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Abstract

The role of ethanolamine plasmalogen extracted from bovine brain (BBEP) in maintaining oxidative stability of bulk soybean oil and liposome made with egg phospholipids (PL) was studied. In a purified soybean oil (PSO), the addition of 200 and 1000 ppm of BBEP promoted lipid oxidation at rates of 0.037 and 0.071 (all rates in $\ln(PV) h^{-1}$, and PV stands for peroxide value), whereas soy lecithin (SL) added in the same amount showed a trend similar to the PSO blank, which had an oxidation rate of 0.025. The PSO with BBEP was susceptible to cupric ion catalyzed oxidation, in that the oil was oxidized much more quickly than the PSO with SL and cupric ion. In commercial soybean oil (CSO) with the presence of tocopherols, SL at 1000 ppm acted synergistically as an antioxidant with the natural tocopherols, but addition of BBEP accelerated lipid oxidation, as evidenced by the oxidative stability index (OSI) test. In the egg PL liposome, the BBEP caused a fast breakdown of the lipid hydroperoxides and consequently promoted more thiobarbituric acid reactive substance (TBARS) formation. The PL oxidation in the presence of copper in the liposome was not affected by the BBEP, which indicates that the hypothesis of ethanolamine plasmalogen (EthPm) chelating cupric ion as the antioxidation mechanism was not supported. The addition of cumene hydroperoxide to the egg PL liposome promoted lipid oxidation, as indicated by a fast development of PV and TBARS. However, the result with cumene hydroperoxide failed to differentiate the effect of BBEP and SL and their concentration on lipid oxidation. On the basis of the observations from this study, we conclude that EthPm is not an antioxidant but rather a pro-oxidant in a bulk lipid system, and it has no significant antioxidant effect for PL oxidation in the liposome.

Keywords

antioxidant, egg lecithin, lipid oxidation, liposome, plasmalogens, soybean oil, soy lecithin, transition-metal ions

Disciplines

Food Chemistry | Food Science | Human and Clinical Nutrition

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The Role of Plasmalogen in the Oxidative Stability of Neutral Lipids and Phospholipids

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The role of ethanolamine plasmalogen extracted from bovine brain (BBEP) in maintaining oxidative stability of bulk soybean oil and liposome made with egg phospholipids (PL) was studied. In a purified soybean oil (PSO), the addition of 200 and 1000 ppm of BBEP promoted lipid oxidation at rates of 0.037 and 0.071 (all rates in $\ln(PV) h^{-1}$, and PV stands for peroxide value), whereas soy lecithin (SL) added in the same amount showed a trend similar to the PSO blank, which had an oxidation rate of 0.025. The PSO with BBEP was susceptible to cupric ion catalyzed oxidation, in that the oil was oxidized much more quickly than the PSO with SL and cupric ion. In commercial soybean oil (CSO) with the presence of tocopherols, SL at 1000 ppm acted synergistically as an antioxidant with the natural tocopherols, but addition of BBEP accelerated lipid oxidation, as evidenced by the oxidative stability index (OSI) test. In the egg PL liposome, the BBEP caused a fast breakdown of the lipid hydroperoxides and consequently promoted more thiobarbituric acid reactive substance (TBARS) formation. The PL oxidation in the presence of copper in the liposome was not affected by the BBEP, which indicates that the hypothesis of ethanolamine plasmalogen (EthPm) chelating cupric ion as the antioxidation mechanism was not supported. The addition of cumene hydroperoxide to the egg PL liposome promoted lipid oxidation, as indicated by a fast development of PV and TBARS. However, the result with cumene hydroperoxide failed to differentiate the effect of BBEP and SL and their concentration on lipid oxidation. On the basis of the observations from this study, we conclude that EthPm is not an antioxidant but rather a pro-oxidant in a bulk lipid system, and it has no significant antioxidant effect for PL oxidation in the liposome.

KEYWORDS: Antioxidant; egg lecithin; lipid oxidation; liposome; plasmalogens; soybean oil; soy lecithin; transition-metal ions

INTRODUCTION

Plasmalogens are a special class of phospholipids (PL) which share a common structure with other PL, except that they have a vinyl ether linkage to the glycerol backbone at the sn-1 position. Plasmalogens are rarely found in plant sources except onion and avocado (1), but they are present in most animal tissues. Ethanolamine plasmalogens (EthPm) are especially rich in brain, testes, and kidney, whereas significant amounts of choline plasmalogens are found in heart and skeletal muscles (2). Several structural and functional properties are associated with plasmalogens in *in vivo* studies, such as increasing membrane fluidity, mediating signal transduction, and relieving free radical induced cell oxidative stress (3).

The antioxidant effect of plasmalogens, especially EthPm, has been studied in both *in vivo* and *in vitro* during the past decade on the basis of the finding that EthPm scavenged reactive oxygen species (ROS) (4,5). It is reported that one vinyl ether double bond protected four double bonds in arachidonate from oxidation (6), which indicates that EthPm may have certain characteristics of

lipid-soluble antioxidants. Zommara et al. (7) demonstrated that, in a liposome system that contained egg choline PL, the EthPm prevented ferrous- and cupric-promoted lipid oxidation in the presence of hydroperoxide initiators. Similar results were reported by Sindelar et al. (8), who found that brain plasmalogens effectively prevented iron-induced lipid peroxidation. The protective role of beef brain lyso-EthPm was claimed by Hahnel et al. (5) in the micelles. They also found that EthPm was more effective in low-density lipoprotein (LDL) particles than in the micelles. In an *in vivo* study, it was found that nerve tissue deficient in plasmalogens was more susceptible to ROS damage, and this deficiency was caused by peroxisomal disorders such as Zellweger syndrome; in the testes, plasmalogens protected spermatocytes from very long chain fatty acid induced degeneration and apoptosis (9). However, the molecular mechanism supporting the plasmalogen-mediated prevention of transition-metal-induced lipid oxidation is still not well understood. A binding stoichiometry of 1:1 for copper ion and vinyl ether was claimed (5), whereas such chelating was not shown in iron-induced lipid oxidation (8). Many oxidation studies were done with the addition of certain hydroperoxide initiators, especially azo compounds that accelerate the production of hydroperoxide by promoting propagation. However, the use of

