JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Amino Acid and Protein Scavenging of Radicals Generated by Iron/Hydroperoxide System: An Electron Spin Resonance Spin Trapping Study

MANUEL PAZOS,* MOGENS L. ANDERSEN, AND LEIF H. SKIBSTED

Department of Food Science, Food Chemistry, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

Reduction of free radicals generated by Fe(II)/cumene-hydroperoxide (CumOOH) by amino acids (Gly, Cys, Met, His, and Trp) and proteins (bovine serum albumin (BSA), β-lactoglobulin, and lactoferrin) was followed by electron spin resonance spectroscopy using α-phenyl-*N*-*tert*-butylnitrone (PBN), 2-methyl-2-nitrosopropane (MNP), and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as spin traps. The radical species detected were mostly carbon-centered radicals from CumOOH fragmentation (methyl/$^{\bullet}H_3$ and ethyl/$^{\bullet}H_2CH_3$), although carbon-centered radicals originated from amino acids could be formed in the presence of Cys, Met, His, or Trp. All proteins and amino acids, except Cys, were effective at inhibiting generation of radicals from the Fe(II)/CumOOH system. Trp was the amino acid with the highest antiradical activity, followed by His > Gly $\sim$ Met. Lactoferrin was the protein showing the most efficient inhibition of radical formation from the Fe(II)/CumOOH system, and BSA and β-lactoglobulin were not significantly different in their antiradical activities. These results suggest that proteins with higher inhibitory activity on lipid oxidation promoted by transition metal catalytic decomposition of hydroperoxides should be those with elevated metal-chelating and radical-scavenging properties as well as low concentration and accessibility of reducing groups from amino acids capable of activating metals, such as sulfhydryl groups.

KEYWORDS: ESR; spin trapping; oxidation; antioxidant activity; amino acids; proteins

INTRODUCTION

Lipid oxidation is the major cause of deterioration of foods during processing and storage, affecting quality and safety and often determining the shelf life of the food product (*1*, *2*). Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), may retard lipid oxidation in food. However, natural antioxidants are getting increasing attention since long-term safety of synthetic antioxidants is being questioned, resulting in negative consumer perception and increasing restriction for their use as food additive.

Amino acids, peptides, and proteins delay lipid oxidation in certain food systems, and they can be obtained as natural products from waste materials from agriculture and food production. Several amino acids, such as Tyr, Met, His, Lys, and Trp, are generally considered as antioxidants in spite of their pro-oxidative effect under certain conditions (*3*−*5*). Carnosine and anserine, histidine-containing dipeptides very abundant in muscle tissues of most vertebrates, have been recognized for their inhibitory activity on lipid oxidation (*6*, *7*). Peptides from hydrolysates of whey (*8*, *9*), soy (*10*, *11*), egg-yolk (*12*), pork (*13*), and fish (*14*, *15*) have been shown to delay lipid oxidation efficiently. Milk proteins such as casein, BSA, and lactoferrin also have potential activity for retarding lipid oxidation in foods (*16*−*18*). In previous studies it was found that the efficiency of amino acids and peptides for improving the oxidative stability largely depends on the presence of pro-oxidant systems, endogenous antioxidants present in the food, and on the nature of the lipid substrate. However, few investigations have focused on the effect of these factors on the antioxidant behavior of proteins (*19*).

Traces of transition metals, from endogenous sources or due to contamination, are important pro-oxidants in food (*2*). Iron is the main transition metal implicated in the oxidation of lipids, although copper can contribute extensively to oxidation processes in seafood (*20*). The ferrous state of iron is especially active promoting oxidation by generation of reactive hydroxyl radicals (HO$^{\bullet}$) in Fenton reactions and lipid radicals (RO$^{\bullet}$) by decomposition of preformed lipid hydroperoxides.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + {}^{-}OH$$

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO^{\bullet} + {}^{-}OH$$

Hydroxyl radicals ($E^{\circ} = 2.31$ V vs NHE) and the lipid-derived radicals, such as peroxyl radical (ROO$^{\bullet}$: $E^{\circ} = 1.00$ V vs NHE) and alkoxyl radical (RO$^{\bullet}$: $E^{\circ} = 1.60$ V vs NHE), can generate new lipid radicals by abstraction of hydrogen atoms from

* To whom correspondence should be addressed. E-mail: mpaz@kvl.dk or mpazos@iim.csic.es. Telephone: +45 35283268. Fax: +45 35283344.

10.1021/jf062134n CCC: $33.50
Published on Web 12/07/2006

bisalyllic positions of polyunsaturated fatty acids (PUFAs: $E° = 0.6$ V vs NHE) (*21*). The auto-oxidative process via radical mechanism will continue by addition of molecular oxygen to the PUFA radical followed by formation of a new lipid hydroperoxide.

The aim of the present work was to investigate the behavior of amino acids and proteins on lipid oxidation promoted by Fe(II) and preformed hydroperoxides. An organic hydroperoxide, cumene hydroperoxide (CumOOH), was used as a model for lipid hydroperoxide, and the effect of amino acids and proteins on the CumOOH-derived free radicals formed were monitored using electron spin resonance (ESR) spectroscopy and the spin trapping technique. Bovine serum albumin, β-lactoglobulin, and lactoferrin were the proteins selected since these bovine whey proteins have different chelating capacities for iron.

MATERIALS AND METHODS

Chemicals. α-Phenyl-*N*-*tert*-butylnitrone (PBN), 2-methyl-2-nitrosopropane (MNP), cumene hydroperoxide (CumOOH), L-histidine (His), bovine serum albumin (BSA), bovine β-lactoglobulin, and bovine lactoferrin were purchased from Sigma (Steinheim, Germany). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) and L-methionine were obtained from Fluka (Buchs, Swizerland). L-Glycine (Gly), L-cysteine (Cys), L-tryptophan (Trp), ferrous sulfate heptahydrate, dibasic sodium phosphate ($Na_2HPO_4{\cdot}2H_2O$), and monobasic sodium phosphate ($NaH_2PO_4{\cdot}H_2O$) were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade, and the water was previously distilled and further deionized using a Milli-Q system (Millipore, Billerica, MA).

