# Inhibition of Proteolysis in Oxidized Lipid-Damaged Proteins

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The proteolysis of bovine serum albumin (BSA) modified by reaction with the lipid peroxidation product 4,5(*E*)-epoxy-2(*E*)-heptenal was studied to better understand the loss of digestibility observed in oxidized lipid-damaged proteins. BSA was incubated for different periods of time with eight concentrations of the epoxyalkenal and, then, treated for 24 h with chymotrypsin, pancreatin, Pronase, or trypsin. The treatment of BSA with the aldehyde always decreased its proteolysis in relation to that of native BSA, and this inhibition of the proteolysis was related to the concentration of the epoxyalkenal and the reaction time. In fact, this inhibition was correlated with the damage suffered by the protein as a consequence of its reaction with the aldehyde: mainly the development of browning, the denaturation of the protein, and the formation of the oxidized lipid/amino acid reaction product $\epsilon$-*N*-pyrrolylnorleucine ($p \leq 0.0011$, 0.0045, and 0.0031, respectively). In addition, $\epsilon$-*N*-pyrrolylnorleucine added at 0.1 or 1 mM inhibited the proteases assayed and suggested that the inhibition of the proteolysis observed in oxidized lipid-damaged proteins may be related to the formation and accumulation of pyrrolized amino acid residues.

**Keywords:** *Lipid peroxidation; carbonyl−amine reactions; Maillard reactions; nonenzymatic browning; oxidized lipid-damaged proteins; pyrrole amino acids; proteolytic enzymes; protease inhibitors; lipofuscins*

## INTRODUCTION

Lipid oxidation is a major cause of food spoilage and is undesirable from sensory acceptability and economic points of view (*1*−*5*). It also can cause oxidative reactions to decrease the nutritional quality of food and generate oxidation products that are potentially toxic. Among the reactions produced by oxidizing lipids in foods, the modification of amines, amino acids, and proteins has received considerable attention (*6*−*8*). This is an important mechanism in the processing and storage of foods with simultaneous formation of both deleterious and beneficial compounds. Thus, these reactions lead to browning, odor and flavor formation, and loss of nutritional quality and compounds with antioxidant effects (*9*−*11*).

The loss of nutritional quality is attributed to the destruction of essential amino acids and a decrease in digestibility and inhibition of proteolytic and glycolytic enzymes. It is clear that a number of essential amino acids, such as tryptophan, lysine, and methionine, are destroyed in addition to the disappearance of essential fatty acids (*12*, *13*). However, the inhibition exhibited by proteolytic and glycolytic enzymes is not at present completely understood.

The present investigation was undertaken in an attempt to clarify this decreased proteolysis observed in oxidized lipid-damaged proteins. As a model protein, bovine serum albumin (BSA) was selected because serum albumins represent a major class of animal proteins; BSA is free of prosthetic groups and other complicating factors, and its primary, secondary, and tertiary structures have been well-characterized in the literature (*14*). As a lipid oxidation product, 4,5(*E*)-epoxy-2(*E*)-heptenal was employed because it is a secondary product of lipid oxidation, it is able to modify amino acids very rapidly, and the mechanisms of the reactions produced with amines, amino acids, and proteins are now well-known (*15*, *16*). Thus, the modifications produced in the structure of BSA following its reaction with 4,5(*E*)-epoxy-2(*E*)-heptenal are initiated by the formation of reaction products between the epoxyalkenal and some amino acid residues, mostly Michael adducts with the histidines and pyrrole derivatives with the lysines. These changes produced in the primary structure of BSA also modified secondary and tertiary structures of the protein, and they were also involved in the development of the color and fluorescence produced in this reaction, which was a consequence of the polymerization of some of the pyrrole derivatives produced (*17*).

## MATERIALS AND METHODS

**Materials.** 4,5(*E*)-Epoxy-2(*E*)-heptenal was prepared in a manner analogous to that of 4,5(*E*)-epoxy-2(*E*)-decenal (*18*). Essentially fatty acid free BSA, chymotrypsin from bovine pancreas (C4129), pancreatin from porcine pancreas (P7545), protease from *Streptomyces griseus* (P8811), and trypsin from bovine pancreas (T8003) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were purchased from reliable commercial sources.

The reaction between 4,5(*E*)-epoxy-2(*E*)-heptenal and lysine produces several monomeric pyrrole amino acids, in addition to different polymers (*19*). Two of these monomeric pyrrole amino acids, $\epsilon$-*N*-pyrrolylnorleucine (**I**) and 1-(5′-amino-1′-carboxypentyl)pyrrole (**II**), were prepared as described previously (*19*) to determine their inhibitory effect on the proteases. Chemical structures of compounds **I** and **II** are collected in Figure 1.

