Effect of pH and Temperature on Comparative Antioxidant Activity of Nonenzymatically Browned Proteins Produced by Reaction with Oxidized Lipids and Carbohydrates

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The antioxidative activity of nonenzymatically browned bovine serum albumin (BSA) produced by reaction with ribose (RI), hydroperoxides of methyl linoleate oxidation (HP), and secondary products of methyl linoleate oxidation (SP), at different pHs (4, 7, and 10) and temperatures (25, 37, 50, 80, and 120 °C), was studied to compare the antioxidative effects of carbohydrate- and oxidized lipids-modified proteins. The modified proteins (RIBSA, HPBSA, and SPBSA) were tested for antioxidative activity (at 100 ppm) in soybean oil using the thiobarbituric acid-reactive substances (TBARS) assay. All of them decreased significantly ($p < 0.05$) the TBARS formation in the oil and exhibited different effectiveness as a function of the temperature and the pH of the medium. In addition, there was a good correlation between the antioxidative activity of the protein and the amino acid losses produced during the nonenzymatic browning. These results are in agreement with an analogous and complimentary contribution of both Maillard and oxidized lipid/protein reactions to the antioxidative activity produced in foods during processing and storage.

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

INTRODUCTION

Lipid oxidation is a major cause of deterioration in foods and feeds, both in those containing substantial amounts of fats, like lard and edible oils, and in those where only minor amounts of lipids occur, as in several vegetable products (Frankel, 1991; Eriksson, 1987). This reaction is responsible for different changes in quality attributes of foods and feeds, including aroma, taste, color, texture, nutritive value, and safety (Ericksson, 1982; Nawar, 1996). Therefore, protection of foods against lipid oxidation has been always a major objective in food technology, and the use of synthetic antioxidants is an old practice in the food industry. However, the safety of these antioxidants has been questioned in recent years (Hemeda and Klein, 1990; Wanasundara and Shahidi, 1994) and the interest toward utilization of natural food constituents with antioxidative properties is increasing (Garewal, 1997; Larson, 1997).

Among the different food constituents that are naturally present in food, or that are produced during food storage and processing, the antioxidant effects of Maillard reaction products (MRP) have been long known (Ledl and Schleicher, 1990; Lingnert and Eriksson, 1981) and, more recently, also the antioxidant effect of the products formed by reaction of oxidized lipids with amino acids and proteins (Alaiz et al., 1995; Zamora and Hidalgo, 1993; Zamora et al., 1997). In fact, a comparative study of the antioxidant activity of Maillard- and oxidized lipid-damaged bovine serum albumin (BSA) concluded that antioxidative activities of both types of modified BSA were very similar (Alaiz et al., 1997). This might be a consequence of the similarity between both types of reactions that are able to produce analogous melanoidin products with similar structures (Hidalgo et al., 1999). As a continuation of those studies, the present investigation was undertaken to study the effect of pH and temperature on the antioxidative activity of proteins modified by both oxidized lipids and carbohydrates. To obtain comparable results, both types of modified proteins were produced by reaction of a carbohydrate or a lipid oxidation product with a protein under specific conditions, and the resulting browned protein was tested for antioxidative activity. The modified proteins used in this study were prepared and characterized previously (Hidalgo et al., 1999).

EXPERIMENTAL PROCEDURES

Materials. Soybean oil was obtained from our Institute's pilot plant (Instituto de la Grasa, CSIC, Sevilla, Spain). 2-Thiobarbituric acid monohydrate was purchased from Merck (Darmstadt, Germany). Other reagents and solvents used were analytical grade and were purchased from reliable commercial sources.

BSA modified by carbohydrates and oxidized lipids was prepared as described previously (Hidalgo et al., 1999). Briefly, BSA (2.5 mg) was dissolved in 2.5 mL of 0.3 M sodium phosphate buffer pH 7.4, sodium acetate pH 4.0, or sodium borate pH 10.0 and was untreated (control) or treated with 10 mM ribose (RI), methyl linoleate hydroperoxides (HP), and secondary products produced in the oxidation of methyl linoleate (SP). The mixtures were incubated for 24 h at 25, 37, 50, 80, or 120 °C, and then, the solutions, which contained the control (COBSA) and the modified proteins (RIBSA, HPBSA, and SPBSA), were desalted using PD-10 columns and freeze-dried.

