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Methyl Linoleate Oxidation in the Presence of Bovine Serum Albumin

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The oxidation of methyl linoleate (LMe) in the presence of bovine serum albumin (BSA) was studied to analyze both the processes involved when lipid oxidation occurs in the presence of proteins and the relative progression of the several reactions implicated. The disappearance of LMe, the formation of primary and secondary lipid oxidation products, the loss of essential amino acids, and the production of oxidized lipid/amino acid reaction products (OLAARPs) were studied as a function of incubation time. During the first steps of lipid oxidation, LMe was converted quantitatively to methyl linoleate hydroperoxides, which were very rapidly degraded to either secondary products of lipid oxidation or OLAARPs. No significant differences were identified in the major lipid oxidation products formed in incubations with or without proteins, indicating that mechanisms for formation of these compounds are similar in both cases. In addition, no significant differences were observed between the time-courses of formation of secondary oxidation products and OLAARPs, suggesting that hydroperoxide decomposition and OLAARP formation occur simultaneously when the lipid oxidation process takes place in the presence of proteins. Furthermore, OLAARP formation seems to be an unavoidable process that should be considered as a last step in the lipid peroxidation process.

KEYWORDS: Amino acid losses; carbonyl−amine reactions; lipid oxidation; nonenzymatic browning; oxidized lipid/protein reactions; protein damage; pyrrole amino acids

INTRODUCTION

Lipid oxidation is a major cause of food spoilage and is undesirable from sensory acceptability and economic points of view. Therefore, extensive research has been done not only to identify the products of lipid oxidation and the conditions that influence their production, but also to study the mechanisms involved (*1*−*8*). Because oxidative reactions in foods are exceedingly complex, simpler model systems, such as pure fatty acids, have been used to ascertain mechanistic pathways, and these data have been extrapolated to more complex food lipid systems. However, other components present in foods are able to play a role in the lipid peroxidation process, which may either modify its kinetics or produce end products that are different from those formed during oxidation of pure lipids.

These last reactions may be of particular interest in the case of proteins. Studies carried out during the past decades have shown that lipid oxidation products are able to react with amines, amino acids, and proteins, producing different compounds which influence food quality (*9*−*11*). Thus, these reactions lead to browning, odor and flavor formation, loss of nutritional quality, and production of compounds with antioxidant effects (*11*−*15*). Among the different compounds produced in these reactions, studies from this laboratory have shown that some lipid oxidation products are able to react with the ϵ-amino groups of the lysine residues producing pyrrole amino acids, and these compounds may be in part responsible for some of the changes produced in foods as a consequence of oxidized lipid/protein reactions (*16*−*19*).

In an attempt to fully understand both the different processes involved when lipid oxidation occurs in the presence of proteins and the relative progression of the several reactions implicated, the present investigation studies the oxidation of methyl linoleate (LMe) in the presence of bovine serum albumin (BSA). The disappearance of LMe and the formation of primary and secondary products of lipid peroxidation, as well as the loss of essential amino acids and the production of oxidized lipid/amino acid reaction products (OLAARPs) were studied as a function of incubation time.

EXPERIMENTAL PROCEDURES

Materials. LMe and essentially fatty-acid-free BSA were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were analytical grade and were purchased from reliable commercial sources.

Sample Preparation. A mixture of LMe (23% w/w) and BSA (77% w/w) was triturated in a mortar until a homogeneous powder was obtained. Triplicate LMe/BSA mixtures (1.5 g) were placed in Petri dishes and incubated for 13 days under air in the dark at 60 °C. This temperature was selected because it is widely employed in accelerated stability tests (such as the Schaal oven test).

Analytical Measurements. At different periods of time, 100-mg samples were removed for analytical measurements. Lipids were extracted twice with 2 mL of chloroform/methanol (2:1), and lipid

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extracts were combined, evaporated, diluted with methanol to 1% solution, and analyzed for LMe disappearance, lipid oxidation, and color changes. Delipidated proteins were analyzed for color changes, amino acid losses, and OLAARP formation.