**Reaction of 4,5(*E*)-Epoxy-2(*E*)-heptenal with BSA.** A solution of 1 mg/mL of BSA in 50 mM sodium phosphate buffer

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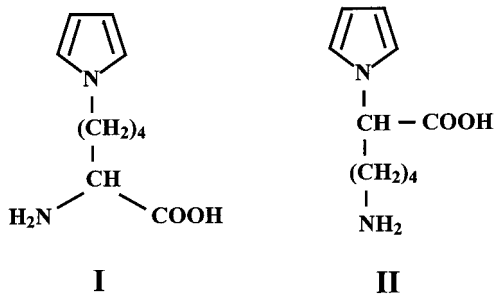


**Figure 1.** Chemical structures of **I**, ϵ-*N*-pyrrolylnorleucine, and **II**, 1-(5′-amino-1′-carboxypentyl)pyrrole.

(pH 7.4) was incubated for different periods of time at 37 °C and in the presence of several concentrations of 4,5(*E*)-epoxy-2(*E*)-heptenal. At the end of the incubation time, modified BSA samples (2.5 mL) were purified by using PD-10 columns.

**Effect of Different Proteases on BSA Modified by 4,5-(*E*)-Epoxy-2(*E*)-heptenal.** Modified BSA (500 μL) was treated at 37 °C for 24 h with 50 μL of a solution of the protease that contained 0.0357 μg of enzyme/μL of 50 mM sodium phosphate buffer (pH 7.4). The effect of proteases was evaluated by both the determination of the remaining protein in the solution and the presence of pyrrole amino acids in the resulting hydrolyzed and nonhydrolyzed proteins. Protein was measured according to the method of Bradford (*20*, *21*) using 50 μL of the hydrolyzed sample, 550 μL of water, and 400 μL of Bradford's reagent. Pyrrole amino acids were determined in the remaining protein and in the produced peptides by using a previously described procedure (*22*). Briefly, 500 μL of the hydrolyzed sample was treated with 79 μL of 2% Ehrlich reagent and incubated at 45 °C for 30 min. The protein was then precipitated with 289 μL of 30% trichloroacetic acid at 0 °C for 30 min and then centrifuged at 4000*g* for 10 min. The supernatant, containing the produced peptides, was measured directly, and the pellet, containing the remaining protein, was collected, dissolved in 1 mL of 6 M guanidine HCl with 20 mM potassium phosphate/trifluoroacetic acid (pH 2.3), and, finally, left for 30 min at 37 °C with vortexing. The absorbance of both supernatant and dissolved pellet at the maximum obtained at 570−585 nm were measured in the next 2 h against a blank prepared under the same conditions but without *p*-dimethylaminobenzaldehyde. For calculations, an extinction coefficient of 37000 $L \cdot mol^{-1} \cdot cm^{-1}$ was used (*11*). Comparisons were carried out by using a percentage of inhibition that was calculated as

$$\text{inhibition (\%)} = 100 \times [(DT - CT)/(C - CT)] \qquad (1)$$

where DT was the value obtained for the modified BSA after proteolysis, CT is the value obtained for the control BSA after proteolysis, and C is the value obtained for the modified or the control BSA without proteolysis.

**Effect of ϵ-*N*-Pyrrolylnorleucine (I) and 1-(5′-Amino-1′-carboxypentyl)pyrrole (II) on Proteases.** BSA samples (200 μL of a solution that contained 1 mg/mL BSA in 50 mM sodium phosphate buffer, pH 7.4) were treated with 20 μL of protease solution (containing 0.0357 μg of enzyme/μL of 50 mM sodium phosphate buffer, pH 7.4) and 20 μL of 1.2 or 12 mM lysine, compound **I**, or compound **II** (the final concentrations of the tested compounds were 0.1 and 1 mM, respectively). The reactions were incubated at 37 °C for 24 h, and the effect on proteases was evaluated by determination of the remaining protein in the solution using the Bradford's procedure as described above. All determinations were carried out in duplicate, and given results represent the mean of these two independent experiments.

## RESULTS

**Effect of Protein/Protease Ratio on the Proteolysis Produced.** The conditions for the proteolysis were optimized by testing different concentrations of the proteases. As expected, the proteolysis of both untreated BSA and modified BSA always increased with the concentration of the protease, and this was independent of the protease assayed. However, the proteolysis observed in the modified BSA was always lower than the proteolysis observed in the control BSA, and this difference depended on the protein/protease ratio employed. Thus, the inhibition of the proteolysis exhibited by the modified BSA with respect to the untreated BSA decreased with the increase in the concentration of the protease for the different proteases assayed (Figure 2). This effect depended on the protease assayed and was very high in Pronase. Pancreatin and chymotrypsin exhibited a decreased inhibition only at a protein/protease ratio lower than 100, and the effect on trypsin was the lowest of the tested proteases. Because the objective of this study was to investigate the loss of digestibility observed in oxidized lipid-damaged proteins, the conditions when a higher inhibition was observed were preferred and the different experiments were carried out using a protein/protease ratio of 200.

