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Changes Produced in the Antioxidative Activity of Phospholipids as a Consequence of Their Oxidation

FRANCISCO J. HIDALGO, FÁTIMA NOGALES, AND ROSARIO ZAMORA*

Instituto de la Grasa, CSIC, Avenida Padre García Tejero 4, 41012 Seville, Spain

The antioxidative activities of native and oxidized soybean phosphatidylcholine (PC), phosphatidylthanolamine (PE), and phosphatidylinositol (PI) in the protection of soybean oil heated in the dark under air at 60 °C were studied in an attempt to clarify the consequences that phospholipid oxidation has on antioxidative activities. The three native phospholipids protected the oil when assayed at 200 ppm, and phospholipid oxidation decreased the antioxidative activity of both PC and PI. However, slightly oxidized PE was more antioxidative than native PE, most likely as a consequence of the formation by amino−carbonyl reactions of pyrrolized phospholipids, which were determined and for which antioxidative properties are known. Nevertheless, further increases in PE oxidation produced a decrease in its antioxidative activity. These results suggest that two opposite reactions are competing in the antioxidative activity of amino phospholipids upon oxidation: fatty acid chain oxidation, which decreases phospholipid antioxidative activity, and amino−carbonyl reactions, which produce derivatives with antioxidant properties. This last property may be useful to increase the antioxidative activity of commercial lecithins containing amino phospholipids.

KEYWORDS: Amino−carbonyl reactions; lipid oxidation; natural antioxidants; nonenzymatic browning; phospholipids; pyrrolized phospholipids

INTRODUCTION

Antioxidative properties of phospholipids have been demonstrated through their addition to processed vegetable oils and animal fats (*1*, *2*). These properties have been proposed to be a consequence in (i) synergism between phospholipids and tocopherol (*3*, *4*); (ii) chelation of pro-oxidant metals by phosphate groups (*5*, *6*); (iii) formation of Maillard-type products between amino phospholipids and oxidation products (*7*); and (iv) action as an oxygen barrier between oil and air interfaces (*8*). However, phospholipids are also very easily oxidized (*9*, *10*), and these oxidative processes are likely to modify some of the phospholipid properties responsible for their antioxidative activities. To our knowledge, no previous studies have been undertaken to determine the changes produced in phospholipid antioxidant properties following oxidation.

In an attempt to clarify the consequences of oxidative processes in phospholipids, the present study determines the antioxidative activities of the three major phospholipids of soybean lecithin when added to soybean oil in both their native forms and after oxidation. In addition, the nonenzymatic browning of phospholipids with oxidation was also studied because of the well-known contribution of phospholipids to nonenzymatic food browning (*11*, *12*) and the formation in these reactions of heterocyclic residues (*13*) with antioxidant properties (*14*, *15*).

EXPERIMENTAL PROCEDURES

Materials. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were isolated from soybean lecithin by column chromatography on silicic acid/silica gel (1:1) using chloroform/methanol (6:1), (9:1), and (1:1), respectively, as eluent. The identity and purity of the obtained phospholipids were confirmed by HPLC and ^{1}H and ^{13}C NMR spectroscopy. Each phospholipid exhibited a single peak in HPLC (*13*) and the characteristic ^{1}H and ^{13}C signals of their polar heads (*16*). The fatty acid composition of PC was as follows: palmitic acid (3.6%), stearic acid (2.3%), oleic acid (8.3%), linoleic acid (75.9), and linolenic acid (9.9%). The fatty acid composition of PE was as follows: palmitic acid (19.8%), stearic acid (3.2%), oleic acid (7.6%), linoleic acid (61.6), and linolenic acid (7.8%). The fatty acid composition of PI was as follows: palmitic acid (29.6%), stearic acid (6.5%), oleic acid (8.4%), linoleic acid (47.7), and linolenic acid (7.8%).

Refined soybean oil was obtained from our Institute's pilot plant (Instituto de la Grasa, CSIC, Seville, Spain). 2-Thiobarbituric acid monohydrate was purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents and solvents used were of analytical grade and were purchased from reliable commercial sources.

Phospholipid Oxidation. The peroxidation of the phospholipids was achieved as described previously (*11*). Briefly, the phospholipid solution was evaporated to dryness under vacuum so that the phospholipid formed a thin layer on the inside surface of a round-bottom flask, which was then incubated under air in the dark at 60 °C.