ESR Spin Trapping Experiments. The spin traps PBN, MNP, or DMPO were dissolved in 10−50 mM phosphate buffer pH 6.8, in presence or absence of 1.0 M ethanol. PBN and MNP were employed in concentrations of 4.0 mM, and DMPO was used in a concentration of 6.7 mM. The spin trap solution (4 mL) was mixed with aqueous ferrous sulfate (50 μL) and aqueous protein/amino acid solutions (100 μL). The reaction was initiated by addition of CumOOH in ethanol (50 μL), and the reaction mixture was subjected to magnetic stirring and pumped into an ESR quartz capillary tube with an internal diameter of 0.75 mm (Wilmad, Buena, NJ). ESR spectra was recorded on a Jeol Jes-FR30 ESR spectrometer (JEOL Ltd., Tokyo, Japan) using the following parameters: microwave power, 4 mW; sweep width, 50 G; sweep time, 2 min; modulation amplitude, 1 G; time constant 0.3 s. Spectra were recorded at room temperature after 2−10 min of incubation.

Peak-to-peak amplitudes were used for the quantification of the signals, and a relative signal intensity was calculated from ratios between the intensity of the sample (a) and the intensity of a Mn(II)-marker (b) attached to the cavity of the spectrometer (**Figure 2**). The inhibition of amino acids on the PBN spin adducts was determined by the percentage of the signal-amplitude decreasing/increasing in presence of amino acids (A_{sample}) compared to the signal-amplitude in control samples without amino acids ($A_{control}$):

$$\text{Inhibition on PBN spin adducts (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Simulation and fitting of ESR spectra were performed by the WinSIM program (*22*).

Statistical Analysis. The experiments were performed at least twice, and data are reported as mean ± standard deviation of two replicates. The data were analyzed by one-way analysis of variance (ANOVA) and the least-squares difference method (*23*). Statistical analyses were performed with the software Statistica 6.0 (*24*).

RESULTS

Influence of Phosphate, Fe(II), CumOOH, and Ethanol on the Formation of PBN Spin Adducts. PBN spin adducts are formed by addition of radical species to the carbon in α position of the PBN nitronyl group, as illustrated in **Figure 1**. The ESR spectrum obtained by incubation of Fe(II)/CumOOH system with the spin trap PBN consisted of an expected triplet of doublets arising from the coupling of the unpaired electron with the nitrogen nucleus (^{14}N, $I = 1$) and the proton (^1H, $I = 1/2$) on the α carbon of the PBN nitronyl group (**Figure 2**).

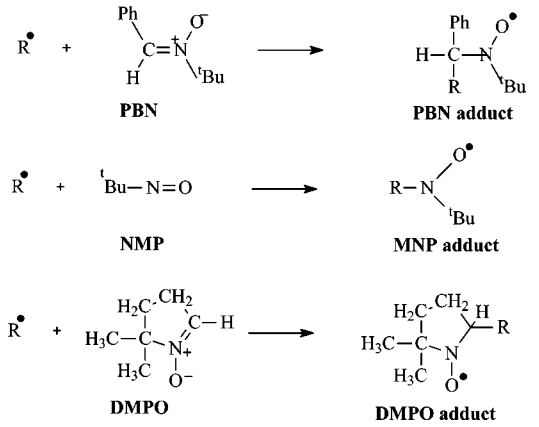


Figure 1. Formation of long-lived nitroxyl radical spin adducts by addition of radicals to the nitrone spin trap, PBN, nitroso spin trap, MNP, and pyrroline-*N*-oxide spin trap, DMPO.

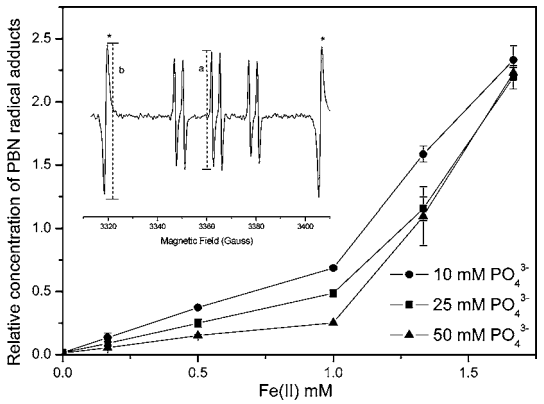


Figure 2. Effect of phosphate and Fe(II) concentrations on the formation of PBN spin adducts. The reaction mixture contained 1.0 M ethanol, 4 mM of PBN, 0–1.7 mM Fe(II), and 0.59 mM CumOOH in 10–50 mM phosphate buffer, pH 6.8, and ESR spectra were recorded after incubation for 2 min at room temperature.

