Antioxidative Activity of Lysine/ 13-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic Acid Reaction Products

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The antioxidative activity of the reaction products formed in the reaction between 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-LOOH) and L-lysine was evaluated to determine if the reaction of lipid hydroperoxides with amino acids produces compounds that are able to protect lipids against oxidation. A reaction mixture of 13-LOOH and lysine was incubated for 8 days at 37 °C and, then, fractionated chromatographically by using silica cartridges. The four fractions obtained in this way were added at two levels of concentration (100 and 200 ppm) to soybean oil, which was oxidized under air in the dark at 60 °C. Oil peroxidation was evaluated by using the thiobarbituric acid-reactive substances (TBARS) assay. The less polar fraction obtained, which was eluted with 2-propanol, did not decrease TBARS production in the soybean oil. However, the other three fractions were able to significantly ($P < 0.05$) reduce TBARS production, suggesting that the reaction of lipid hydroperoxides with amino acids produces compounds with antioxidative properties. These reactions may contribute to a decrease of the lipid peroxidation process in foods containing proteins.

Keywords: *Antioxidants; nonenzymatic browning; lipid peroxidation; lysine modification; soybean oil*

INTRODUCTION

Lipid oxidation is a complicated process leading to the formation of many compounds (Gardner, 1989; Kubow, 1992). It is one of the major causes of food spoilage and is undesirable not only from an aesthetic and economic point of view but also because oxidative reactions can decrease the nutritional quality of foods and generate oxidation products that are potentially toxic (Eriksson, 1987; Nawar, 1985). In addition, lipid oxidation products seem to be directly involved in the development of many physical disorders including coronary artery diseases, atherosclerosis, and cancer, as well as the aging process (Rice-Evans and Burdon, 1993).

Nowadays, antioxidants are commonly used as food additives to extend the shelf life of oils and fatty foods during storage and processing. The antioxidants to be used are determined by various factors including legislation, effectiveness, and cost. In addition, consumer preference for natural additives has encouraged the development of natural antioxidants. Thus, much research has been conducted to find safe antioxidants with high activity from natural resources (Nishiyama et al., 1993; Kanner et al., 1994). Furthermore, other studies have been dedicated to isolate and characterize components, normally present in foods, that possess antioxidative properties. In this context, the antioxidative properties of Maillard reaction products have long been known (Lingnert and Eriksson, 1981; Ledl and Schleicher, 1990) and, more recently, also the antioxidative activity of the products of reaction between some oxidized lipids and amino acids (Zamora and Hidalgo, 1993).

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The reaction of (*E*)-2-octenal with lysine or histidine produced different compounds that exhibited antioxidative properties (Alaiz et al., 1995a). Analogous results were obtained with (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction products (Alaiz et al., 1995b), and this protective effect seems to be general for most of the heterocyclic derivatives produced in the reaction between short-chain aldehydes and amino acids (Alaiz et al., 1996). In addition, these compounds have also been shown to delay the peroxidation initiated in a soybean oil, suggesting that these compounds could be acting at the same time that they are being produced when the lipid peroxidation process occurs in the presence of proteins (Alaiz et al., 1995c). However, all of these studies have involved aldehydes, which are only a minor fraction of the lipid peroxidation products, and no studies have been dedicated to the major products of lipid peroxidation.

In a search for new antioxidants that are normally produced in foods, the present study has evaluated for antioxidative activity several fractions of the compounds produced in the reaction between 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-LOOH) and L-lysine. The objective of this study is to find cheap antioxidants that can be easily prepared. Therefore, we did not attempt to isolate and characterize individual components in these mixtures.

EXPERIMENTAL PROCEDURES

Materials. Soybean oil was obtained from our Institute's pilot plant (Institute de la Grasa, CSIC, Sevilla, Spain). Linoleic acid and soybean lipoxygenase were purchased from Fluka Chemie AG (Buchs, Switzerland). 2-Thiobarbituric acid monohydrate (TBA) was purchased from Merck (Darmstadt, Germany). L-Lysine, butylated hydroxytoluene (BHT), and *n*-propyl gallate were purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents and solvents used were of analytical grade and were purchased from reliable commercial sources.

13-LOOH Synthesis. The oxidation of linoleic acid with soybean lipoxygenase was carried out according to the proce-

S0021-8561(96)00126-4 CCC: $12.00

dure described by Hidalgo et al. (1992), using the modification reported by Zamora and Hidalgo (1995). The purity of the obtained 13-LOOH was tested by TLC with hexane/diethyl ether/acetic acid (50:50:1) as eluent. For most experiments, the hydroperoxide obtained was pure and was used without further purification.

