from Goldstar Co. (Korea).

2.3. Heat treatment

Several cooking methods, including baking, blanching, steaming and microwave cooking were used to heat spinach leaves.

1. *Baking*: Approximately 1000 g spinach leaves were divided into four portions of 250 g each. Each portion was placed on a separate aluminum plate and heated at 105°C for 20, 40, 60, or 80 min in an oven.
2. *Blanching*: Approximately 1250 g spinach leaves were divided into five portions of 250 g each. Each portion was cooked in boiling water for 3, 6, 9, 12, or 15 min.
3. *Steaming*: 1250 g spinach leaves were divided into five portions of 250 g each. Each portion was placed on a paper towel and then steamed over boiling water for 7.5, 15, 30, 45, or 60 min.
4. *Microwave cooking*: Approximately 1250 g spinach leaves were divided into 25 portions of 50 g each. Each portion was placed in a separate heat-resistant plastic bag and cooked in a microwave oven at 2450 MHz for 1, 3, 5, 7, or 9 min with an output power of 700 W.

After heating, spinach leaves were collected at intervals, drained, and chopped into ground pieces. For control treatment, 30 g ground pieces of spinach leaf samples were used. The dry weight of spinach leaves was measured by placing 5 g spinach leave samples in a hot oven at 105°C for 5 h.

2.4. Extraction of chlorophylls and their derivatives

Extraction of chlorophylls and their derivatives was performed under dim light and conducted as soon as spinach leaves were heated. Approximately 0.5 g spinach leaves were placed in a test tube, to which 3 ml methanol were added. The mixture was homogenized with a homogenizer for 1 min and centrifuged at 765 ***g*** (4°C) for 3 min. The methanol extract was collected in another test tube and the residue was vortexed with 3 ml methanol. This procedure was repeated several times until the extract became colorless. All the methanol extracts were pooled and brought to volume in a 10 or 20 ml volumetric flask, and then the solution was filtered through a 0.2 μm membrane filter for HPLC analysis.

2.5. Analysis of chlorophylls and their derivatives by HPLC

A solvent system of acetonitrile/methanol chloroform/n-hexane (75:12.5:7.5:7.5, v/v/v/v) (Chen & Chen, 1993) was used to separate chlorophylls and their derivatives with detection at 430 nm and flow rate at 1 ml min^{-1}. The injection volume was 20 μl. The absorption spectrum of each pigment was determined on line using a photodiode-array detector. In most cases, chlorophylls and their derivatives were identified by comparison of the absorption spectra and retention times of unknown peaks with reference standards, and by cochromatography with added standards. Some pigments, such as pyrochlorophylls a and b, were further identified by positive ion FAB mass spectrometry (FAB–MS). An external calibration method was used to

quantify each pigment. The calibration curves of chlorophyll a, chlorophyll b, pheophytin a and pheophytin b were prepared by plotting five concentrations of each against peak area and high linearity was obtained with correlation coefficient of 0.99. Chlorophylls a, a′ and pyrochlorophyll a were quantified using the calibration curve of chlorophyll a; likewise, chlorophyll b′ and pyrochlorophyll b used that of chlorophyll b; pheophytin a′ and pyropheophytin a used that of pheophytin a, and pyropheophytin b used that of pheophytin b.

2.6. Analysis of pyrochlorophylls by FAB–MS

The eluates of pyrochlorophylls a and b were collected 50 times individually at the outlet of the HPLC system. The solution was evaporated to dryness under a stream of nitrogen and approximately 1 μg of each compound was obtained. Each pigment was dissolved in methanol, and then added to the FAB matrix, 3-nitrobenzyl alcohol for MS analysis. Xenon was served as collision gas. The accelerating voltage was 10 keV, and the resolving power was 1000. Mass spectra of both compounds were determined in duplicate.

