Agric. Biol. Chem., **48** (4), 985~989, 1984

The Photoxidative Alteration of Chlorophylls in Methyl Linoleate and Prooxidant Activity of Their Decomposition Products

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Received September 28, 1983

Methyl linoleate containing chlorophylls and/or pheophytins was exposed to light in the presence of oxygen. The photooxidative reaction of both chlorophylls *a* and *b* was first-order, and the reaction rate for chlorophyll *a* was higher than that for chlorophyll *b*. On the other hand, pheophytins *a* and *b* hardly decomposed even after irradiation for 24 hr, and retained a green or a brownish-green color. In qualitative analysis of the photooxidation products of chlorophylls *a* and *b*, no pheophytins or pheophorbides were detected, while green and polar red pigments were observed on a thin layer chromatogram near the spot of chlorophyll and the origin, respectively. These photooxidation compounds also had prooxidant effects as well as did chlorophyll.

Chlorophyll (Chl) remaining in plant oils is known to promote the oxidation of oils as a photosensitizer.[1,2] Previously we observed that even refined edible oils contained more pheophytins (Phys) than chlorophylls (Chls) [3] and that pheophytin (Phy) had photooxidative activities as well as did Chl.[4] This observation suggested that the Chl oxidation products which are expected to exist in plant oils might have prooxidant effects or photooxidative activities. Therefore, we investigated the decomposition pathway of Chl in oils to estimate the oxidative stabilities of oils.

There have been many papers published[5~8] regarding the decomposition rates and products of Chl in various solvent systems. Jen & Mackinney[9] showed that no Phy was detected in the reaction products of irradiated Chl solutions in the absence of water. Hynninen *et al.*[10,11] found that allomerization compounds and traces of Phy were formed during the irradiation of Chl-pyridine solutions.

In several laboratories, the relationship between Chl decomposition and oil deterioration has also been studied. In one of these studies Chl was oxidized by lipoxygenase in the presence of highly unconjugated fatty acids.[12] When bleached by lipoxygenase, Chl is expected to change to colorless low molecular weight compounds without the formation of detectable Phy and pheophorbide (Pho) within short period.[13] In addition, some investigators[14~16] reported that Chl may react with intermeditate compounds of lipid peroxidation and peroxides to be bleached. Thus the mechanism of photodegradation of Chl in oils has not been completely elucidated. In this experiment, the photodegradation rates of Chl and Phy, and spectroscopic and chromatographic characteristics of Chl photodecomposition products in methyl linoleate (ML) were investigated in order to clarify the mechanism of Chl photooxidation.

MATERIALS AND METHODS

Materials and equipment. Preparation of Chl, Phy, and ML were described in a previous paper.[4] For the analysis, peroxide-free diethyl ether and acetone of spectrometric analytical grade were used. Other solvents, of reagent grade purity, were used after distillation.

The fluorometric analysis of Chl and Phy was performed with a Hitachi Spectrophotofluorometer Model 204, according to the procedure developed in our laboratory.[3]

Photooxidation of chlorophylls and pheophytins. ML (1.00 g) containing 2.2×10^{-7} mol of Chl or Phy was put in a glass beaker (27 mmϕ), and irradiated with transmitted light through the water layer from a 15 W fluorescent lamp at 0°C with occasionally stirring. Light intensity on the surface of the sample was 500 μW/cm^2 sec. The degradation rates for Chl and Phy in ML were determined by measuring decreased optical density at specific wavelengths (Chl *a*, 662; Chl *b*, 646; Phy *a*, 665; Phy *b*, 653 nm).

Thin layer chromatography (TLC). A commercial cellulose layer (Funakoshi Chemicals Co., Ltd.; 5 × 20 cm) was used as the plate, which was developed in *n*-heptane–pyridine (7 : 3, v/v) under nitrogen gas, in the dark at room temperature. Identification of Chl derivatives was made by their *Rf* values, color, and the red fluorescence under the UV light.[17] For the measurement of visible absorption spectra,[18,19] Chl derivatives were eluted with diethyl ether from the TLC plate.