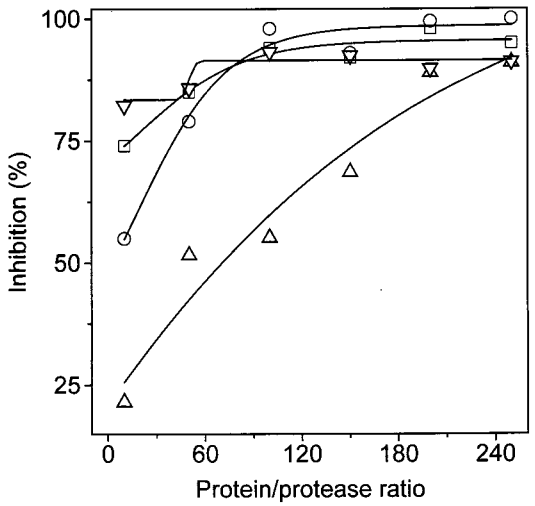


**Figure 2.** Effect of protein/protease ratio on the inhibition of the proteolysis observed for a modified BSA incubated in the presence of chymotrypsin (□), pancreatin (○), Pronase (△), and trypsin (▽). BSA was incubated overnight in the presence of 5 mM 4,5(*E*)-epoxy-2(*E*)-heptenal and, then, passed through PD-10 columns to obtain the modified protein. Modified proteins were hydrolyzed for 24 h in the presence of the proteases.

**Relationship between the Damage Suffered by the BSA and the Inhibition of the Proteolysis.** Analogously to the concentration of the protease, the inhibition of the proteolysis depended on the extent of damage to the protein. According to previous studies (*15*), the damage suffered by the protein depended on the concentration of the aldehyde and the reaction time. Therefore, both effects were studied to determine their influence on the inhibition of the proteolysis. Figure 3 shows the effect of the incubation time between the BSA and the aldehyde at two concentrations of 4,5(*E*)-epoxy-2(*E*)-heptenal: 1 and 10 mM. The damage produced by the epoxyaldehyde at 10 mM was always higher than that produced by the aldehyde at 1 mM (*15*, *16*), as was the inhibition of the proteolysis observed. For most proteases, the main changes in the inhibition were observed during the first 6 h of the incubation between the epoxyalkenal and the protein, and higher incubation times did not produce additional increases in the inhibition. All of these time courses were parallel to the changes produced in BSA as a consequence of its reaction with 4,5(*E*)-epoxy-2(*E*)-heptenal, which were

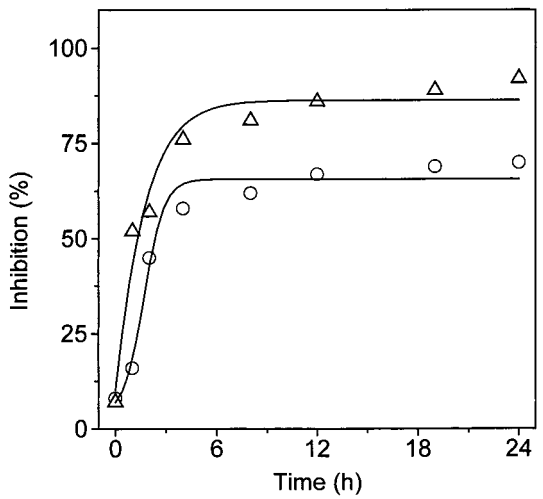


**Figure 3.** Effect of 4,5(*E*)-epoxy-2(*E*)-heptenal/BSA reaction time on the inhibition of the proteolysis observed for the modified BSA incubated in the presence of chymotrypsin (pancreatin, Pronase, and trypsin produced analogous results). BSA was incubated for the indicated periods in the presence of 1 (○) or 10 mM (△) 4,5(*E*)-epoxy-2(*E*)-heptenal and, then, passed through PD-10 columns to obtain the modified proteins. Modified proteins were hydrolyzed for 24 h in the presence of the proteases.

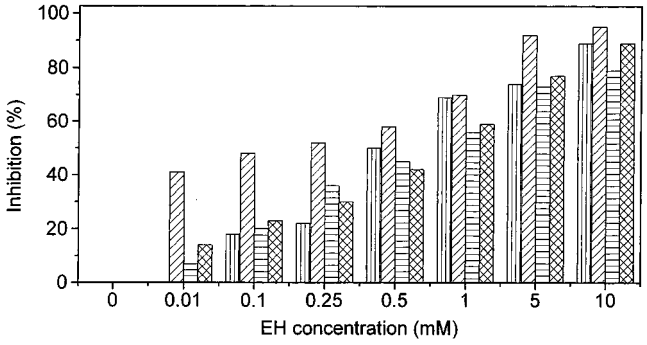


**Figure 4.** Effect of 4,5(*E*)-epoxy-2(*E*)-heptenal concentration on the inhibition of the proteolysis observed for the modified BSA incubated in the presence of chymotrypsin (vertically stripled bars), pancreatin (slashed bars), Pronase (horizontally striped bars), and trypsin (crossh-hatched bars). BSA was incubated in the presence of 4,5(*E*)-epoxy-2(*E*)-heptenal for 19 h and, then, passed through PD-10 columns to obtain the modified proteins. Modified proteins were hydrolyzed for 24 h in the presence of the proteases.

described previously (*15*, *16*): development of browning and fluorescence, losses of lysine and histidine, formation of ϵ-*N*-pyrrolylnorleucine and protein carbonyls, and protein denaturation and polymerization.

