Antioxidative Activity of Nonenzymatically Browned Proteins Produced in Oxidized Lipid/Protein Reactions

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The antioxidative activity of nonenzymatically browned proteins was studied to analyze the contribution of oxidized lipid/amino acid reaction products (OLAARP) to the antioxidative activities observed for proteins and protein hydrolysates. Bovine serum albumin (BSA) was incubated overnight with (*E*)-4,5-epoxy-(*E*)-2-heptenal at 37 °C and later fractionated on Sephacryl S-200-HR. Two modified protein fractions were obtained, which corresponded to monomeric and dimeric modified BSA (MBSA and DBSA, respectively). Both MBSA and DBSA exhibited decreased basic amino acid residues and the presence of OLAARP residue ϵ-*N*-pyrrolylnorleucine. BSA, MBSA, DBSA, and butylated hydroxytoluene (BHT) (added at 10, 30, and 50 ppm) were tested for antioxidative activity in soybean oil using the thiobarbituric acid-reactive substances (TBARS) assay, and all of them significantly ($p < 0.05$) decreased TBARS formation. The order of effectiveness obtained was BSA < MBSA < DBSA < BHT and was parallel to the number of OLAARP residues in the protein. These results suggest that OLAARP formation conferred to the modified protein their antioxidative activities.

Keywords: *Oxidized lipid/protein reaction products; amino acid modification; antioxidative activity; nonenzymatic browning; lipid peroxidation*

INTRODUCTION

Analogous to peptides and amino acids, proteins and protein hydrolysates have been shown to have significant antioxidative properties (Rajalakshmi and Narasimhan, 1996). Thus, gluten, egg albumin, and casein were effective in safflower and sardine oil model systems (Taguchi et al., 1988). In addition, when used alone, 10% autolyzed yeast protein was equivalent to 0.02% butylated hydroxyanisole (BHA) in corn oil, but when combined with only 0.005% BHA, the total effect was as great as that of 50% protein isolate or twice that of 0.02% BHA (Hayes et al., 1977; Bishov and Henick, 1972). Therefore, proteins and protein hydrolysates seem to function as synergists or primary antioxidants.

However, recent studies from this laboratory have pointed out that amino acids, in addition to their function as primary antioxidants, have a much more important effect due to their ability to react with some lipid oxidation products to produce stable oxidized lipid/amino acid reaction products (OLAARP) with high antioxidative activities (Alaiz et al., 1995a, 1996a; Zamora and Hidalgo, 1993). This protective effect seems to be general for most of the heterocyclic derivatives produced in oxidized lipid/amino acid browning reactions (Alaiz et al., 1996b), and natural OLAARP have been used to protect vegetable oils against oxidation (Alaiz et al., 1995b). They have also been shown to delay the peroxidation initiated in a soybean oil, suggesting that OLAARP could be acting at the same time that they are being produced when the lipid peroxidation process occurs in the presence of amino acids (Alaiz et al., 1995c).

Analogously to amino acids, OLAARP formation should also be expected in proteins, because lipid peroxidation products are able to react with the protein reactive groups producing modified residues (Chio and Tappel, 1969; Gardner, 1979; Hidalgo and Kinsella, 1989). These modified residues should have significant antioxidative activities by analogy with the same derivatives produced in free amino acids, and, therefore, they should confer antioxidative properties to the modified proteins.

The objective of this study was to analyze if OLAARP formation in proteins might be contributing to the overall antioxidative activities observed for proteins and protein hydrolysates, by evaluating the different antioxidative activities exhibited by a model protein and the same protein modified by a lipid peroxidation product. (*E*)-4,5-Epoxy-(*E*)-2-heptenal (EH) was selected as the model lipid peroxidation product because of its high reactivity with free amino groups in amino acids and proteins (Zamora and Hidalgo, 1992; Zamora et al., 1995) and because the mechanisms involved in these reactions are now well-known (Hidalgo and Zamora, 1993; Zamora and Hidalgo, 1994).