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Table 1. Effect of BBEP and SL on the Oxidation of Purified Soybean Oil at 32 °C^a

treatment	PL type	PL, ppm	Cu ²⁺ , ppm	oxidn rate, slope of ln(PV) h ⁻¹	R ² for the exponential regression
control	N/A	0	0	0.025	0.918
free copper	N/A	0	8	0.026 ± 0.0004	0.890
SL200	SL	200	0	0.026	0.886
BBEP200	BBEP	200	0	0.037	0.989
SL1000	SL	1000	0	0.025	0.906
BBEP1000	BBEP	1000	0	0.071	0.939
SL200/Cu	SL	200	8	0.034 ± 0.005	0.944
BBEP200/Cu	BBEP	200	8	0.030 ± 0.002 ^b	0.940
SL1000/Cu	SL	1000	8	0.041 ± 0.002	0.949
BBEP1000/Cu	BBEP	1000	8	0.033 ± 0.010 ^b	0.966

^a Abbreviations: BBEP, beef brain ethanolamine plasmalogen; SL, soy lecithin; R² is the coefficient of determination. ^b Oxidation rate for BBEP200/Cu and BBEP1000/Cu only represents oxidation rate after initiation stage because of their fast oxidation.

a hydroperoxide initiator is highly questionable, because it is an artificial system that is not found in either food or biological systems (10).

While some researchers showed that EthPm functioned as an antioxidant, others showed different results based on the fact that the vinyl ether bond is highly sensitive to oxidative attack (11, 12). The breakdown of the vinyl ether bond was observed following the oxygen consumption, and the degradation of plasmalogen was faster in the presence of polyunsaturated fatty acids (PUFA). Rancidity development in meat might also be attributed to the instability of plasmalogens (13). A class of decomposed plasmalogen epoxidation products, α -hydroxyaldehydes, was confirmed, and their high reactivity also raised doubts as to the protective effect of EthPm in biological systems (14). Felde and Spiteller (15) also reported the α -hydroxy aldehydes as oxidation products of plasmalogen in lipoprotein systems. Another study conducted using plasmalogen-deficient fibroblasts did not show a major role of plasmalogen in protecting cells against ROS as proposed by Zoeller et al. (4). Kehrer and Biswal (16) reported a deleterious effect of the Schiff base adducts derived from the aldehydes on the neural cell membrane. The Schiff base adducts were verified by using GC-MS after the EthPm-containing rat brain homogenates were oxidized under UV and Fe²⁺/ascorbate (17).

In the present study, we evaluated the antioxidant properties of a plasmalogen using classical lipid oxidation methods, e.g., bulk oil oxidation, as indicated by peroxide value (PV) and oxidative stability index (OSI). The oxidative stability of purified soybean oil was compared with that of commercial soybean oil in the presence of BBEP and soy lecithin (SL) in order to determine the possible antioxidant synergy between tocopherols and BBEP. A pure liposome model containing only egg PL (EPL) and water was also used to simulate cell membranes. We were interested in the chelating mechanism as proposed by Hahnel et al. (5); therefore, cupric ion was added to both models to verify the proposed mechanism of antioxidation.

MATERIALS AND METHODS

Materials and Reagents. As a source of EthPm, beef brain was taken from two 21-month-old cows, which were slaughtered in the Meat Lab at Iowa State University. Egg phospholipids (EPL) PL-100 M was purchased from Q.P. Corporation (Tokyo, Japan) with a claimed purity of above 99%. Soy phospholipids (SL) was purchased from Sigma-Aldrich (St. Louis, MO). Standard egg phosphatidylethanolamine (eggPE), lyso-eggPE, phosphatidylcholine (soyPC), lyso-soyPC, and phosphatidylinositol (soyPI) were purchased from Avanti Polar Lipid (Alabaster, AL). Soybean oil (Crisco, J.M. Smucker Company, Orrville, OH) was purchased from a local grocery store. All other chemicals were purchased from either Fisher Scientific or Sigma unless otherwise noted.

PE Plasmalogen Extraction. Fresh beef brain tissue was first ground in a Smart Power blender (Cuisinart, East Windsor, NJ) by adding a small

portion of water. The resulting homogenates were then dehydrated by adding pure ethyl alcohol with manual stirring. The total phospholipids were extracted by using 100% ethyl alcohol and then hexanes as described by Polacios and Wang (18) with slight modification. The polar lipid fraction was fractionated by liquid partitioning, and the polar lipid fraction was subjected to an acetone wash to remove neutral lipids. The crude polar lipid was further subjected to Folch wash (19) to obtain the pure PL fraction. A further separation of the PE fraction was achieved by thin-layer chromatography (TLC). An aliquot of the resulting PL, about 20 mg, was loaded on a 2000 μ m Uniplate silica G thin-layer plate (Whatman, Piscataway, NJ), which was developed with chloroform/methanol/water (25/10/1, v/v/v). The PE band was scraped into a centrifuge tube, and lipid was extracted twice by following the Bligh–Dyer procedure (20). The resulting PE fraction was collected, weighed, and verified by TLC as compared with standard eggPE, soyPC, soyPI, and lyso-soyPC. The final PE fraction (containing PE and EthPm) was dissolved in chloroform/methanol (1/1, v/v) and designated as BBEP. The existence of EthPm was verified by base hydrolysis, and the resulting lyso-EthPm was verified by the same TLC method as compared with standard lyso-eggPE.