Fatty acid analysis was carried out by capillary GC (*20*) using the methanolic solution and methyl heptadecanoate acid which was added as internal standard. GC−MS analysis was performed with a Finnigan MAT 95 double-focusing mass spectrometer (Finnigan, Bremen, Germany) operating in the electron ionization mode. Electron energy was 70 eV, multiplier voltage was 1500 V, source temperature was 200 °C, and transfer line temperature was 250 °C. Spectral data were acquired over a mass range of 28−600 amu at a scan rate of 1 s/scan.

Three determinations were used to evaluate lipid peroxidation: the absorbances at 234 and 270 nm, and the thiobarbituric acid-reactive substances (TBARS) assay. Absorbances at 234 and 270 nm were measured using 0.01% solutions in methanol. Values are expressed as specific extinctions, conventionally indicated as K_{234} and K_{270} (*21*). The TBARS assay was carried out by the method of Kosugi et al. (*22*) using the 0.01% solutions in methanol.

Protein damage was evaluated by determining amino acid losses and OLAARP formation. Amino acid analysis was carried out by hydrolyzing the delipidated proteins overnight in the presence of 6 N HCl. The produced amino acids were derivatized with diethyl ethoxymethylmalonate, and fractionated by HPLC using a previously described gradient (*23*, *24*). OLAARP formation was determined spectrophotometrically after derivatization of protein pyrroles produced with *p*-dimethylaminobenzaldehyde (*18*, *25*). Pyrrole determination is a good measurement of OLAARPs because these compounds have been shown to be produced both in the reaction of protein amino groups with many lipid oxidation products, including 4,5-epoxy-2-alkenals (*24*), 4-hydroxy-2-alkenals (*26*, *27*), unsaturated epoxyoxo fatty acids (*28*), and lipid hydroperoxides (*29*), as well as in the oxidation of fish microsomes in the presence of reactive oxygen species (*30*, *31*).

Color changes were determined spectrophotometrically in both lipid extracts and delipidated proteins. Lipid extracts were prepared at 1% in methanol and delipidated proteins were solubilized in 6 M guanidine HCl with 20 mM potassium phosphate/trifluoroacetic acid, pH 2.3 (1 mg/mL). Yellowness index (YI) was expressed according to Francis and Clydesdale (*32*) as follows:

$$\mathrm{YI} = 142.86 \times \left(\frac{b^*}{L^*}\right)$$

Statistical Analysis. All results are expressed as mean values ± SD of three experiments, except for times 0 and 24 h which are mean values of six 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 (*33*). 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

Lipid Peroxidation in the LMe/BSA Model System. The method described in the Experimental Procedures section allowed quantitative extraction of all lipid compounds present in the incubations (23.7 ± 1.0% of the incubation mixture, $n = 45$). GC analysis of this extract showed that the incubation of the LMe in the presence of the BSA at 60 °C rapidly produced the disappearance of the fatty acid. **Figure 1** shows the GC chromatograms obtained at the initial time, after 14 and 32 h of incubation, and after 13 days. LMe (peak a) disappeared completely after about 1 day, and its disappearance was parallel to the appearance to some other peaks in the chromatogram. Although no exhaustive characterization of peaks was intended, major peaks did not differ from lipid oxidation products formed in incubations of the lipid carried out in the absence of proteins (data not shown) and were tentatively identified by using GC−MS. Thus, the rapid formation of methyl 9-oxononanoate (peak c), a typical decomposition product of LMe hydroperoxides, and epoxy (peaks d) and hydroxy (peaks e) derivatives was observed. These compounds might be present in the incubation mixture or produced in the injection port of the chromatograph. In addition, most of these compounds decreased or disappeared with the incubation time, and the appearance of conjugated triunsaturated derivatives (peak f) was observed.