The concentration of phosphate, Fe(II), CumOOH, and ethanol did not affect the shape of the ESR signal, but they influenced the concentration of spin adducts as evidenced by the spectral intensity. Phosphate and Fe(II) concentrations affected the amount of PBN spin adducts generated (**Figure 2**). PBN spin adducts were not detected without the addition of Fe(II). Higher Fe(II) levels enhanced the formation of PBN adducts, while phosphate reduced the signal intensity, especially at lower Fe(II) levels. With the highest Fe(II) concentration (1.75 mM), the signal intensity was not significantly different for the different phosphate levels used. For concentrations up to 1.0 mM Fe(II), the linear relationship between the radical species and Fe(II) concentrations is notable for all phosphate levels. For higher Fe(II) concentration, radical generation becomes more extensive. CumOOH affected substantially different on spin adducts formation, since spin adducts were formed very efficiently at lower CumOOH concentration, while higher concentrations increased less extensively spin adducts (**Figure 3**). These results showed that Fe(II) is the limiting factor on the generation of radical species by Fe(II) and hydroperoxides,

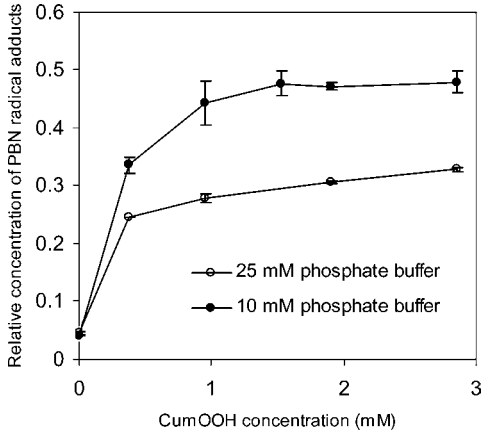


Figure 3. Effect of hydroperoxide concentration on the formation of PBN spin adducts. The reaction mixture contained 1 M ethanol, 4 mM of PBN, 0.95 mM Fe(II), and 0–2.6 mM CumOOH in 10 or 25 mM phosphate buffer, pH 6.8.

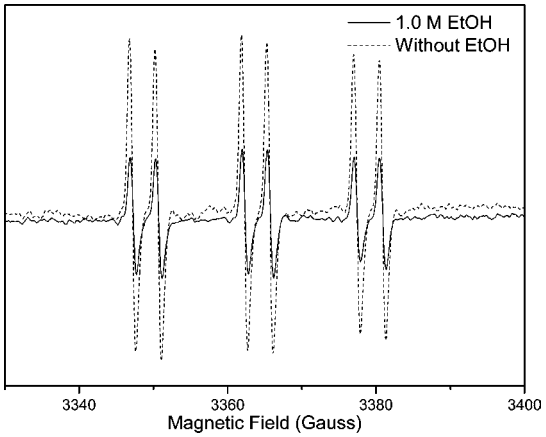


Figure 4. Effect of ethanol on the formation of PBN spin adducts. ESR spectra of samples containing 1 M ethanol and without ethanol (dashed line), 4 mM of PBN, 0.95 mM Fe(II), and 0.95 mM CumOOH in 10 mM phosphate buffer, pH 6.8, were recorded after 2 min of incubation at room temperature.

and a low level of hydroperoxide concentration is sufficient for the development of oxidation processes.

Ethanol influenced the amount of radical species detected in the Fe(II)/hydroperoxide systems. A concentration of 1.0 M ethanol decreased the formation of radical species, as compared to a system without ethanol (**Figure 4**). The peak-to-peak amplitude was 60% lower in presence of 1.0 M ethanol after 2 min of incubation.

Amino Acids and Protein Spin Adducts. The ESR spectra obtained by incubation of the Fe(II)/CumOOH system with the spin trap PBN for 2 min were successfully simulated with a spin adduct with coupling constants $a_N = 15.1$ G and $a_H = 3.4$ G (**I**) (**Figure 5A**,**B**). This spin adduct **I** has previously been reported and assigned to an adduct PBN/carbon-centered radical, probably generated by β-scission of cumyloxyl radicals (Cum-O$^\bullet$) (*25*) (**Table 1**). No significant differences ($p < 0.05$) were found in these coupling constants in the presence of amino acids (Gly, Cys, Met, His, and Trp) and proteins (BSA, β-lactoglobulin, and lactoferrin). However, a new PBN spin adduct (**II**) with coupling constants $a_N = 16.2$ G and $a_H = 3.4$ G was detected with the addition of Cys (**Figure 5C**,**D**) or His (**Figure 5E**,**F**). The spin adduct **II** contributed 62−75% to the ESR signal with 0.30−1.19 mM Cys, while with 0.59 mM His, the contribution of **II** was about 20%. Experiments indicated that

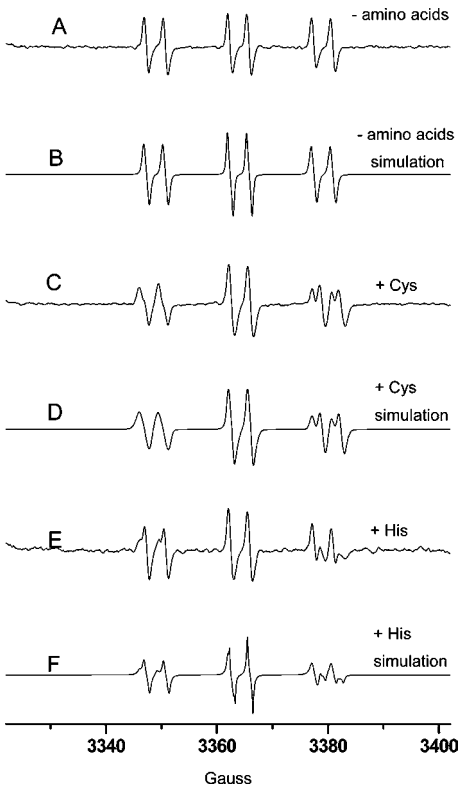


Figure 5. ESR spectra and computer simulations of PBN spin adducts formed from the Fe(II)/CumOOH system. (**A**) ESR spectrum in absence of amino acids and (**B**) its computer simulation, (**C**) ESR spectrum in presence of Cys and (**D**) its computer simulation, and (**E**) ESR spectrum in presence of His and (**F**) its computer simulation. The reaction mixture containing 10 mM phosphate, pH 6.8, 1.0 M ethanol, 4 mM PBN, 0.95 mM Fe(II), 0.95 mM CumOOH, and 0.59 mM Cys or His was subjected to ESR measurement after incubation for 2 min at room temperature.