EXPERIMENTAL PROCEDURES

Materials. Soybean oil was obtained from our Institute's Pilot Plant (Instituto de la Grasa, CSIC, Sevilla, Spain). EH was prepared from (*E*)-2-(*E*)-4-heptadienal analogously to (*E*)-4,5-epoxy-(*E*)-2-decenal (Zamora and Hidalgo, 1995). (*E*)-2-(*E*)-4-Heptadienal and 3-chloroperoxybenzoic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). 2-Thiobarbituric acid monohydrate was purchased from Merck (Darmstadt, Germany). Essentially fatty acid free bovine serum albumin (BSA), Sephacryl S-200-HR, PD-10 columns packed with Sephadex G-25 medium, butylated hydroxytoluene (BHT), β-amylase from sweet potato, alcohol dehydrogenase from yeast, soybean lipoxygenase, ovalbumin from chicken egg, and carbonic anhydrase from bovine erythrocytes 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.

Modified Protein Preparation. BSA (30 mg) was dissolved in 3 mL of 50 mM sodium phosphate buffer, pH 7.4, and treated with 10 mM EH. The mixture was allowed to

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S0021-8561(96)00698-X CCC: $14.00

react for 19 h at 37 °C, and then the brown solution was fractionated chromatographically on a Sephacryl S-200-HR column, previously calibrated with β-amylase (MW 200 000), alcohol dehydrogenase (MW 150 000), soybean lipoxygenase (MW 100 000), BSA (MW 66 000), ovalbumin (MW 45 000), and carbonic anhydrase (MW 29 000). One milliliter of the protein solution was injected in the column (1 × 56 cm) and eluted with 50 mM sodium phosphate buffer, pH 7.4, at a flow rate of 11 mL/h. Five hundred microliter fractions were collected and tested for protein by absorption at 280 nm. This sequence was used repeatedly until the whole sample was fractionated. Fractions corresponding to monomeric (MBSA) and dimeric (DBSA) modified BSA were pooled, desalted using a PD-10 column, and freeze-dried. This procedure yielded 16.2 mg of MBSA and 4.7 mg of DBSA. Both MBSA and DBSA were studied for amino acid composition, using a previously described procedure (Alaiz et al., 1992), and for ϵ-*N*-pyrrolylnorleucine formation (an OLAARP marker), as described previously (Zamora et al., 1995).

Measurement of Antioxidative Activity. Stripped soybean oil with no antioxidant was compared with samples containing BSA, MBSA, DBSA, and BHT, added at concentrations of 10, 30, and 50 ppm. Oil samples (10 g) were weighed into 90 × 20 mm Petri dishes and oxidized for 360 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, both induction period (IP) and protection index (PI) were used. IP was determined (in hours) by the method of tangents to the two parts of the kinetic curve. PI was defined according to the following equation:

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

PI equal to 100 meant that the compound tested was as effective as BHT. 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 can only be applied at $t > 0$, because TBARS of oil should be higher than TBARS of BHT.

Statistical Analysis. All antioxidative activity results are expressed as mean values of three experiments. Statistical comparisons between two groups were made using Student's *t*-test. With several groups, ANOVA was used. 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, Inc., New York). Significance level is $p < 0.05$ unless otherwise indicated.

RESULTS

Modified Protein Preparation. EH is a secondary oxidation product of $n-3$ fatty acid oxidation, and it is very reactive with amines and amino acids, producing a variety of pyrrole derivatives; some of these derviatives are able to polymerize, producing brown color and fluorescence (Hidalgo and Zamora, 1993; Zamora and Hidalgo, 1994). When EH was incubated in the presence of BSA, the solution rapidly turned brown and the modification of the protein was observed. Figure 1 shows the gel filtration fractionation on Sephacryl S-200-HR obtained for a BSA/EH incubation mixture. Two main fractions corresponding to MBSA and DBSA, respectively, were observed. Both fractions, which exhibited fluorescence at 440 nm when excitated at 350 nm (data not shown), were isolated and studied for amino acid composition.