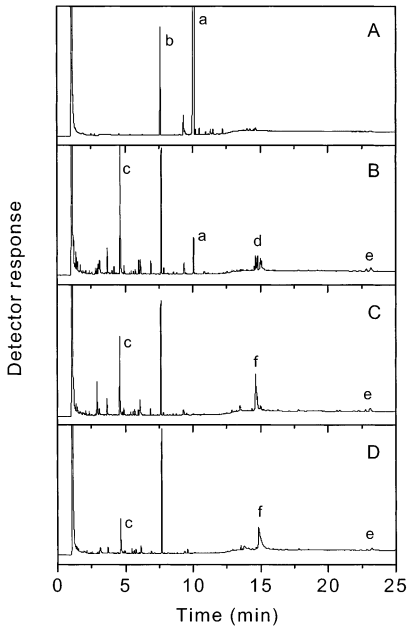

Figure 1. GC chromatograms of lipid extracts obtained from LMe/BSA incubations in the dark at 60 °C after: A, 0 h; B, 14 h; C, 32 h; and D, 13 days. Major peaks were tentatively assigned by GC–MS as follows: a, LMe; b, methyl heptadecanoate (internal standard); c, methyl 9-oxononanoate; d, epoxy esters; e, hydroxy esters; and f, conjugated triunsaturated fatty esters.

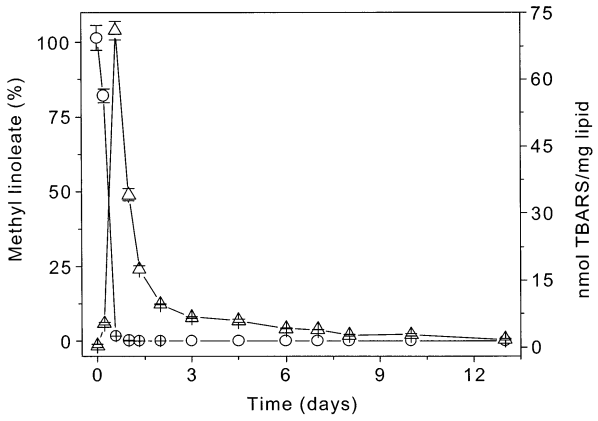

Figure 2. Time-course of LMe disappearance (○) and TBARS formation (△) in the lipid extracts isolated from LMe/BSA incubations in the dark at 60 °C. Data are mean values ± SD of three independent experiments.

These results are in agreement with the data obtained by measuring K_{234}, K_{270}, and TBARS formation. **Figure 2** shows the time-course of LMe disappearance and TBARS formation. Both processes exhibited a small lag time during the first few hours, more clearly observed for TBARS. This lag time implied that neither LMe decreased nor TBARS increased linearly for the first 14 h. This short period was followed by a rapid decrease of LMe concentration at the same time that TBARS increased very rapidly to arrive at its highest value. This point was observed after 14 h of incubation, the same incubation time at which the highest concentration of peak c

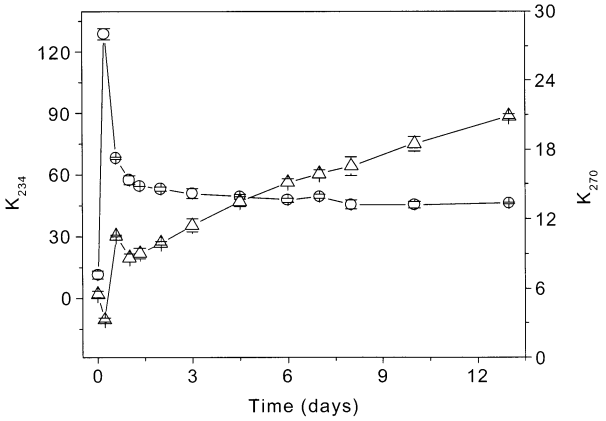

Figure 3. Time-course of K_{234} (○) and K_{270} (△) in the lipid extracts isolated from LMe/BSA incubations in the dark at 60 °C. Data are mean values ± SD of three independent experiments.

(methyl 9-oxononanoate) was observed (**Figure 2B**). After this time LMe disappeared completely in the next few hours and TBARS began to decrease, first very rapidly (the next 2 days) and then much more slowly.

