JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Biochemical Changes in Myofibrillar Protein Isolates Exposed to Three Oxidizing Systems

DONKEUN PARK,† YOULING L. XIONG,*,† AMY L. ALDERTON,† AND TOORU OOIZUMI‡

Department of Animal and Food Sciences, University of Kentucky, Lexington, Kentucky 40546, and Department of Marine Bioscience, Fukui Prefectural University, 1-1 Gakuen-Cho, Obama 917-003, Japan

The objective of the study was to compare three different oxidizing systems commonly present in muscle foods for their influence on the biochemical properties of muscle proteins. Myofibrillar protein isolate (MPI) prepared from pork serratus ventralis muscle was suspended (30 mg protein/mL) in 15 mM piperazine-*N*,*N*-bis(2-ethane sulfonic acid) buffer (pH 6.0). Oxidation was induced by incubating the protein suspension at 4 °C for 24 h with (i) a hydroxyl radical-generating system (HRGS: 10 μM $FeCl_3$, 0.1 mM ascorbic acid, and 0.05−5.0 mM H_2O_2), (ii) a lipid-oxidizing system (LOS: 0.05−5.0 mM linoleic acid and 3750 units of lipoxidase/mL), or (iii) a metmyoglobin-oxidizing system (MOS: 0.05−0.5 mM metmyoglobin). Changes in oxidized MPI were measured as Ca- and K-ATPase activities, formation of protein carbonyls and 2-thiobarbituric acid-reactive substances (TBARS), loss of protein thermal stability, and protein aggregation. The three oxidizing matrixes induced complex MPI changes; for example, the Ca- and K-ATPase activities were altered mainly by low-concentration oxidants, but the changes were unique for each oxidizing system. The carbonyl content in MOS-treated MPI was the highest, while the TBARS production, changes in thermal properties, and loss of the myosin heavy chain were the greatest in HRGS-treated MPIs. Overall, the hydroxyl radical-producing medium appeared to be the most oxidative to myofibrillar proteins under the experimental conditions employed in the study.

KEYWORDS: Myofibrils; protein oxidation; myosin; ATPase; TBARS; carbonyls; SDS−PAGE; DSC

INTRODUCTION

Protein oxidation is one of the main indicators of meat quality deterioration. Meats and meat products are known to be susceptible to oxidation that occurs during processing and storage. Yet, the current understanding of protein oxidation has been derived primarily from biomedical research, which demonstrates that structure and properties of proteins and enzymes could be readily modified by reactive oxygen species generated via lipid oxidation, metal- or enzyme-catalyzed oxidative reactions, and other chemical and biochemical processes (*1*−*3*).

Protein oxidation via oxidized lipids results either from reactions with lipid free radicals or from interactions with secondary products of lipid oxidation (*4*). Other oxidizing agents, such as metmyoglobin (*5*−*7*), transition metal ions (*8*−*12*), oxidative enzymes (*13*), and harsh processing conditions including γ-irradiations (*14*), are also known initiators of protein oxidation. The oxidation of proteins almost always leads to a change in protein physical and chemical properties and, ultimately, alterations in protein functional behavior in food systems. In muscle food products, the oxidation-induced protein functionality changes corresponded well to an augmented UV absorbance, the formation of protein carbonyl compounds, and an increase in the number of high molecular weight oligomers and polymers (*15*). Increased gelling ability, water-holding capacity, and solubility have been observed under mild oxidizing conditions (*16*−*18*).

Myosin, the most abundant protein in the myofibril complex, is susceptible to reactive oxygen species, including lipid free radicals, lipid hydroperoxides, iron or copper/ascorbate/H_2O_2, and myoglobin/H_2O_2, and can form large, insoluble aggregates when oxidized (*7*, *9*, *19*−*22*). Martinaud et al. (*10*) examined oxidative changes in beef myofibrillar proteins induced by different oxidative systems, including three metal-catalyzed oxidizing solutions, a xanthine/xanthine oxidase solution, and a metmyoglobin/H_2O_2 solution. The authors showed that the changes in protein carbonyl and sulfhydryl content and in the protein aggregation pattern were dependent upon muscle type and the oxidizing systems. Also, Ooizumi and Xiong (*12*) reported major biochemical changes in chicken myofibrillar protein exposed to a nonenzymatic, hydroxyl radical-generating

* To whom correspondence should be addressed. Tel: 859-257-3822. Fax: 859-257-5318. E-mail: ylxiong@uky.edu.
† University of Kentucky.
‡ Fukui Prefectural University.