In accordance with previous studies (Zamora et al., 1995), the reaction of EH with BSA only produced changes in basic amino acids, and, particularly, lysine. Table 1 shows the number of these residues that could be recovered after acid hydrolysis. Control BSA had

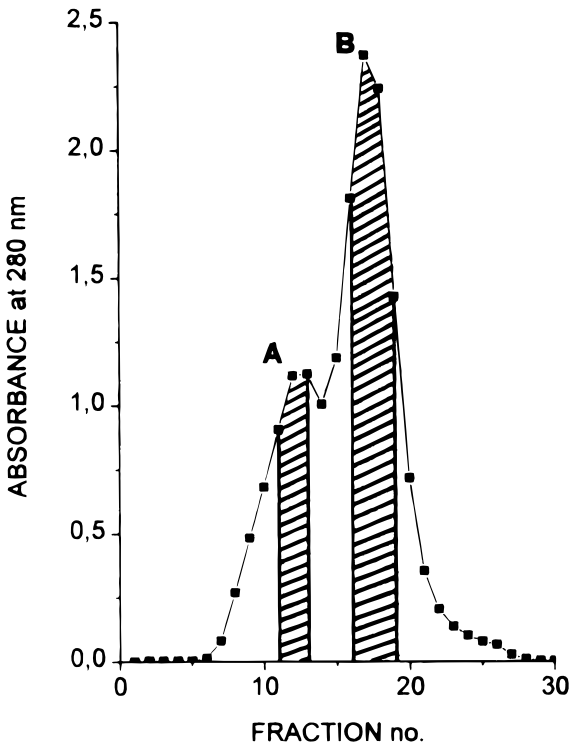

Figure 1. Gel filtration fractionation of an incubation mixture of EH and BSA on a Sephacryl S-200-HR. The column was equilibrated with 50 mM sodium phosphate buffer, pH 7.4, and proteins were eluted with the same buffer and monitored by UV at 280 nm. A drain of 15 mL was used prior to the collection. Two fractions were isolated, which mainly corresponded to (A) DBSA and (B) MBSA.

Table 1. Comparison of the Basic Amino Acid Residues of BSA, MBSA, and DBSA Determined by HPLC after Acid Hydrolysis

	number of residues to nearest integer		
amino acid	BSA (sequence)[a]	MBSA	DBSA[b]
histidine	17 (17)	15	17
arginine	23 (23)	22	21
lysine	56 (59)	40	38

[a] Obtained from Cheftel (1985). [b] Per half-molecule.

three fewer residues (5%) than expected according to the sequence. In addition, its treatment with EH produced an important decrease (30%) in this residue. This decrease was smaller for the other two basic amino acids (0−10%). Both MBSA and DBSA showed very similar amino acid compositions, with higher losses in lysine and arginine for DBSA and in histidine for MBSA.

Analogous results, with some significant differences, were obtained by high-performance capillary electrophoresis (HPCE) after basic hydrolysis. With the method employed (Zamora et al., 1995) only arginine and lysine could be determined, but this method allowed the determination of the OLAARP Pnl. After basic hydrolysis (Table 2), the BSA used in this study had only 753 μmol of lysine/g of protein (about 49 residues from the hypothetical 59). Its treatment with EH decreased lysine residues by 39% and arginine residues by 6−11%. On the contrary, the transformation of some lysine residues into Pnl was also observed. MBSA had 88 μmol of Pnl/g of protein (about 6 residues per molecule) and DBSA had 117 μmol of Pnl/g of protein (about 8 residues per half-molecule). The existence of Pnl in the modified proteins indicated the presence of

