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Antioxidant Effects of Chlorophyll and Pheophytin on the Autoxidation of Oils in the Dark. II. The Mechanism of Antioxidative Action of Chlorophyll

YASUSHI ENDO*, RIICHIRO USUKI[1] and TAKASHI KANEDA[2], Department of Food Chemistry, Faculty of Agriculture, Tohoku University, Sendai, Japan 980

ABSTRACT

To understand the mechanism of the antioxidant effect of chlorophyll on the autoxidation of oils in the dark, antioxidant activities of several derivatives of chlorophyll were compared. Antioxidant activities were observed in chlorophyll derivatives such as protoporphyrin methyl ester and its magnesium chelated compound. Porphyrin seems to be an essential chemical structure for the antioxidant activity of chlorophyll. Chlorophyll did not decompose the hydroperoxides, but reduced free radicals such as 1,1-diphenyl-2-picrylhydrazyl. Electron spin resonance spectrum of the π-cation radical was recorded during the oxidation of chlorophyll in methyl linoleate solution. These observations suggest that chlorophyll may act as a hydrogen donor to break the chain reaction.

INTRODUCTION

In a previous paper (1), we reported the antioxidant activities of chlorophyll (CHL) and pheophytin (PHY) on the autoxidation of methyl linoleate (ML) in the dark. The antioxidant effect of CHL also was observed in triglycerides such as rapeseed and soybean oils. In this paper, to understand antioxidant effects in CHL and PHY on the autoxidation of oils in the dark, the following studies were carried out:

(i) The relationship between structure and antioxidant effects of CHL derivatives;

(ii) The reaction of CHL and PHY with hydroperoxides and 1,1-diphenyl-2-picrylhydrazyl (DPPH), and

(iii) The electron spin resonance (ESR) spectrum of CHL in ML during autoxidation

Based on the results obtained, we discuss the possible mechanism of the antioxidant effect of CHL on the autoxidation of oils in the dark.

MATERIALS AND METHODS

Materials

The preparation of CHL, PHY and ML has been described previously (2).

Protoporphyrin methyl ester (PRO) was obtained by esterification of sodium protoporphyrin purchased from Nakarai Chemical Ltd. Magnesium chelated porphyrin methyl ester (Mg-PRO) was prepared after inducing magnesium (Mg) in PRO by the degradative Grignard reaction (3).

Pyrrole and magnesium chloride ($MgCl_2$) were purchased from Tokyo Kasei Industries Ltd. and Wako Pure Chemical Industries Ltd. (Tokyo, Japan), respectively.

Methyl linoleate hydroperoxides (MLHPO) were prepared from autoxidized ML by silicic acid column chromatography with a series of n-hexane-diethyl ether mixtures as solvent system.

Autoxidation

Some derivatives and structural constituents of CHL were diluted with n-hexane-diethyl ether solutions, except $MgCl_2$, which was diluted with methanol, and added to one gram of ML in a small beaker (ϕ 27 mm). These samples were incubated at 30 C in the dark. In addition to CHL A, PHY A, PRO and Mg-PRO as typical CHL derivatives, $MgCl_2$ as Mg ion and pyrrole as one structural component of porphyrin were used, respectively, for oven tests. Addition levels of derivatives and structural constituents of CHL were 2.2×10^{-7} mol/g ML, except for pyrrole, which was of 8.8×10^{-8} mol/g ML. Peroxide value (PV) and carbonyl value (CV) of each sample were determined after autoxidation.

Degradation Test of Methyl Linoleate Hydroperoxides

1% (w/w) CHL A and PHY A were added to 10% (w/w) MHLPO in ML solution in a small beaker and then incubated at 30 C in the dark.

Reduction Test of 1,1-diphenyl-2-picrylhydrazyl

CHL A and PHY A (4.4×10^{-5} and 1.1×10^{-4} M) in carbon tetrachloride (CCl_4) solutions were added to 4.0×10^{-4} M DPPH in CCl_4 solution, and absorbance at 516 nm of DPPH of these mixtures was monitored during incubation at room temperature (4).