10.1021/jf0531813 CCC: $33.50
Published on Web 05/17/2006

system (HRGS). However, despite these findings, the relative importance of hydroxyl radicals, oxidized lipids, and oxidized heme pigments, which are expected to coexist in processed muscle food products, is not well-understood. The objective of the present study was to elucidate the biochemical changes in myofibrillar protein subjected to these three common oxidizing environments.

MATERIALS AND METHODS

Materials. Ten pork Boston shoulders (4 days postmortem) in five separate vacuum packages were obtained from a commercial packing plant through a local meat purveyor. Whole serratus ventralis muscle was removed from each shoulder and diced into approximately 15 g pieces. The muscle dices were mixed well by hand and subsequently divided into 20 equal portions. Each portion, weighing approximately 250 g, was placed in Cryovac vacuum bags, vacuum-sealed, and stored in a −30 °C freezer for less than 2 months before use. Unless specified otherwise, all of the data reported herein represent the means from three to five independent trials (i.e., replicate myofibril preparations). For each experimental replication, one random frozen bag of the meat samples was removed from the freezer and thawed in a 4 °C refrigerator for 16 h before use.

Preparation of Myofibrils. Myofibril isolates were prepared in a 2 °C walk-in cooler. Thawed muscle was finely chopped with a knife, mixed with 4 vol (w/v) of isolation buffer, and blended in a Waring blender for 30 s at high speed. The isolation buffer contained 0.1 M NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 6.1 mM sodium phosphate dibasic (Na_2HPO_4), and 3.9 mM sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) (pH 7.0). After centrifugation at 2000*g* for 15 min, the crude myofibril pellet was washed two more times with 4 vol of the same isolation buffer using the same blending and centrifugation conditions indicated above. The myofibril pellet was then washed three more times with 4 vol of 0.1 M NaCl under the same conditions as above except that in the last wash, the myofibril suspension was filtered through four layers of cheesecloth to remove connective tissue and its pH was adjusted to 6.0 (to closely simulate the pH condition in processed meats) with 0.1 N HCl prior to centrifugation. Myofibrillar protein isolate (MPI) was kept in a tightly capped bottle and stored on ice before use (in 18 h). The protein concentration of the myofibril pellet was measured by the Biuret method (*23*) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard.

Crude Fat Content and Fatty Acid Analysis. The total crude fat in MPI was determined from freeze-dried MPI by extracting approximately 1.0 g of the dry MPI with 20 mL of petroleum ether for 4 h using a Soxtec 1043 extraction unit (Tecator AB, Höganäs, Sweden). After extraction, the solvent was evaporated at 21 °C in a fume hood. The crude fat was dried in an oven at 110 °C for 30 min, desiccated for 20 min, and weighed.

To determine the fatty acid profile in the crude fat, aliquots of approximately 100 mg of extracted crude fat were dissolved in 2 mL of hexane. Methyl esters of fatty acids were prepared according to the modified Boron trifluoride method of the AOAC (*24*). One microliter of diluted fatty acid methyl esters was analyzed with a Perkin-Elmer AutoSystem Gas Chromatograph equipped with a DB-225 capillary column (30 m × 0.32 mm i.d. with a 0.25 μm film thickness, J&W Scientific, Folsom, CA) and a flame ionization detector. The fatty acids in the sample were identified using methyl ester standard mixtures. The helium carrier gas flow rate was 1.0 mL per min. The injector and detector in the machine were set at 240 and 260 °C, respectively. The initial oven temperature was 190 °C, which was held for 7.5 min and then increased at a rate of 3 °C per min to 220 °C. The final temperature was held for 2.5 min. In the injector, a split valve was set at a 1:60 ratio.

Oxidation of Myofibrils. MPI was suspended (40 mg protein/mL) in a 15 mM piperazine-*N*,*N*-bis(2-ethane sulfonic acid) (PIPES) buffer containing 0.6 M NaCl (pH 6.0). Suspended MPI was oxidized for 24 h at 4 °C with (i) a HRGS (10 μM $FeCl_3$, 0.1 mM ascorbic acid, and 0.05−5.0 mM H_2O_2), (ii) a lipid (lineolic acid)-oxidizing system (LOS: 0.05−5.0 mM linoleic acid and 3750 units of lipoxidase/mL), or (iii) a metmyoglobin-oxidizing system (MOS: 0.05−0.5 mM metmyoglobin). Oxidation was terminated by adding propyl gallate/Trolox C/EDTA (1 mM each). The protein concentration in MPI after oxidation treatment was adjusted to 30 mg/mL with the 15 mM PIPES buffer (pH 6.0) containing 0.6 M NaCl.