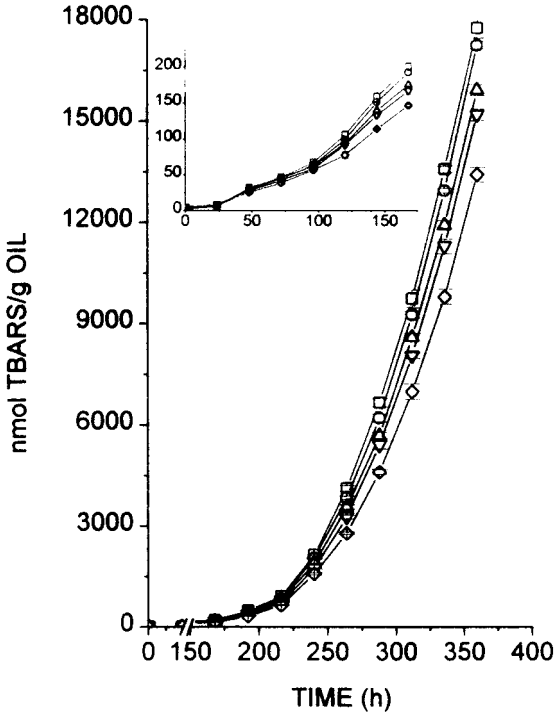

Figure 2. Effects of BSA (○), MBSA (△), DBSA (▽), and BHT (◇), added at 50 ppm, on soybean oil oxidation (□), measured as TBARS formation. Results represent the mean ± SD of three assays. (Inset) Expanded scale.

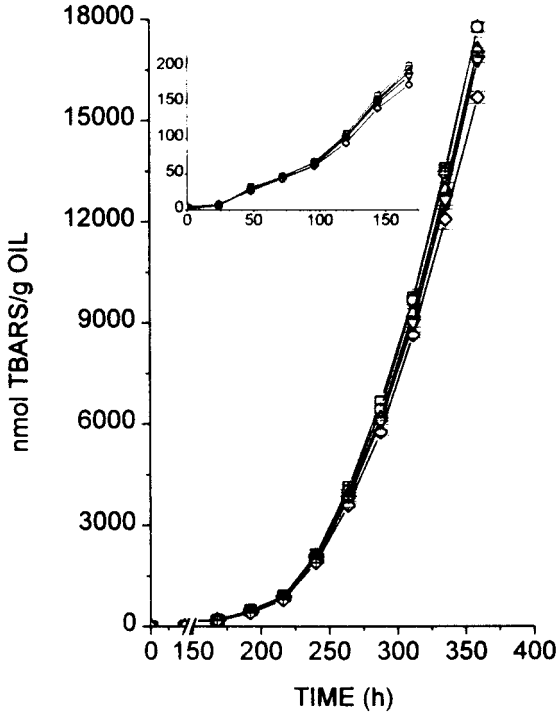

Figure 3. Effects of BSA (○), MBSA (△), DBSA (▽), and BHT (◇), added at 30 ppm, on soybean oil oxidation (□), measured as TBARS formation. Results represent the mean ± SD of three assays. (Inset) Expanded scale.

Table 2. Determination of Pnl, Arginine, and Lysine in BSA, MBSA, and DBSA after Basic Hydrolysis

	means, μmol/g of protein, ± SEM		
amino acid	BSA	MBSA	DBSA
Pnl		88 ± 10^a	117 ± 6^b
arginine	354 ± 15	316 ± 15	333 ± 16
lysine	753 ± 6^a	461 ± 13^b	457 ± 18^b

[a,b]Means in the same row with different letters are significantly different ($p < 0.05$).

Table 3. Induction Periods (in Hours) of Untreated Soybean Oil and the Oil Treated with BSA, MBSA, DBSA, and BHT

	concentration		
additive	10 ppm	30 ppm	50 ppm
none	255.0	255.0	255.0
BSA	256.1	256.3	257.9
MBSA	256.6	259.3	265.5
DBSA	257.6	262.9	267.3
BHT	259.0	264.5	272.0

OLAARP in both MBSA and DBSA. DBSA had a significantly higher level of Pnl ($p = 0.043$) than MBSA.