Peroxidation was evaluated by using the thiobarbituric acid-reactive substances (TBARS) assay as described by Kosugi et al. (*17*), which was slightly modified. Briefly, samples were diluted with water (600 μL) and treated with 1.1 mL of acetic acid (20% solution, pH 3.5), 1.3 mL of thiobarbituric acid (0.71% solution), and 40 μL of BHT (0.8% solution in acetic acid). Solutions were heated at 100 °C for 60 min, then cooled, and, finally, extracted with 3 mL of *n*-butyl alcohol. Organic layers were separated by centrifugation, and TBARS were determined by fluorescence using $\lambda_{ex} = 535$ nm and $\lambda_{em} = 550$ nm.

* Author to whom correspondence should be addressed [telephone +(34) 954 611 550; fax +(34) 954 616 790; e-mail rzamora@ig.csic.es].

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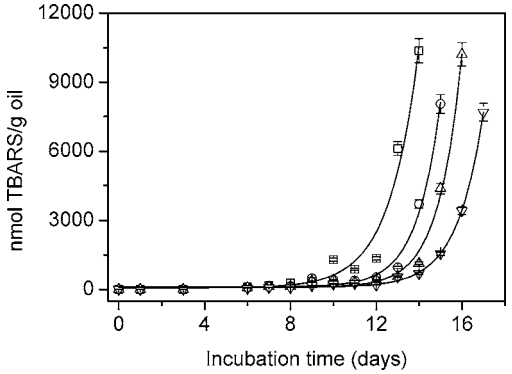


Figure 1. Effect of native soybean PE (○), oxidized PE (△), and BHT (▽) added at 200 ppm on soybean oil oxidation (□) measured as TBARS formation.

Phospholipid pyrrolization, which was determined as described previously (*18*), was employed as an index of nonenzymatic browning development (*19*). Briefly, samples were diluted with 150 mM sodium phosphate, pH 7.0 (1 mL), containing 3% of sodium dodecyl sulfate, and treated with 160 μL of Ehrlich reagent [the reagent was prepared by suspending 200 mg of *p*-(dimethylamino)benzaldehyde in 2 mL of ethanol and adding 8 mL of 1.25 N HCl]. The resulting solution was incubated at 45 °C for 30 min. The absorbance of the maximum at ~570 nm was measured in the next 4 h against a blank prepared under the same conditions but without *p*-(dimethylamino)benzaldehyde.

Mesurement of Antioxidative Activity. Soybean oil with no phospholipids was compared with samples containing native or oxidized phospholipids or BHT, which were dissolved in the oil at 200 ppm. Oil samples (10 g) were weighed into 90 × 20 mm Petri dishes and oxidized for 18 days under air in the dark at 60 °C. Peroxidation was evaluated by using the TBARS assay as described above. For comparison purposes, both induction periods (IPs) and protection indexes (PInds) were employed. IPs were determined (in hours) by using the method of tangents to the two parts of the kinetic curve. PInds were determined, at the end of the incubation period, according to the following equation (*20*):

$$\text{PInd} = 100 - [100 \times (\text{IP sample} - \text{IP BHT})/(\text{IP oil} - \text{IP BHT})]$$

PInd is the percentage of protection of the tested antioxidant in relation to BHT. Thus, a PInd equal to 100% meant that the compound tested was as effective as BHT. PInd equal to 0% meant that the compound tested had no protective effect. A PInd < 0 meant that the tested compound had a pro-oxidant effect.

Statistical Analyses. TBARS and pyrrole determinations are expressed as mean ± standard deviation (SD) values of three experiments. IPs were calculated from the mean kinetic curves of the three experiments. Statistical comparisons among different groups were made using ANOVA. When significant *F* values were obtained, group differences were evaluated according to the Student−Newman−Keuls test (*21*). All statistical procedures were carried out using *Primer of Biostatistics: The Program* (*22*). The significance level was $p < 0.05$ unless otherwise indicated.

RESULTS

Changes in Phospholipid Antioxidative Activities Following Oxidation. As expected, the addition of 200 ppm of PC, PE, or PI to a soybean oil increased the IP of the oil when heated under air in the dark at 60 °C. **Figure 1** shows the typical curves obtained for the TBARS determined in the oil with or without the addition of the phospholipids. The IPs calculated for the oil treated with the different phospholipids assayed are collected in **Figure 2**. Although the three phospholipids protected the oil, this protection was more effective for PC (PInd = 58.8%) than for PE (PInd = 45.2%), and PI exhibited only a small protection (PInd = 5.2%).

