# Addition of Oxidized Lipid/Amino Acid Reaction Products Delays the Peroxidation Initiated in a Soybean Oil

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The antioxidative activity of two amino acid residue analogs, $N$-(carbobenzyloxy)-L-histidine (Z-His) and $N^2$-(carbobenzyloxy)-L-lysine (Z-Lys), and their products of reaction with $(E)$-2-octenal, $N$-(carbobenzyloxy)-1(3)-[1'-(formylmethyl)hexyl]-L-histidine dihydrate (1), 1-[$N^2$-(carbobenzyloxy)-L-lysyl]-2-[3'-carboxy-2'-$(E)$-propen-1'-yl]-4-pentylpyridinium betaine (2), and bis[1-($N^2$-carbobenzyloxy)-L-lysyl]-2-(3'-carboxy-2'-propene-1',2'-diyl)-4-pentylpyridinium betaine (isomeric mixture) (3), were studied to evaluate if some oxidized lipid/amino acid reaction products might play a role in delaying an initiated peroxidative process in a vegetable oil. All of these compounds were added at two levels of concentration (100 and 200 ppm) to partly oxidized soybean oil which was further oxidized under air in the dark at 60 °C. Oil peroxidation was evaluated by using the thiobarbituric acid-reactive substances (TBARS) assay. Compounds 1–3 significantly delayed new TBARS formation on soybean oil ($p < 0.05$) and showed diverse activity as compared with synthetic antioxidants. The order of effectiveness obtained was Z-His < Z-Lys < 1 < 2 ≈ BHT ≈ 3 < propyl gallate. These results suggest that some oxidized lipid/amino acid reaction products might be playing an antioxidative role in foods by delaying the peroxidative process at the same time that they are being produced.

## INTRODUCTION

The oxidative degradation of polyunsaturated fatty acids, in addition to microbial spoilage and browning reactions, contributes significantly to the shelf life of many products (Allen, 1987; Frankel, 1991). Lipid oxidation is a complicated process leading to the formation of many compounds (Gardner, 1989; Kubow, 1992). Some of its effects include development of off-flavors and odors, changes in texture, and loss of nutritive value (Eriksson, 1987). In addition, lipid oxidation products seem to be directly involved in the development of a number of diseases including coronary artery disease, atherosclerosis, and cancer and the aging process (Rice-Evans and Burdon, 1993).

Preservation of food products with additives is an ancient practice. In particular, antioxidants are principal ingredients that protect food quality by retarding oxidative breakdown of lipids (Frankel, 1993; Madhavi and Salunkhe, 1995). Thus, synthetic phenolic antioxidants are commonly used in fats and oils because of their effectiveness. However, in the past 15–20 years, special attention has been given to the use of natural antioxidants because of the possible, but not yet well established, hazardous effects of synthetic antioxidants (Wanasundara and Shahidi, 1994).

In a search for new natural antioxidants, previous research from this laboratory has shown that some naturally formed oxidized lipid/amino acid reaction products (OLAARP) are able to protect bulk vegetable oils against oxidation (Alaiz et al., 1995a,b). The formation of these products considerably increased the antioxidative activity of unreacted amino acids, and these reaction products might play a role in the stability

of foods during their processing or storage. However, because OLAARP are formed during the peroxidation process, it was unclear if this antioxidative activity might also play a role in slowing an initiated peroxidative process or in preventing further oxidations. The objective of this study was to analyze if OLAARP are able to stop or slow a peroxidative process initiated in a vegetable oil.
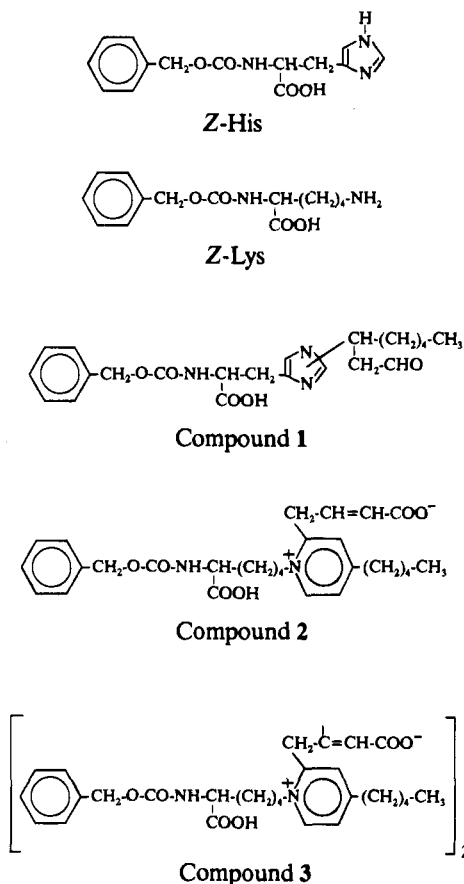
## EXPERIMENTAL PROCEDURES