ESR Measurement

200 μl of 10^{-5} mol CHL A in benzene solution and 10^{-5} mol iron chloride ($FeCl_3$) as an oxidative initiator were mixed in 300 μl of ML. After vigorously shaking this mixed solution, its ESR spectrum was measured at room tempera-

*To whom correspondence should be addressed at Department of Food Chemistry, Faculty of Agriculture, Tohoku University, 1-1 Amamiyamachi-Tsutsumidori, Sendai, Japan 980
[1] Present address Shokei Women's Junior College, Sendai, Japan.
[2] Present address Koriyama Women's College, Koriyama, Japan.

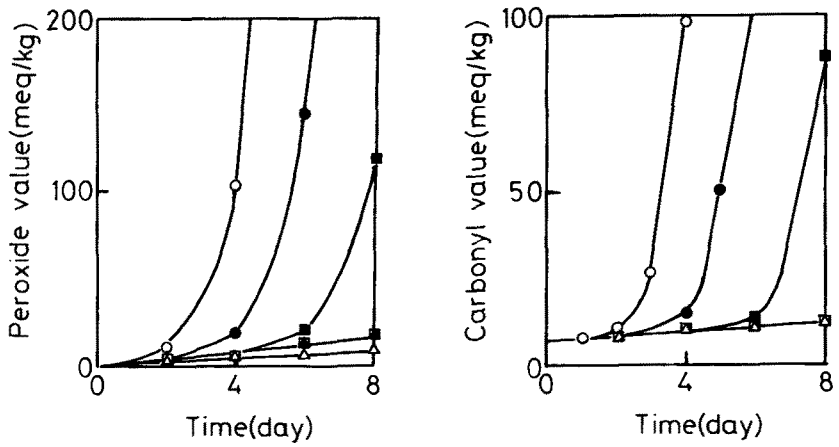

FIG. 1. Effects of derivatives and structural constituents of chlorophyll on the autoxidation of methyl linoleate. Some derivatives and structural constituents of CHL were added to ML and were incubated at 30 C in the dark. Addition levels were 2.2×10^{-7} mol/g ML except for pyrrole, which was 8.8×10^{-7} mol/g ML. ○, control; ●, pyrrole; △, CHL A; ▲, PHY A; □, Mg-PRO, and ■, PRO.

ture with a Varian E-4 spectrometer with 100 KH_z magnetic field modulation (5).

RESULTS AND DISCUSSION

In order to understand the mechanism of antioxidative action of CHL, in particular the essential chemical structure for the appearance of antioxidant activity of CHL, we examined whether the derivatives and structural constituents of CHL possess antioxidant activities. Effects of derivatives and structural constituents of CHL on the autoxidation of ML were compared with those of CHL, as shown in Figure 1. CHL derivatives such as PHY, PRO and Mg-PRO also retarded the formation of peroxides and carbonyl compounds during autoxidation of ML as CHL showed the effect at concentrations of 2.2×10^{-7} mol/g ML. PHY, PRO and Mg-PRO showed antioxidant activity as CHL did, but Mg-chelated compounds such as CHL and Mg-PRO showed stronger antioxidant activities than PHY and PRO did as far as the inhibition of peroxide formation is concerned. For the data of CV, antioxidant activity of PHY was the same as that of CHL at 2.2×10^{-7} mol/g ML. However, we had observed earlier that antioxidant activity of CHL was higher than that of PHY on the autoxidation of ML and vegetable oils (1). At the concentration of 2.2×10^{-8} mol/g ML, it was observed that PHY and PRO showed very low antioxidant activity on the autoxidation of ML in comparison with CHL and Mg-PRO, in the determination of both PV and CV changes. On the other hand, pyrrole, a constituent of the porphyrin structure, did not show as strong an antioxidant effect as CHL did. A similar observation also was made at the concentration of 2.2×10^{-8} mol/g ML. These results suggest that the porphyrin structure may be necessary for the antioxidative action of CHL, and the presence of Mg may activate the antioxidant activity of CHL.