Table 1. Structural Assignment for the Spin Adducts

		coupling constants		
	spin trap	a_N (G)	a_H (G)	assignment
I	PBN	15.1	3.4	PBN/carbon-centered radical (*25*)
II	PBN	16.2	3.4	PBN/carbon-centered radical (*37*)
III	NMP	17.4	14.3	MNP/CH_3 (*40*)
IV	NMP	17.0	11.2	MNP/CH_2R (*34*, *35*)
V	DMPO	16.2	23.3	DMPO/carbon-centered radical (*34*, *35*)
VI	DMPO	14.4	10.5	DMPO/OCH_3 (*36*)

spin adduct **II** was also formed in the presence of Trp and Met after 5 min of incubation, but it was not detected in samples without amino acids or with Gly present after 10 min of reaction. The spin adduct **II** has been tentatively assigned to an adduct of PBN and a carbon-centered radical by similarity with coupling constants previously reported (*26*). The assignment of the spin adduct **II** as 1-hydroxyethyl spin adduct (PBN/$^\bullet$-$CHOHCH_3$), which gives similar coupling constants and can be found in presence of ethanol and a radical-generating source (*27*), was discarded since it was also detected in the absence of ethanol (data not shown).

The carbon-centered radicals formed from the Fe(II)/CumOOH system were further characterized using the nitroso spin trap MNP. With nitroso spin traps, the radical adds directly to the nitroxide nitrogen, giving more information with regard to which type of carbon-centered has been trapped (**Figure 1**). After 2 min of incubation, MNP spin radical adducts presented

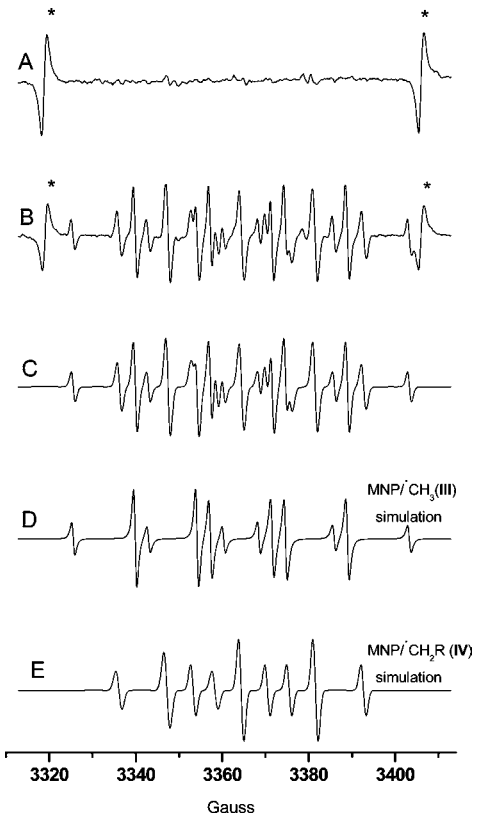


Figure 6. ESR spectra and computer simulations of MNP spin adducts formed from the Fe(II)/CumOOH system. ESR spectra after incubation for (**A**) 2 min and (**B**) 10 min, (**C**) composite computer simulation of the ESR spectrum after 10 min of incubation, (**D**) individual computer simulations for the spin radical adducts MNP/•CH_3 (**III**) and (**E**) for MNP/•CH_2R (**IV**). The reaction mixture contained 4 mM of MNP, 0.95 mM Fe(II), and 0.95 mM CumOOH in 10 mM phosphate buffer, pH 6.8. The signals marked by stars correspond to the Mn(II) internal standard.

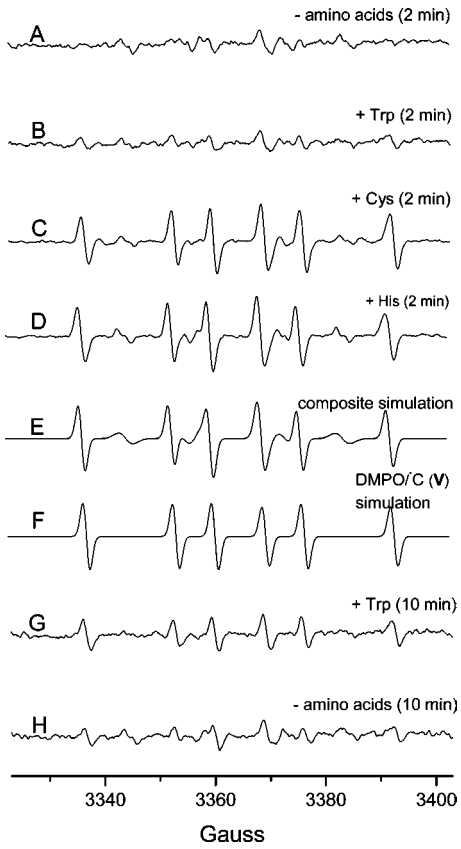


Figure 7. ESR spectra and computer simulations of DMPO spin adducts formed from the Fe(II)/CumOOH system. (**A**) ESR spectrum at 2 min in absence and presence of (**B**) Trp, (**C**) Cys, and (**D**) His. (**E**) Composite computer simulation and (**F**) computer simulation for the DMPO/carbon-centered spin adducts (**V**). (**G**) ESR spectrum at 10 min in presence and (**H**) absence of Trp. The reaction mixture containing 10 mM phosphate, pH 6.8, 6.7 mM DMPO, 0.95 mM Fe(II), 0.95 mM CumOOH, and 0.59 mM Cys, His, or Trp was subjected to ESR measure after incubation for 2 min or 10 min at room temperature.

a very weak background signal (**Figure 6A**). However, a complex ESR spectrum was found after 10 min (**Figure 6B**), and the deconvolution of this ESR spectrum gave a mixture of two species **III** and **IV** (**Figure 6C**−**E**). On the basis of the pattern of splitting and on the coupling constant values of the ESR spectra, the spin adducts **III** and **IV** were assigned to MNP/•CH_3 and MNP/•CH_2R radical adducts, respectively (**Table 1**). MNP/•CH_3 and MNP/•CH_2R were found in both samples with and without amino acids. The contribution of methyl adduct to the total spectrum was 50−63%.

