Comparative Antioxidant Activity of Maillard- and Oxidized Lipid-Damaged Bovine Serum Albumin

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The antioxidative activity of nonenzymatically browned proteins produced by reaction with both carbohydrates and oxidized lipids was studied to compare the effect of formation of Maillard reaction products (MRP) and oxidized lipid/amino acid reaction products (OLAARP) in the stabilities of foods during processing and storage. Bovine serum albumin (BSA) was incubated at 80 °C for 24 h in the absence (control, CO) or in the presence of 10 mM glucose (GL), fructose (FR), ribose (RI), methyl linoleate hydroperoxides (HP), and secondary products produced in the oxidation of methyl linoleate (SP). The modified proteins (COBSA, GLBSA, FRBSA, RIBSA, HPBSA, and SPBSA) were isolated, characterized by amino acid analysis, and tested for antioxidative activity (at 100 ppm) in soybean oil using the thiobarbituric acid-reactive substances (TBARS) assay. All of them significantly ($p < 0.05$) decreased TBARS formation and exhibited the following order of effectiveness: COBSA < GLBSA < FRBSA ≈ HPBSA < SPBSA < RIBSA < BHT. No big differences among antioxidative properties of OLAARP- and MRP-containing proteins were observed under the assayed conditions. Therefore, both Maillard and oxidized lipid/protein reactions may be contributing analogously to increase the stability of foods during processing and storage.

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

INTRODUCTION

A most difficult aspect of food preservation is the control of oxidation that most natural and processed foods undergo, which results in the formation of characteristic undesirable odors and flavors (Nawar, 1996). The most general approach for improving flavors of oxidizable foods is through the use of additives. Antioxidants most frequently used include synthetic additives such as butylated hydroxyanisole and butylated hydroxytoluene (BHT), but these and related additives are becoming less acceptable to the consuming public and food legislators (Bailey and Um, 1992). One solution used by manufacturers for decreasing risk to consumers and increasing food acceptability is the use of natural products as additives. Therefore, much research has been conducted to find safe antioxidants with high activity from natural sources (Kanner et al., 1994; Nishiyama et al., 1993; Six, 1994). Moreover, many other studies have been dedicated to the isolation and characterization of components, normally present in foods, that possess antioxidative properties. In this context, the antioxidative properties of Maillard reaction products (MRP) have long been known (Lingnert and Eriksson, 1981; Bailey, 1988), and, more recently, the antioxidative properties of oxidized lipid/amino acid reaction products (OLAARP) have also been described (Zamora and Hidalgo, 1993; Alaiz et al., 1995a).

OLAARP are produced as a final step in the lipid peroxidation process when oxidation of lipids occurs in the presence of amino acids and proteins (Gardner, 1979). OLAARP production involves both volatile and nonvolatile lipid oxidation products (Zamora and Hidalgo, 1995; Hidalgo and Zamora, 1993a), and all OLAARP studied exhibited antioxidative properties when tested in edible oils (Alaiz et al., 1996a; Ahmad et al., 1996). In addition, when OLAARP are produced between oxidized lipids and amino acid residues of a protein, the resulting browned protein also exhibited antioxidative activity (Alaiz et al., 1997).

The objective of this study was to analyze comparatively antioxidative activities of both MRP and OLAARP to evaluate their influence in the stabilities of foods during processing and storage. To obtain comparable products, both MRP and OLAARP were produced by reaction of a carbohydrate or a lipid oxidation product with a protein, and the resulting browned protein was tested for antioxidative activity.

EXPERIMENTAL PROCEDURES

Materials. Soybean oil was obtained from our Institute's pilot plant (Instituto de la Grasa, CSIC, Sevilla, Spain). Chromatographically pure methyl linoleate was a gift from Prof. Eduardo Vioque. D-Fructose (FR), D-glucose (GL), and D-ribose (RI) 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), PD-10 columns packed with Sephadex G-25 medium, and BHT 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.