³¹P NMR Determination of PL in BBEP. Triphenyl phosphate (TPP) was used as an internal standard for the quantification of PL in the BBEP. Purified BBEP (~50 mg) and TPP (~10 mg) were dissolved in chloroform-*d* (1 mL), methanol (1 mL), and 0.2 N cesium ethylenediaminetetraacetic acid (EDTA) solution (1 mL, pH 8.5). After vigorous shaking, the sample was centrifuged, and the lower phase was transferred to a NMR tube (5 mm). The NMR spectra were obtained from a Bruker VXR-400 spectrometer, and it was operated at 400 MHz. Samples were analyzed with inverse gated decoupling. The NMR spectroscopic scan conditions were as follows: pulse width, 22 μ s; sweep width, 9718 Hz; acquisition time, 1.2 s; relaxation delay, 10 s; number of scans, 256. The chemical shifts were reported relative to TPP (δ -17.8). The data processing was completed using MestReNova software (Santiago de Compostela, Spain).

Fatty Acid Profile of PL. The PL fatty acid profile was determined using a Hewlett-Packard Model 5890 Series II gas chromatograph (GC) with a flame ionization detector. A 10 mg portion of PL was first dissolved in 4 mL of sodium methoxide (1 M in methanol). The reaction was conducted for 30 min under ambient temperature and was stopped by adding a few drops of water. Fatty acid methyl esters (FAME) were extracted by using hexanes and applied to the GC for analysis. The conditions used for GC analysis were as follows: injection temperature, 230 °C; detector temperature, 230 °C. The oven temperature was programmed from 110 to 220 °C with a heating rate of 10 °C/min. The column was a Supelco SP-2330 (Bellefonte, PA) capillary column, 15 m (length) \times 0.25 mm (i.d.) \times 0.2 μ m (film thickness).

Effect of BBEP and Cupric Ion on the Oxidation of Purified Soybean Oil (PSO). Commercial soybean oil was purified by subjecting it to an activated alumina column (the oil to alumina ratio was 1:1) that was eluted with distilled petroleum ether to remove tocopherols and other polar impurities following the method of Jensen et al. (21). All glassware was soaked in 10% nitric acid and washed with deionized water prior to use. A 5 g portion of PSO was then weighed into a 250 mL Erlenmeyer flask with addition of different amounts of cupric sulfate and BBEP or SL. The treatment arrangement is shown in Table 1. Since antioxidant-free oil

oxidizes very quickly, a low-temperature oxidation method had to be used. Samples were stored in a dark forced-air oven at 32 °C. The oxidation experiment was conducted for 160 h with a sampling interval of about 12 h. At each sampling time, a drop of 2% butylated hydroxytoluene (BHT) in ethanol was added into the storage vial. All samples were sealed with nitrogen and stored in a -20 °C freezer until analysis. Cupric ion treated samples were prepared in duplicate. PV and TBARS (thiobarbituric acid reactive substances) were measured according to the methods mentioned in the following section. The natural logarithm of PV was also plotted against time in hours, and the slope of the linear line was used as the oxidation rate.

Effect of BBEP and Cupric Ion on Oxidation of Commercial Soybean Oil (CSO). All treatments were prepared in the same manner as that for the purified soybean oil, but soybean oil was not treated and used as is in order to examine the effect of tocopherols on lipid oxidation with BBEP, SL, and cupric ion additions. Oxidative stability was measured as an oxidative stability index (OSI, in h) using an ADM Oxidative Stability Instrument (Omniion, Rockland, MA) at 100 °C, according to AOCS Official Method Ca 5a-40 (22). All treatments were in duplicate. Since commercial oil is much more stable than purified oil, a higher temperature for oxidation experiments can be used to compare oxidative stabilities.

Effect of BBEP and Cupric Ion on Oxidation of EPL in Two Liposome Systems. The method to prepare liposome was modified from that reported by Zommará et al (7). Briefly, EPL PL-100 M was first dissolved in a small quantity of chloroform/methanol (1/1). The solution was transferred to a round-bottom flask, a thin film was formed, and then the solvent was rotary evaporated. Residual solvent was further removed using a vacuum oven at ambient temperature overnight. The dried lipid film was then dispersed into a solution containing 0.1 M tris-HCl buffer and 0.1 M sodium chloride at pH 7.4, and the dispersion was vortexed to facilitate lipid dispersion. Then, BBEP (1000 ppm, based on EPL weight) and cupric sulfate (8 ppm, based on EPL weight) were added to the above dispersion. The molar ratio of BBEP to cupric ion was 10:1. The resulting dispersion was sonicated for 5 min with 30 s pause for every 1 min sonication in an ice-water bath by using a Misonix XL sonicator (Farmingdale, NY) with a power setting at 9. The final concentrations were 25 mg/mL, 25 µg/mL, and 0.2 µg/mL for EPL, BBEP, and cupric ion, respectively. The size distribution of liposome particles (without adding BBEP and SL) was measured using a Hydro 2000 MU laser scattering particle size analyzer (Malvern, U.K.) by following the method of Wang and Wang (23). The oxidation of such liposomes was conducted in a dark forced-air oven at 60 °C for 2 weeks with periodic samplings. This temperature was desirable to examine PL oxidation because it is more stable than pure oil. Lipid oxidation was terminated by adding a drop of reagents containing BHT (butylated hydroxytoluene) and EDTA with approximate concentrations of 1 and 10 mM, respectively. All samples were filled with nitrogen and stored in a -20 °C freezer until quantification for PV and TBARS. All treatments were in duplicate.