Figure 4 shows the effect of eight different concentrations of 4,5(*E*)-epoxy-2(*E*)-heptenal (0, 0.01, 0.1, 0.25, 0.5, 1, 5, and 10 mM) on the inhibition of the proteolysis observed in the modified protein with respect to untreated BSA. As expected, the inhibition increased with the concentration of the aldehyde and was similar for the four assayed proteases. Analogously to the time courses described above, these results were also parallel to the changes produced in BSA as a consequence of its reaction with 4,5(*E*)-epoxy-2(*E*)-heptenal. In fact, there were good correlations among the results obtained for the inhibition of the proteolysis using different proteases and the development of browning and fluorescence, the losses of lysine and histidine, the production of ϵ-*N*-pyrrolylnorleucine and protein carbonyls, or the denaturation and the polymerization of the protein (Table 1). With independence of the proteases assayed, the best correlations were always obtained with the development of browning, the formation of ϵ-*N*-pyrrolylnorleucine, and the denaturation of the protein.

**Effect of Proteases on Pyrrolyzed Proteins.** Many of the changes exhibited by the BSA following its reaction with 4,5(*E*)-epoxy-2(*E*)-heptenal are a consequence of the formation of pyrrole derivatives between the ϵ-amino group of lysine residues and the aldehyde and the later polymerization of the compounds produced (*15*, *17*). In an attempt to better understand the decreased proteolysis observed in the modified BSA, these pyrrole amino acids were determined in both the remaining protein fraction and the hydrolyzed peptides resulting from the action of the proteases. Figure 5 shows the pyrroles determined in the remaining protein fraction of the modified BSA as a function of the incubation time between the epoxyalkenal and the protein. The incubation time between the protein and the epoxyalkenal increased both the concentration of pyrroles (*15*) and the inhibition of the proteases (see above). Therefore, the pyrroles determined in the nonhydrolyzed fraction always increased as a function of the incubation time and were higher in the BSA treated with 10 mM 4,5(*E*)-epoxy-2(*E*)-heptenal than in the protein treated with 1 mM aldehyde. In addition, there were not big differences among the different proteases and between the proteases and the control, suggesting that most pyrroles remained in the nonhydrolyzed part of the modified protein. The only protease that exhibited a clear decrease in the pyrroles determined in this fraction was the Pronase.

More information was obtained when pyrroles were determined in the supernatant (Figure 6). Modified BSA in the absence of proteases did not liberate any pyrroles. However, the treatment with the proteases produced

**Table 1. Correlations between the Inhibition of the Proteolysis Observed in the Modified BSA and the Changes Produced in This BSA after Reaction with 0−10 mM 4,5(*E*)-Epoxy-2(*E*)-heptenal[a]**

| | protease | | | |
|---|---|---|---|---|
| | chymotrypsin | pancreatin | Pronase | trypsin |
| browning | 0.969 (<0.0001) | 0.922 (0.0011) | 0.987 (<0.0001) | 0.990 (<0.0001) |
| fluorescence | 0.908 (0.0018) | 0.866 (0.0054) | 0.924 (0.0010) | 0.949 (0.00033) |
| lysine content | −0.868 (0.0052) | −0.864 (0.0056) | −0.889 (0.0031) | −0.933 (0.00070) |
| histidine content | −0.770 (0.0255) | −0.745 (0.0339) | −0.783 (0.0215) | −0.834 (0.0100) |
| ϵ-*N*-pyrrolylnorleucine | 0.936 (0.00061) | 0.889 (0.0031) | 0.946 (0.00038) | 0.972 (<0.0001) |
| protein carbonyls | 0.875 (0.0045) | 0.830 (0.0107) | 0.885 (0.0035) | 0.926 (0.00097) |
| denaturation | 0.931 (0.00078) | 0.874 (0.0045) | 0.934 (0.00067) | 0.963 (0.00012) |
| polymerization | 0.877 (0.0042) | 0.814 (0.0139) | 0.869 (0.0051) | 0.918 (0.0013) |

[a] The concentrations of 4,5(*E*)-epoxy-2(*E*)-heptenal employed were 0, 0.01, 0.25, 0.5, 1, 5, and 10 mM. First values are correlation coefficients. Significances are given in parentheses. Changes produced in BSA as a consequence of its reaction with 4,5(*E*)-epoxy-2(*E*)-heptenal were described previously (*15*, *16*).