Prooxidant activities of chlorophyll derivatives. Diethyl ether solutions of Chl derivatives pooled from TLC plates were added to ML, and irradiated with a fluorescent lamp at 0°C, as mentioned above. Peroxide values of samples were determined after photooxidation for 12 and 24 hr.

RESULTS AND DISCUSSION

The photodegradation rates of chlorophylls and pheophytins

The photodegradation rates of Chl and Phy in ML were determined to estimate their stabilities against exposure to light. As shown in Fig. 1, Phys *a* and *b* in ML were stable and did not decompose at all during irradiation for 24 hr. On the other hand, Chls *a* and *b* in ML were very unstable and decomposed readily. The degradative reaction of Chls *a* and *b* in ML were found to be first-order and their rate constants were -8.5×10^{-2} and -6.9×10^{-2} (h^{-1}) respectively, which indicated a high stability of Chl *b* compared to Chl *a*. We have shown that greater amounts of Phys than Chls were contained in refined edible plant oils by fluorospectrometric analysis.[3] These observations agree closely with the data obtained in this experiment. Thus the decomposition products of Chls are expected to exist in commercial plant oils in addition to Chls and Phys.

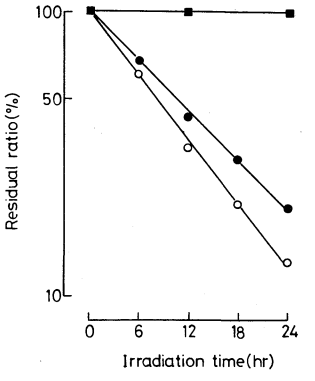

FIG. 1. The Degradation of Chlorophylls and Pheophytins in Methyl Linoleate during Photooxidation.

Chlorophyll or pheophytin (2.2×10^{-7} mol) was added to methyl linoleate (1.0 g) and irradiated with a fluorescent lamp (500 μW/cm^{2}sec) at 0°C. Residual ratios were estimated by measuring decreased optical density at the absorption maxima. ○, Chl *a*; ●, Chl *b*; ■, Phy *a* and *b*.

Changes in visible absorption and fluorescence spectrum

Changes in the visible absorption spectrum of Chl during photooxidation in ML are illustrated in Fig. 2. The optical density at the absorption maxima of Chls *a* and *b* decreased, while absorption in the blue region of Chl increased with irradiation time. However, no formation of an absorption maximum for Phy

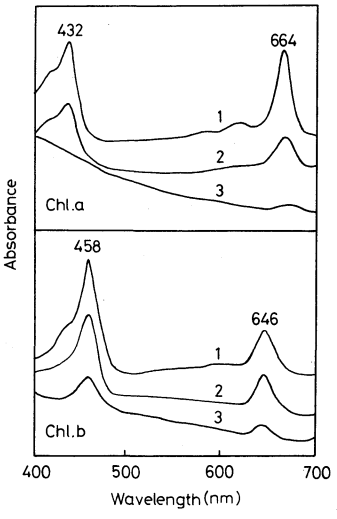

FIG. 2. Changes in Spectra of Chlorophylls *a* and *b* in Methyl Linoleate during Photooxidation.

The change in visible absorption spectra of chlorophylls *a* and *b* in methyl linoleate was measured after irradiation for (1) 0 hr, (2) 12 hr, (3) 24 hr at 0°C.

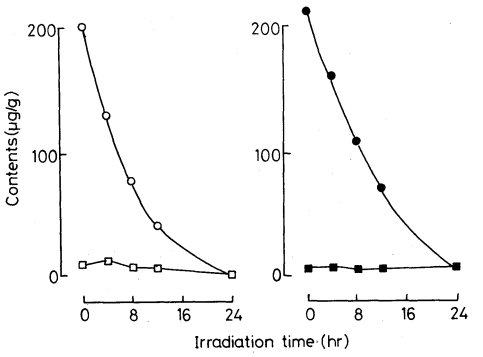

FIG. 3. Compositional Variation of Chlorophylls and Pheophytins in Methyl Linoleate during Photooxidation.