Reaction between 13-LOOH and Lysine. A solution of 13-LOOH (93 mg, 0.3 mmol) in 4.5 mL of acetonitrile/water (2:1) was oxygenated for a few minutes and, then, treated with L-lysine (86.5 mg, 0.6 mmol). The reaction mixture was incubated for 8 days at 37 °C. The reaction color changed from colorless to light brown as a function of the incubation time until no further browning was observed at the end of the incubation period. The reaction mixture was then evaporated, dissolved in 4.5 mL of methanol/2-propanol (1:1), and fractionated chromatographically by using 3 mL Baker silica cartridges (J. T. Baker Inc., Phillipsburg, NJ). The chromatographic procedure consisted of passing 500 μL of the reaction mixture through the silica cartridge. The first fraction (fraction I) was eluted with 3 mL of 2-propanol, and, subsequently, three other fractions were obtained with increasing polarity of the solvent. Fraction II was eluted with 3 mL of 2-propanol/water (9:1), fraction III was eluted with 3 mL of 2-propanol/water (8:2), and fraction IV was eluted with 3 mL of water. This sequence of elution was used repeatedly until the whole reaction mixture was fractionated. The yields obtained for fractions I−IV were 41, 27.5, 22.9, and 71.9 mg, respectively.

Fractions I−IV were analyzed by high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE) to determine the presence of lysine. The HPLC system consisted of a 126 programmable delivery module and a 168 diode array detector module (Beckman, Fullerton, CA). Lysine was determined on a 300 × 3.9 mm i.d. reversed-phase column (Nova-Pack C_{18}, 4 μm; Waters, Milford, MA) using a previously described gradient for amino acid analysis (Alaiz et al., 1992). The HPCE system consisted of a Beckman 5000 P/ACE unit equipped with a UV detector. Lysine was determined by micellar electrokinetic capillary chromatography according to the method of Zamora et al. (1995).

Measurement of Antioxidative Activity. Oxidative stability of refined soybean oil with no antioxidant added was compared with oil samples containing fractions I−IV, BHT, *n*-propyl gallate, or L-lysine added at concentrations of 100−200 ppm. Oil samples (10 g) were weighed into 90 × 20 mm Petri dishes and oxidized for 216 h under air in the dark at 60 °C. Peroxidation was evaluated periodically by using the thiobarbituric acid-reactive substances (TBARS) assay as described by Kosugi et al. (1989). For comparison purposes a protection index (PI) was defined according to the following equation:

$$PI = 100 - [100 \times (\text{TBARS sample} - \text{TBARS gallate})/(\text{TBARS oil} - \text{TBARS gallate})]$$

PI equal to 100 meant that the compound tested was as effective as gallate. PI equal to 0 meant that the compound tested had no protective effect. A PI < 0 meant that the compound tested had a prooxidant effect. This index cannot be applied at the initial time because TBARS of oil should be higher than TBARS of gallate. PI values used in this study were calculated after an incubation of 192 h. This incubation time gave TBARS values similar to those obtained in previous studies (Alaiz et al., 1995c), and, therefore, the calculated PI values were comparable with the PI values obtained in those studies.

Statistical Analysis. All of the results are means of three replicates. Statistical comparisons between several groups were made using ANOVA. When significant *F* values were obtained, group differences were evaluated by the Student-Newman-Keuls test (Snedecor and Cochran, 1980). All statistical procedures were carried out using Primer of Biostatistics, The Program (McGraw-Hill, New York). Significance level is $P < 0.05$ unless otherwise indicated.

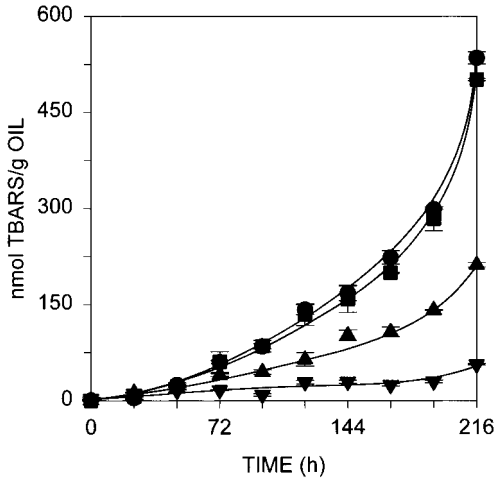

Figure 1. Effects of fraction I at 100 ppm (■), BHT at 200 ppm (▲), and propyl gallate at 200 ppm (▼), on soybean oil oxidation (●) measured as TBARS formation. Results represent the mean ± SD of three assays.