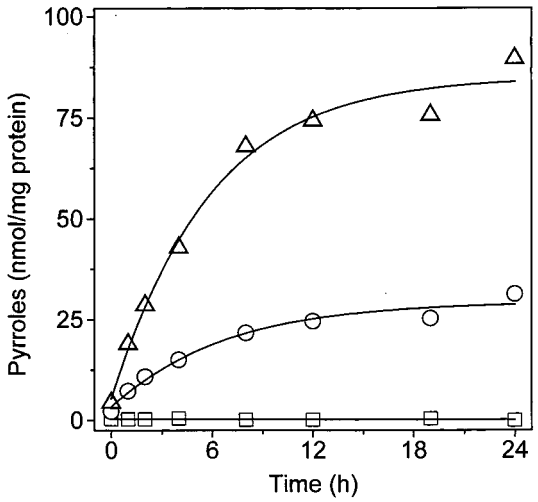


**Figure 5.** Effect of 4,5(*E*)-epoxy-2(*E*)-heptenal/BSA reaction time on pyrroles present in the nonhydrolyzed proteins that resulted from the incubation of the modified BSA with chymotrypsin (pancreatin, Pronase, and trypsin, as well as BSA incubated in the absence of proteases, produced analogous results). BSA was incubated for the indicated periods in the presence of 0 (□), 1 (○), or 10 mM (△) 4,5(*E*)-epoxy-2(*E*)-heptenal and, then, passed through PD-10 columns to obtain the modified proteins. Modified proteins were hydrolyzed for 24 h in the presence of the proteases.

pyrrole-containing peptides. The order observed for the liberation of pyrroles was chymotrypsin < trypsin < pancreatin ≪ Pronase. For most of the proteases assayed, there were no differences between the pyrroles determined in the protein treated with 1 or 10 mM concentrations of the aldehyde. Only the treatment with Pronase liberated more pyrroles in the protein treated with a 10 mM concentration of the aldehyde. In addition, proteases liberated fewer pyrrole-containing peptides in highly damaged proteins than in less damaged proteins (see, for example, the slight decrease of pyrroles liberated in the protein treated with 10 mM aldehyde as a function of time for the four proteases).

The effect of the concentration of the epoxyalkenal on the pyrroles determined in the remaining proteins and the hydrolyzed peptides is given in Figures 7 and 8, respectively. Analogously to the time course results shown in Figure 5, most of the pyrroles appeared in the protein that was not hydrolyzed. The concentration of pyrroles increased with the concentration of the epoxyalkenal and was similar for the control modified BSA or the BSA treated with most proteases. Only the treatment with Pronase produced a clear decrease in the concentration of pyrroles determined in the remaining proteins.

Similarly to the results obtained in the time course studies, the analysis of the hydrolyzed fraction produced clearer results (Figure 8). The incubation of the treated BSA in the absence of proteases did not liberate any pyrroles. However, these were liberated by any of the proteases, and these followed an order analogous to that observed in time course experiments: chymotrypsin ≤ trypsin < pancreatin < Pronase. In addition, the concentration of pyrroles liberated for most proteases was only slightly dependent on the concentration of the epoxyalkenal employed to modify the BSA, suggesting that a more damaged protein, containing more pyrroles, was less hydrolyzed than a less damaged protein, which contained fewer pyrroles. All of these results always related the number of pyrroles to the decrease of the

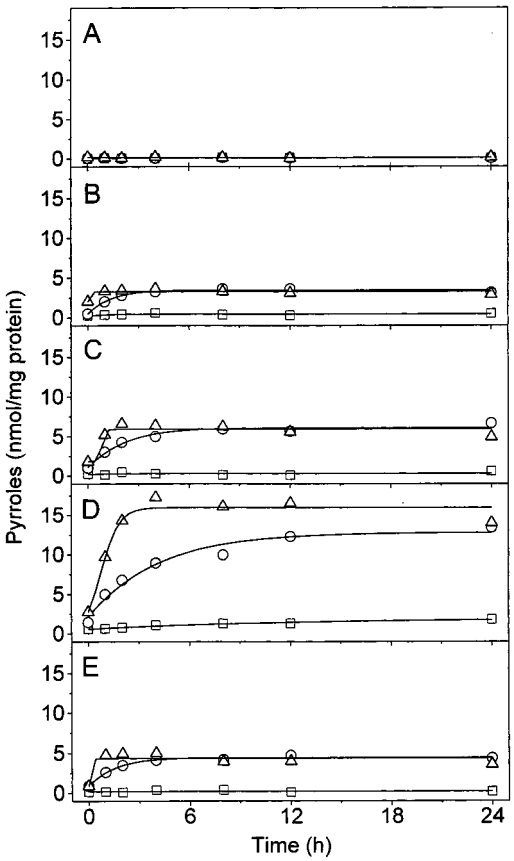


**Figure 6.** Effect of 4,5(*E*)-epoxy-2(*E*)-heptenal/BSA reaction time on pyrroles present in the soluble peptides that resulted from the incubation of the modified BSA with (A) no protease, (B) chymotrypsin, (C) pancreatin (D) Pronase, and (E) trypsin. BSA was incubated for the indicated periods in the presence of 0 (□), 1 (○) or 10 mM (△) 4,5(*E*)-epoxy-2(*E*)-heptenal and, then, passed through PD-10 columns to obtain the modified proteins. Modified proteins were hydrolyzed for 24 h in the presence of the proteases.