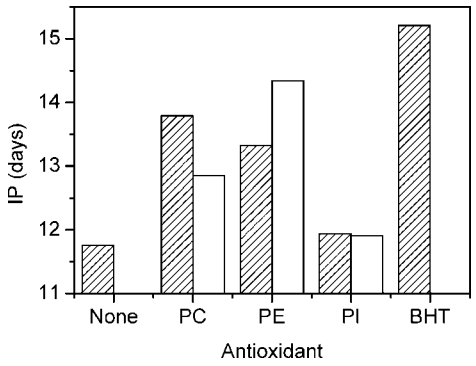


Figure 2. IP of soybean oil heated under air in the dark at 60 °C and not treated (control) or treated with 200 ppm of PC, PE, PI, and BHT. IPs were determined for both native (slashed bars) and oxidized phospholipids (open bars).

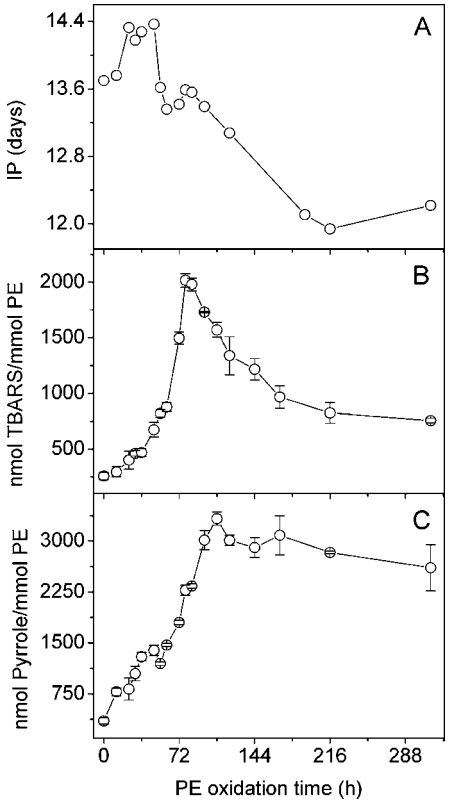
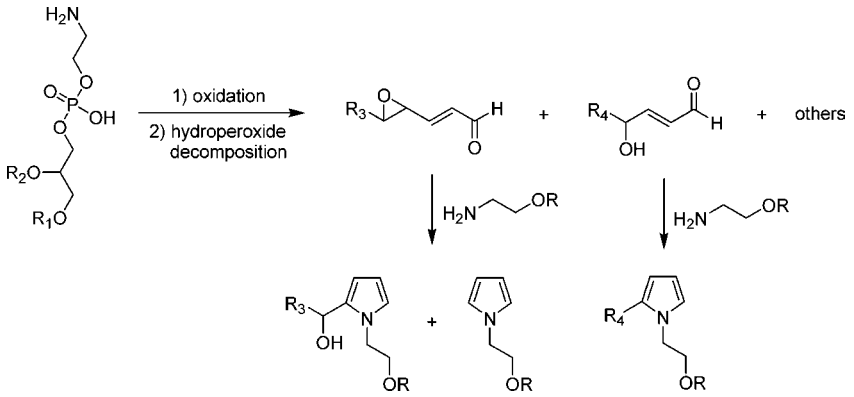


Figure 3. Effect of PE oxidation time on (**A**) IP of a soybean oil heated under air in the dark at 60 °C and treated with 200 ppm of the phospholipid, (**B**) TBARS formation, and (**C**) formation of pyrrole derivatives.

This phospholipid protection changed when phospholipids were oxidized (**Figure 2**). Thus, the protection offered by either oxidized PC or PI decreased with oxidation (the PInds for PC and PI oxidized for 96 h were 31.6 and 4.3%, respectively). On the other hand, slightly oxidized PE was more antioxidant than native PE. Thus, the PInd determined for an oxidized PE having 674 ± 66 nmol of TBARS/mmol of PE was 74.8%.

Effect of Oxidation Degree on Antioxidative Activity of PE. In contrast to PC and PI, for which the antioxidative activity decreased with oxidation, the antioxidative activity of PE depended on its oxidation degree (**Figure 3A**). Thus, the native PE antioxidative activity (PInd ~ 45%) was maintained during the first 12 h of oxidation at 60 °C and, then, increased to PInd ~ 73%. This increased protection was observed between 24 and 48 h of oxidation and, then, decreased to a PInd analogous to that of native PE (between 54 and 96 h of oxidation). A

Scheme 1. Production of Reactive Carbonyls during PE Oxidation and the Later Formation of Pyrrolized Phospholipids by Amino–Carbonyl Reactions[a]



[a] R is the phospholipid without the polar head; R_1 and R_2 are fatty acid chains; and R_3 and R_4 are alkyl chains.

further oxidation of PE decreased its antioxidative activity, which was almost null (PInd = 5−10%) after >192 h of oxidation.

