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Antioxidant Activity of a Novel Phosphatidyl Derivative of Vitamin E in Lard and Its Model System

Takuro Koga*,† and Junji Terao‡

Noda Institute for Scientific Research, 399 Noda, Noda-shi, Chiba 278, Japan, and National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki 305, Japan

The antioxidant activity of a novel phosphatidyl derivative of vitamin E [1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′,8′-tetramethyl-6′-hydroxychroman; PCh] was investigated. The induction period with PCh was longer than that with the mixture of vitamin E and phosphatidylcholine (PC) when lard was allowed to autoxidize at 60 °C in the dark. There was little difference in the antioxidant activities between vitamin E and PCh in an azo compound-induced radical chain reaction of the mixture of methyl linoleate and methyl laurate. However, PCh suppressed the occurrence of ferric ion-induced oxidation of this model oil more efficiently than vitamin E. Therefore, the inhibition of iron-mediated oxidation is likely to be responsible for the superiority of PCh in lard. It is also suggested that the synergistic effect of PC is related to the elevation of vitamin E activity for iron-mediated oxidation.

INTRODUCTION

Vitamin E (Figure 1) is used for increasing the oxidative stability of edible oils and oil-containing foods. A considerable number of studies have shown that phospholipids enhance the antioxidant activity of vitamin E, although their own antioxidant activities are insignificant (Privett and Quackenbush, 1954; Olcott and Veen, 1963; Linow and Mieth, 1976; Bhatia et al., 1978; Hudson and Mahgoub, 1981; Hildebrand et al., 1984; Dziedzic and Hudson, 1984; Kashima et al., 1991; Oshima et al., 1993). However, there seems to be a disagreement on the effectiveness of each phospholipid class because of the differences of the oxidation conditions in the individual studies. In addition, the mechanism of the synergistic effect of each phospholipid is still a subject of discussion.

We recently succeeded in the synthesis of phosphatidylchromanol as a novel vitamin E analogue [1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′,8′-tetramethyl-6′-hydroxychroman; PCh] in which a phytyl chain was replaced by a phosphatidyl moiety (Koga et al., 1994) (Figure 1). The peroxyl radical–scavenging ability of this compound was found to be close to that of vitamin E. Thus, it is of interest to know the effectiveness of PCh as a potential additive for preventing oxidative deterioration of edible oils. Furthermore, PCh is a useful compound for understanding the mechanism of the synergistic antioxidant effect between phospholipids and vitamin E. The objective of this work is to evaluate the effect of PCh on the oxidative stability of lard. We measured the effect of vitamin E, phosphatidylcholine (PC), and PCh on the oxidative stability of lard and its model system comprising methyl linoleate and methyl laurate.

MATERIALS AND METHODS

Chemicals. Egg yolk PC was purchased from Sigma Chemical Co. (St. Louis, MO). Vitamin E (*d*-α-tocopherol) was obtained from Eisai Co. (Tokyo, Japan). 2,5,7,8-Tetramethyl-6-hydroxy-2-hydroxyethyl)chroman (Toc-Et) was kindly supplied from Kuraray Co. Ltd. (Kurashiki, Japan). Phospholipase D (PLD) from *Streptomyces lydicus* was kindly provided from Honen Co. (Yokohama, Japan). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) was the product of Wako Pure Chemical Co. (Osaka, Japan). Purified lard without additives was a kind gift from Tsukishima Food Industrial Co. Ltd. (Tokyo, Japan). It contained trace amounts of water (0.03% by weight) and iron (0.03 ppm) as contaminants. The fatty acid composition of the lard was as follows (by mol): 16:0, 26.5%; 18:0, 7.7%; 18.1, 50.1%; and 18:2, 15.7%. Methyl linoleate (99%) and methyl laurate (99%) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Methyl linoleate was purified to remove contaminant hydroperoxide by column chromatography (Terao and Matsushita, 1986). Other chemicals were of reagent grade.