After chlorophylls *a* or *b* (2.2×10^{-7} mol) was added to methyl linoleate (1.0 g) and photooxidized at 0°C, contents of chlorophyll and pheophytin were estimated by the fluorometric method. ○, Chl *a*; ●, Chl *b*; □, Phy *a*; ■, Phy *b*.

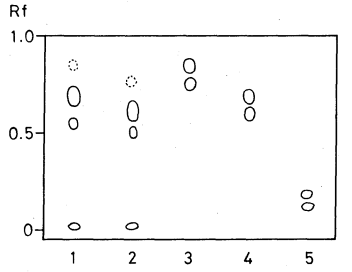

FIG. 4. Thin Layer Chromatogram of Photooxidation Products of Chlorophylls *a* and *b*.

Plate, cellulose; solvent, *n*-heptane–pyridine (7 : 3); (1) Chl *a* photooxidized for 12 hr; (2) Chl *b* photooxidized for 12 hr; (3) Phy *a* and *b*; (4) Chl *a* and *b*; (5) Pho *a* and *b*.

or Pho was observed during photooxidation. The ratios of absorbance at the Soret band (A_S) to that at the Red band (A_I) of Chls *a* and *b* were estimated during photooxidation.[19,20] The ratio, A_S/A_I, in Chl *a* after irradiation for 0, 12, and 24 hr was 1.32, 1.66, and 3.72, respectively. While the ratios in Chl *b* were 2.10, 2.33, and 2.67, respectively. These results suggested that chelated magnesium might be removed from Chl during photooxidation. Next, variation in Phy content in ML was monitored by measurement of its fluorescence intensity during photooxidation of Chls. As shown in Fig. 3, Phys *a* and *b* were almost undetectable in contrast with the considerable decrease of Chl.

Thin layer chromatogram after photooxidation

Thin layer chromatograms of Chls *a* and *b* after photooxidation for 12 hr are shown in Fig. 4. Four spots on the plate were observed in photooxidized Chl *a*. The spot at *Rf* 0.68 was in accord with the *Rf* value of Chl *a* in the literature[17] and other spots seemed to be photooxidation products of Chl *a*. Three spots with red fluorescence at *Rf* 0.84, 0.53, and 0 were grey-green, green, and red, respectively. The smallest spot at *Rf* 0.84 was identified as Phy *a* due to its *Rf* value and visible absorption spectrum. Visible absorption spectra of the other green spot (*Rf* 0.53) and the red spot (*Rf* 0) were shown in Table I. The green pigment exhibited absorption maxima at 415 and 662 nm in acetone, and its ratio of A_S/A_I was 2.29. This suggested that no chelated magnesium exist in the molecule. On the other hand, the red pigment exhibited absorption maxima at 410 and 662 nm in acetone and the ratio of A_S/A_I was 5.47. Probably the red pigment also does not seem to possess a chelated magnesium. As photooxidation proceeded, the size of the red spot gradually increased, while the green spot disappeared.

Three spots were observed as the photooxidation products of Chl *b* (*Rf* 0.62) as in the case of the photooxidized Chl *a* (Fig. 4). Three spots with red fluorescence at *Rf* 0.76, 0.50, and 0 were green, yellow-green, and red, respectively. The spot at *Rf* 0.76 was identified as Phy *b* by its *Rf* value and visible absorption spectrum. Other two spots were pooled from the plate and their visible absorption spectra

TABLE I. SPECTRAL PROPERTIES OF PHOTOOXIDATION PRODUCTS OF CHLOROPHYLLS IN ACETONE

Compound	λ_{max} nm (Soret)	λ_{max} nm (Red)	A_S/A_I (Soret/Red)
Photooxidized Chl *a*			
Rf 0.53	415	662	2.29
Rf 0	410	662	5.47
Photooxidized Chl *b*			
Rf 0.50	450	643	2.17
Rf 0	455	643	3.47

were monitored (Table I). The yellow-green pigment (*Rf* 0.50) exhibited absorption maxima at 450 and 643 nm in acetone and a A_S/A_1 ratio of 2.17, suggesting the presence of chelated magnesium. The polar red pigment (*Rf* 0) exhibited absorption maxima at 455 and 643 nm and the A_S/A_1 ratio was 3.47. This red pigment increased with irradiation time as in the case of Chl *a*.