Hydroperoxides (HP) and secondary products (SP) of methyl linoleate oxidation were prepared and purified according to the method of Frankel et al. (1989), following a procedure that was slightly modified. A sample of methyl linoleate (0.2 g) was autoxidized with pure oxygen at 40 °C for 7 days. At the end of this period the absorbance at 231 nm was 0.2798, which corresponded to 21.9% of HP using an absorptivity of 25 738. The oxidized sample was dissolved in 0.5 mL of 5% ether in hexane and fractionated by solid-phase extraction chromatography using 3 mL silica columns (J. T. Baker Inc., Phillipsburg, NJ). The first fraction, which contained unoxidized methyl linoleate (22.2 mg), was eluted with 4 mL of 5% ether in hexane. The second fraction, which contained the purified HP

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Table 1. Amino Acid Composition of MRP- and OLAARP-Containing Proteins Prepared in This Study

	μmol/g of protein (number of residues to nearest integer)						
amino acid	BSA	COBSA	GLBSA	FRBSA	RIBSA	HPBSA	SPBSA
Ala	690 (46)	705 (47)	700 (46)	722 (48)	749 (49)	693 (46)	693 (46)
Arg	342 (23)	336 (22)	132 (9)	140 (9)	137 (9)	253 (17)	162 (11)
Asx[a]	734 (48)	788 (52)	705 (47)	785 (52)	761 (50)	791 (52)	770 (51)
cystine	250 (17)	77 (5)	118 (8)	118 (8)	138 (9)	122 (8)	211 (14)
Glx[a]	1143 (75)	1150 (76)	1135 (75)	1170 (77)	1165 (77)	1152 (76)	1160 (77)
Gly	234 (15)	260 (17)	256 (17)	264 (17)	266 (18)	246 (16)	280 (19)
His	259 (17)	197 (13)	159 (10)	201 (13)	211 (14)	153 (10)	187 (12)
Ile	219 (14)	211 (14)	204 (13)	213 (14)	214 (14)	202 (13)	197 (13)
Leu	918 (61)	917 (61)	900 (59)	952 (63)	955 (63)	923 (61)	921 (61)
Lys	866 (57)	766 (51)	561 (37)	497 (33)	336 (22)	334 (22)	305 (20)
Met	62 (4)	58 (4)	55 (4)	53 (3)	51 (3)	53 (3)	65 (4)
Phe	400 (26)	409 (27)	394 (26)	424 (28)	422 (28)	400 (26)	400 (26)
Ser	417 (28)	407 (27)	407 (27)	407 (27)	406 (27)	415 (27)	443 (29)
Thr	508 (34)	485 (32)	508 (34)	512 (34)	510 (34)	494 (33)	517 (34)
Tyr	294 (19)	270 (18)	249 (16)	247 (16)	253 (17)	247 (16)	280 (18)
Val	538 (36)	539 (36)	545 (36)	555 (37)	562 (37)	537 (35)	524 (35)

[a] Asx, aspartic acid + asparagine; Glx, glutamic acid + glutamine.

(48.0 mg), was eluted with 4 mL of 25% ether in hexane. The third and final fraction, which contained SP (113.9 mg), was eluted with 4 mL of 25% ether in hexane. These fractions were characterized by gas chromatography coupled with mass spectrometry (GC/MS). The HP fraction consisted of a 1:1 mixture of methyl 9-hydroperoxy-10,12-octadecadienoate and methyl 13-hydroperoxy-9,11-octadecadienoate, which could be identified after reduction and silylation (Hidalgo and Zamora, 1995). The SP fraction was free of hydroperoxides and consisted of a very complex mixture of compounds. No attempts were made to identify the compounds present in the mixture, although many short-chain aldehydes could be easily detected. It is important to point out the presence of 4,5-epoxy-2-decenal in the mixture, because epoxyalkenals are very reactive with protein reactive groups (Hidalgo and Zamora, 1993b) and produce modified proteins with antioxidative properties (Alaiz et al., 1997).