The spin trap DMPO forms more stable spin adducts with oxygen centered radicals than PBN or MNP (*28*). Small amounts of DMPO spin adducts were detected in samples without amino acids or samples with Trp after 2 min of incubation (**Figure 7A**,**B**). However, the incubation of Fe(II) and CumOOH with Cys or His increased the formation of DMPO spin adducts (**Figure 7C**,**D**). The spectra were successfully simulated as a mixture of the spin adducts **V** and **VI** (**Table 1**). Spin adduct **V** was assigned to an adduct of DMPO and a carbon-centered radical which explained the six higher signals in these spectra, while a DMPO/methoxyl adduct (**VI**) can explain the lower signals (**Figure 7E**,**F**). The spin adduct **V** was also detected in samples with Trp after 10 min, but it was not significantly generated in samples without amino acids (**Figure 7G**,**H**).

Evaluation of Scavenger Activity of Amino Acids and Proteins Based on PBN Adducts. All amino acids tested except Cys (Gly, Met, His, and Trp) decreased the detected amount of PBN adducts formed by incubating Fe(II) and CumOOH

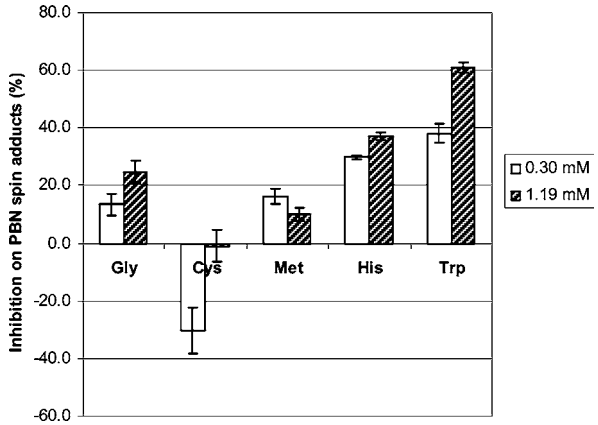


Figure 8. Effect of Gly, Cys, His, Met, and Trp on the PBN spin adducts formed in the presence of Fe(II)/CumOOH. ESR spectra of samples containing 1.0 M ethanol, 4 mM of PBN, 0.95 mM Fe(II), and 0.95 mM CumOOH in 10 mM phosphate buffer, pH 6.8, were recorded after 2 min of incubation at room temperature. Antiradical activity was determined as the reduction of the peak-to-peak amplitude in the presence of amino acids relative to the samples without amino acids.

(**Figure 8**). The amino acids were tested at two concentration levels: 0.30 and 1.19 mM. Trp was significantly more effective in inhibiting the formation of radical species for both amino acid concentrations, followed by His ($p < 0.05$). Gly and Met

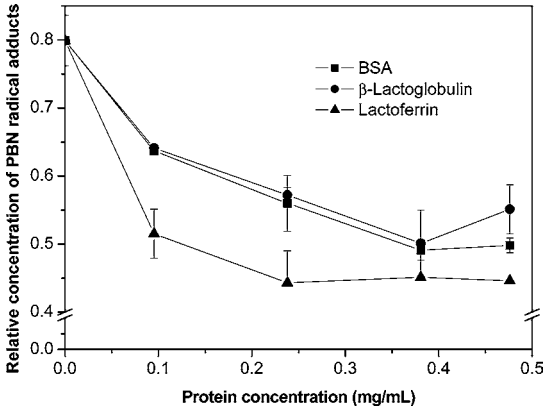


Figure 9. Effect of BSA, β-lactoglobulin, and lactoferrin on the PBN spin adducts generated from Fe(II) and CumOOH incubation. ESR spectra of samples containing 0–0.48 mg/mL proteins, 1.0 M ethanol, 4 mM of PBN, 0.95 mM Fe(II), and 0.95 mM CumOOH in 10 mM phosphate buffer, pH 6.8, were recorded after 2 min of incubation.

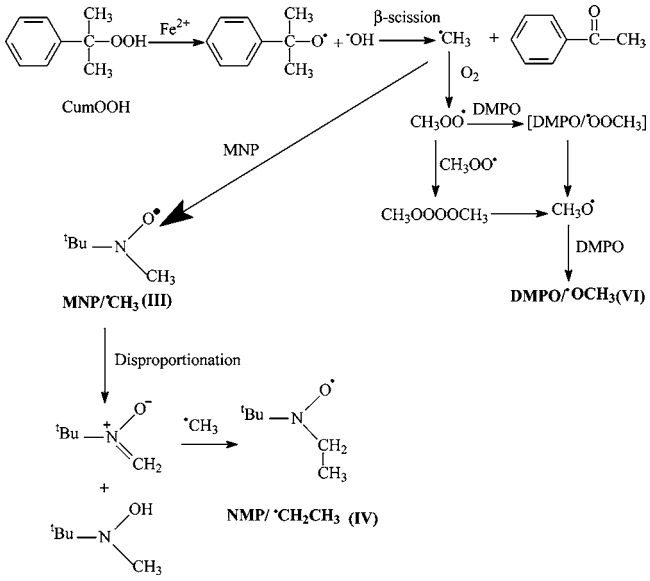
showed similar activity reducing the generation of radicals 13−16% at lower amino acid concentrations (0.30 mM). For higher concentrations (1.19 mM), Gly was significantly better than Met ($p < 0.05$) in reducing the formation of PBN spin adducts. Two different behaviors were found for Cys depending on the Cys concentration. The addition of 0.30 mM of Cys significantly increased the generation of PBN spin adducts, while the samples with higher concentration of Cys (1.19 mM) did not show significant differences ($p < 0.05$) on the generation of PBN adducts compared to samples without amino acids (**Figure 8**).