Figure 3 shows the time-course of K_{234} and K_{270} during LMe/BSA incubation. The hydroperoxide formation (K_{234}) followed a time-course similar to that observed for the TBARS formation, but the maximum K_{234} value was observed after only 5 h. Considering an absorptivity for the hydroperoxide of 26,000 (*34*), the results obtained indicated the presence of 16.2% of hydroperoxides after 5 h, a value which is in agreement with the loss of about 18% of LMe observed at this time by GC (**Figure 1**). After this time, LMe hydroperoxides were decomposed very rapidly, according to the well-known capacity of proteins to promote hydroperoxide decomposition (*35*). This decrease in K_{234} was very rapid during the next 24 h and decreased much more slowly afterward.

Hydroperoxide decomposition was parallel to the increase observed in K_{270} value, at least during the first incubation times. K_{270} value is related to the formation of conjugated trienes, and, therefore, is indicative of formation of secondary products of lipid oxidation. The time-course of K_{270} was not lineal during the first incubation day. Thus, it decreased for the first 5 h, then increased very rapidly for the next 9 h, at the same time that K_{234} decreased very rapidly, and after that a new decrease was observed. From the second incubation day to the end K_{270} always increased, indicating that either secondary products were continuously produced during the whole incubation period, despite the low decrease observed for K_{234} or TBARS during this period, or the formation of other products that contributed to the increase of absorbance observed at this wavelength.

Protein Damage in the LMe/BSA Model System. The incubation of BSA in the presence of the LMe decreased the number of some amino acid residues recovered after acid hydrolysis at the same time that showed the OLAARP production. **Figure 4** shows the time-course of disappearance of lysine residues and the formation of pyrrole rings in the lysine residues of the protein. Lysine residues recovered after acid hydrolysis decreased very rapidly during the first 2 days and, then, much more slowly during the next 11 days. Inverse behavior was observed for the pyrrole formation. In fact, there was a correlation between lysine losses and pyrrole formation ($r = -0.913$, $p < 0.0001$), therefore confirming the origin of pyrrole derivatives in the protein (*24*, *31*). The increase of pyrrole derivatives observed during protein incubation was 0.3−0.4 nmol/mg protein. By using a molecular weight of 66,411 for

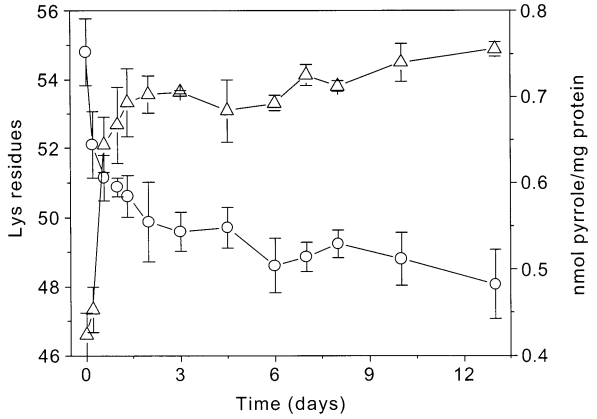

Figure 4. Time-course of lysine residues recovered after acid hydrolysis (○) and protein pyrroles (△) determined in the delipidated proteins isolated from LMe/BSA incubations in the dark at 60 °C. Data are mean values ± SD of three independent experiments.