In order to verify the effect of hydroperoxide initiator on the oxidative behavior of BBEP and SL in liposome, a second experiment was conducted in a different liposome system where the concentration of EPL was 4.0 mg/mL, and the concentrations of BBEP and SL were still 1000 ppm based on EPL; the molar ratio of cupric ion to BBEP was 1:10, and the concentration of cumene hydroperoxide as a lipid-soluble hydroperoxide initiator was 40 µM in liposome. Because the ratio of plasmalogen to other PL is found to be high in brain and testes, another BBEP treatment was included with a concentration of 10%, with 10% of SL as a comparison. The liposome samples for all treatments were prepared in the same manner as mentioned above and were stored in a forced-air conventional oven at 37 °C. Sampling was done every 6 h for a total of 30 h, and PV and TBARS were determined.

Peroxide Value and TBARS Determination. For all the oxidation experiments mentioned above, except for that with commercial soybean oils, both PV and TBARS were measured as indications of lipid oxidation. To measure the PV of oxidized soybean oil (PSO), a ferric thiocyanate colorimetric method was used following a previously established procedure (23). Briefly, previously oxidized soybean oil with 19.8 mequiv/kg of PV, measured by the AOCS Official Method Cd 8-53 (24), was used to establish the standard curve of absorbance versus hydroperoxide (µequiv/kg). PSO samples of 10–100 mg were weighed into 10 mL volumetric flasks, and then the flasks were filled with chloroform/methanol (2/1, v/v).

Table 2. Fatty Acid Composition (mol %) of Phospholipids from Different Sources^a

PL source/ FA type	C16:0	C18:0	C18:1	C18:2	C18:3	C20:4	C22:6	others
BBEP	12	11	31	20	nd	7	11	8
EPL	30	16	26	16	nd	6	2	5
SL	21	4	11	57	7	nd	nd	2

^a Abbreviations: BBEP, beef brain ethanolamine plasmalogen; SL, soy lecithin; EPL, egg phospholipid; nd, not detected.

Serial dilution was used for individual samples so that all absorbance values fell within the linear range as specified by the standard curve. Then, 100 µL of ferrous chloride (0.014 M) and 50 µL of ammonium thiocyanate (3.75 M) were added to 4 mL of diluted samples and vortexed. The reaction mixture was allowed to stand for 20 min, and the absorbance at 500 nm was recorded by using a Genesys 20 spectrophotometer (Cambridge, U.K.). The PV of EPL liposome was measured in the same manner. For these samples, an oxidized EPL (PV of 29.1 mequiv/kg) was used to establish the standard curve. An amount of water equivalent to that of the liposome was added to the PL standards so that any interference in absorption was controlled.

The method used for determining TBARS was described in Wang and Wang's procedure (23), and quantification of TBARS was conducted at 532 nm using the same spectrophotometer as that for PV measurement.

Statistical Analysis. All experiments were conducted with duplicate treatments unless otherwise noted. Data analyses were done by using the SAS program (version 9.1, SAS Institute Inc., Cary, NC). One-way analysis of variance (ANOVA) was used for mean comparisons, and Fisher's least significant differences were calculated at $P < 0.05$ (LSD_{0.05}).

RESULTS AND DISCUSSION

Chemical Composition of BBEP. BBEP was a mixture of EthPm and PE, among which EthPm was 78.2% (mol %), and PE was 17.3%, as quantified by ³¹P NMR. With respect to the fatty acid profile, EthPm is a rich source of arachidonic acid (AA, 20:4, ω -6, 7%) and docosahexaenoic acid (DHA, 22:6, ω -3, 11%) (Table 2). In the plasmalogen molecule, AA and DHA have a tendency to attach at the sn-2 position, which is usually considered to be less susceptible to oxidation than that on the outer position. A lipid oxidation study with individual FFA micelles (25) showed that linoleate produced much more lipid peroxide and conjugated dienes but much less TBARS than DHA and AA. Both the vinyl ether linkage and FA composition make EthPm significantly different from SL, which had undetectable AA and DHA and no plasmalogens.

Effect of BBEP on Oxidative Stability of PSO. Commercial soybean oil contains mainly C16 and C18 fatty acids, with 52–55% linoleic acid being the major acid, 8% linolenic acid, and 22–25% oleic acid (26). The ratio of oxidation rate was reported as about 1:12:25 in the order of oleic, linoleic, and linolenic acids in a free radical autoxidation system (27). Therefore, the soybean oil after removal of tocopherols is a good model for studying antioxidant effects because of its richness in double bonds. Our previous test with the same procedure showed that at least 99% of the naturally occurring tocopherols were removed by purification with activated alumina (28).

The oxidation development of PSO, expressed as PV versus time, is shown in Figure 1. The relationships between PV and time for all treatments were fitted with exponential regressions, shown as the smooth curves in this figure. The coefficients of determination (R^2) are present in Table 1.

All treatments generally fell into three groups on the basis of PV development and PL class. The first group contains PSO only (blank or control) and PSO with cupric ion. It was unexpected that the sample containing cupric ion showed low PV development during the storage at 32 °C, as was the case for the blank.