2.7. Statistical analysis

With the exception of MS analysis, all the other tests were conducted in five replications. Data were subjected to analysis of variance and Duncan's multiple range test using SAS (1985). The degradations of chlorophylls a and b during heating of spinach leaves were kinetically studied (Chen & Huang, 1998; Chen et al., 1994). The correlation coefficient (r^2) was measured from the plot of the logarithm of the chlorophyll a concentration or chlorophyll b concentration vs time. The degradation rate constant (min^{-1}) was obtained using the following formula:

$$K = -\ln(CA/CA_0)/t$$

where CA: the concentration of chlorophyll a or chlorophyll b after heating, CA_0: the initial concentration of chlorophyll a or chlorophyll b, t: heating time.

3. Results and discussion

3.1. HPLC analysis of chlorophylls and their derivatives

Fig. 1 shows the HPLC chromatogram of chlorophylls and their derivatives by employing a quaternary solvent system of acetonitrile/methanol/chloroform/n-hexane (75:12.5:7.5:7.5, v/v/v/v) (Chen & Chen, 1993). A total of 10 pigments, including chlorophyll b, chlorophyll b′, pyrochlorophyll b, chlorophyll a, chlorophyll a′, pyrochlorophyll a, pyropheophytin b, pheophytin a, pheophytin a′ and pyropheophytin a were detected in microwave-cooked spinach leaves. The retention times of pyrochlorophylls and pyropheophytins were greater than those of chlorophylls and pheophytins, respectively. This is probably because the elimination of the carbomethoxy group reduced the polarity of these pigments, and thus resulted in greater interaction with the C_{18} stationary phase (Suzuki et al., 1987).

Chlorophylls a, a′, and pyrochlorophyll a had the same maximum absorption at 232, 383, 428, 619 and 662 nm, and these pigments differed from each other only in shape of absorption spectra. Likewise, chlorophylls b, b′ and pyrochlorophyll b had maximum absorption at 253, 311, 341, 460 and 646 nm. This similarity in light absorption properties was also observed for both pheophytin a and pyropheophytin a, and both pheophytin b and pyropheophytin b. Since the absorption spectra of chlorophylls and their derivatives can be attributed to the primary porphyrin structure, the C-10 epimerization or decarbomethoxylation should not affect the color change of spinach leaves.

Chlorophylls and their derivatives are routinely detected at wavelengths ranging between 640 and 660 nm (Daood et al., 1989; Khachik et al., 1986; Schwartz et al., 1981; von Elbe et al., 1986). However, in this range of wavelengths chlorophylls and their derivatives have relatively low extinction values and some derivatives, such as pyrochlorophylls, may fail to be detected. The absorption spectra of chlorophylls and their derivatives revealed that the optimal detection wavelengths for chlorophylls and chlorophyll epimers, and pyrochlorophyll b were 428 and 460 nm, respectively; those for pheophytin a and pyropheophytin a, and pyropheophytin b, were 409 and 436 nm, respectively. For the simultaneous detection of these compounds, 430 nm was found to be the most appropriate.

3.2. FAB–MS of pyrochlorophylls

FAB–MS was used for the identification of pyrochlorophylls. Typical fragments (m/z) for pyrochlorophylls a and b were 835.4, 813.5, and 556.1, and 849.4, 827.4, and 570.1, respectively. The molecular peaks [M^+] for pyrochlorophylls a (835.4) and b (849.4) were 57.1 weight units smaller than chlorophylls a and b, respectively. Loss of magnesium ion resulted in fragments (m/z) of 813.5 and 827.4. Cleavage of the phytyl ester at C-7 produced the ion peaks of 556.1 and 570.1. All these observations suggested that peaks 3 and 6 (Fig. 1) are pyrochlorophylls b and a, respectively.