Preparation of MRP- and OLAARP-Containing Proteins. BSA (2.5 mg) was dissolved in 2.5 mL of 0.3 M sodium phosphate buffer (pH 7.4) and was untreated (control, COBSA) or treated with 10 mM GL, FR, RI, HP, or SP (the average molecular weight used for SP was 200). The mixtures were incubated for 24 h at 80 °C, and then the solutions, which contained the modified proteins (COBSA, GLBSA, FRBSA, RIBSA, HPBSA and SPBSA), were desalted using PD-10 columns and freeze-dried. MRP- and OLAARP-containing proteins were studied for amino acid composition, using a previously described procedure (Alaiz et al., 1992), and for ϵ-*N*-pyrrolylnorleucine (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 COBSA, GLBSA, FRBSA, RIBSA, HPBSA, SPBSA, and BHT, which were dissolved in the oil at 100 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

Preparation of MRP- and OLAARP-Containing Proteins. The reaction between carbohydrates and proteins and between oxidized lipids and proteins produces browned proteins with antioxidative properties (Ledl and Schleicher, 1990; Alaiz et al., 1997). To compare antioxidative activities of browned proteins produced by both reactions, five modified proteins were prepared by using three carbohydrates (GL, FR, and RI) and two fractions of oxidized lipids (HP and SP), which were selected as models of the different types of carbohydrates and oxidized lipids that are present in foods. The reaction mixtures were incubated at 80 °C for 24 h to produce the modified proteins (GLBSA, FRBSA, RIBSA, HPBSA, and SPBSA). Such incubation produced extensive damage in the protein and increased its antioxidative activity.

Table 1 shows the amino acid composition of MRP- and OLAARP-containing proteins prepared in this study. These results were obtained after acid hydrolysis and important losses in some amino acids were observed. Incubation of BSA at 80 °C for 24 h produced some losses in lysine, histidine, and cyst(e)ine residues when compared with the native protein. However, amino acid losses were much higher when the incubation was carried out in the presence of carbohydrates (GL, FR, or RI) or oxidized lipids (HP or SP). Incubation with carbohydrates highly decreased basic amino acids, essentially arginine and lysine. Thus, recovered arginine residues after acid hydrolysis decreased from 22 in COBSA to 9 in all GLBSA, FRBSA, and RIBSA; and lysine residues decreased from 51 in COBSA to 37, 33, and 22 in GLBSA, FRBSA, and RIBSA, respectively. In addition, lysine losses depended on the carbohydrate employed and were higher for RI. Similar losses were obtained for basic amino acid residues in BSA treated with HP or SP. Thus, recovered arginine residues decreased from 22 to 17 and 11 for HP and SP, respectively; and lysine residues decreased from 51 to

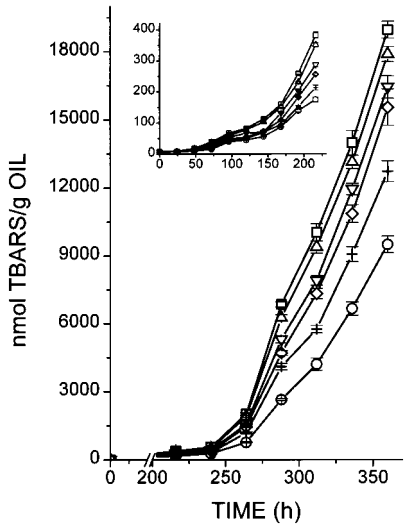

Figure 1. Effects of COBSA (△), GLBSA (▽), FRBSA (◇), RIBSA (+), and BHT (○), added at 100 ppm, on soybean oil oxidation (□), measured as TBARS formation. Results represent the mean ± SD of three assays. (Inset) Expanded scale.