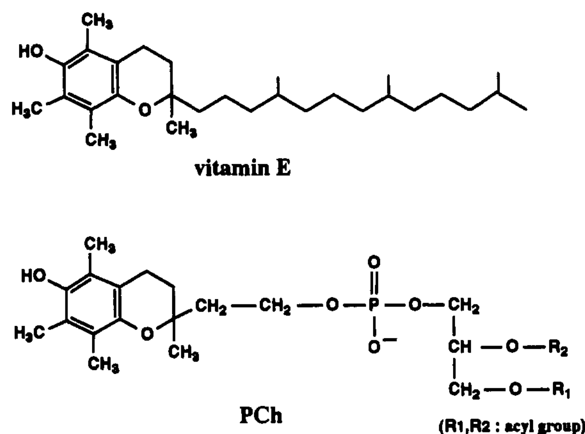

Figure 1. Molecular structures of vitamin E and PCh.

Preparation of PCh. PCh was prepared from egg yolk PC and Toc-Et by PLD in a biphasic system with diethyl ether and aqueous solution (Nagao et al., 1991; Koga et al., 1994). The standard reaction mixture contained 2.5 mL of 25 mM egg yolk PC and 25 mM Toc-Et in diethyl ether and 25 mL of 10 units of PLD and 10 mM $CaCl_2$ in 10 mM acetate buffer (pH 5.1). The enzymatic reaction was performed at 37 °C with continuous shaking at 120 rpm. After 2 h of incubation, 3 mL of 6 N HCl was added to stop the reaction. PCh was then extracted three times from the reaction mixture, each with 30 mL of chloroform/methanol (2:1 v/v). The combined chloroform layer was washed with 20 mL of water and evaporated in a rotary evaporator. The residue was dissolved in chloroform/methanol (95:5 v/v) at a concentration of 10 mg/mL and put onto a silica gel column (Lichroprep Si 60, 40–63 μm, Merck, Darmstadt, Germany) that had previously been equilibrated with the same solvent. The

* Author to whom correspondence should be addressed (telephone 81-471-23-5569; fax 81-471-23-5959).

† Noda Institute for Scientific Research.

‡ National Food Research Institute.

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Table 1. Effect of Additives on Autoxidation of Lard at 60 °C

	expt 1		expt 2	
	inductn period[a] (days)	antiox effect[b]	inductn period[a] (days)	antiox effect[b]
control	11.0	1.00	8.0	1.00
PC	9.5	0.86	7.0	0.88
vitamin E	17.0	1.55	14.0	1.75
vitamin E + PC	21.0	1.91	17.5	2.19
PCh	28.5	2.59	22.5	2.81

[a] Induction period was defined as the days required to increase the weight of oil by 1%. Data are the average values of two samples. [b] Values were calculated on the basis of the induction period of the treated *vs* control.

product was eluted at a flow rate of 1.8 mL/min and fractionated to each 1.0 mL. Each fraction was monitored by developing TLC plates (silica gel 60, Merck) with chloroform/methanol (8:2 v/v). Phospholipids were visualized by spraying with Dittmer's reagent (Dittmer and Lester, 1964). Fractions containing PCh alone were collected and redissolved in methanol after evaporation. PCh was obtained at the yield of 48%. The purity of PCh was estimated to be approximately 98% by high-performance liquid chromatography (HPLC). The purified PCh synthesized from 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine was identified as 1,2-dimyristoyl-*sn*-glycero-2'-(hydroxyethyl)-2',5',7',8'-tetramethyl-6'-hydroxychroman by spectral data (Koga et al., 1994): SIMS-MS [*m/z* 825 (M + H$^+$)]; ^{13}C NMR (67.9 MHz, $CDCl_3$) δ 62.85 (CH_2O, *sn*-1), 70.78 (CHO, *sn*-2), 63.86 (CH_2OP, *sn*-3), 174.27 and 173.90 (CO ester), 34.48–22.99 (CH_2), 14.22 (CH_3 terminal), 73.63 (C-2'), 32.40 (C-3'), 20.92 (C-4'), 117.25 (C-5'), 145.40 (C-6'), 120.93 (C-7'), 122.51 (C-8'); IR (KBr, cm^{-1}) 1745 (C=O), 1457 (-OH), 1246 (P=O), 1027 (P-O-C).