3.3. Degradation of chlorophylls

The average concentrations of chlorophylls a and b in fresh spinach leaves were 14.1 and 6.2 mg g^{-1} on a dry

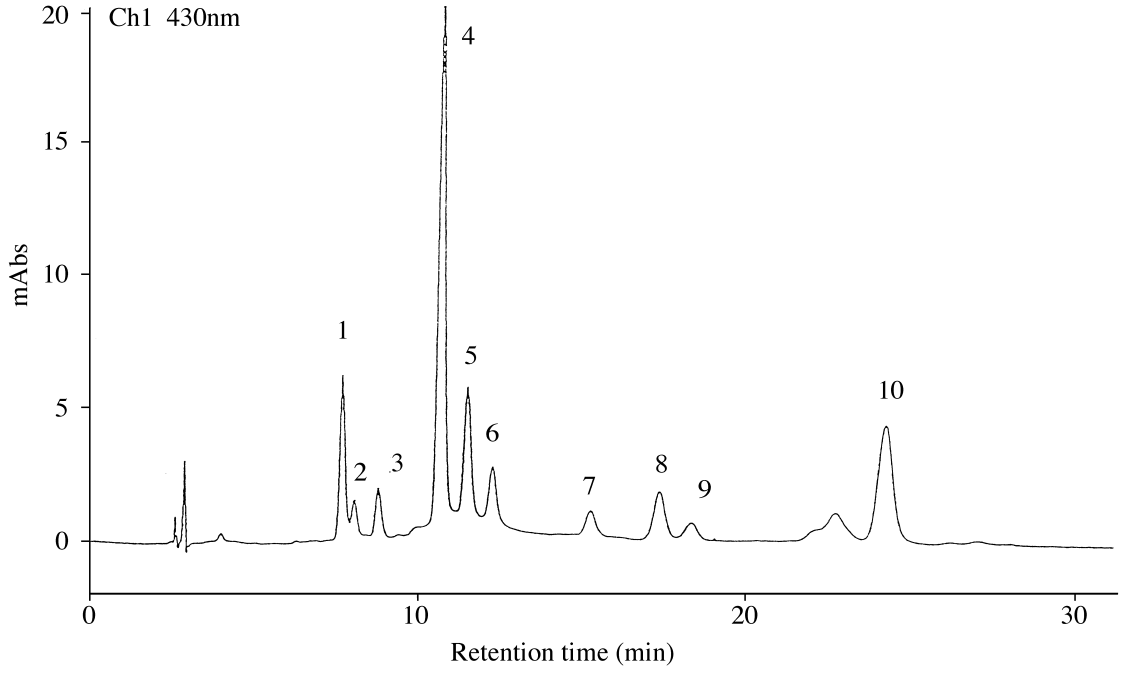

Fig. 1. HPLC chromatogram of chlorophylls and their derivatives in spinach leaves cooked in a microwave oven (700 W) for 8 min. Chromatographic conditions are described in the text. Peaks: 1, chlorophyll b; 2, chlorophyll b′; 3, pyrochlorophyll b; 4, chlorophyll a; 5, chlorophyll a′; 6, pyrochlorophyll a; 7, pyropheophytin b; 8, pheophytin a; 9, pheophytin a′, 10, pyropheophytin a.

weight basis, respectively. The amounts of chlorophylls decreased with increasing heating time. For each treatment the degradations of both chlorophylls a and b fit the first-order model because a linear correlation was observed for the plot of the logarithm of the concentrations of both chlorophylls a and b vs time. Similar results were reported previously (Canjura et al., 1991; Schwartz & Lorenzo, 1991; Schwartz & von Elbe, 1983).

Table 1 shows the rate constants of chlorophyll degradations in spinach leaves during baking, blanching, steaming and microwave cooking. Of the four heat treatments, blanching and microwave cooking resulted in the highest degradation rate of both chlorophylls a and b, followed by steaming and baking. This also showed that the degradation rate constants of chlorophyll a by wet-heating methods (blanching and steaming), were about twice as high as that of chlorophyll b. The same phenomenon was observed by Schwartz & Lorenzo (1991), who studied the chlorophyll stability during continuous aseptic processing of spinach puree. However, only slight difference in the degradation rate constants between chlorophylls a an b was observed by dry heating methods (baking and microwave cooking). These observations implied that by wet heating methods chlorophyll a is more susceptible to degradation than chlorophyll b. It is also possible that chlorophyll a can be leached into water more readily than chlorophyll b during cooking of spinach leaves.