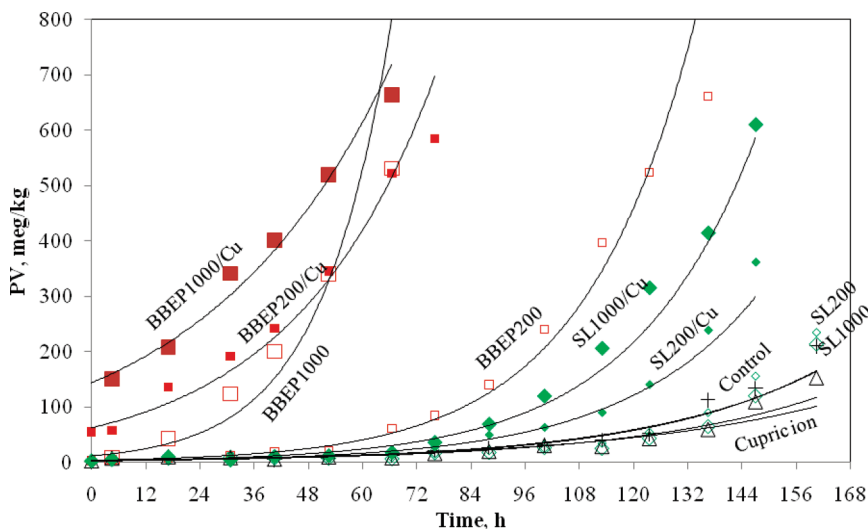


Figure 1. PV development of purified soybean oil at 32 °C in the presence of SL and BBEP fractions (200 and 1000 ppm) and cupric ion (8 ppm). Trend lines are drawn by following exponential regression models. For treatment abbreviations, refer to the footnotes of **Tables 1** and **2**.

The low temperature and the lack of initiators (transition-metal ions, peroxides, etc.) might have prevented PSO from starting oxidation. However, even after cupric ion was added, PSO still showed no change in PV development as compared with the blank.

The second group of curves (**Figure 1**) contains SL treatments. Two treatments with 200 and 1000 ppm of SL (SL200 and SL1000) but without copper showed almost the same PV development as the blank. This indicates that addition of SL had no obvious pro- or antioxidant effect on PSO. However, when 8 ppm of cupric ion was added to these two treatments (SL200/Cu and SL1000/Cu), an increase in oxidation rate was observed. Cupric ion accelerated lipid oxidation in the presence of SL, and SL acted as pro-oxidant because SL1000/Cu was oxidized much more quickly than SL200/Cu. SL may have helped the dispersion of cupric ion in the oil system; thus, the pro-oxidant effect of cupric ion was observed.

The third group is BBEP treatments with and without cupric ion addition. As 200 ppm of BBEP was added to PSO (BBEP200), the oxidation rate of PSO was increased in comparison with the first two groups. On the basis of the fact that SL200 and SL1000 did not promote oxidation of bulk oil but BBEP did, it suggests that BBEP alone acted as a pro-oxidant. When 1000 ppm of BBEP was added (BBEP1000), the oxidation was even more significantly accelerated, validating its pro-oxidant effect. When 8 ppm of cupric ion was added to BBEP treatments (BBEP200/Cu, BBEP1000/Cu), the “lag” or initiation phase of the oxidation disappeared. However, the effect of cupric ion on hydroperoxide propagation (the rate of increase) seemed to be less dramatic than the dose effect of BBEP. This may indicate the main effect of cupric ion in the presence of plasmalogen was to accelerate the initiation of lipid oxidation. **Figure 1** also shows that the cupric treatments with BBEP had much higher initial PV values than the rest of the treatments. These data clearly demonstrate that lipid oxidation in the presence of EthPm or BBEP was much more susceptible to cupric ion. Therefore, these results failed to support the claim of protective effect of EthPm against transition metal ions induced lipid oxidation.

Another parameter, the oxidation rate derived from data in **Figure 1**, is also shown in **Table 1** for a quantitative comparison. The oxidation rate is defined as the slope of the linearized natural logarithm of PV plotted against time in hours ($\ln(\text{PV}) \text{ h}^{-1}$; units omitted in further discussion). It was found that generally SL200,

SL200/Cu, and SL1000 had oxidation rates similar to that of the blank, which was 0.025, whereas the oxidation rate for BBEP200 (0.037) was almost equal to that of SL1000/Cu. Also, the oxidation rate for BBEP1000 almost doubled from that of BBEP200 and reached 0.071. The value for the treatments BBEP200/Cu and BBEP1000/Cu might not reflect the true oxidation process because both treatments oxidized much more quickly than others, and the initiation stage was lost. Therefore, the curve is somewhat atypical.

It should be noted that all cupric ion treated samples were duplicated, and the others were just single measurements. Because of the total number of treatments and sampling and study feasibility, we decided to duplicate half of the treatments to show our treatment variation, as shown in **Table 2**, the standard deviation of the oxidation rate. For these duplicates, the reproducibility in general is acceptable (about 12% average coefficient of variation).

Effect of BBEP on Oxidative Stability of Commercial Soybean Oil (CSO). Commercial soybean oil contains significant amounts of tocopherols (about 1000 ppm) (29), and a synergistic effect was reported between tocopherols and phospholipids contributing to lipid oxidative stability (30). As shown in **Figure 2**, the OSI of CSO blank (14.1 h) was the same as that with cupric ion treated CSO (13.6 h), and this observation was the same as that in the purified soybean oil. When 1000 ppm of SL was added to CSO, a significant increase in OSI (16.2 h) was observed in comparison to the blank, and this is different from the oxidation of purified soybean oil. This provides a confirmation of a moderate synergistic effect with tocopherols, as reported by other researchers (31). In comparison to SL and CSO blank, the addition of BBEP at the two concentrations (200 and 1000 ppm) significantly reduced the OSI, and this reduction was dose-dependent (10.8 h at 200 ppm and 7.9 h at 1000 ppm). When cupric ion was added to the corresponding treatments, both BBEP and SL treatments showed significant reduction in OSI compared with all non-cupric treatments. Furthermore, 1000 ppm of BBEP with cupric ion showed more significant reduction in OSI than its SL counterpart at 1000 ppm. This demonstrates that BBEP failed to chelate cupric ion, and it acted as a pro-oxidant in the bulk commercial oil system. All the observations in the commercial soybean oil were consistent with those obtained from purified soybean oil with respect to BBEP performance. With regard to the antioxidant synergy of tocopherols and