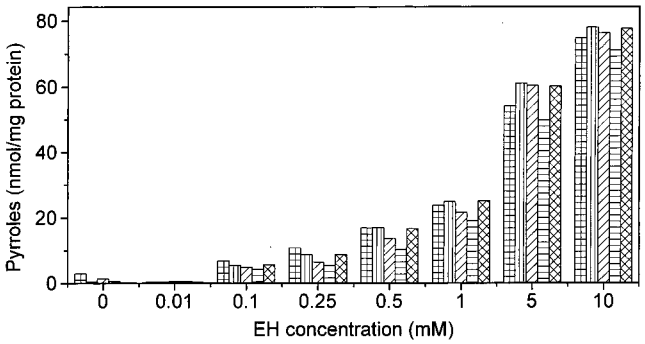


**Figure 7.** Effect of 4,5(*E*)-epoxy-2(*E*)-heptenal concentration on pyrroles present in the nonhydrolyzed proteins that resulted from the incubation of the modified BSA with no protease (grid bars), chymotrypsin (vertically striped bars), pancreatin (slashed bars), Pronase (horizontally striped bars), and trypsin (cross-hatched bars). BSA was incubated in the presence of 4,5(*E*)-epoxy-2(*E*)-heptenal for 19 h and, then, passed through PD-10 columns to obtain the modified proteins. Modified proteins were hydrolyzed for 24 h in the presence of the proteases.

proteolysis, suggesting an inhibitory effect for the pyrroles produced on the proteases.

**Inhibition of the Proteases by the Pyrrole Amino Acids Produced in the Reaction between 4,5(*E*)-Epoxy-2(*E*)-heptenal and Lysine.** To test the hypothesis of the inhibitory role of pyrroles on proteases, one of the pyrroles are produced with the ϵ-amino group of

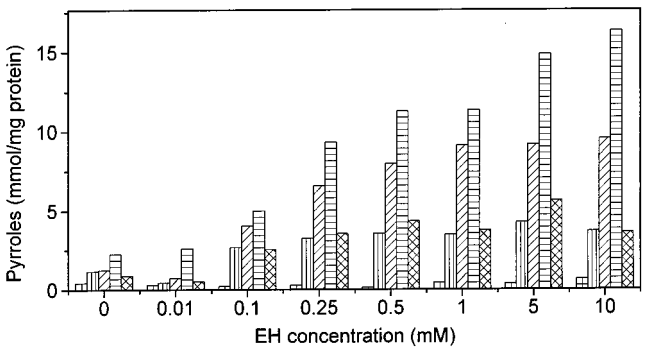


**Figure 8.** Effect of 4,5(*E*)-epoxy-2(*E*)-heptenal concentration on pyrroles present in the soluble peptides that resulted from the incubation of the modified BSA with no protease (grid bars), chymotrypsin (vertically striped bars), pancreatin (slashed bars), Pronase (horizontally striped bars), and trypsin (cross-hatched bars). BSA was incubated in the presence of 4,5(*E*)-epoxy-2(*E*)-heptenal for 19 h and, then, passed through PD-10 columns to obtain the modified proteins. Modified proteins were hydrolyzed for 24 h in the presence of the proteases.

**Table 2. Inhibition (Percent) of Proteases by Some Amino Acids**

| | protease | | | |
|---|---|---|---|---|
| compound | chymotrypsin | pancreatin | Pronase | trypsin |
| lysine (0.1 mM) | 0 | 0 | 0 | 0 |
| lysine (1 mM) | 1.0 | 0 | 0.1 | 0 |
| **I** (0.1 mM) | 16.3 | 13.4 | 13.8 | 50.1 |
| **I** (1 mM) | 56.5 | 47.0 | 18.8 | 83.5 |
| **II** (0.1 mM) | 8.1 | 5.2 | 1.9 | 12.9 |
| **II** (1 mM) | 14.9 | 7.1 | 0 | 7.4 |

lysine residues (ϵ-*N*-pyrrolylnorleucine, **I**) and other produced with the α-amino group of lysine residues [1-(5′-amino-1′-carboxypentyl)pyrrole, **II**] were assayed at two concentrations (0.1 and 1 mM) for determining their inhibitory effect on proteases. The results obtained are collected in Table 2. As expected, treatment of the proteases with the amino acid lysine, which was used as a control, did not produce any inhibitory effect. However, the activity of the proteases was reduced when they were treated with compound **I** at either 0.1 or 1 mM. This amino acid had a great influence on the activity of trypsin and exhibited a lower effect on chymotrypsin or pancreatin. The lowest effect was observed on Pronase. Different from compound **I**, compound **II**, which differed only in the position of the carboxyl group, exhibited a very small inhibitory effect.