The oxidation degree of PE as a function of oxidation time is collected in **Figure 3B**. TBARS increased exponentially during the first 78 h ($r^2 = 0.994$) and, then, decreased also in an exponential way ($r^2 = 0.992$). There was not a relationship between the antioxidative activity of PE and its oxidation degree during the first 78 h. However, after that, both IP and TBARS decreased in parallel ($r = 0.972$, $p < 0.0001$). The increase in the PE antioxidative activity was produced in the first steps of the oxidative process, when TBARS values were in the range of 400−675 nmol of TBARS/mmol of PE.

Phospholipid fatty acid chain oxidation produces reactive carbonyls, which are able to react with the primary amino group of either native or oxidized PE, producing pyrrolized phospholipids (**Scheme 1**) (*13*, *18*). Therefore, PE pyrrolization should be expected as a function of oxidation time (**Figure 3C**). In contrast to TBARS production, an almost linear increase ($r = 0.96$, $p < 0.0001$) of pyrrolized PE was observed for the first 108 h of oxidation, and this was followed by a slow decrease. The maximum values of PE antioxidative activity were observed when the pyrrole content was in the range of 800−1400 nmol of pyrrole/mmol of PE.

DISCUSSION

An antioxidant is usually defined as any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substance (*23*). However, during its antioxidative action, antioxidants are degraded and their antioxidative properties cease. This is particularly important for phospholipids, which are composed of highly unsaturated fatty acid chains in addition to the polar heads responsible for their antioxidative properties. These fatty acid chains are easily oxidized, and this oxidation is likely to produce significant changes in the phospholipid antioxidative properties.

The results obtained in this study show that PC and PI oxidation, and more likely also the oxidation of other phospholipids with no primary amino groups, decreased their antioxidative activity. However, when there was a primary amino group in the phospholipid structure, as has been shown for PE and should also be expected for the analogous phosphatidylserine, the formation of heterocyclic residues with antioxidative properties was observed. This reaction increased the antioxidative properties of PE. Nevertheless, this increase was produced only during the first steps of oxidation, therefore suggesting that in amino phospholipids, two types of reactions are competing: the decrease in antioxidative activity produced as a consequence of fatty acid chain oxidation and the increase in antioxidative activity produced as a consequence of amino−carbonyl reactions.

All of these results confirm the complexity of the antioxidative activity of phospholipids, which seems to be the result of diverse phospholipid properties. Among them, the ability of amino phospholipids to produce heterocyclic residues with antioxidative properties should be considered. This ability can be employed, for example, to increase the antioxidative activity of lecithins that are usually employed as food additives, analogously to the increase in the stability observed for lipid/protein samples submitted to slight oxidation (*24*).

ABBREVIATIONS USED

IP, induction period; PC, soybean phosphatidylcholine; PE, soybean phosphatidylethanolamine; PI, soybean phosphatidylinositol; PInd, protection index; SD, standard deviation; TBARS, thiobarbituric acid-reactive substances.

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LITERATURE CITED

(1) List, G. R.; Friedrich, J. P. Oxidative stability of seed oils extracted with supercritical carbon dioxide. *J. Am. Oil Chem. Soc.* **1989**, *66*, 98−101.

(2) King, M. F.; Boyd, L. C.; Sheldon, B. W. Antioxidant properties of individual phospholipids in a salmon oil model system. *J. Am. Oil Chem. Soc.* **1992**, *69*, 545−551.

(3) Koga, T.; Terao, J. Phospholipids increase radical-scavenging activity of vitamin E in a bulk oil model system. *J. Agric. Food Chem.* **1995**, *43*, 1450−1454.

(4) Judde, A.; Villeneuve, P.; Rossignol-Castera, A.; le Guillou, A. Antioxidant effect of soy lecithins on vegetable oil stability and their synergism with tocopherols. *J. Am. Oil Chem. Soc.* **2003**, *80*, 1209−1215.

(5) Privett, O. S.; Quackenbush, F. W. Reaction of phosphoric acid in the autoxidation of fats. *J. Am. Oil Chem. Soc.* **1954**, *31*, 225−227.