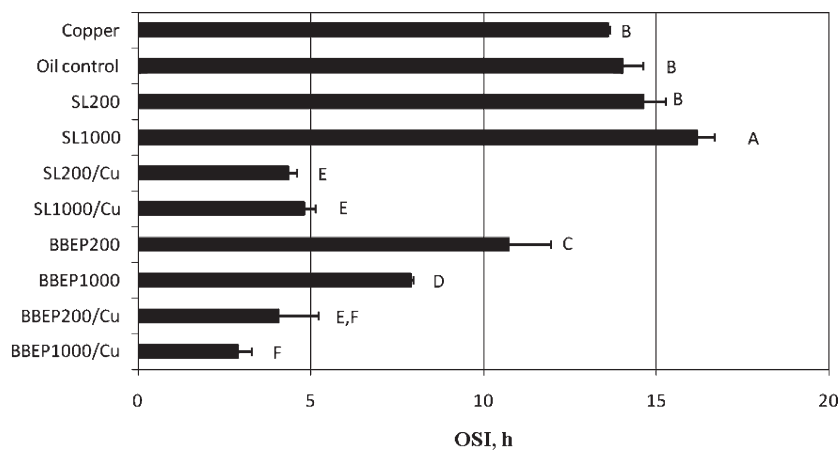


Figure 2. OSI value (time, h) of commercial soybean oil (CSO) at 100 °C in the presence of SL and BBEP fraction (200 and 1000 ppm) and cupric ion (8 ppm). Error bars represent SD from duplicate preparations. For treatment abbreviations, refer to the footnotes of **Tables 1** and **2**. Different letters represent the least significant difference at $P < 0.05$.

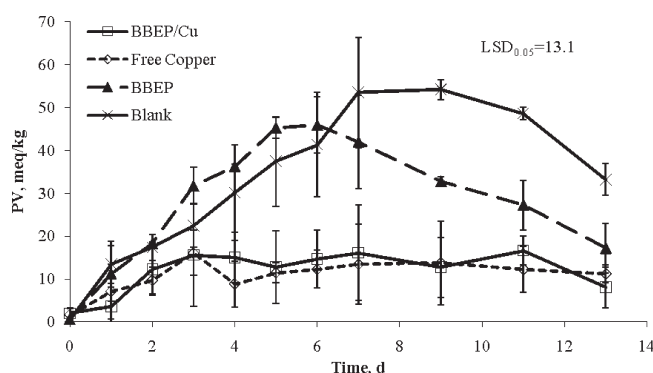


Figure 3. PV development of egg phospholipids in liposome at 60 °C in the presence of BBEP (1000 ppm) and cupric ion (8 ppm). Error bars represent SD from duplicate preparations. For treatment abbreviations, refer to the footnotes of **Tables 1** and **2**.

PLs, Bandarra et al. (30) proposed that the Maillard reaction is possibly involved. We have no reason to believe that BBEP will not have a Maillard reaction similar to that of PL, but the strong pro-oxidant effect of BBEP might have masked any possible synergistic antioxidant effect due to the Maillard mechanism.

Effect of BBEP on the Oxidative Stability of EPL Liposome in the Absence of Cumene Hydroperoxide. The prepared liposome had a pH of 7.4 and a particle size (volume moment mean of the particle, $D[4,3]$) of about 20 μm with three size distribution peaks centered at 0.5, 8.0, and 50.0 μm . It was assumed that adding small amounts of BBEP or SL would not dramatically change the particle size distribution. The liposome appeared milky, and no visually detectable separation was found when it was stored at 60 °C. However, spectrophotometric examination indicated a daily increase in turbidity at 410 nm, which was related to liposome instability. All samples were therefore revortexed at each sampling time to ensure homogeneity for sampling.

PV developments for these liposome samples are shown in **Figure 3**. The oxidative behavior of lipid in an aqueous environment is usually more complicated than it is in the bulk system because many external factors may significantly influence lipid oxidation, such as impurities, particle size, pH, and net charge of particles (32, 33), in addition to the physicochemical properties of the lipids themselves. The large variations in PV measurement could possibly be due to hydroperoxide instability in this environment. Such variation should not be due to sampling error, because no such significant variation was observed for TBARS

measurements, which was done on the same samples. The complexity of the liposome aqueous system might also be responsible for the absence of the classic oxidation curve as seen in bulk oil. Similar PL oxidation curve was also reported by Palacios and Wang (18).

The four treatments can clearly be divided into two groups based on the presence of cupric ion (**Figure 3**, $\text{LSD}_{0.05} = 13.1$). The egg PL liposome with 8 ppm cupric ion (free copper) showed no difference as compared to that with BBEP (1000 ppm) and cupric ion (8 ppm) (BBEP/Cu). PV of both free copper and BBEP/Cu had a maximum value (approximately 15 mequiv/kg) at day 3, and thereafter, they tended to stabilize with extended storage at 60 °C. This indicates that BBEP did not promote PV accumulation in cupric ion induced lipid oxidation. In the second group, both blank and BBEP showed generally higher PV values than that of the first group containing cupric ion. The PV of BBEP seemed to reach a lower peak value earlier than that of the blank, even though no statistical significance could be shown. An apparent peroxide breakdown was observed for both BBEP and the blank at relatively lower PV (50 mequiv/kg) than that typically observed in bulk lipid systems (~400 mequiv/kg) (18). These data indicate that the addition of BBEP to EPL promoted faster hydroperoxide breakdown as compared to the blank. The reason the addition of copper caused significant reduced PV development is not quite clear by just examining this figure alone. Transition-metal ions are known to be good catalysts for the breakdown of hydroperoxides. Therefore, further analysis was conducted to examine products of lipid hydroperoxide breakdown by using the TBARS method.