Table 1
Rate constants (min^{-1}) of chlorophyll a (Chl a) and chlorophyll b (Chl b) degradations during heating of spinach leaves

Heat treatment	Rate constant (min^{-1})	
	Chl a	Chl b
Baking	0.012 ± 0.001[a]	0.010 ± 0.002
Blanching	0.112 ± 0.007	0.058 ± 0.009
Steaming	0.050 ± 0.007	0.024 ± 0.001
Microwave cooking	0.092 ± 0.005	0.095 ± 0.007

[a] Values are expressed as regression coefficient ± standard deviation. All the regression coefficients are significant at the 0.05 level.

3.4. Formation of chlorophyll derivatives

Table 2 shows the concentration changes of chlorophylls and their derivatives during heating of spinach leaves. The amounts and varieties of chlorophyll derivatives formed was found to be dependent upon cooking methods and length of heat treatment. Chlorophyll derivatives were not detected in fresh spinach leaves. In general, the amounts of chlorophyll derivatives increased gradually at the beginning and then reached a plateau during heating. However, they decreased again after prolonged cooking. For most cooking methods, chlorophylls a′ and b′ may undergo isomerization and degradation simultaneously as soon as they are formed from chlorophylls during heating. Nevertheless, both chlorophylls a′ and b′ were formed in greatest amount by blanching, followed by microwave cooking, steaming and baking. It was also observed that chlorophyll a′ can be more readily formed than chlorophyll b′ during

Table 2
Concentration (mg/g dry weight) changes of chlorophylls and their derivatives during heating of spinach leaves (mg/g dry weight)[a,b]