**Materials.** Soybean oil was obtained from our Institute's Pilot Plant (Instituto de la Grasa, CSIC, Sevilla, Spain). The oil was stored for 3 months at 4 °C before its use, to obtain an initial peroxidation. 2-Thiobarbituric acid monohydrate (TBA) was purchased from Merck (Darmstadt, Germany). $(E)$-2-Octenal, and $N^2$-(carbobenzyloxy)-L-lysine (Z-Lys) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Butylated hydroxytoluene (BHT), $n$-propyl gallate (PG), and $N$-(carbobenzyloxy)-L-histidine (Z-His) were purchased from Sigma Chemical Co. (St. Louis, MO). MN-Kiesegel 60 (0.063–0.2 mm particle size) for column chromatography and Alugram analytical plates (20 × 20 cm) with fluorescent indicator for TLC were obtained from Macherey Nagel (Düren, Germany). Other reagents and solvents used were of analytical grade and were purchased from reliable commercial sources.

**Chemical Synthesis of $(E)$-2-Octenal/Amino Acid Reaction Products.** $N$-(Carbobenzyloxy)-1(3)-[1'-(formylmethyl)hexyl]-L-histidine dihydrate (1), as a mixture of two Michael adducts (structures for the compounds used in this study are given in Figure 1), was obtained by reaction of Z-His with $(E)$-2-octenal as described previously (Alaiz and Girón, 1994). The synthesis of the quaternary pyridinium salts produced in the reaction between $(E)$-2-octenal and Z-Lys; 1-($N^2$-(carbobenzyloxy)-L-lysyl]-2-[3'-carboxy-2'-$(E)$-propen-1'-yl]-4-pentylpyridinium betaine (2) and bis[1-($N^2$-carbobenzyloxy)-L-lysyl]-2-(3'-carboxy-2'-propene-1',2'-diyl)-4-pentylpyridinium betaine (isomeric mixture) (3), was also described previously (Alaiz and Barragán, 1995).

**Measurement of Antioxidative Activity of Compounds 1–3 in an Oxidized Soybean Oil.** Oil samples (10 g) were

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Z-His

Z-Lys

Compound 1

Compound 2

Compound 3

**Figure 1.** Chemical structures of compounds tested for antioxidative activity in this study.



**Figure 2.** Effects of BHT at 100 (■) and 200 (▲) ppm and PG at 100 (▼) and 200 (♦) ppm on soybean oil oxidation (●) measured as TBARS formation. Results represent the mean ± SD of three assays. Tested compounds were added after 24 h of oxidation.

weighed into 90 × 20 mm Petri dishes and oxidized for 24 h under air in the dark at 60 °C. This time is needed for production of (*E*)-2-octenal and its reaction products with amino acids (Esterbauer, 1982; Alaiz and Barragán, 1995; Alaiz and Girón, 1994). After that time, some oil samples were treated with compounds 1–3 and also, for comparison purposes, with BHT, PG, Z-His, and Z-Lys, added at concentrations of 100 and 200 ppm by weight. Oil samples were then oxidized for another 72 h under air in the dark at 60 °C. Peroxidation was evaluated periodically during the whole process (96 h) 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 zero meant that the compound tested had no protective effect. A PI < 0 meant that the compound tested had a prooxidant effect. This index could only be applied at $t$ > 24 h in this study because TBARS of oil should be higher than TBARS of gallate.