As shown in **Figure 4**, the TBARS development of the four treatments fell into two groups as well ($\text{LSD}_{0.05} = 0.13$), but with a trend opposite that seen in PV development. Free copper and BBEP/Cu samples had a significant elevation in TBARS compared to the other two treatments. Combining the TBARS with PV, it is clear that the cupric ion promoted the breakdown of EPL hydroperoxide in the liposome system, therefore causing less peroxide accumulation. The fact that BBEP did not slow down the breakdown of hydroperoxide in the presence of copper also indicates that BBEP did not chelate copper or act as an antioxidant. The molar ratio of cupric ion to BBEP in liposome was 1:10 when copper was added at 8 ppm, and theoretically cupric ion should have been fully chelated according to the 1:1 chelating mechanism. In comparison to the blank, BBEP alone also promoted the formation of TBARS, as evidenced by its peak value being higher than that of blank at day 3. TBARS measures

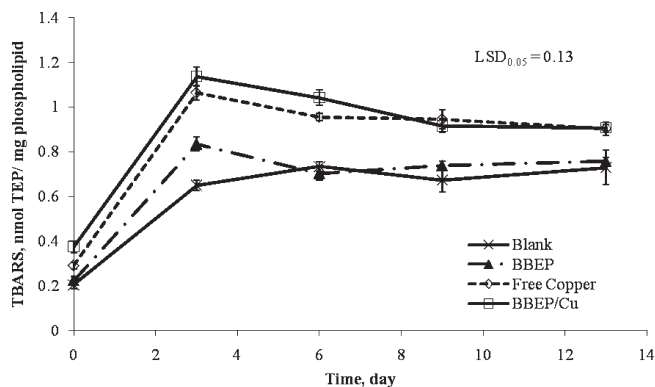


Figure 4. TBARS development of egg PL in liposome system at 60 °C in the presence of 1000 ppm BBEP and 8 ppm cupric ion. Error bars represent SD from duplicate preparations. For treatment abbreviations, refer to the footnotes of **Tables 1** and **2**.

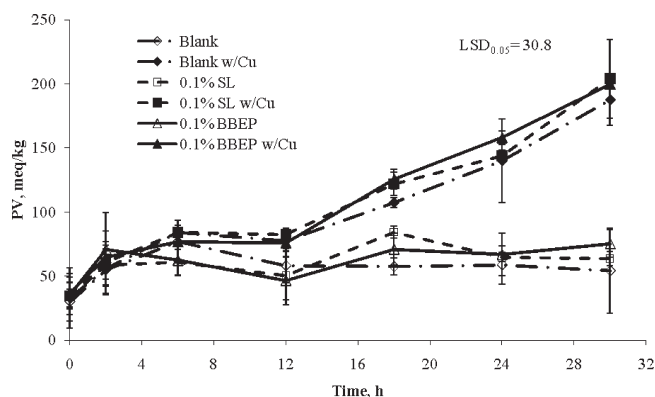


Figure 5. PV development of egg phospholipids in liposome at 37 °C in the presence of cumene hydroperoxide (40 μ M in liposome). Error bars represent SD from duplicate preparations. The concentration of SL and BBEP is 1000 ppm based on EPL. For treatment abbreviations, refer to the footnotes of **Tables 1** and **2**.

malondialdehyde, formed from the breakdown of hydroperoxides of fatty acids with three or more double bonds. It was reported that the oxidation of AA and DHA produces maximum TBARS, and they were 8–10 times higher than linoleate in a model system (25). This may partially explain why BBEP had a slightly higher peak value than blank, if the effect of the vinyl ether bond is not considered. However, the amount of BBEP used was quite low in comparison with that of the egg PL base in liposome therefore, BBEP may have truly accelerated PL oxidation.

As an overall result from PV and TBARS developments, the lipid hydroperoxides in liposome were easily decomposed in the presence of cupric ion. In addition, plasmalogen failed to prevent cupric ion induced PL oxidation. Plasmalogens also tended to be a pro-oxidant compared to blank without cupric ion.

Effect of BBEP on Oxidative Stability of EPL Liposome in the Presence of Cumene Hydroperoxide. Cumene hydroperoxide is a fat-soluble oxidizing agent and has been used to accelerate lipid oxidation in other studies (7). As shown in **Figure 5**, 40 μ M of cumene peroxide greatly increased lipid oxidation in two aspects: first, the overall starting PV values for all treatments were much higher compared to the results without cumene as shown in **Figure 3**, and second, PV development was much faster and the values were much higher at lower temperature (37 °C) for shorter time than those treatments without cumene (60 °C). **Figure 5** also shows that all treatments with cupric ion addition had much

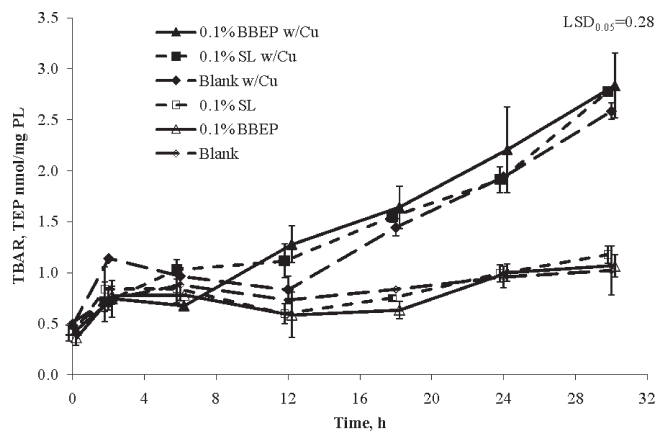


Figure 6. TBARS development of egg phospholipid in liposome at 37 °C in the presence of cumene hydroperoxide (40 μ M in liposome). Error bars represent SD from duplicate preparations. The concentration of SL and BBEP is 1000 ppm based on EPL. For treatment abbreviations, refer to the footnotes of **Tables 1** and **2**.