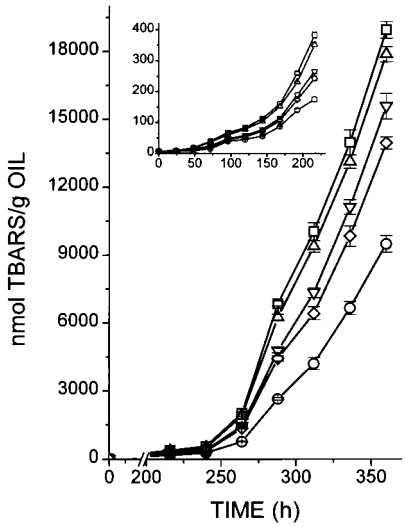

Figure 2. Effects of COBSA (△), HPBSA (▽), SPBSA (◇), and BHT (○), added at 100 ppm, on soybean oil oxidation (□), measured as TBARS formation. Results represent the mean ± SD of three assays. (Inset) Expanded scale.

22 and 20 for HP and SP, respectively. Therefore, the highest losses in arginine were observed for the three carbohydrates assayed, and the highest losses in lysine were obtained for RI, HP, and SP.

When MRP- and OLAARP-containing proteins were analyzed for the OLAARP marker Pnl after basic hydrolysis, most of them exhibited negligible amounts of this amino acid. Only SPBSA had 22 μmol of Pnl/g of protein (about 1.5 residues per molecule of damaged protein).

Antioxidative Activity of COBSA, GLBSA, FRBSA, RIBSA, HPBSA, and SPBSA. Figure 1 shows the TBARS produced in the untreated oil and in the oil samples treated with 100 ppm of COBSA, GLBSA, FRBSA, RIBSA, and BHT. The treatment of the oil with COBSA produced a decrease in the TBARS production, which was significant after 72 h. However, this decrease was much higher when a MRP-containing protein was added. Thus, addition of 100 ppm of GLBSA, FRBSA, or RIBSA significantly decreased TBARS production after 48 h. The effect produced by RI was more important than that produced by GL or FR, and this difference was significant after 48 h. Nevertheless, the highest effect was observed with the addition of 100 ppm of BHT.

Analogous results were obtained for the OLAARP-containing proteins assayed. Thus, Figure 2 shows the TBARS produced in the untreated oil and in the oil samples treated with 100 ppm of COBSA, HPBSA, SPBSA, and BHT. Addition of 100 ppm of HPBSA or SPBSA produced a significant decrease in TBARS production after 48 h, and this decrease was significantly higher than that produced by COBSA. In addition, the effect produced by SPBSA was significantly higher than that produced by HPBSA.

Comparison among all of these TBARS values can be made by using either IP or PI. Table 2 shows the IP calculated for the untreated oil and the oil treated with 100 ppm of COBSA, GLBSA, FRBSA, RIBSA, HPBSA, SPBSA, and BHT. Addition of COBSA increased IP by 0.8%, and this difference was higher when MRP- or OLAARP-containing proteins were added. Thus, IP increased by 3.5, 4.6, 5.4, 4.3, and 5.0% for GLBSA, FRBSA, RIBSA, HPBSA, and SPBSA, respectively. The highest increase was observed with the addition of BHT

Table 2. IP of Untreated Soybean Oil and Oil Treated with 100 ppm of COBSA, GLBSA, FRBSA, RIBSA, HPBSA, SPBSA, and BHT

additive	IP, h	additive	IP, h	additive	IP, h
none	261.0	FRBSA	273.1	SPBSA	274.1
COBSA	263.0	RIBSA	275.1	BHT	277.1
GLBSA	270.1	HPBSA	272.1		

(6.2%). The relative protections of COBSA, GLBSA, FRBSA, RIBSA, HPBSA, and SPBSA when compared with that of BHT were 12, 57, 75, 88, 69, and 81%, respectively.

Similar results were obtained when PI values were used. At the end of the incubation period, PI values calculated for COBSA, GLBSA, FRBSA, RIBSA, HPBSA, and SPBSA were 11, 27, 36, 66, 36, and 53, respectively.