RESULTS

The refined soybean oil used in this study was stored in a freezer prior to its use; it had 0.56 nmol of TBARS/g of oil at the initial time, suggesting an insignificant peroxidation in the oil. At this time, the four isolated fractions, BHT, and propyl gallate were added, and TBARS production was determined every 24 h for the whole incubation period. Figure 1 shows TBARS production in untreated oil and in the oil treated with fraction I, added at 100 ppm, and with BHT and propyl gallate, added at 200 ppm. Incubation of untreated oil at 60 °C showed a gradual increase in TBARS production for the first 192 h and, then, it experienced a sudden increase. This behavior was not modified by the addition of fraction I at 100 ppm. Therefore, fraction I, which contained only traces of lysine according to HPLC and HPCE analyses (data not shown), should be composed of products either with no antioxidative activities or with inverse activities that produced a null result. On the contrary, addition of both BHT and propyl gallate at 200 ppm significantly reduced TBARS production in the oil. The protection exhibited by BHT was partial, and it was only able to delay the peroxidation process. On the contrary, propyl gallate stopped the process, and TBARS at the end of the incubation period were only slightly higher than those measured at the beginning. These results are in agreement with the general rule postulated by Porter (1993) that in foods of low surface-to-volume ratio (e.g., the soybean oil used) polar antioxidants, such as propyl gallate, are more effective than nonpolar antioxidants.

In contrast to fraction I, fractions II−IV significantly reduced TBARS production when they were added to the soybean oil at the two concentrations assayed. Figure 2 shows the TBARS production in the untreated oil and in the oil treated with fraction II at 100 and 200 ppm. This fraction, which also contained negligible amounts of lysine, exhibited antioxidative activity at the two concentrations assayed by decreasing TBARS production in the oil. This decrease was significant after only 48 h and was higher when a higher concentration of fraction II was tested. The PI obtained for this fraction after 192 h of incubation was 43 when it was added at 200 ppm (Table 1).

A smaller protection was observed when fraction III was tested. Figure 3 shows the TBARS production in

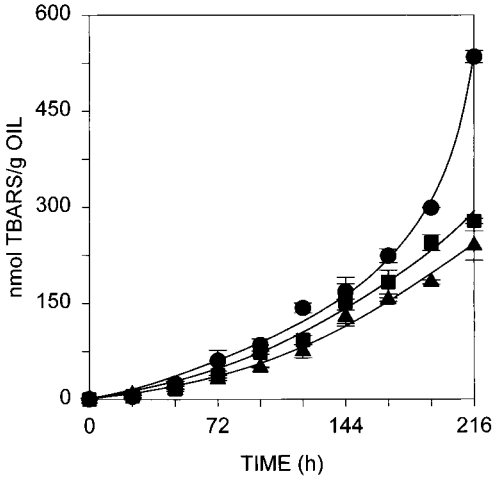

Figure 2. Effects of fraction II at 100 (■) and 200 ppm (▲) on soybean oil oxidation (●) measured as TBARS formation. Results represent the mean ± SD of three assays.

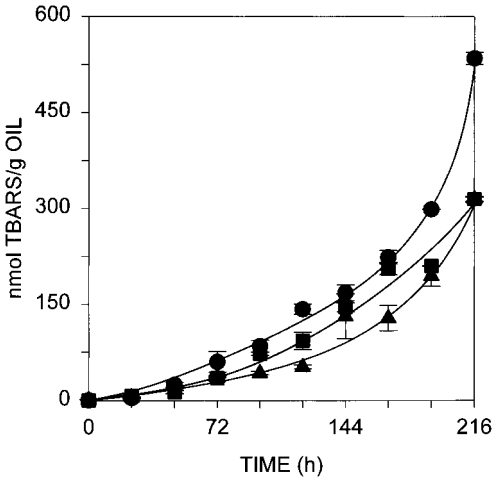

Figure 3. Effects of fraction III at 100 (■) and 200 ppm (▲) on soybean oil oxidation (●) measured as TBARS formation. Results represent the mean ± SD of three assays.