higher PVs than those of noncupric ion treatments. This observation is different from what was found in the absence of cumene hydroperoxide. Lower temperature (37 °C) might have delayed the decomposition of hydroperoxide, whereas cupric ion may have accelerated the release of free radical from cumene hydroperoxide, therefore greatly accelerating lipid oxidation.

Different from the experiment conducted at 60 °C without cumene hydroperoxide, 1000 ppm of SL was also added to this experiment. It was found that it was the presence of cupric ion that mainly contributed to the higher PV development, and types of phospholipids did not. Data in **Figure 5** also indicate that BBEP or SL acted neither as a pro-oxidant nor as an antioxidant in the cumene hydroperoxide induced oxidation system. For the other three treatments with 10% addition of SL, BBEP, and EPL to liposome, no significant differences were found among the PL types either. All three treatments showed trends similar to those of 1000 ppm of treatments (data not shown).

TBARS development for those cumene-added treatments is presented in **Figure 6**. The same trend as in **Figure 4** was found, except that the addition of cumene peroxide accelerated the formation of TBARS and cupric ion was still the dominant factor contributing to the higher TBARS. The type of PL (SL and BBEP) had no impact on TBARS development. The strong oxidizing effect of cumene hydroperoxide might have masked any difference between the types of PLs.

Antioxidant or Pro-oxidant? Food antioxidants can be categorized into two classes based on their antioxidant mechanisms: e.g., type I and type II. From the literature, plasmalogens were thought to be a primary antioxidant (type I). Like tocopherols and polyphenols, they scavenge free radicals and prevent free radicals from attacking double bonds. Phenolic compounds are usually good proton donors, and the phenolic ring structures make themselves resonance-stable upon losing the proton radicals. In reality, plasmalogens do not possess such structural characteristics. On the contrary, its vinyl ether bond is much more sensitive to oxidative attack (11). Our observations in bulk oil confirmed that it was BBEP, not PL, that accelerated the lipid oxidation. Other studies also described the decomposed products, α -hydroxyaldehydes, from EthPm oxidation being reactive compounds (14, 15). Felde and Spittler (15) used human serum lipoproteins, which contain significant levels of EthPm, and proposed EthPm oxidation by lipid peroxy radicals (LOO \cdot) through an epoxide-mediated mechanism at the vinyl double bond. Other researchers (17, 34) found that EthPm oxidation

mainly produced α -hydroxyaldehydes of 16:0 and 18:0 rather than aldehydes of 16:0 and 18:0 following a Fe^{2+} /ascorbate oxidation by using rat brain homogenates. The highly reactive hydroxyaldehydes further reacted with the amine group in PE or EthPm to produce Schiff base adducts. Further controversy also might arise from the Schiff base adducts, because these Schiff base/Maillard reaction products can either be antioxidants, as reviewed by Zamora and Hidalgo (35), or be responsible for deleterious effects on cells (17). It is possible that EthPm may act differently in vivo than in vitro, as the environments can be very different.

Other studies (5–7) indicated that EthPm may act as a secondary antioxidant (type II), such as a chelating agent, like EDTA, phosphoric acid, and citric acid. This class of antioxidants usually chelates certain transition-metal ions by sterically hindering formation of the metal hydroperoxide complex or precipitating metal ion from the lipid systems (36). Structurally this class of compounds all have bi- or multidentate ligands containing multiple O, P, or N atoms. It is theoretically doubtful that vinyl ether bonds have such chelating properties. More study is needed to validate this hypothesis. Furthermore, some of the plasmalogen oxidation studies employed a significant level of azo type compound as a free radical initiator. Such practices and validity of results may be questionable.

Considering the fact that the cupric ion accelerated the lipid oxidation in the presence of EthPm, we see the oxidation mechanism as: vinyl ether bond of BBEP is first oxidized because of its low activation energy followed by the formation of epoxide or peroxide. Cupric ion further promotes the breakdown of epoxide and peroxide with the formation of more free radicals that propagate further lipid oxidation.

ABBREVIATIONS USED

^{31}P NMR, phosphorus-31 nuclear magnetic resonance; AA, arachidonic acid; ANOVA, analysis of variance; AOCS, American Oil Chemists' Society; BBEP, bovine brain ethanolamine plasmalogens; BHT, butylated hydroxytoluene; CSO, commercial soybean oil; DHA, docosahexaenoic acid; EDTA, ethylenediaminetetraacetic acid; EPL, egg phospholipids; EthPm, ethanolamine plasmalogen; FAME, fatty acid methyl ester; FFA, free fatty acid; GC, gas chromatography; GC-MS, gas chromatography–mass spectrometry; LDL, low-density lipoprotein; $\text{LSD}_{0.05}$, least significant difference at $P < 0.05$; mequiv, milliequivalent; OSI, oxidative stability index; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; ppm, parts per million; PSO, purified soybean oil; PUFA, polyunsaturated fatty acids; PV, peroxide value; ROS, reactive oxygen species; SL, soy lecithin; TBARS, thiobarbituric acid reactive substances; TLC, thin-layer chromatography; TPP, triphenyl phosphite.

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