Measurement of Antioxidative Activity. Stripped soybean oil with no antioxidant was compared with samples containing COBSA, RIBSA, HPBSA, SPBSA, and BHT, which

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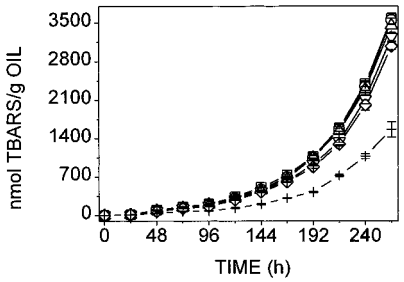

Figure 1. Effects of COBSA (○), RIBSA (△), HPBSA (▽), and SPBSA (◇), prepared at 25 °C and pH 7 and added at 100 ppm, on soybean oil oxidation (□). The effect of 100 ppm of BHT (+) has also been included for comparison purposes. Results represent the mean ± SD of three assays.

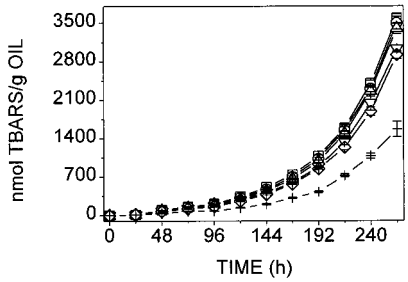

Figure 2. Effects of COBSA (○), RIBSA (△), HPBSA (▽), and SPBSA (◇), prepared at 37 °C and pH 7 and added at 100 ppm, on soybean oil oxidation (□). The effect of 100 ppm of BHT (+) has also been included for comparison purposes. Results represent the mean ± SD of three assays.

were dissolved in the oil at 100 ppm. Oil samples (10 g) were weighed into 90 × 20 mm Petri dishes and oxidized for 264 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, at the end of the incubation period a protection index (PI) was determined according to the following equation:

$$PI = 100 - [100(\text{TBARS sample} - \text{TBARS BHT})/(\text{TBARS oil} - \text{TBARS BHT})] \quad (1)$$

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 tested compound had a prooxidant effect.

Statistical Analysis. All results are expressed as mean values of three experiments. Statistical comparisons among different 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, Inc., New York). The significance level is $p < 0.05$ unless otherwise indicated.

RESULTS

Effect of Temperature on Antioxidant Activity of BSA Incubated with Carbohydrates and Oxidized Lipids. The incubation of BSA with RI, HP, or SP at different temperatures and pH 7 always increased the antioxidant activity of the protein. However, the relative antioxidative effects observed at each temperature were different if the modified protein was produced with the carbohydrate or the oxidized lipids. Figure 1 shows the effect of these modified proteins when they were prepared at 25 °C. At this temperature, the protections exhibited by COBSA or RIBSA were not significant. However, both HPBSA and SPBSA protected significantly, and no significant differences in the protection were observed between them. PI values obtained at the end of the incubation period were 1.31, 6.31, 16.78, and 25.75 for COBSA, RIBSA, HPBSA, and SPBSA, respectively.

The incubation of BSA with RI, HP, and SP at 37 °C produced modified proteins with relative antioxidative activities similar to the obtained values at 25 °C. Figure 2 shows the TBARS produced in an oil incubated at 60 °C in the absence and in the presence of 100 ppm of COBSA, RIBSA, HPBSA, and SPBSA prepared at 37 °C. The figure also includes the TBARS production in the oil incubated with 100 ppm of BHT for comparison purposes. No significant decreases in TBARS production was observed for the oil treated with COBSA or RIBSA. However, both HPBSA and SPBSA decreased significantly TBARS production in the oil, and SPBSA exhibited a significantly higher protection than HPBSA. PI values obtained at the end of the incubation period were 4.03, 8.96, 27.20, and 32.73 for COBSA, RIBSA, HPBSA, and SPBSA, respectively.

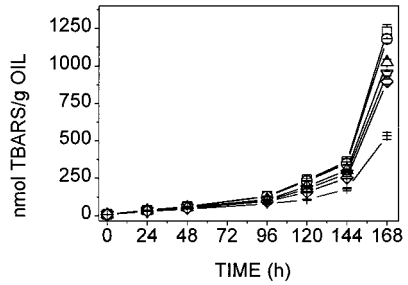

Figure 3. Effects of COBSA (○), RIBSA (△), HPBSA (▽), and SPBSA (◇), prepared at 50 °C and pH 7 and added at 100 ppm, on soybean oil oxidation (□). The effect of 100 ppm of BHT (+) has also been included for comparison purposes. Results represent the mean ± SD of three assays.