Measurement of Lipid Oxidation. The oxidation of residual lipids in the MPI was assessed by measuring 2-thiobarbituric acid-reactive substances (TBARS) using a modified distillation procedure (*25*). Quantitatively, 5.0 g of each oxidized or nonoxidized MPI sample (30 mg/mL protein) was transferred into a 125 mL flask. Then, 2.5 mL of distilled deionized water, 2.5 mL of 1% propyl gallate/EDTA solution, 1 mL of 1 N HCl solution, and three drops of antifoam reagent (Antifoam B, Sigma Chemical Co.) were added to the sample flask and mixed by swirling. The flask was connected to a distillation setup and heated at 100 °C to collect distillate. An equal amount (3.0 mL) of the distillate and 0.02 M thiobarbituric acid reagent was mixed in a screw cap test tube, followed by heating in a boiling water bath (100 °C) for 35 min. Absorbance at 530 nm was read against a blank, and the amount of TBARS was calculated from a malonaldehyde (MDA) standard curve prepared with a series of 1,1,3,3-tetraethoxypropane [malonaldehyde bis(diethyl acetal)] (Fisher Scientific, Hampton, NH) solution (0.0−1.0 μM). Results were expressed as mg MDA equivalent/kg sample protein.

ATPase Assay. Myosin ATPase activities of control and oxidized MPI were determined according to Wells et al. (*26*) and Katoh et al. (*27*). Briefly, MPI samples were diluted to a 3.0 mg/mL protein concentration. Aliquots of 0.2 mL of the protein suspension were mixed with 2.0 mL of the reaction solution (for Ca-ATPase: 7.6 mM ATP, 15 mM $CaCl_2$, 150 mM KCl, and 180 mM Tris-HCl, pH 7.4; for K-ATPase: 7.6 mM ATP, 300 mM KCl, 5.0 mM EDTA, and 180 mM Tris-HCl, pH 7.4). After reaction at 25 °C for 10 min, 1.0 mL of 10% trichloroacetic acid was added to stop the reaction. The mixture was subsequently centrifuged at 2500*g* for 5 min, and 1 mL of the supernatant was reacted with 3.0 mL of 0.66% ammonium molybdate in 0.75 N sulfuric acid. One-half-milliliter of freshly prepared 10% $FeSO_4$ in 0.15 N sulfuric acid was then added, and the mixture was allowed to react for 2 min for color development. The absorbance of the liberated inorganic phosphate was read at 700 nm to determine the Ca-ATPase and K-ATPase activities. Results were expressed as μmol phosphate/mg protein/10 min. A series of NaH_2PO_4 solutions (0.0−1.0 mM) were used to prepare the standard curve for phosphate calculation.

Protein Carbonyls. Protein carbonyls in MPI samples were measured following the procedure described by Levine et al. (*28*). Diluted MPI samples (7.5 mg/mL protein) were incubated with 2,4-dinotrophynylhydrazine reagent for 30 min at room temperature (22 °C). The spectra at 355−390 nm were measured. The carbonyl content was calculated from the peak absorbance (355−390 nm) using an absorption coefficient of 22000 M^{-1} cm^{-1}. Because some protein was lost in the various washing steps, the protein content in the final pellet after all washings was determined by dissolving the pellet in 6 M guanidine hydrochloride and then reading the absorbance at 280 nm. A standard curve was prepared by measuring the absorbance (280 nm) of bovine serum albumin solutions (0.0−10.0 mg/mL) dissolved in the same guanidine hydrochloride. Results were expressed as μmol carbonyl/g protein.

Differential Scanning Calorimetry (DSC). The oxidation-induced protein structural stability change was analyzed by a model 2920 DSC machine (TA Instruments, New Castle, DE). Accurately weighed MPI samples (12−16 mg) were placed in polymer-coated aluminum pans and hermetically sealed. An empty pan was used as a reference. A heating rate of 10 °C/min was used to thermally scan samples from 20 to 95 °C. The enthalpy change (ΔH) associated with the unfolding of the individual proteins was estimated by measuring the area above the DSC transition curve with a straight baseline constructed from the start to the end of the endotherm. The temperature maximum (T_m) for a transition was determined by constructing a tangent to the leading edge of the transition and determining the temperature at the point of intersection with the baseline using Universal analysis Version 1.2 N software (TA Instruments) as described in the DSC user manual.