## DISCUSSION

The above results have shown that the reaction between the lipid oxidation product 4,5(*E*)-epoxy-2(*E*)-heptenal and BSA produced a modified protein that was degraded with more difficulty by proteases than the native protein. Analogous findings were obtained previously for other products of lipid peroxidation both in foods and in other biological systems (*23*−*28*), and this was considered a consequence of the denaturation and polymerization suffered by the protein. The results found in the present study suggest, however, that this inhibition may be a consequence of the formation of some oxidized lipid/amino acid reaction products that both contribute to the denaturation and polymerization of the protein and, in some cases, are also inhibitors of the proteases.

When the changes suffered by the BSA as a consequence of its reaction with the 4,5(*E*)-epoxy-2(*E*)-heptenal were correlated with the inhibition of the proteolysis observed, the best correlations were obtained with the development of browning, the protein denaturation, and the formation of ϵ-*N*-pyrrolylnorleucine. The browning development in this system is a consequence of the pyrrole formation and polymerization produced between the aldehyde and the ϵ-amino group of lysine residues (*15*, *17*). In addition, these reactions also contribute to the denaturation of the protein and are responsible for the formation of ϵ-*N*-pyrrolylnorleucine (*15*). Therefore, the formation of these pyrrole amino acid residues in a protein treated with an epoxyalkenal seems to be the origin of both most of the changes exhibited by the protein and its decreased proteolysis.

Different pyrrole amino acid residues have been described to be produced in the reaction of oxidized lipids with amines, amino acids, and proteins (*17*, *29*, *30*), and one of them (compound **I**) has been shown to be present in >20 fresh food products (*31*). The above results have shown that this compound **I** is also an inhibitor for the proteases assayed. Thus, it inhibited trypsin by 50.1 and 83.5%, chymotrypsin by 16.3 and 56.5%, pancreatin by 13.4 and 47.0%, and Pronase by 13.8 and 18.8%, at 0.1 and 1 mM, respectively. On the contrary, compound **II**, which differed from compound **I** only in the position of the carboxyl group, did not exhibit a great inhibition of any of the proteases assayed. In addition, Pronase, which was the protease least inhibited by compound **I**, liberated many more pyrrole-containing peptides than any other of the proteases assayed.

Protease inhibition by substituted pyrroles has been known for many years (*32*, *33*). However, to our knowledge, this inhibitory effect was not previously related to the loss of digestibility observed in oxidized lipid-damaged proteins. In addition, these results also may explain in part the in vivo accumulation of some oxidatively damaged cellular components observed in organisms with age or as a consequence of oxidative stress.

## ACKNOWLEDGMENT

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## LITERATURE CITED

(1) Frankel, E. N. *Lipid Oxidation*; The Oily Press: Dundee, Scotland, 1998.
(2) German, J. B. Food processing and lipid oxidation. *Adv. Exp. Med. Biol.* **1999**, *459*, 23−50.
(3) Kubow, S. Routes of formation and toxic consequences of lipid oxidation products in foods. *Free Radical Biol. Med.* **1992**, *12*, 63−81.
(4) Shahidi, F. Indicators for evaluation of lipid oxidation and off-flavor development in food. *Dev. Food Sci.* **1998**, *40*, 55−68.
(5) St. Angelo, A. J. Lipid oxidation in foods. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 175−224.
(6) Chio, K. S.; Tappel, A. L. Synthesis and characterization of the fluorescent products derived from malondialdehyde and amino acids. *Biochemistry* **1969**, *8*, 2821−2827.
(7) Gardner, H. W. Lipid hydroperoxide reactivity with proteins and amino acids: A review. *J. Agric. Food Chem.* **1979**, *27*, 220−229.
(8) Hidalgo, F. J.; Zamora, R. The role of lipids in nonenzymatic browning. *Grasas Aceites* **2000**, *51*, 35−49.