Measurement of Oxidative Stability of Lard. Antioxidants dissolved in *n*-hexane were added to the purified lard at a concentration of 0.4 μmol/g. The oils containing antioxidant solution were evaporated with a rotary evaporator at 30 °C for 30 min and then *in vacuo* for 1 h to remove *n*-hexane completely. One gram oil was measured accurately and spread out in a glass dish (90-mm diameter). These dishes were stored at 60 °C in the dark under circulating air. The weight grain was recorded at an appropriate interval (Olcott and Einset, 1958).

Measurement of Oxidation of Methyl Linoleate. The antioxidant and AMVN were dissolved in a solution of chloroform and methanol (2:1 v/v) and were placed in a test tube. The solvent was removed with a stream of nitrogen and then *in vacuo*. The residue was dissolved in the mixture of methyl linoleate and methyl laurate (15:85 by mol) followed by mixing with a vortex mixer for 30 s. When the oxidation was initiated by ferric ion, 1.25 μmol for $Fe(NO_3)\cdot 9H_2O$ dissolved in water (2 μL) was added to the mixture of methyl linoleate and methyl laurate (1 mL) and dispersed by a vortex mixer for 30 s followed by ultrasonic irradiation in a Heat System sonifier (Model W-380, Farmingdale, NY) for 1 min. The reaction mixture was incubated in the dark at 37 °C with continuous shaking at 120 rpm. At appropriate intervals, aliquots of the reaction mixture were withdrawn and injected into the HPLC column to determine methyl linoleate hydroperoxides (MeL-OOH). The HPLC conditions were the same as described previously (Terao and Matsushita, 1986).

RESULTS

Effect of PCh on the Oxidative Stability of Lard. Purified lard was stored at 60 °C in the dark, and the weight gain was measured. The end of the induction period was easily recognized by a sharp weight gain. The induction periods of lard with and without antioxidants were defined as the days required to increase the weight of the oil by 1% and summarized in Table 1. The addition of vitamin E increased the induction period. Although the induction period was slightly shortened by the addition of PC, the combination of vitamin E and PC apparently increased the induction period. Thus, PC acted as a

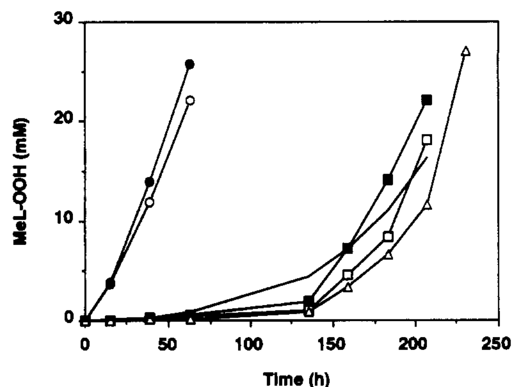


Figure 2. Effects of vitamin E, PC, and PCh on AMVN-induced oxidation of the mixture of methyl linoleate and methyl laurate. The reaction system consisted of additives (20 nmol/mL) and AMVN (50 nmol/mL) in a mixture of methyl linoleate and methyl laurate (15:85 by mol) and was incubated with continuous shaking at 37 °C in the dark. (—) Autoxidation; (○) no addition; (●) PC; (□) vitamin E; (■) vitamin E and PC; (△) PCh.

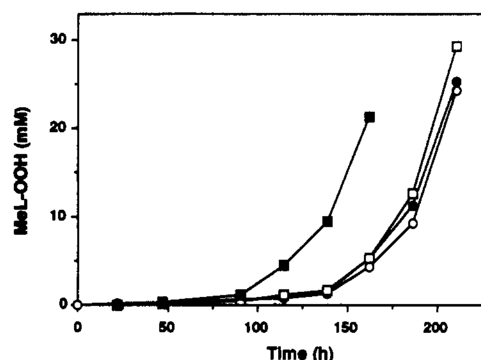


Figure 3. Effects of water on the inhibition by vitamin E and PCh of AMVN-induced MeL-OOH formation in the mixture of methyl linoleate and methyl laurate. The reaction system consisted of additives (20 nmol/mL) and AMVN (50 nmol/mL) in a mixture of methyl linoleate and methyl laurate (15:85 by mol), with or without water (0.5% v/v), and was incubated with continuous shaking at 37 °C in the dark. (○) Vitamin E; (●) vitamin E in the presence of water; (□) PCh; (■) PCh in the presence of water.

synergist with vitamin E. The induction period with PCh was the longest among all experiments.