**Statistical Analysis.** All results are expressed as mean values ± SD 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* (Glantz, 1987). Significance level is $p$ < 0.05 unless otherwise indicated.
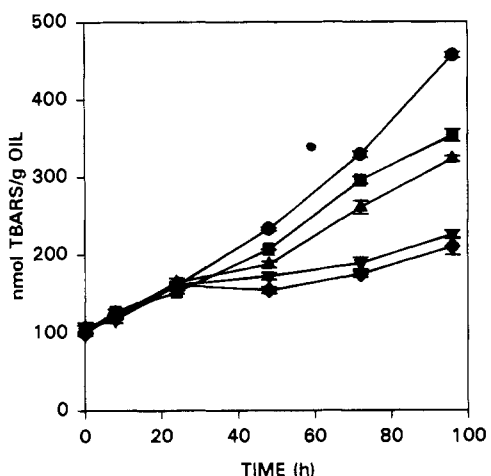
## RESULTS AND DISCUSSION

The stripped soybean oil used in this study was stored in a refrigerator for 3 months previous to its use, and it
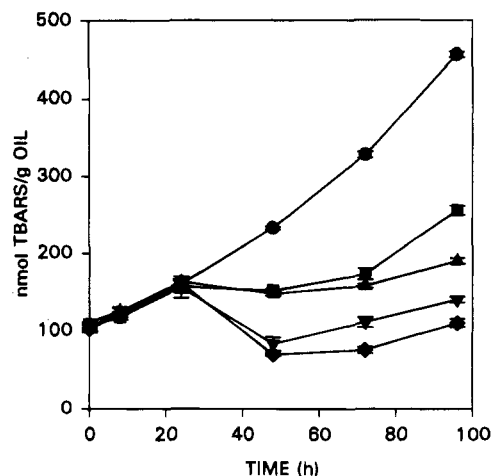
had 104 nmol of TBARS/g of oil, which suggested an initiated peroxidation process in the oil. In addition, it was heated for 24 h at 60 °C before the compounds that were going to be tested were added, and its TBARS increased to 160 nmol/g of oil, showing a new increase in lipid peroxidation. This oxidation time is needed for production of (*E*)-2-octenal and its later reaction with amino acids. At this time, the different compounds were added and TBARS production was determined for another 72 h. Figure 2 shows TBARS production in untreated oil and oil treated with BHT and PG, added at 100 and 200 ppm. The untreated oil had significantly increased TBARS during the whole heating time. On the contrary, addition of either BHT or PG stopped TBARS production. Thus, when 100 ppm of BHT was added, no significant increase in TBARS was observed for the following 24 h, suggesting that BHT inhibited further peroxidation. This effect was also observed for another 24 h; only in the last 24 h of the study was a significant increase in TBARS production observed. At the end of the incubation period the PI for BHT added at 100 ppm was 63. When BHT was added at 200 ppm, a similar effect was observed. No increase in TBARS production was observed for 48 h after addition; only in the last 24 h did TBARS production show a significant increase. Its PI, at the end of the heating period, was 77.

The antioxidative effect of PG on TBARS production was more pronounced. Addition of PG not only stopped TBARS production, but TBARS measured after the antioxidant was added were lower than the initial value. This effect might be a consequence of PG, in addition to stopping lipid peroxidation, being able to react with the secondary products of lipid oxidation and, therefore, diminishing its TBARS value. This effect was not observed in any of the other assayed compounds. At 100 ppm, the addition of PG significantly decreased TBARS, and, although later heating increased TBARS value, TBARS measured during the whole heating period were always lower than the value obtained at the time of addition of the antioxidant. At 200 ppm, a greater decrease was observed and TBARS did not show any increase in the following 48 h. TBARS increased only in the last 24 h of the experiment, but final TBARS were lower than TBARS measured when the antioxidant was added.
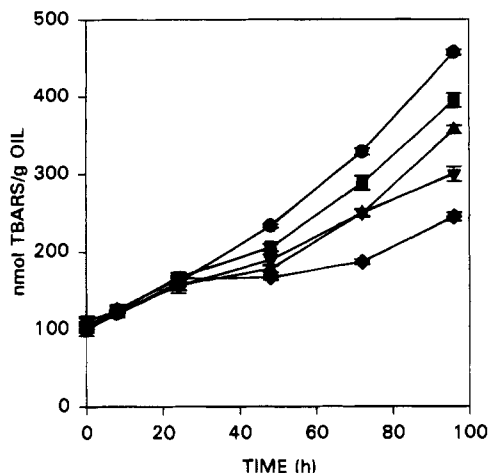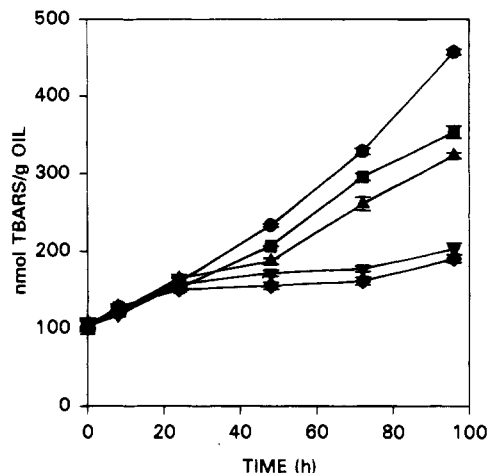
**Figure 3.** Effects of Z-His at 100 (■) and 200 (▲) ppm and compound **1** at 100 (▼) and 200 (♦) ppm on soybean oil oxidation (●) measured as TBARS formation. Results represent the mean ± SD of three assays. Tested compounds were added after 24 h of oxidation.