The effects of BSA, β-lactoglobulin, and lactoferrin on radicals generated by Fe(II) and CumOOH were evaluated by using PBN as spin trap. The ESR spectra obtained in the presence of these proteins showed only the PBN spin adduct **I** after 2 min of incubation (figure not shown). The proteins effectively inhibited the formation of PBN spin adduct **I** at protein concentrations up to 0.5 mg/mL (**Figure 9**). Lactoferrin inhibited the generation of spin radical adducts most efficiently, while BSA and β-lactoglobulin were not significantly different ($p < 0.05$). The same behavior was found for these proteins: increasing the concentration of proteins reduced the amount of PBN spin adduct but a considerable amount of the spin adducts was still formed.

DISCUSSION

The breakdown of lipid hydroperoxides by transition metal ions or metalloproteins is considered to be of considerable importance in the development of lipid oxidation in muscle based foods. Using the organic cumene hydroperoxide as model, we have investigated the factors that control the generation of radical species from the breakdown of lipid hydroperoxides by Fe(II) and characterized the radical species formed. This study indicates that Fe(II) concentration is a limiting factor for the release of free radicals from Fe(II)/CumOOH systems and that the increase in hydroperoxide concentration above molar ratios 1:1 for Fe(II)/CumOOH did not significantly enhance the generation of radical species. This finding supports previous investigations, which found that washed cod muscle activated by hemoglobin did not accelerate the development of lipid oxidation after increasing the concentration of hydroperoxides more than 80 times with the supplementation of 15% menhaden oil (*29*). On the basis of these results, it was suggested that the hydroperoxide concentration in the membrane lipid fraction already was a sufficient substrate for hydroperoxide breakdown by the active hemoglobin level. The results of the present investigation also showed that ethanol and phosphate effectively reduced the amount of free radicals formed from the breakdown of hydroperoxides by Fe(II). The reduction of hydroperoxide derived radicals by ethanol could be result of the direct donation of hydrogen atoms from ethanol to radical species, mainly to form the 1-hydroxyethyl radical. However, 1-hydroxyethyl radical was not detected during our experiments, and it could be caused by the oxidation of 1-hydroxyethyl radicals ($E = -0.94$ V vs SHE) in the presence of high concentration of ferric iron (*30*). Ethanol also exhibited scavenging activity toward hydroxyl radicals (*27*) and has been reported to behave like an antioxidant in human low-density lipoprotein (*31*) and in fish muscle (*32*). The inhibition of Fe(II)-catalyzed oxidation by phosphate buffer seems to be explained because phosphate catalyzes the autoxidation of Fe(II), and Fe(III) formed is quite insoluble, which decreases the Fe(II) content in the reaction medium.

Scheme 1. Spin Radical Adducts Detected from Cumene Hydroperoxide in the Presence of Fe(II)



Spin trapping with MNP allowed the identification of two carbon-centered radicals released from CumOOH in the presence of Fe(II): $^{\bullet}CH_3$ and $^{\bullet}CH_2R$ radicals. The tertiary alkoxyl radical (CumO$^{\bullet}$), formed by the breakdown of CumOOH in the presence of Fe(II), is not stable and undergoes fragmentation through β-scission to form the methyl radical and a ketone (*33*) (**Scheme 1**). The formation of alkyl radicals centered on secondary carbons has previously been reported and assigned to ethyl radicals ($^{\bullet}CH_2CH_3$). Mason and co-workers suggested that MNP adducts corresponding to ethyl radicals can be formed by disproportionation of the MNP/$^{\bullet}CH_3$ adduct followed by the trapping of a second methyl radical, as shown **Scheme 1** (*34*, *35*). An unexpected observation was the absence of the MNP/carbon-centered adducts after 2 min of reaction. However, the PBN spin adducts **I**, tentatively assigned as a PBN/carbon-centered adduct, were detected after 2 min of incubation. Given that the MNP spin trap reacts faster in spin trapping reaction of carbon-centered radicals than PBN (*28*), the higher instability of the MNP radical adducts led to the production of non-radical adducts via a disproportionation reaction or consecutive addition of two radical species could explain this finding. With DMPO spin trapping an oxygen-centered radical was detected, and it was assigned to the adduct of DMPO with methoxyl radical based on HPLC-ESR and MS/MS studies (*36*). This adduct was

previously assigned wrongly to cumylperoxyl radical (CumOO•) which has similar coupling constants to the DMPO/superoxide radical adduct. The carbon-centered radical that forms the PBN spin adduct **II** seems to be an amino acid derived radical, since the spin adduct **II** was not detected in samples without amino acids. Davies et al. (*37*) have observed the formation of spin adducts with similar hyperfine coupling constants after incubating γ-radiated amino acids with Fe(II). These protein derived radical species should be formed from the degradation of protein hydroperoxides. Moreover, the carbon-centered radical detected by DMPO spin trapping seems to be the same type as the radical species that form the PBN adduct **II**, since both adducts were only detected with Cys and His after 2 min and with Trp after 10 min of incubation, while their formation in absence of amino acids was very low or undetected.