Antioxidative Activity of BSA, MBSA, and DBSA. Figure 2 shows the TBARS produced in the untreated oil and in the oil samples treated with 50 ppm of BSA, MBSA, DBSA, and BHT. The treatment of the oil with BSA produced a decrease in the TBARS production, which was significant after 144 h. However, this decrease was much higher when MBSA or DBSA was added. Addition of 50 ppm of MBSA or DBSA produced a significant decrease in TBARS production after 96 h. The effect produced by DBSA was more important than that produced by MBSA, and this difference was significant after 168 h. Nevertheless, the highest effect was observed with the addition of 50 ppm of BHT. The decrease produced by BHT was significant after 24 h.

Comparison among these TBARS values can be made by using either IP or PI. Table 3 shows the IP calculated for the untreated oil and the oil treated with 50 ppm of BSA, MBSA, DBSA, and BHT. Addition of BSA increased IP by 1%, and this difference was higher when MBSA or DBSA was added (4 and 5%, respectively). The highest increase was observed with the addition of BHT (7%). The relative protections of BSA, MBSA, and DBSA when compared with that of BHT were 17, 62, and 72%, respectively.

Analogous results were obtained when PI values were used. At the end of the incubation period, the PI values calculated for BSA, MBSA, and DBSA were 12, 42, and 59%, respectively.

The treatment of the oil with 30 or 10 ppm of BSA, MBSA, DBSA, and BHT produced analogous results, although, as should be expected, the effects were significantly lower. Figure 3 shows the TBARS produced in the untreated oil and in the oil samples treated with 30 ppm of BSA, MBSA, DBSA, and BHT. At this level BSA did not exhibit any protective effect by lowering TBARS. However, this protection was significant for MBSA after 192 h, for DBSA after 168 h, and for BHT after 24 h. The relative protections observed for MBSA and DBSA when compared with that of BHT were 45 and 83%, respectively, when using IP data (Table 3), and 31 and 46%, respectively, when using PI data.

Figure 4 shows the TBARS produced in the untreated oil and in the oil samples treated with 10 ppm of BSA, MBSA, DBSA, and BHT. At this level, only BHT exhibited a significant protective effect, and the increases observed in IP (Table 3) were also very small.

DISCUSSION

The reaction of epoxyalkenals with amino compounds is a complex process that produces in a first step 1-substituted 2-(1′-hydroxyalkyl)pyrroles and 1-substituted pyrroles (Zamora and Hidalgo, 1994, 1995). 1-Sub-

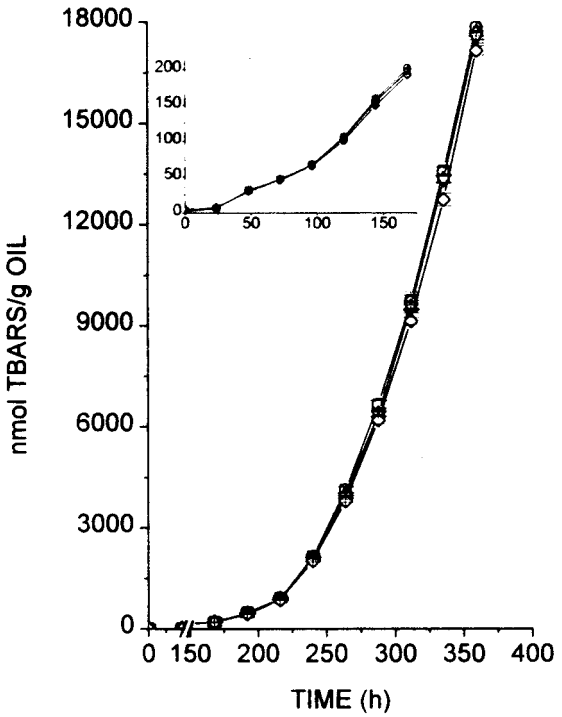

Figure 4. Effects of BSA (○), MBSA (△), DBSA (▽), and BHT (◇), added at 10 ppm, on soybean oil oxidation (□), measured as TBARS formation. Results represent the mean ± SD of three assays. (Inset) Expanded scale.