The incubation of the proteins at 50 °C increased their antioxidative activities, and at this temperature, higher differences among their antioxidative activities were observed. Figure 3 shows the TBARS production in an oil treated with 100 ppm of COBSA, RIBSA, HPBSA, and SPBSA prepared at 50 °C. COBSA exhibited a small degree of protection of the oil. However, the treatment with RIBSA significantly increased its stability. More protection than that exhibited by RIBSA was observed by HPBSA. Nevertheless, the highest protection of the prepared proteins was exhibited by SPBSA. The PI values obtained at the end of the incubation period were 6.86, 29.38, 40.30, and 47.83, for COBSA, RIBSA, HPBSA, and SPBSA, respectively.

When the modified proteins were prepared at 80 °C, the order of effectiveness among them changed, and RIBSA was the most effective. TBARS values obtained for an oil treated with 100 ppm of COBSA, RIBSA, HPBSA, and SPBSA, prepared at 80 °C, are shown in Figure 4. At this temperature, COBSA exhibited a small significant protection for the oil, but this protection was much less than that exhibited by any other of the modified proteins. Thus, HPBSA reduced TBARS production in the oil by 44%, when compared with the TBARS decrease exhibited by 100 ppm of BHT, and was less protective than SPBSA. The highest degree of protection among these proteins was observed for RIBSA, which reduced TBARS production by 66% when compared with BHT. The PI values obtained for COBSA, RIBSA, HPBSA, and SPBSA incubated at 80 °C were 7.84, 65.94, 44.34, and 53.11, respectively.

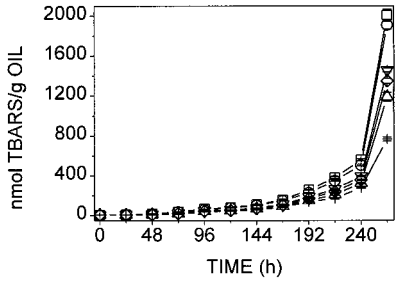

Figure 4. Effects of COBSA (○), RIBSA (△), HPBSA (▽), and SPBSA (◇), prepared at 80 °C and pH 7 and added at 100 ppm, on soybean oil oxidation (□). The effect of 100 ppm of BHT (+) has also been included for comparison purposes. Results represent the mean ± SD of three assays.

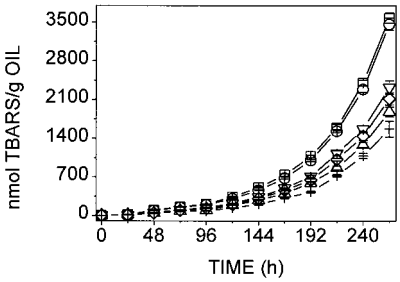

Figure 5. Effects of COBSA (○), RIBSA (△), HPBSA (▽), and SPBSA (◇), prepared at 120 °C and pH 7 and added at 100 ppm, on soybean oil oxidation (□). The effect of 100 ppm of BHT (+) has also been included for comparison purposes. Results represent the mean ± SD of three assays.

Table 1. Effect of Temperature on PI Values of BSA Incubated at pH 7 with Carbohydrates and Oxidized Lipids

	PI values			
temp (°C)	COBSA	RIBSA	HPBSA	SPBSA
25	1.33 ± 4.16^a	6.31 ± 6.42^a	16.78 ± 3.79^b	25.75 ± 3.44^c
37	4.03 ± 5.60^a	8.96 ± 5.05^a	27.20 ± 3.19^b	32.73 ± 3.54^b
50	6.86 ± 4.33^a	29.38 ± 3.04^b	40.30 ± 2.03^c	47.83 ± 2.73^d
80	7.84 ± 3.21^a	65.94 ± 3.39^b	44.34 ± 1.61^c	53.11 ± 1.95^d
120	5.77 ± 5.51^a	84.59 ± 4.48^b	62.69 ± 5.16^c	72.86 ± 4.87^d

$^{a-d}$Means values in the same row with different superscripts are significantly different ($p < 0.05$).

A new increase in the incubation temperature used to prepare the modified proteins increased the antioxidative effects observed at 80 °C but did not change the relative activities of the modified proteins. Figure 5 shows the TBARS production in an oil treated with 100 ppm of COBSA, RIBSA, HPBSA, and SPBSA, which were prepared at 120 °C. COBSA exhibited a small degree of protection, which was much smaller than that exhibited by HPBSA, SPBSA, or RIBSA. The PI values obtained for COBSA, RIBSA, HPBSA, and SPBSA at the end of the incubation period were 5.77, 84.59, 62.69, and 72.86, respectively.