To make clear whether Mg ion or chelated Mg might be responsible for the strong antioxidant activity of CHL, the effects of $MgCl_2$ were examined next. The concentrations of $MgCl_2$ were 2.2×10^{-7} and 2.2×10^{-8} mol/g ML. Figure 2 shows the changes in PV and CV of ML with $MgCl_2$ were the same as those in the control. At the concentration of 2.2×10^{-8} mol/g ML, $MgCl_2$ did not exhibit the remarkable inhibitory effect against the formation of peroxides and carbonyl compounds during autoxidation. Moreover,

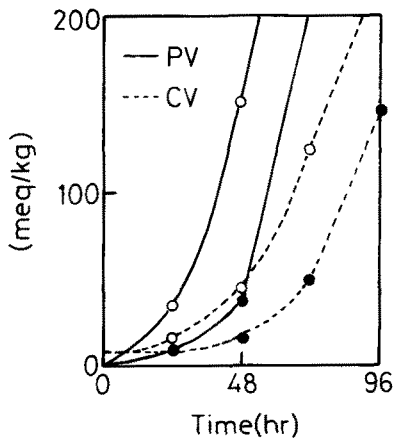

FIG. 2. Effects of magnesium chloride on the autoxidation of methyl linoleate. ML with $MgCl_2$ was oxidized at 30 C in the dark. Addition levels of $MgCl_2$ were 2.2×10^{-7} and 2.2×10^{-8} mol/g ML, respectively. ○, control and $MgCl_2$ (2.2×10^{-7} mol/g ML). ●, $MgCl_2$ (2.2×10^{-8} mol/g ML).

$MgCl_2$ did not strengthen the antioxidant activity of PHY when ML with 2.2×10^{-8} mol PHY A and 2.2×10^{-8} mol $MgCl_2$ was oxidized in the dark. From these results, the requirement for Mg to give a strong antioxidant activity with CHL seems to be not the ionic form but rather a chelated form.

Generally, the antioxidant effect has been explained by the two following mechanisms. One mechanism is ascribed to scavenging free radicals such as peroxy and alkyl radicals to break the chain reaction (6-10). Another one is ascribed to decomposition of hydroperoxides (11-14).

To study the decomposition rate of hydroperoxides by CHL and PHY, 10% MHLPO and 1% CHL A or PHY A were added to ML before incubation, and changes in PV and CV were evaluated at intervals during incubation at 30 C in the dark (Fig. 3). CHL and PHY did not decrease the PV or advance the CV. This result was in accord with that obtained using BHT as an antioxidant. We conclude the antioxidant effect of CHL may not be ascribed to the decomposition of hydroperoxides, because neither CHL nor PHY could decompose the MLHPO.

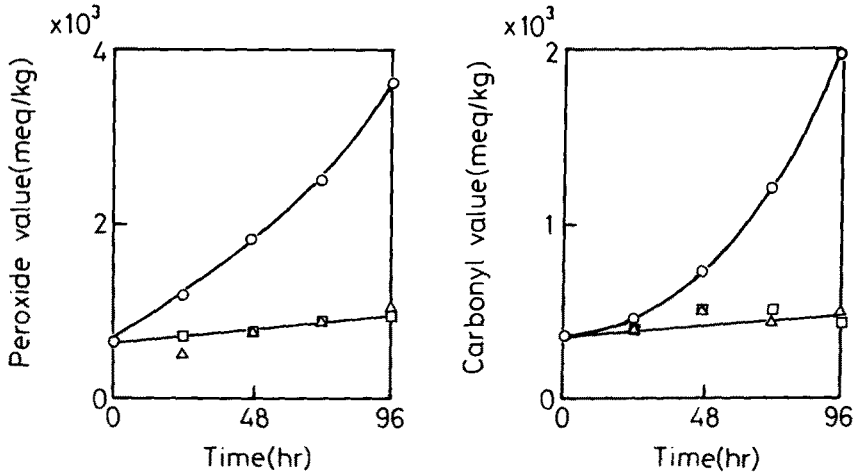

FIG. 3. Effects of chlorophyll and pheophytin on the autoxidation of methyl linoleate with methyl linoleate hydroperoxides. After MLHPO was diluted with pure ML in a concentration of 10% (w/w) and CHL A or PHY A was added at the level of 1% (w/w), the mixture was incubated at 30 C in the dark. ○, control; △, CHL A, and □, PHY A.