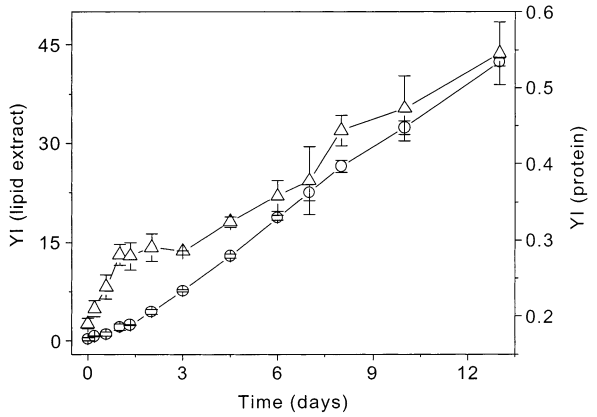

Figure 5. Time-course of YI of lipid extracts (○) and delipidated proteins (△) isolated from LMe/BSA incubations in the dark at 60 °C. Data are mean values ± SD of three independent experiments.

the protein (*36*), the pyrroles found suggest that the 2.0−2.7% of lysine residues (between 1.2 and 1.6 residues) were transformed into pyrroles. Because the results found showed that between 5 and 8 residues of lysine were lost during incubation, pyrrole formation is responsible for the loss of 15−32% of lysine residues. The rest of lysine residues may be either decomposed by the free radicals produced during LMe hydroperoxide decomposition or by formation of other OLAARPs.

Color Changes in the LMe/BSA Model System. As a consequence of all these changes produced in both the lipid and the protein during the incubation time, the formation of brown color was observed. Both lipid and protein developed color changes that were parallel. **Figure 5** shows the time-course of YI for both the lipid extract and the delipidated protein as a function of the incubation time. Both indexes increased almost linearly during the whole incubation period ($r = 0.983$, $p < 0.0001$, for the lipid extract; and $r = 0.966$, $p < 0.0001$, for the delipidated protein), and there was a correlation between YI data obtained for the lipid extract and for the delipidated protein at the different incubation times ($r = 0.977$, $p < 0.0001$). In addition, YI also correlated with K_{270} for the last 12 days ($r = 0.991$, $p < 0.0001$, for the lipid extract; and $r = 0.958$, $p < 0.0001$, for the delipidated protein).

DISCUSSION

When lipid oxidation takes place in the presence of other food components, these last compounds are able to modify the

lipid oxidation process by reacting with the lipid oxidation products. All these processes are simultaneous and not all the lipid has to be oxidized and degraded before oxidized lipid/amino acid reactions take place. Thus, when the oxidation of LMe was carried out in the presence of BSA, the first step was the production of lipid hydroperoxides. These products were formed quantitatively from the lipid within the first few hours of incubation with little or no influence of the protein, because after 5 h the concentration of lipid hydroperoxides was approximately the same as the loss of unoxidized lipid.

These hydroperoxides were decomposed very rapidly, and, although the maximum of TBARS was observed after 14 h, the concentration of hydroperoxides was much reduced by then. This maximum of TBARS, which should be indicative of the maximum concentration of secondary products of lipid oxidation, also coincided with the highest increase of OLAARP formation and the highest lysine losses. These results suggest that OLAARP formation is parallel to secondary products production and they are formed either directly from lipid hydroperoxides or very rapidly from secondary products. These results are also in agreement with previous results indicating that antioxidant effects of OLAARPs may be employed to delay lipid oxidation in mixtures containing lipids and proteins (*37*).

Although the main chemical changes in the reaction (lipid oxidation and OLAARP formation) were produced very rapidly during the first few hours of incubation, changes in the appearance of the samples were produced much more slowly, when most of the analyses carried out did not show significant changes. This is in agreement with the mechanisms proposed for development of browning (*11*). Browning in lipids is supposed to be produced by aldol condensations which should not affect either the TBARS value or the absorbance at 234. An increase in the absorbance at 270 nm may be expected as a consequence of the increase in the conjugation of the products formed. Browning in proteins is supposed to be produced by pyrrole polymerization (*18*, *24*). This reaction does not imply the increase in pyrrole concentration, although the extinction coefficients of monomers and dimers are not the same (*18*). However, an increase in the absorbance at 270 nm may also be expected.

The results obtained in this study suggest that the mechanisms of lipid oxidation in the presence of proteins do not differ greatly from those produced in the absence of these last compounds. However, OLAARPs are always produced, and the formation of these compounds should be considered a final step in the lipid peroxidation process, simultaneously to the formation of other secondary lipid oxidation products.

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