Heat treatment	Chlorophyll		Chlorophyll		Pheophytin		Pyrochlorophyll		Pyropheophytin	
	a	b	a′	b′	a	a′	a	b	a	b
Control	14.1 ± 0.53	6.23 ± 0.11	–[c]	–	–	–	–	–	–	
Baking										
20 min	9.70 ± 0.7a	4.30 ± 0.13a	0.78 ± 0.07a	0.50 ± 0.01a	2.18 ± 0.93a	0.28 ± 0.04a	–	–	–	–
40 min	6.25 ± 0.3b	3.33 ± 0.21b	0.92 ± 0.07b	0.58 ± 0.04b	1.33 ± 0.10b	0.10 ± 0.02b	–	–	–	–
60 min	6.07 ± 0.5b	2.68 ± 0.29c	0.75 ± 0.07a	0.46 ± 0.06a	1.06 ± 0.19b	0.10 ± 0.00b	–	–	–	–
80 min	4.67 ± 0.6c	2.71 ± 0.25c	0.60 ± 0.08b	0.60 ± 0.08b	1.56 ± 0.20c	0.16 ± 0.03c	–	–	–	–
Blanching										
3										
6 min	4.93 ± 0.25b	4.74 ± 0.10b	2.72 ± 0.09b	1.38 ± 0.02a	2.11 ± 0.39b	0.08 ± 0.02a	–	–	–	–
9 min	4.29 ± 0.06c	3.98 ± 0.10c	3.83 ± 0.24c	1.88 ± 0.04b	2.58 ± 0.01b	0.27 ± 0.06b	–	–	–	–
12 min	3.83 ± 0.39d	3.55 ± 0.12d	4.41 ± 0.20d	1.93 ± 0.06b	4.43 ± 0.30c	0.51 ± 0.11c	–	–	–	–
15 min	2.82 ± 0.23e	2.45 ± 0.22e	4.53 ± 0.29d	1.66 ± 0.22c	5.51 ± 0.11d	0.69 ± 0.04d	–	–	–	–
Steaming										
7.5 min	2.20 ± 0.16a	2.79 ± 0.08a	0.51 ± 0.03a	0.80 ± 0.09a	4.53 ± 0.16a	0.54 ± 0.02a	–	–	–	–
15 min	1.81 ± 0.06b	2.90 ± 0.15a	0.67 ± 0.05b	0.77 ± 0.06a	7.66 ± 0.49b	1.69 ± 0.04b	0.03 ± 0.00a	–	–	–
30 min	1.73 ± 0.07b	1.78 ± 0.06b	1.53 ± 0.09c	0.51 ± 0.02b	9.91 ± 0.30c	2.41 ± 0.05c	0.26 ± 0.01b	0.05 ± 0.00a	0.72 ± 0.09a	0.15 ± 0.02a
45 min	1.08 ± 0.08c	1.49 ± 0.17c	2.01 ± 0.48d	0.34 ± 0.05c	12.6 ± 4.47d	4.35 ± 0.07d	0.37 ± 0.05c	0.07 ± 0.01b	2.61 ± 0.44b	0.89 ± 0.03b
60 min	0.18 ± 0.04d	0.96 ± 0.06d	0.53 ± 0.03a	0.22 ± 0.02d	9.14 ± 0.01e	2.03 ± 0.03e	0.11 ± 0.01d	0.06 ± 0.01b	1.97 ± 0.07c	0.49 ± 0.01c
Microwave cooking										
1 min	11.9 ± 1.56a	4.87 ± 0.48a	0.95 ± 0.23a	0.85 ± 0.22a	1.81 ± 0.26a	0.24 ± 0.05a	0.05 ± 0.02a	–	–	–
3 min	10.83 ± 0.36a	4.29 ± 0.17a	2.31 ± 0.45b	1.01 ± 0.18a	3.00 ± 0.46b	0.32 ± 0.05b	0.10 ± 0.04b	0.07 ± 0.00a	–	–
5 min	8.38 ± 0.38b	3.47 ± 0.22b	3.16 ± 0.08c	1.20 ± 0.10a	2.70 ± 0.29b	0.70 ± 0.06c	0.38 ± 0.02c	0.32 ± 0.01b	0.25 ± 0.05a	0.03 ± 0.00a
7 min	6.94 ± 1.58c	3.21 ± 0.92b	2.37 ± 0.59b	0.81 ± 0.32a	1.32 ± 0.27c	0.16 ± 0.03d	1.19 ± 0.35d	0.93 ± 0.01c	1.37 ± 0.59b	0.16 ± 0.13b
9 min	5.62 ± 0.32c	2.36 ± 0.15c	2.19 ± 0.25b	0.72 ± 0.00b	1.80 ± 0.10a	0.41 ± 0.10e	0.88 ± 0.25d	1.13 ± 0.40c	1.29 ± 0.31b	0.30 ± 0.20c

[a] For each treatment values with different letters in the same column are significantly different ($p < 0.05$).
[b] Values are expressed in mean ± standard deviation.
[c] – = not detectable.