No Phy was detected by fluorometric estimation in spite of its detection by TLC, so we assumed that the Chl underwent oxidation other than photooxidation in the oil system, resulting in transformation to Phy during TLC or column chromatography. We actually found a greater amount of Phy on the plate when the TLC of Chl was carried out in a chamber full of air. Accordingly the Phy observed on TLC under N_2 is not expected to be a photooxidation product of Chl in ML.

The spots of Pho and chlorophyllide were not detected in photooxidized Chl. This might be caused by the absence of chlorophyllase that catalyzes the transformation of Chl to chlorophyllide and the no acidic circumstance that transforms Chl to Phy and Pho.

Prooxidant activity of photooxidation products

Peroxide values of ML to which the photooxidation products of Chl were added were determined after photooxidation for 12 and 24 hr. As shown in Table II, photooxidation products of Chls also exhibited prooxidant effects.

Chl and Phy were photooxidized in a ML system as a model compound of oils. The photooxidative reaction of Chl obeyed first-order kinetics and the reaction rate of Chl *a* was higher than that of Chl *b*. On the other hand, Phy was insensitive to oxygen and was hardly decomposed at all during irradiation. Huff[21] showed that Chl *a* in potassium phosphate buffer was more easily decomposed than Chl *b*, and that Phy hardly reacted with oxygen when Chl and Phy were bleached in the presence of peroxides.

Ishitani and Kimura[8] reported that relative stabilities of Chls *a* and *b* and Phys *a* and *b*

TABLE II. EFFECTS OF PHOTOOXIDIZED CHLOROPHYLLS ON PHOTOOXIDATION OF METHYL LINOLEATE

The pigment (OD 1.0 at 662 or 643 nm) was added to methyl linoleate (1.0 g) and photooxidized under the conditions described in METHODS.

Sample	POV (meq/kg)	
	12 hr	24 hr
Control	42	123
Photooxidized Chl *a*		
Rf 0.53	198	472
Rf 0	201	467
Photooxidized Chl *b*		
Rf 0.50	85	222
Rf 0	80	227

depend on the polarity of the solvent. We also observed a high stability of Chl *a* in comparison with that of Chl *b* during photooxidation of triglycerides[22] in contrast to the result in ML. Moreover, in our previous paper[23] a relatively constant compositional ratio of the four pigments was observed in refined plant edible oils. Thus, from the practical view point it is of little significance to consider the *a*- and *b*-types of Chl or Phy separately. The concentration and photooxidative behavior of Chl and Phy in ML were discussed in our other paper.[4]

The qualitative analysis of photooxidation products of Chl indicated that neither Phy nor Pho was formed during the photooxidation of Chl, agreeing with the result reported by Jen *et al.*[9] However, our other paper[23] revealed that most plant edible oils contain higher amounts of Phy than Chl and that Phy is not transformed from Chl during oil-refining. This Phy may be transferred from plant materials in which Phy has been produced from Chl enzymatically or nonenzymatically.

Of the two spots observed on TLC, the green pigment seemed to be one of the allomerization compounds of Chl indicated by Hynninen *et al.*[10,11,19] and Strain.[24] But the visible absorption spectrum of this pigment did not coincide with the reported ones. The characteristics of another polar red pigment

have been studied by Mackinney and co-workers,[25,26] but its detailed structure has not been clarified. We found that these photooxidation products of Chl could promote the oxidation of oils. It is possible that these photooxidation and decomposition products may exist in some plant oils. Thus the structure and contents of Chl decomposition products in oils should be further investigated to determine the quality of the oils.

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