(9) Alaiz, M.; Hidalgo, F. J.; Zamora, R. Effect of pH and temperature on comparative antioxidant activity of nonenzymatically browned proteins produced by reaction with oxidized lipids and carbohydrates. *J. Agric. Food Chem*. **1999**, *47*, 748−752.
(10) Friedman, M. Food browning and its prevention: an overview. *J. Agric. Food Chem*. **1996**, *44*, 631−653.
(11) Zamora, R.; Alaiz, M.; Hidalgo, F. J. Contribution of pyrrole formation and polymerization to the nonenzymatic browning produced by amino-carbonyl reactions. *J. Agric. Food Chem*. **2000**, *48*, 3152−3158.
(12) Gillat, P. N.; Rossell, J. B. The interaction of oxidized lipids with proteins. In *Advances in Applied Lipid Research*; Padley, F. B., Ed.; JAI Press: Greenwich, CT, 1992; Vol. 1, pp 65−118.
(13) Nielsen, H. K.; Finot, P. A.; Hurrell, R. F. Reactions of proteins with oxidizing lipids. 2. Influence on protein quality and on the bioavailability of lysine, methionine, cyst(e)ine and tryptophan as measured in rat assays. *Br. J. Nutr*. **1985**, *53*, 75−86.
(14) Peters, T., Jr. *All About Albumin. Biochemistry, Genetics and Medical Applications*; Academic Press: San Diego, CA, 1996.
(15) Hidalgo, F. J.; Zamora, R. Modification of bovine serum albumin structure following reaction with 4,5(*E*)-epoxy-2(*E*)-heptenal. *Chem. Res. Toxicol*. **2000**, *13*, 501−508.
(16) Zamora, R.; Alaiz, M.; Hidalgo, F. J. Modification of histidine residues by 4,5-epoxy-2-alkenals. *Chem. Res. Toxicol*. **1999**, *12*, 654−660.
(17) Hidalgo, F. J.; Zamora, R. Fluorescent pyrrole products from carbonyl-amine reactions. *J. Biol. Chem*. **1993**, *268*, 16190−16197.
(18) Zamora, R.; Hidalgo, F. J. Linoleic acid oxidation in the presence of amino compounds produces pyrroles by carbonyl amine reactions. *Biochim. Biophys. Acta* **1995**, *1258*, 319−327.
(19) Zamora, R.; Hidalgo, F. J. Modification of lysine amino groups by the lipid peroxidation product 4,5(*E*)-epoxy-2(*E*)-heptenal. *Lipids* **1994**, *29*, 243−249.
(20) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248−254.
(21) Buroker-Kilgore, M.; Wang, K. K. W. A Coomassie Brilliant Blue G-250-based colorimetric assay for measuring of Calpain and other proteases. *Anal. Biochem*. **1993**, *208*, 387−392.
(22) Hidalgo, F. J.; Alaiz, M.; Zamora, R. A spectrophotometric method for the determination of proteins damaged by oxidized lipids. *Anal. Biochem*. **1998**, *262*, 129−136.
(23) Davies, K. J. Protein damage and degradation by oxygen radicals. I. General aspects. *J. Biol. Chem*. **1987**, *262*, 9895−9901.
(24) Friguet, B.; Stadtman, E. R.; Szweda, L. I. Modification of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. Formation of cross-linked protein that inhibits the multicatalytic protease. *J. Biol. Chem*. **1994**, *269*, 21639−21643.
(25) Friguet, B.; Szweda, L. I. Inhibition of the multicatalytic proteinase (proteasome) by 4-hydroxy-2-nonenal cross-linked protein. *FEBS Lett*. **1997**, *405*, 21−25.
(26) Pokorny, J. Interactions of oxidized lipids with protein. *Riv. Ital. Sostanze Grasse* **1977**, *54*, 389−393.
(27) Rivett, A. J. Regulation of intracellular protein turnover: Covalent modification as a mechanism for marking proteins for degradation. *Curr. Top. Cell Regul*. **1986**, *28*, 291−337.
(28) Uchida, K.; Stadtman, E. R. Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate deshydrogenase. A possible involvement of intra- and intermolecular cross-linking reaction. *J. Biol. Chem*. **1993**, *268*, 6388−6393.
(29) Hidalgo, F. J.; Zamora, R. In vitro production of long chain pyrrole fatty esters from carbonyl-amine reactions. *J. Lipid Res*. **1995**, *36*, 725−735.
(30) Xu, G.; Liu, Y.; Kansal, M. M.; Sayre, L. M. Rapid cross-linking of proteins by 4-ketoaldehydes and 4-hydroxy-2-alkenals does not arise from the lysine-derived monoalkylpyrroles. *Chem. Res. Toxicol*. **1999**, *12*, 855−861.
(31) Zamora, R.; Alaiz, M.; Hidalgo, F. J. Determination of $\epsilon$-*N*-pyrrolylnorleucine in fresh food products. *J. Agric. Food Chem*. **1999**, *47*, 1942−1947.
(32) Davis, J. S.; Waters, J. A.; Parmar, S. S. Anti-inflammatory, antyproteolytic, and antihemolytic properties of pyrrole carboxylic acids. *Pharmacology* **1981**, *23*, 287−292.
(33) Oste, R. Effect of Maillard reaction products on protein digestion. *Prog. Clin. Biol. Res*. **1989**, *304*, 329−342.

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