**Figure 5.** Effects of Z-Lys at 100 (■) and 200 (▲) ppm and compound **3** at 100 (▼) and 200 (♦) ppm on soybean oil oxidation (●) measured as TBARS formation. Results represent the mean ± SD of three assays. Tested compounds were added after 24 h of oxidation.



**Figure 4.** Effects of Z-Lys at 100 (■) and 200 (▲) ppm and compound **2** at 100 (▼) and 200 (♦) ppm on soybean oil oxidation (●) measured as TBARS formation. Results represent the mean ± SD of three assays. Tested compounds were added after 24 h of oxidation.

Addition of Z-His or its reaction product with (E)-2-octenal, compound **1**, also delayed TBARS production (Figure 3). The effect of Z-His was less than that observed for BHT or gallate, and TBARS increased with time at both concentrations assayed. This increase was, however, lower than that observed for TBARS production in untreated oil. At the end of the heating period, the PI values obtained for Z-His at 100 and 200 ppm were 20 and 29, respectively. When compound **1** was tested, its effect on stopping TBARS production was greater than that observed with addition of Z-His. Thus, at 200 ppm, no significant increase on TBARS production was observed for the first 24 h after addition, and only a low increase was observed at later stages. The PI values obtained at the end of the heating period were 50 and 61 at 100 and 200 ppm, respectively.

Analogous results were obtained when Z-Lys or its reaction products with (E)-2-octenal, compounds **2** and **3**, were analyzed. Figure 4 shows TBARS production as a function of incubation time for Z-Lys and compound **2**. Z-Lys delayed TBARS production at both 100 and 200 ppm as compared with untreated soybean oil, but this delay was small. At the end of the incubation time its PI values were 33 and 39 at 100 and 200 ppm,

respectively. The reaction of Z-Lys with (E)-2-octenal produced a pyridinium salt, compound **2**, that inhibited TBARS production much more efficiently than Z-Lys itself. After addition of compound **2** at 100 ppm, TBARS did not change for 24 h and only slightly increased in the following 48 h. The effect of compound **2** at 200 ppm was higher. TBARS did not change for the first 48 h after addition and only increased in the last 24 h. The PI values for compound **2** at 100 and 200 ppm at the end of the incubation period were 73 and 71, respectively.

Figure 5 shows the effect of addition of compound **3** on TBARS production. The effect of this compound was slightly greater than the effect of compound **2**, and TBARS did not change for 48 h after addition at the two concentrations assayed. They only increase in the last 24 h of the heating period. Its PI values at this time were 80 and 77, respectively, at the two concentrations assayed.

The results obtained in this study show that amino acid analogs Z-His and Z-Lys are able to delay a peroxidation process initiated in a soybean oil. However, when these compounds reacted with the lipid peroxidation product (E)-2-octenal, the produced compounds exhibited an increased antioxidative activity. The order of effectiveness obtained was Z-His < Z-Lys < **1** < **2** ≈ BHT ≈ **3** < PG. All of them were able to delay or inhibit lipid peroxidation after initiation, and, therefore, they might play a role in foods by delaying the peroxidative process at the same time that they are being produced. This mechanism may be contributing to the well-known antioxidative effect of amino acids and proteins. Thus, for example, oils and fats are less susceptible to oxidation when they are mixed with whey powder, wheat flour, casein, or amino acids and then heated at temperatures ranging from 100 to 300 °C (Lips, 1951; Janicek and Pokorny, 1961; Kawashima et al., 1977; Dworschák and Szabó, 1986).

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