Next, the reduction of DPPH by CHL and PHY was examined. It is well known that the reduction of DPPH, which possesses a stable free radical, can be indicative of the presence of a hydrogen donor in the reaction system (15,16). Bolland suggested that a hydrogen donor could break the chain reaction involving a free radical (6). Then we tried to confirm the possibility that CHL acts as a chain breaking antioxidant. Figure 4 shows the timecourse of residual DPPH in the reaction of DPPH with CHL A and PHY A. Residual amounts of DPPH are represented as a relative ratio (%) of absorbance at 516 nm between sample and control. As shown in Figure 4, DPPH decreased after CHL A was mixed with a DPPH CCl_4 solution. At higher CHL addition levels, DPPH disappeared rapidly. On the other hand, a slight decrease was observed in the DPPH after addition of PHY A to the DPPH CCl_4 solution, instead of CHL A. This observation means that CHL provides the hydrogen donor to reduce free radicals such as DPPH, supposing the possibility that CHL may scavenge lipid radicals produced during the oxidation of oils to break the chain reaction.

On the other hand, CHL showed antioxidant activity for ML with a low hydroperoxide content, but not for ML with a high hydroperoxide content. This result means that CHL shows antioxidant activity only in the initial stage of autoxidation before hydroperoxides are formed. We suppose that CHL may react with free radicals such as lipid radicals produced in the initial stage of autoxidation of oils, to break the chain reaction.

To learn what CHL site reacts with the free radicals such as lipid radicals, ESR spectrum was obtained for CHL A in ML during the oxidation at room temperature, using iron chloride ($FeCl_3$) as an initiator. As shown in Figure 5, an ESR singlet of gaussian shape and 11 G linewidth was observed at 2.00 G for CHL A in ML during oxidation. This ESR spectrum was identified as a π-cation radical of CHL A by comparison with references (17-20). This singlet signal also was observed in the uncatalyzed reaction, so π-cation radical of CHL A may be formed in the ordinary autoxidation of oils. These π-cation radicals also have been observed in porphyrin compounds such as tetraphenylporphyrin perchlorate (17) and bacteriochlorophyll and bacteriopheophytin (21) after electrolysis or photolysis, and it is easy to generate the π-cation radical from porphyrin compounds.

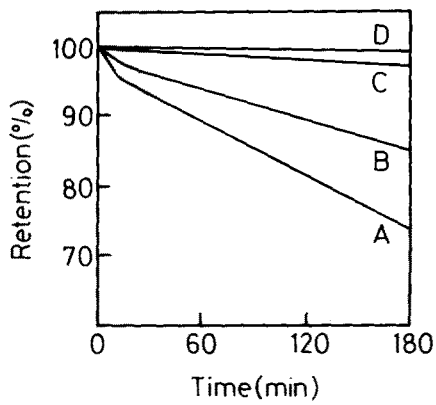

FIG. 4. Reduction of 1,1-diphenyl-2-picrylhydrazyl by chlorophyll and pheophytin. After 4.4×10^{-5} M (or 1.1×10^{-4} M) CHL A and PHY A were added to 4.0×10^{-4} M DPPH CCl_4 solution, absorbance of DPPH in each sample was monitored at room temperature. A, 1.1×10^{-4} M CHL A; B, 4.4×10^{-5} M CHL A; C, 1.1×10^{-4} M PHY A, and D, 4.4×10^{-5} M PHY A.