Table 1 collects the PI values obtained for the different proteins at the five assayed temperatures. Patterns similar to those observed for the TBARS data were also observed for the PI values. In addition, PI values for COBSA were not clearly related to the incubation temperature. However, PI values were correlated linearly with the temperature for RIBSA ($r = 0.977$, $p = 0.004$), HPBSA ($r = 0.965$, $p = 0.008$), and SPBSA ($r = 0.975$, $p = 0.005$).

Effect of pH on Antioxidant Activity of BSA Incubated with Carbohydrates and Oxidized Lipids. Analogously to the effect of the temperature, the incubation of BSA with RI, HP, or SP at different pHs always produced modified proteins with antioxidative activities. However, the relative effectiveness of the modified proteins depended on the pH at which they were prepared. A temperature of 80 °C was selected for these experiments because at this temperature the three modified proteins had a substantial antioxidative activity. Figure 6 shows the effect of COBSA, RIBSA, HPBSA, and SPBSA, prepared at pH 4, on the TBARS production in a soybean oil which was heated in the dark at 60 °C. The addition of COBSA slightly decreased TBARS production. However, the addition of RIBSA, HPBSA, or SPBSA protected much more efficiently the oil against oxidation. The order of effectiveness observed was COBSA < RIBSA < HPBSA < SPBSA, and the PI values were 9.43, 35.59, 46.26, and 57.53, respectively.

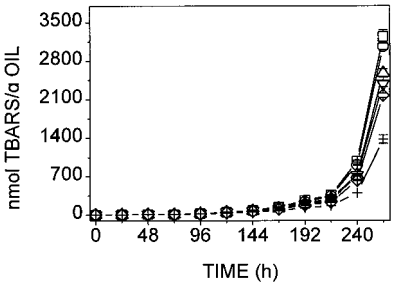

Figure 6. Effects of COBSA (○), RIBSA (△), HPBSA (▽), and SPBSA (◇), prepared at 80 °C and pH 4 and added at 100 ppm, on soybean oil oxidation (□): The effect of 100 ppm of BHT (+) has also been included for comparison purposes. Results represent the mean ± SD of three assays.

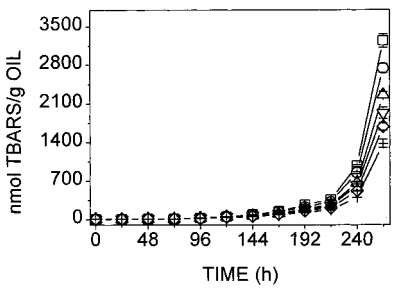

Figure 7. Effects of COBSA (○), RIBSA (△), HPBSA (▽), and SPBSA (◇), prepared at 80 °C and pH 10 and added at 100 ppm, on soybean oil oxidation (□). The effect of 100 ppm of BHT (+) has also been included for comparison purposes. Results represent the mean ± SD of three assays.

When the pH was increased to 7, the order of effectiveness changed and RIBSA resulted as the most antioxidant protein (Figure 4). However, when proteins were prepared at pH 10, the relative antioxidative effect observed for these proteins was the same than the found for proteins prepared at pH 4. Figure 7 shows the effect of proteins prepared at pH 10 on TBARS production in a soybean oil when the tested proteins were added at 100 ppm. This treatment increased the antioxidative activities of the four proteins assayed that exhibited a much higher antioxidative activity than at pH 4. The PI values obtained for COBSA, RIBSA, HPBSA, and SPBSA at the end of the incubation period were 26.75, 52.78, 70.34, and 83.57, respectively.

Table 2 collects the PI values obtained for the different proteins at the three assayed pHs. Differently from the temperature, the antioxidative efficiency of the modified proteins did not show a clear correlation with the pH and was different for proteins modified by the

Table 2. Effect of pH on PI Values of BSA Incubated at 80 °C with Carbohydrates and Oxidized Lipids

	PI values			
pH	COBSA	RIBSA	HPBSA	SPBSA
4	9.43 ± 3.40^a	35.59 ± 4.21^b	46.26 ± 2.51^c	57.53 ± 2.05^d
7	7.84 ± 3.21^a	65.94 ± 3.39^b	44.34 ± 1.61^c	53.11 ± 1.95^d
10	26.75 ± 4.51^a	52.78 ± 3.40^b	70.34 ± 5.33^c	83.57 ± 3.60^d

[a–d]Means values in the same row with different superscripts are significantly different ($p < 0.05$).

carbohydrate and the oxidized lipids. Thus, RIBSA exhibited the highest antioxidative effect when it was prepared at neutral pH. However, COBSA, HPBSA, and SPBSA showed very similar activities when prepared at acid or neutral pHs, and their antioxidative effects increased considerably when they were prepared at basic pH. The PI of SPBSA prepared at 80 °C and pH 10 was very similar to the PI of RIBSA prepared at 120 °C and pH 7 ($p = 0.774$).