DISCUSSION

The above results show that the incubation of BSA at 80 °C for 24 h in the presence of carbohydrates or oxidized lipids produces modified proteins with antioxidative activities. These reactions produced significant losses in basic amino acids, especially arginine and lysine, which depended on the compounds that were incubated with the protein. Thus, incubation of carbohydrates decreased recovered arginine residues by 60−61% and lysine residues by 35−61%. These results are in agreement with previous studies, which pointed out arginine and lysine as the major damaged amino acid residues in proteins after Maillard reaction (Ledl and Schleicher, 1990; Cho et al., 1986). In addition, lysine losses depended on the carbohydrate employed and were much higher for RI, according to the higher reactivity of this pentose for the reaction (Namiki and Hayashi, 1983). Similar losses were obtained for basic amino acid residues in BSA treated with HP or SP. Thus, recovered arginine residues decreased by 26−53% and lysine residues by 61−65%. Some of lysine losses produced by SP were a consequence of the formation of Pnl, which could be determined after basic hydrolysis. This amino acid was likely produced by reaction of the 4,5-epoxy-2-decenal detected in the SP fraction with the lysine ϵ-amino group. Although HP have also been shown to produce Pnl by reaction of HP with the amino groups

of amino acids (Zamora and Hidalgo, 1995), the conditions used in this study did not seem to induce that reaction. BSA modification should be mainly produced with the oxidized lipids without fractionation (Hidalgo and Zamora, 1995).

The oxidation of lipids produces many compounds that are able to interact with reactive groups in proteins, producing modified proteins with altered color and nutritive values (Eriksson, 1987; Gardner, 1979). However, the results obtained in this and in a previous study (Alaiz et al., 1997) have shown that proteins modified by oxidized lipids exhibited antioxidative properties when tested in edible oils, suggesting a positive role for the lipid peroxidation process. In addition, these antioxidative properties were independent of the reaction's occurring with the primary products of lipid oxidation (HP) or their decomposition products (SP), suggesting that if the lipid peroxidation process takes place in the presence of proteins, it will always produce modified proteins with antioxidative properties, which will delay the process. Because OLAARP compounds are able to retard the lipid peroxidation process at the same time that they are being produced (Alaiz et al., 1995b, 1996b), the above results suggest that the lipid peroxidation process is controlled, in some way, by OLAARP formation.

All assayed MRP- and OLAARP-containing proteins exhibited a decrease in TBARS that was higher than that produced by the untreated protein. In addition, TBARS values obtained for the oil incubated in the presence of MRP-containing proteins were similar to TBARS values obtained for the oil incubated in the presence of proteins containing OLAARP, although some significant differences were observed. These differences were much more related to lysine losses than to arginine losses, and the order of effectiveness observed was COBSA < GLBSA < FRBSA ≈ HPBSA < SPBSA < RIBSA < BHT. No big differences among antioxidative properties of OLAARP- and MRP-containing proteins were observed under the assayed conditions; therefore, both Maillard and oxidized lipid/protein reactions seem to be contributing analogously to increase the stability of foods during processing and storage. Additional studies are needed to evaluate the influence of reaction conditions in the antioxidative properties of proteins produced by both types of processes. These studies are being developed at present in this laboratory.

ABBREVIATIONS USED

BHT, butylated hydroxytoluene; BSA, bovine serum albumin; COBSA, control BSA; FR, fructose; FRBSA, BSA modified by FR; GL, glucose; GLBSA, BSA modified by glucose; HP, hydroperoxides of methyl linoleate; HPBSA, BSA modified by HP; IP, induction period; MRP, Maillard reaction products; OLAARP, oxidized lipid/amino acid reaction products; PI, protection index; Pnl, ϵ-*N*-pyrrolylnorleucine; TBARS, thiobarbituric acid-reactive substances; RI, ribose; RIBSA, BSA modified by ribose; SP, secondary products of methyl linoleate; SPBSA, BSA modified by SP.

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