(6) Jewell, N. E.; Nawar, W. W. Thermal oxidation of phospholipids. 1,2-Dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine. *J. Am. Oil Chem. Soc.* **1980**, *57*, 398−402.

(7) Husain, S. R.; Terao, J.; Matsushita, S. Comparison of phosphatidylcholine and phosphatidylethanolamine affecting the browning reaction of heated oil. In *Amino−Carbonyl Reactions in Food and Biological Systems*; Fugimaki, M., Namiki, M., Kato, H., Eds.; Elsevier: New York, 1984; pp 301−309.

(8) Porter, W. L. Recent trends in food applications of antioxidants. In *Autoxidation in Food and Biological Systems*; Simic, M. G., Karel, M., Eds.; Plenum Press: New York, 1980; pp 295−365.
(9) Passi, S.; Ricci, R.; Cataudella, S.; Ferrante, I.; de Simone, F.; Rastrelli, L. Fatty acid pattern, oxidation product development, and antioxidant loss in muscle tissue of rainbow trout and *Dicentrarchus labrax* during growth. *J. Agric. Food Chem.* **2004**, *52*, 2587−2592.
(10) Undeland, I.; Kristinsson, H. G.; Hultin, H. O. Hemoglobin-mediated oxidation of washed minced cod muscle phospholipids: effect of pH and hemoglobin source. *J. Agric. Food Chem.* **2004**, *52*, 4444−4451.
(11) Hidalgo, F. J.; Zamora, R.; Alaiz, M.; Maza, M. P.; Millán, F.; Vioque, E. Non-enzymatic browning reactions of phospholipids. *Fat Sci. Technol.* **1990**, *92*, 185−188.
(12) Zamora, R.; Olmo, C.; Navarro, J. L.; Hidalgo, F. J. Contribution of phospholipid pyrrolization to the color reversion produced during deodorization of poorly degummed vegetable oils. *J. Agric. Food Chem.* **2004**, *52*, 4166−4171.
(13) Zamora, R.; Hidalgo, F. J. Phosphatidylethanolamine modification by oxidative stress product 4,5(*E*)-epoxy-2(*E*)-heptenal. *Chem. Res. Toxicol.* **2003**, *16*, 1632−1641.
(14) Alaiz, M.; Zamora, R.; Hidalgo, F. J. Antioxidative activity of pyrrole, imidazole, dihydropyridine, and pyridinium salt derivatives produced in oxidized lipid/amino acid browning reactions. *J. Agric. Food Chem.* **1996**, *44*, 686−691.
(15) Hidalgo, F. J.; Nogales, F.; Zamora, R. Effect of pyrrole polymerization mechanism on the antioxidative activity of nonenzymatic browning reactions. *J. Agric. Food Chem.* **2003**, *51*, 5703−5708.
(16) Gunstone, F. D. High resolution ^{13}C NMR spectroscopy of lipids. In *Advances in Lipid Methodology−Two*; Christie, W. W., Ed.; The Oily Press: Dundee, Scotland, 1993; pp 1−68.
(17) Kosugi, H.; Kojima, T.; Kikugawa, K. Thiobarbituric acid-reactive substances from peroxidized lipids. *Lipids* **1989**, *24*, 873−881.
(18) Hidalgo, F. J.; Nogales, F.; Zamora, R. Determination of pyrrolized phospholipids in oxidized phospholipid vesicles and lipoproteins. *Anal. Biochem.* **2004**, *334*, 155−163.
(19) Zamora, R.; Alaiz, M.; Hidalgo, F. J. Contribution of pyrrole formation and polymerization to the nonenzymatic browning produced by amino−carbonyl reactions. *J. Agric. Food Chem.* **2000**, *48*, 3152−3158.
(20) Alaiz, M.; Hidalgo, F. J.; Zamora, R. Effect of pH and temperature on comparative antioxidant activity of nonenzymatically browned proteins produced by reaction with oxidized lipids and carbohydrates. *J. Agric. Food Chem.* **1999**, *47*, 748−752.
(21) Snedecor, G. W.; Cochran, W. G. *Statistical Methods*, 7th ed.; Iowa State University Press: Ames, IA, 1980.
(22) Glantz, S. A. *Primer of Biostatistics: The Program*; McGraw-Hill: New York, 1997.
(23) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 2nd ed.; Clarendon Press: Oxford, U.K., 1989; p 236.
(24) Alaiz, M.; Hidalgo, F. J.; Zamora, R. Effect of initial slight oxidation on stability of polyunsaturated fatty acid/protein mixtures under controlled atmospheres. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1127−1133.

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