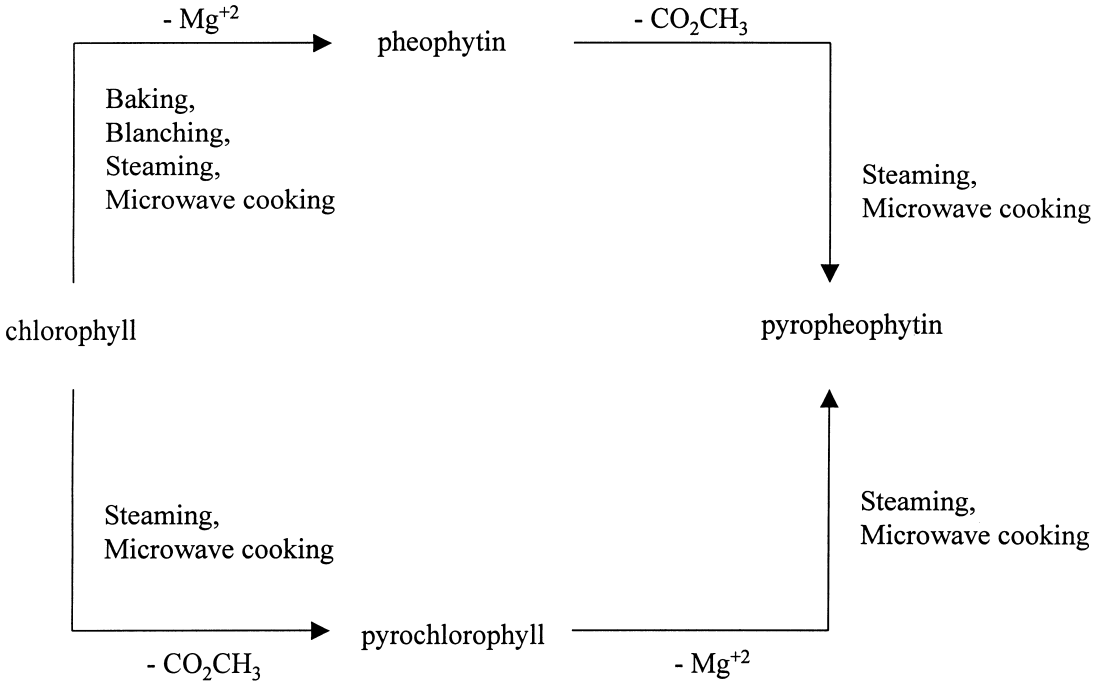

Fig. 2. Formation pathway of pyrochlorophylls and their derivatives in spinach leaves during heating.

cooking. Pheophytins a and a′ were formed in highest amount by steaming, followed by blanching, microwave cooking and baking. In addition, pheophytin a was formed in a higher amount than pheophytin a′. Less pheophytins were also found in spinach leaves by dry heating methods (baking and microwave cooking) than that by wet heating methods (blanching and steaming). This is probably because, with moist heat, the liberation of organic acids from the matrix of spinach leaves can facilitate formation of pheophytins; however, with dry heat, the matrix was dehydrated and less organic acids were thus available for formation of pheophytins. Furthermore, both pyrochlorophylls and pyropheophytins can only be detected in steamed or microwave-cooked spinach leaves, probably because of less liberation of organic acids. In addition, the greater heat penetration by steaming or microwave cooking may facilitate the removal of the carbomethoxyl group from chlorophylls. Neither pyrochlorophyll, a or b, were detected until steaming time reached 30 min or microwave cooking time reached 1 min, respectively. Pyropheophytins a and b could be detected only after steaming or microwave cooking time reached 30 and 5 min, respectively. These observations indicated that chlorophylls could be converted to pyropheophytins only after they were changed to pyrochlorophylls or pheophytins. Similarly, pyrochlorophyll a was formed in a higher amount than pyrochlorophyll b while pyropheophytin a was higher than pyropheophytin b.

Fig. 2 illustrates the formation pathway of pyrochlorophylls and other chlorophyll derivatives during cooking of spinach leaves. As discussed in the previous section, pheophytins can be formed through dry-heating and moist-heating. Pyrochlorophylls were observed only by steaming and microwave cooking. Pyropheophytins could be formed from pheophytins through decarbomethoxylation or from pyrochlorophylls through elimination of magnesium ion during steaming or microwave cooking. Thus, the dominant reaction was dependent mainly upon the heating methods. Also it may be affected by the concentrations of both pheophytins and pyrochlorophylls, and the activation energy of both reactions. The major chlorophyll derivatives during blanching and steaming were pheophytins a and b, which were further converted to pyropheophytins in the presence of a low concentration of pyrochlorophylls. In a similar study Schwartz and von Elbe (1983) heated spinach puree in a retort and found that pyropheophytins a and b were the predominant chlorophyll derivatives. From the above discussion it can be concluded that microwave cooking favors the formation of pyrochlorophylls while steaming favors that of pyropheophytins. For baking and blanching, both chlorophyll epimers and pheophytins are the dominant chlorophyll derivatives. Further research is necessary to study the mechanism of formation of pyro-derivatives of chlorophylls during heating of vegetables.

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