DISCUSSION

Previous studies have suggested an analogy between some browned products produced in the reaction of proteins with both carbohydrates and oxidized lipids (Tressl at al., 1998; Hidalgo et al., 1999). This similarity between both reactions has been shown by determining color changes, amino acid losses, and protein pyrrolization (Hidalgo et al., 1999). In addition, because products of both reactions are similar, an equivalent antioxidant activity for both reactions should be also expected. The results obtained in this study confirm this hypothesis and very similar antioxidant effects were observed for a BSA incubated with RI or oxidized lipids.

The protective effect exhibited by RIBSA, HPBSA, and SPBSA was a function of the incubation pH and temperature and was different for RI and for the oxidized lipids. Thus, at 25−50 °C the highest antioxidative effects were observed for HPBSA and SPBSA, and at 80−120 °C the highest antioxidative effects were observed for the RIBSA. These results are likely a consequence of the lower reactivity of carbohydrates at low temperature when compared with the oxidized lipids, and therefore, RIBSA should be less modified at 25−50 °C than HPBSA or SPBSA. In fact, at 25−50 °C RI produced less browning and lysine losses in BSA than HP or SP, and these effects were reversed at 80−120 °C (Hidalgo et al., 1999).

This correlation between antioxidative activity and protein changes observed at the different temperatures was not so clear when the antioxidative activities were analyzed at the three pH studied. Thus, the highest antioxidative activity of RIBSA was observed at pH 7, which was also the pH at which the highest browning and lysine losses were produced (Hidalgo et al., 1999). However, the highest antioxidative activities of HPBSA and SPBSA were observed at pH 10, and this was not correlated with the protein damage produced at this pH (Hidalgo et al., 1999). Nevertheless, when PI values were compared with the values of color difference, fluorescence, lysine recovered after acid hydrolysis, and arginine recovered after acid hydrolysis obtained for the different proteins (Hidalgo et al., 1999), there was a good correlation between PI and some of these determinations (Table 3). The best correlation was always obtained between PI and the amino acid losses, suggesting that to obtain high antioxidative activities a high number of amino acid residues (mainly lysine) should be modified. On the other hand, no correlation was observed between fluorescence development and antioxidative activity.

Table 3. Correlation of PI with Color Difference, Fluorescence, Lysine Recovered after Acid Hydrolysis, and Arginine Recovered after Acid Hydrolysis in BSA Incubated with Carbohydrates and Oxidized Lipids at Different pHs and Temperatures[a]

	modified protein		
correlation	RIBSA	HPBSA	SPBSA
PI/color difference	$r = 0.853$	$r = 0.784$	$r = 0.539$
	$p = 0.015$	$p = 0.037$	$p = 0.212$
PI/fluorescence	$r = 0.614$	$r = 0.368$	$r = 0.068$
	$p = 0.143$	$p = 0.417$	$p = 0.885$
PI/lysine recovered	$r = -0.984$	$r = -0.824$	$r = -0.787$
	$p < 0.0001$	$p = 0.023$	$p = 0.036$
PI/arginine recovered	$r = -0.911$	$r = -0.870$	$r = -0.742$
	$p = 0.0043$	$p = 0.011$	$p = 0.056$

[a] Data for color difference, fluorescence, lysine recovered after acid hydrolysis, and arginine recovered after acid hydrolysis were determined in a previous study (Hidalgo et al., 1999).

The results obtained in this and in the previous paper (Hidalgo et al., 1999) are in agreement with an analogous contribution of both Maillard and oxidized lipid/protein reactions to the nonenzymatic browning, fluorescence, and antioxidative activity produced in foods during processing and storage. However, both reactions are not favored under the same reaction conditions, and at low or moderate temperatures oxidized lipid/protein reactions will be more important for causing these effects than carbohydrate/protein reactions, and these roles will be inverted at high temperatures.

ABBREVIATIONS USED

BSA, bovine serum albumin; COBSA, control BSA; HP, hydroperoxides of methyl linoleate; HPBSA, BSA modified by HP; RI, ribose; RIBSA, BSA modified by RI; SP, secondary products of methyl linoleate oxidation; SPBSA, BSA modified by SP; YI, yellowness index.

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