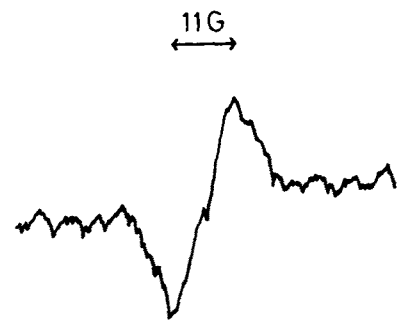

FIG. 5. Electron spin resonance spectrum of chlorophyll a in methyl linoleate during oxidation by iron chloride ($FeCl_3$). ESR spectrum was measured at room temperature in the reaction mixture of 300 μl ML and 200 μl benzene solution containing 10^{-5} mol CHL A and 10^{-5} mol $FeCl_3$.

Moreover, an essential structure for antioxidant activity of CHL derivatives was found to be the porphyrin but not pyrrole, phytol, metal and isocyclic ring, from our results mentioned above and according to some references (22,23). These assumed that π-cation radicals of porphyrin compounds involving CHL have something to do with their antioxidant activity. On the other hand, general antioxidants possess the hydrogen donor which transfers hydrogen atoms to free radicals and interrupts the chain reaction involving radicals, but no possible hydrogen donor is found in the chemical structure of porphyrin. Thus, we suppose the π-cation radical of porphyrin compounds involving CHL, in particular, may play the role of electron donor scavenging free radicals. In fact, the signal intensity of the π-cation radical of CHL A was decreased after addition of a high level of oxidized ML, indicating the π-cation radical may react with oxidation products of ML (probably free radicals).

Antioxidant activities of CHL and PHY were found in oven tests with ML as substrate as described in a previous paper (1). Therefore, we tried some experiments to make clear the mechanism of the antioxidative action of CHL. We obtained interesting observations, as follows: (i) The principal antioxidant effect of CHL was ascribed to the porphyrin structure. (ii) Mg could strengthen the antioxidant activity of porphyrin compounds only in the chelated form. (iii) CHL reduced free radicals such as DPPH. (iv) The π-cation radical was produced in CHL when CHL was oxidized in the ML system. From these results, we propose the following possible mechanism for the antioxidant effect of CHL on the autoxidation of oils:

$$ROO^{\cdot} + CHL \longrightarrow ROO:^{(-)}CHL^{(+)}$$

$$ROO:^{(-)}CHL^{(+)} + ROO^{\cdot} \longrightarrow \text{Inactive products}$$

($ROO^{\cdot}$; peroxy radical)

We suppose the mechanism of the antioxidative action of CHL can be explained by the "molecular complex" proposed by Boozer et al. (7,8), because we found the reduction of DPPH with CHL A obeyed the third order reaction [$k = 1 \times 10^6$ (l^2/mol^2 min) at ca. 20 C] such as that with primary arylamines (24). This observation resembles the result reported by Boozer et al., that the initial oxidation rates for cumene and tetralin catalyzed by azodi-iso-butyronitrile with N-methylaniline as an inhibitor were inversely proportional to the square root of the inhibitor concentrations.

Antioxidative action of CHL may be carried out as a following process; CHL reacts with the peroxy radical produced in the initial stage of oxidation of oils to transform to a π-cation radical. The cation radical of CHL connects with the (-) negative charged peroxy radical loosely to form the charge transfer complex. Furthermore, the charge transfer complex reacts with another peroxy radical leading to inactive products. Chain reaction involving free radicals stops with this reaction.

On the other hand, the mechanism for antioxidative action of PHY is supposed to be similar to that of CHL. However, it was hard to demonstrate because the ESR signal of PHY was very weak and was not identified as a π-cation radical.

Matsushita et al. showed that hematic compounds are prooxidants, but that the porphyrin structure may provide antioxidant activity (22). Sato et al. also reported that copper chlorin, which consists of the porphyrin structure, is responsible for the antioxidant activity of sodium copper chlorophyll (23). These reports are in accord with our results. However, the mechanism of the antioxidant effect of CHL derivatives has not been established yet. We believe that our data is applicable in discussing the mechanism of the antioxidant effect of other CHL derivatives.

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