Antioxidant Activity of PCh on the Radical Chain Reaction of the Mixture of Methyl Linoleate and Methyl Laurate. Antioxidant activity of PCh or other compounds was monitored by measuring the accumulation of MeL-OOH, and a typical example is illustrated in Figure 2. A lipid-soluble radical initiator, AMVN, was added to accelerate the chain propagation reaction of autoxidation (Niki, 1990). Vitamin E retarded the accumulation of MeL-OOH with a clear induction period. PC showed neither inhibitory effect in its own right nor synergistic effect with vitamin E. On the other hand, the inhibition of the accumulation of MeL-OOH by PCh was comparable to that of vitamin E. The inhibition by PCh was weakened by the addition of 0.5% (v/v) of water to the mixture, although the antioxidant activity of vitamin E was not affected (Figure 3).

Antioxidant Activity of PCh on Ferric Ion-Induced Oxidation of the Mixture of Methyl Linoleate and Methyl Laurate. The mixture comprising methyl linoleate and methyl laurate was oxidized by the addition of ferric ion dissolved in water. Ferric ion accelerated the accumulation of MeL-OOH as shown in Figure 4. Although vitamin E could suppress the accumulation of MeL-OOH, MeL-OOH increased gradually with the

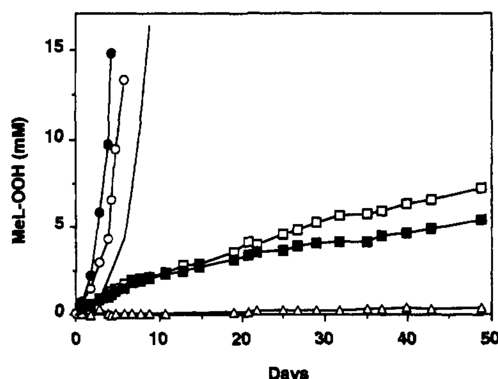


Figure 4. Effects of vitamin E, PC, and PCh on ferric ion-induced oxidation of the mixture of methyl linoleate and methyl laurate. The reaction system consisted of additives (20 nmol/mL), $Fe(NO_3)_3 \cdot 9H_2O$ (50 nmol/mL), and water (0.25% v/v) in a mixture of methyl linoleate and methyl laurate (15:85 by mol) and was incubated with continuous shaking at 37 °C in the dark. (—) Autoxidation; (○) no addition; (●) PC; (□) vitamin E; (■) vitamin E and PC; (△) PCh.

elapse of incubation time. Egg yolk PC did not show any inhibitory effect in this system. However, MeL–OOH accumulated more slowly in the presence of PC and vitamin E than in the presence of vitamin E alone. PCh completely suppressed the accumulation of MeL–OOH throughout the incubation time.

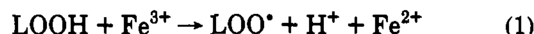
DISCUSSION

It is well-known that phospholipids exert a synergistic antioxidant action with vitamin E in edible oil. Some workers have suggested that phospholipids enhance the effectiveness of vitamin E (Linow and Mieth, 1976; Hildebrand et al., 1984; Ishikawa et al., 1984; Kashima et al., 1991). It is also implied that the synergistic effect of phospholipids is derived from chelation of metal ions (Hudson and Mahgoub, 1981). However, little is clarified about the mechanism of the synergism by phospholipids and vitamin E.

In our results, PC itself had a prooxidant action in both the autoxidation of lard (Table 1) and the accelerated oxidation of the model oil (Figures 2 and 4). The prooxidant effect of PC seems to be due to the presence of polyunsaturated fatty acids in egg yolk PC used in this experiment, as pointed out by Husain et al. (1986).