stituted pyrroles seem to be final products in the reaction. However, 1-substituted 2-(1′-hydroxyalkyl)-pyrroles polymerize with time to produce brown polymers. These polymers are responsible for the color and the fluorescence observed in these reactions (Hidalgo and Zamora, 1993). Analogously, the reaction between an epoxyalkenal (EH) and a protein (BSA) produced similar results. The reaction mixture became brown, and the polymerization of the protein was observed. In addition, the formation of the corresponding 1-substituted pyrrole (Pnl) was detected. Two fractions of the damaged protein were isolated: one that corresponded to a monomeric protein (MBSA) and another that corresponded to a dimeric protein (DBSA). However, in both fractions the protein was modified, because both were colored and fluorescent, and they had Pnl. These results suggest that the reaction between EH and the lysine residues follows a mechanism similar to the reaction between EH and the free amino acid. Therefore, this reaction, in addition to Pnl, would produce the corresponding bound 1-substituted 2-(1′-hydroxyalkyl)-pyrrole, which would be able to polymerize. This polymerization occurred both intra- and intermolecularly, producing MBSA and DBSA, respectively. Although both fractions had very similar amino acid compositions, the damage produced in DBSA was higher, as indicated by the higher number of lysine residues transformed into Pnl.

Because the same OLAARP are produced in the reaction with either amino acids or proteins, analogous antioxidative properties of both modified amino acids and modified proteins should be expected. Thus, the results obtained showed that, analogously to free OLAARP (Alaiz et al., 1996b), both MBSA and DBSA exhibited antioxidative properties in a vegetable oil when added at 30 or 50 ppm. Therefore, the antioxidative effect of OLAARP is independent if these compounds are studied alone or as a part of a protein, and their formation may be contributing to the antioxidative activity observed for proteins. In addition, DBSA was more antioxidant than MBSA, and this may be related to the higher presence of Pnl in DBSA.

Nowadays, there is an increasing interest in the potential beneficial effects obtained from consuming antioxidant-rich nutrients (Awasthi et al., 1996; Deshpande et al., 1996; James, 1996). The results obtained in this study, and in previous studies (Alaiz et al., 1995a−c, 1996a,b; Zamora and Hidalgo, 1993), suggest that, in addition to the well-known antioxidants, other compounds are also able to act as antioxidants, and they may either be taken unknowingly in the diet or even produced during normal *in vivo* oxidative stress. Preliminary studies from this laboratory have shown that the OLAARP Pnl is present in some foods at levels higher than those used in this series of studies (Zamora et al., unpublished results). Therefore, its presence should be contributing to the antioxidative activity of those foods. Additional studies are needed to know the foods that are richer in OLAARP and the importance of OLAARP in the total antioxidative activity of those foods. These studies are being developed at present in this laboratory.

ABBREVIATIONS USED

BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; DBSA, dimeric modified BSA; EH, (*E*)-4,5-epoxy-(*E*)-2-heptenal; HPCE, high-performance capillary electrophoresis; IP, induction period; MBSA, monomeric modified BSA; OLAARP, oxidized lipid/amino acid reaction products; PI, protection index; Pnl, ϵ-*N*-pyrrolylnorleucine; TBARS, thiobarbituric acid-reactive substances.

ACKNOWLEDGMENT

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

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Received for review September 13, 1996. Accepted January 27, 1997.[⊗] 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).

JF960698T

[⊗] Abstract published in *Advance ACS Abstracts*, March 1, 1997.