Our ESR spin trapping experiments demonstrate that amino acids and proteins can effectively lessen the generation of radical species from Fe(II)/hydroperoxides systems. In homogeneous systems, the inhibition of oxidative processes promoted by traces of transition metals can be generally explained by two mechanisms: (i) chelating the metallic traces with the subsequent lower accessibility to lipid hydroperoxides or reducing agents and (ii) the scavenger activity on radical species. Lactoferrin showed a more efficient inhibition of radical generation compared to BSA and β-lactoglobulin, in agreement with its high metal iron-chelating properties (*18*). The radical-scavenging and chelating properties may also help to explain the inhibitory activity observed for amino acids: Trp > His > Met ~ Gly > Cys. Wu et al. (*38*) reported that Cu(II) is effectively chelated by Gly and Ala, although His shows higher chelating effect. This investigation demonstrates that amine and carboxylic groups in α positions relate to α-amino acids chelating properties on metals, but other functional groups (the imidazole group in His) can enhance their chelating abilities. Regarding radical-scavenging ability of amino acids, it is mainly associated with the presence of phenolic, sulfhydryl, and amine-heterocyclic groups where hydrogen atoms can be easily abstracted by radical species. Thus, Trp, His, and Cys have the ability to scavenger DPPH radical, but this property was not found in Gly, Ala, and Met (*5*, *38*). Therefore, the higher inhibitory activity observed for Trp and His can be related to their dual behavior as chelating and radical-scavenging agent, while Gly and Met, which were less effective reducing radicals generated by Fe(II) and CumOOH, only exhibit chelating properties. Trp showed higher inhibitory activity than His in agreement with its superior radical-scavenging activity (*5*). However, Cys promoted the generation of radicals despite its high ability to scavenger radicals. This promotion of radical species by Cys has been previously reported in a dry model system (*5*), herring oil emulsions (*3*), and linoleic emulsions (*4*), and it can be attributed to the capacity of a sulfhydryl group of Cys to activate the reduction of Fe(III) to Fe(II), which has a higher reactivity with hydroperoxides (*39*).

In conclusion, an ESR spin trapping method was developed to evaluate the antioxidant capacity of amino acids and peptides on lipid oxidation promoted by metallic traces and preformed lipid-hydroperoxides. These studies confirmed that the supplementation with amino acids and related compounds can give effective and consumer friendly strategies for inhibiting lipid oxidation promoted by metallic traces. In general, the amino acids and proteins with higher metal-chelating and radical-scavenging properties were more efficient in inhibiting the generation radical species promoted by Fe(II) and hydroperoxides. However, these results also emphasize the requirement of optimizing the antioxidant properties of amino acids and proteins in the particular food system, since they can activate a generation of free radicals in the presence of metals, depending on the concentration and accessibility of reducing groups from amino acids capable to activate metals (like sulfhydryl groups) and the concentration of metals.

ACKNOWLEDGMENT

The continuing support from LMC-Centre for Advance Food Studies to the oxidation research of the Food Chemistry group is gratefully acknowledged.

LITERATURE CITED

(1) Frankel, E. N. *Lipid oxidation*; The Oily Press: Dundee, Scotland, 1998.
(2) Kanner, J. Oxidative processes in meat and meat products quality implications. *Meat Sci.* **1994**, *36*, 169−189.
(3) Marcuse, R. Antioxidative effect of amino-acids. *Nature* **1960**, *186*, 886−887.
(4) Marcuse, R. The effect of some amino acids on the oxidation of linoleic acid and its methyl ester. *J. Am. Oil Chem. Soc.* **1962**, *39*, 97−103.
(5) Park, E. Y.; Murakami, H.; Matsumura, Y. Effects of the addition of amino acids and peptides on lipid oxidation in a powdery model system. *J. Agric. Food Chem.* **2005**, *53*, 8334−8341.
(6) Boldyrev, A. A.; Dupin, A. M.; Pindel, E. V.; Severin, S. E. Antioxidative properties of histidine-containing dipeptides from skeletal-muscles of vertebrates. *Comp. Biochem. Phys. B.* **1988**, *89*, 245−250.
(7) Decker, E. A.; Crum, A. D. Antioxidant activity of carnosine in cooked ground pork. *Meat Sci.* **1993**, *34*, 245−253.
(8) Pena-Ramos, E. A.; Xiong, Y. L. L.; Arteaga, G. E. Fractionation and characterisation for antioxidant activity of hydrolysed whey protein. *J. Sci. Food Agric.* **2004**, *84*, 1908−1918.
(9) Tong, L. M.; Sasaki, S.; McClements, D. J.; Decker, E. A. Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *J. Agric. Food Chem.* **2000**, *48*, 1473−1478.
(10) Chen, H. M.; Muramoto, K.; Yamauchi, F.; Nokihara, K. Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *J. Agric. Food Chem.* **1996**, *44*, 2619−2623.
(11) Park, E. Y.; Murakami, H.; Mori, T.; Matsumura, Y. Effects of protein and peptide addition on lipid oxidation in powder model system. *J. Agric. Food Chem.* **2005**, *53*, 137−144.
(12) Sakanaka, S.; Tachibana, Y.; Ishihara, N.; Juneja, L. R. Antioxidant activity of egg-yolk protein hydrolysates in a linoleic acid oxidation system. *Food Chem.* **2004**, *86*, 99−103.
(13) Saiga, A.; Tanabe, S.; Nishimura, T. Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. *J. Agric. Food Chem.* **2003**, *51*, 3661−3667.
(14) Kim, S. K.; Kim, Y. T.; Byun, H. G.; Nam, K. S.; Joo, D. S.; Shahidi, F. Isolation and characterization of antioxidative peptides from gelatin hydrolysate of alaska pollack skin. *J. Agric. Food Chem.* **2001**, *49*, 1984−1989.
(15) Jun, S. Y.; Park, P. J.; Jung, W. K.; Kim, S. K. Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (limanda aspera) frame protein. *Eur. Food Res. Technol.* **2004**, *219*, 20−26.
(16) Viljanen, K.; Kivikari, R.; Heinonen, M. Protein-lipid interactions during liposome oxidation with added anthocyanin and other phenolic compounds. *J. Agric. Food Chem.* **2004**, *52*, 1104−1111.
(17) Diaz, M.; Decker, E. A. Antioxidant mechanisms of caseinophosphopeptides and casein hydrolisates and their application in ground beef. *J. Agric. Food Chem.* **2004**, *52*, 8208−8213.
(18) Huang, S.-W.; Satué-Gracia, M. T.; Frankel, E. N.; German, J. B. Effect of lactoferrin on oxidative stability of corn oil emulsions and liposomes. *J. Agric. Food Chem.* **1999**, *47*, 1356−1361.