Table 1. Protection Index (PI) Calculated for the Fractions Obtained from a Lysine/ 13-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic Acid Reaction Mixture

fraction	PI	fraction	PI
I	none	IV	36
II	43	lysine[a]	28
III	39		

[a] Obtained from Alaiz et al. (1995b).

a soybean oil treated with 100 and 200 ppm of fraction III. Analogously to fraction II, the protection exhibited by fraction III was higher at 200 ppm than at 100 ppm. However, this difference was not significant at the end of the incubation period. After 192 h, the PI obtained for fraction III added at 200 ppm was 39. This protection was significantly smaller than that exhibited by fraction II. Fraction III was rich in lysine, and a part of its antioxidative activity was likely due to this amino acid. The PI calculated for lysine at 200 ppm in this system is 28 (Alaiz et al., 1995b).

Figure 4 shows the TBARS production in the soybean oil treated with 100 and 200 ppm of fraction IV. Analogously to the above fractions II and III, this fraction also exhibited antioxidative activity by decreasing TBARS production. This protection was higher at 200 ppm than at 100 ppm, and, after 192 h of incubation, the PI obtained for fraction IV was 36 when added at 200 ppm. This fraction was also rich in lysine, which, analogously to results the observed in fraction III, may contribute to the antioxidative activity of the fraction.

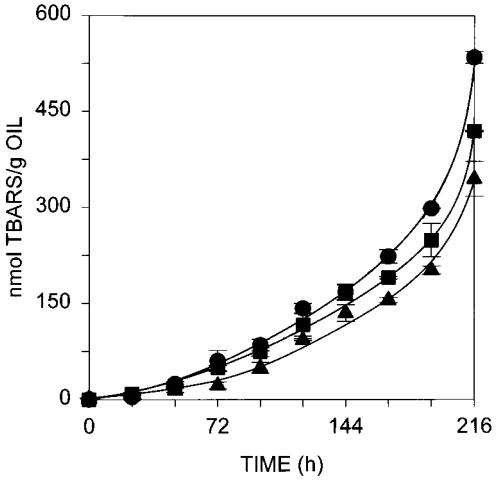

Figure 4. Effects of fraction IV at 100 (■) and 200 ppm (▲) on soybean oil oxidation (●) measured as thiobarbituric acid-reactive substances (TBARS) formation. Results represent the mean ± SD of three assays.

DISCUSSION

The results obtained in this study show that three fractions obtained from a 13-LOOH/lysine reaction mixture exhibited an antioxidative activity when added at 100 and 200 ppm to a soybean oil which was heated at 60 °C. These results are in agreement with previous results that showed an antioxidative activity of some oxidized lipid/amino acid reaction products (Zamora and Hidalgo, 1993; Alaiz et al., 1996) and extend the conclusions obtained in those studies to the major products of lipid oxidation.

These results also suggest that the antioxidative activity of oxidized lipid/amino acid reaction products are related to the polarity of the fractions. Fraction I, which was eluted with the less polar solvent and should include most of the unreacted oxidized lipids, did not show any antioxidative activity. On the contrary, an increase in the polarity of the solvent, which should elute more polar products such as those containing the amino acid residue, produced fractions that were able to decrease the TBARS produced in the heated oil.

The activities measured for fractions II−IV were different, but these results should be carefully analyzed because fractions III and IV include some unreacted lysine, and its presence may diminish the activity measured for the fraction (Alaiz et al., 1995a,b). Additional studies are needed to analyze the different compounds contained in these fractions and their relative contributions to the antioxidative activity observed. However, the results obtained in this study show it is not necessary to isolate individual components to obtain antioxidative activity in oxidized lipid/amino acid reaction products. An easy fractionation by polarity allowed us to obtain fractions that exhibited this antioxidative activity. These mixtures, which are produced naturally in foods during processing and storage (Zamora and Hidalgo, unpublished results), may play a role in the stability of stored or processed foods and might also be used as antioxidants to extend the shelf life of oils and fatty foods.

ACKNOWLEDGMENT

We are indebted to Dr. M. V. Ruíz-Méndez for providing the soybean oil used in this study and to Ms. M. D. García and Mr. J. L. Navarro for technical assistance.

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Received for review February 27, 1996. Revised manuscript received August 20, 1996. Accepted August 29, 1996.[⊗] This study was supported in part by the Comisión Interministerial de Ciencia y Tecnología of Spain (Project ALI94-0763) and the Junta de Andalucía (Project 2075). I.A. is recipient of a fellowship from the Ministerio de Educación y Ciencia of Spain (SAB94-0239).

JF9601269

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1996.