PCh is a novel phospholipid containing a chromanol structure which is responsible for the radical scavenging activity of vitamin E (Burton and Ingold, 1981). Our preceding work demonstrated that the ability of PCh as a chain-breaking antioxidant is comparable to that of vitamin E (Koga et al., 1994). In addition, PCh may possess a property of synergism of phospholipids and vitamin E because their structures are included in its molecule. The result that PCh prolonged the induction period more efficiently than vitamin E (Table 1) indicates that PCh itself exerts this synergism. Interestingly, the antioxidant activity of PCh was more effective than that obtained by the combination of vitamin E and PC.

In general, edible oils contain a trace amounts of iron and water as contaminants. The lard used in this study also contained iron (0.03 ppm) and water (0.03%). It is known that metals such as iron and their derivatives are mostly responsible for the initiation of autoxidation of edible oils (Pokorny, 1987). For example, the reaction of iron with preformed lipid hydroperoxides (LOOH) yields chain-initiating lipid alkoxyl radicals (LO•) or lipid peroxyl radicals (LOO•) as shown in the following equations (Schaich, 1992).



$$LOOH + Fe^{3+} \rightarrow LOO^{\bullet} + H^{+} + Fe^{2+} \quad (1)$$

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + OH^{-} + Fe^{3+} \quad (2)$$

This reaction is likely to occur in lard, resulting in radical chain oxidation. Vitamin E is known to act as chain-breaking antioxidant by scavenging chain-propagating LOO• (Burton and Ingold, 1981; Niki et al., 1984). Vitamin E may also suppress the iron ion-catalyzed initiation reaction by scavenging chain-initiating LO• or LOO•. Thus, we measured the antioxidant activity of PCh on an azo compound-mediated radical chain reaction and on a ferric ion-mediated initiation reaction. The model system for lard consisted of methyl linoleate and methyl laurate, in which the ratio of methyl linoleate was adjusted to the linoleic acid content in lard. AMVN used here is a lipid-soluble azo compound and thereby causes radical chain reaction by generating peroxyl radicals in the lipid phase (Barclay et al., 1984a; Yamamoto et al., 1984). Vitamin E and PCh can act as chain-breaking antioxidant because both compounds possess a peroxyl radical scavenging activity (Koga et al., 1994). On the other hand, no antioxidant activity of PC in AMVN-mediated chain oxidation confirms that PC cannot act as chain-breaking antioxidant. Furthermore, little synergistic effect of PC and little difference on the antioxidant activity between PCh and vitamin E were observed in AMVN-mediated chain oxidation (Figure 2). It is therefore unlikely that the synergistic effect of phospholipid participates in the radical chain reaction.

The antioxidant activity of PCh was lowered by the addition of water in the model systems subjected to an azo compound-induced radical chain reaction (Figure 3). The mechanism of this lowered activity of PCh is not fully explained. However, the fact that the inhibition by PCh on ferric ion-induced oxidation is superior to that by vitamin E (Figure 4) is not based on the activity of chain-breaking antioxidant, because this reaction system contained a trace amoutn of water. Since the anion of phosphoric acid in PCh is not compensated by the base, PCh seems to be an acidic phospholipid. Therefore, a possible mechanism for the effective activity of PCh on ferric ion-induced oxidation is that an acidic property of PCh acts as an iron chelator similar to phosphatidylserine (Yoshida et al., 1991). In addition, PCh may form reverse micelles in oil with a trace amount of water, resulting in better accessibility of the chromanol group to the polar site where an iron-dependent initiation reaction takes place. In fact, it has been reported that phospholipids form reverse micelles in apolar solvent (Kanamoto et al., 1981) and that the addition of a trace amount of water increases the aggregation (Barclay et al., 1984b). Thus, the synergistic action of PC may be derived from its aggregation to form reverse micelles.

In conclusion, PCh can act as an effective antioxidant for lard. It is likely that its antioxidant mechanism involves the synergism between phospholipids and vitamin E.

ABBREVIATIONS USED

AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile): HPLC, high-performance liquid chromatography; LO•, lipid alkoxyl radical; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxides; MeL–OOH, methyl linoleate hydroperoxides; PC, phosphatidylcholine; PCh, phosphatidylchromanol; PLD, phospholipase D; Toc-Et, 2,5,7,8-tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman.

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