(19) Baron, C. P.; Refsgaard, H. H. F.; Skibsted, L. H.; Andersen, M. L. Oxidation of bovine serum albumin initiated by the fenton reaction-effect of EDTA, *tert*-butylhydroperoxide and tetrahydrofuran. *Free Radical Res.* **2006**, *40*, 409−417.

(20) Decker, E. A.; Hultin, H. O. Factors influencing catalysis of lipid oxidation by the soluble fraction of mackerel muscle. *J. Food Sci.* **1990**, *55*, 947−950.

(21) Buettner, G. R. The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol and ascorbate. *Arch. Biochem. Biophys.* **1993**, *300*, 535−543.

(22) Duling, D. R. Simulation of multiple isotropic spin-trap EPR spectra. *J Magn. Reson. B* **1994**, *104*, 105−110.

(23) Sokal, R.; Rohlf, F. *Biometry*; Freeman and Co.: San Francisco, CA, 1981.

(24) Statsoft. *Statistica for macintosh*; Statsoft and its licensors: Tulsa, OK, 1994.

(25) Ueda, J.-I.; Daito, N.; Ozawa, T. ESR spin trapping studies on the reactions of hydroperoxides with Cu(II) complex. *J. Inorg. Biochem.* **1996**, *64*, 197−206.

(26) Augusto, O.; Ortiz de Montellano, J. o. A. a. F. C. P.; Quintanilha, A. Spin-trapping of free radicals formed during microsomal metabolism of ethylhydrazine and acetylhydrazine. *Biochem. Biophys. Res. Commun.* **1981**, *101*, 1324−1330.

(27) Andersen, M. L.; Skibsted, L. H. Electron spin resonance trapping identification of radicals formed during aerobic forced aging of beer. *J. Agric. Food Chem.* **1998**, *46*, 1272−1275.

(28) Rosen, G. M.; Britigan, B. E.; Halpern, H. J.; Pou, S. *Free radicals: Biology and detection by spin trapping*; Oxford University Press: Oxford, 1999.

(29) Undeland, I.; Hultin, H. O.; Richards, M. P. Added triacylglycerols do not hasten hemoglobin-mediated lipid oxidation in washed minced cod muscle. *J. Agric. Food Chem.* **2002**, *50*, 6847−6853.

(30) Lilie, J.; Beck, G.; Henglein, A. Pulse radiolysis and polarography - half-wave potentials for oxidation and reduction of short-lived organic radicals on hg electrode. *Ber. Bunsen-Ges. Phys. Chem.* **1971**, *75*, 458.

(31) Rousselot, D. B.; Rouscilles, A.; Bizard, C.; Delattre, J.; Jore, D.; Albert, M. G. Antioxidant effect of ethanol toward in vitro peroxidation of human low-density lipoproteins initiated by oxygen free radicals. *Radiat. Res.* **2000**, *155*, 279−287.

(32) Raghavan, S.; Hultin, H. O. Model system for testing the efficacy of antioxidants in muscle foods. *J. Agric. Food Chem.* **2005**, *53*, 4572−4577.

(33) Kalyanaraman, B.; Perezreyes, E.; Mason, R. P. Reduction of nitroso-spin traps in chemical and biological-systems - cautionary note. *Tetrahedron Lett.* **1979**, 4809−4812.

(34) Taffe, B. G.; Takahashi, N.; Kensler, T. W.; Mason, R. P. Generation of free-radicals from organic hydroperoxide tumor promoters in isolated mouse keratinocytes - formation of alkyl and alkoxyl radicals from tert-butyl hydroperoxide and cumene hydroperoxide. *J. Biol. Chem.* **1987**, *262*, 12143−12149.

(35) Chamulitrat, W.; Takahashi, N.; Mason, R. P. Peroxyl, alkoxyl, and carbon-centered radical formation from organic hydroperoxides by chloroperoxidase. *J. Biol. Chem.* **1989**, *264*, 7889−7899.

(36) Guo, Q.; Qian, S. Y.; Mason, R. P. Separation and identification of DMPO adducts of oxygen-centered radicals formed from organic hydroperoxides by HPLC-ESR, ESI-MS and MS/MS. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 862−871.

(37) Davies, M. J.; Fu, S. L.; Dean, R. T. Protein hydroperoxides can give rise to reactive free-radicals. *Biochem. J.* **1995**, *305*, 643−649.

(38) Wu, H. C.; Shiau, C. Y.; Chen, H. M.; Chiou, T. K. Antioxidant activities of carnosine, anserine, some free amino acids and their combination. *J. Food Drug Anal.* **2003**, *11*, 148−153.

(39) O'Brien, P. J. Intracellular mechanisms for the decomposition of a lipid peroxide. I. Decomposition of a lipid peroxide by metal ions, heme compounds and nucleophiles. *Can. J. Fish Aquat. Sci.* **1969**, *47*, 485−492.

(40) Barr, D. P.; Martin, M. V.; Guengerich, F. P.; Mason, R. P. Reaction of cytochrome p450 with cumene hydroperoxide: ESR spin trapping evidence for the homolytic scission of the peroxide o-o bond by ferric cytochrome p450 1a2. *Chem. Res. Toxicol.* **1996**, *9*, 318−325.

Received for review July 27, 2006. Revised manuscript received October 11, 2006. Accepted October 17, 2006. The Spanish Ministry of Science and Technology is gratefully acknowledged for a Ph.D. grant to M.P.

JF062134N