Table 1. Fatty Acid Profile in Pork MPIs

fatty acid	relative quantity (%)
myristic acid (C14:0)	3.1 ± 0.5[a]
palmitic acid (C16:0)	37.9 ± 1.5
palmitoleic acid (C16:1)	1.8 ± 0.2
stearic acid (C18:0)	20.8 ± 0.7
oleic acid (C18:1)	28.9 ± 2.1
linoleic acid (C18:2)	5.5 ± 0.7
linolenic acid (C18:3)	0.7 ± 0.0
arachidic acid (C20:0)	1.3 ± 0.2

[a] Standard error of the mean.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Oxidized MPI samples were subjected to SDS-PAGE (*29*) to determine covalent protein cross-linking. Dilute samples (2 mg protein/mL) were mixed (1:1, v/v) with the SDS-PAGE sample buffer (4% SDS, 20% glycerol, and 0.125 M Tris, pH 6.8), with or without 5% of 2-mercatoethanol, to obtain a final protein concentration of 1.0 mg/mL. For the sample without 2-mercatoethanol, 0.5 mM *N*-ethylmaleimide (a thiol blocking agent) was added to prevent disulfide artifacts. The mixture was heated in boiling water (100 °C) for 3 min. Electrophoresis was run using an SE 250 Mighty Small II vertical slab gel electrohoresis units (Hoefer Scientific Instruments, San Fransisco, CA). An aliquot of 20 μL (20 μg of protein) of samples was loaded to each well in the 3% polyacrylamide stacking gel, and individual proteins were separated in the 10% resolving gel. The electrophoresis was initiated with a 20 mA constant current per gel, and after the sample front line reached the resolving gel, it was increased to 40 mA per gel. After electrophoresis, the gels were immersed in an aqueous staining solution (50% methanol, 6.8% glacial acetic acid, and 0.1% Coomassie Brilliant Blue R) for 4 h and subsequently destained to remove the background stain with an aqueous destaining solution (7.5% of glacial acetic acid and 5% methanol).

Statistical Analysis. Significance of the main effects (type and concentration of oxidizing agents) was determined by the analysis of variance test using SAS/STAT (SAS Institute Inc., Cary, NC). Differences between means were compared by Student−Newman−Kuels multiple comparison using SAS at a significance level of 0.05.

RESULTS

Protein and Fat in MPI. A total of six MPI extractions were prepared, and the average protein content in the wet MPI pellets was 6.40 ± 0.34% (w/w). The crude fat content in MPI was minimal (0.49 ± 0.02% on a dry basis or 0.034 ± 0.001% on a wet basis). The fatty acid profile of the extracted fat is shown in **Table 1**. The main constituents were palmitic and oleic acids. Linoleic acid and linolenic acid (a main precursor of MDA) constituted, respectively, 5.5 and 0.7% of the total fatty acid content.

Lipid Oxidation. The production of TBARS was unique, both in pattern and in intensity, to each of the three oxidizing systems. In the metal-catalyzed oxidizing systems (HRGS and MOS), the amount of TBARS produced increased ($P < 0.05$) with the oxidant concentration, reaching a maximum at 0.1 mM metmyoglobin and at 1.0 mM H_2O_2 (**Figure 1**), and then declined at higher oxidant concentrations. In contrast, the TBARS formation in LOS-oxidized MPI continued with increasing oxidant concentrations. While TBARS production in the metal-catalyzed oxidizing systems was facilitated by low-concentration oxidants, at high dosages of the oxidants, the process was likely accompanied by secondary reactions that resulted in either degradation of MDA or its formation of a complex with proteins. The lipid oxidation by lipoxidase (the LOS system) at refrigerated temperature was conspicuously slow but was promoted by increasing the concentration of the substrate (linoleic acid). Notwithstanding the fact that the content of residual fat in MPI was low, the amount of TBARS produced in HRGS was overall higher ($P < 0.05$) than those in MOS and LOS, probably due to the reaction of the TBA reagent with protein carbonyls.

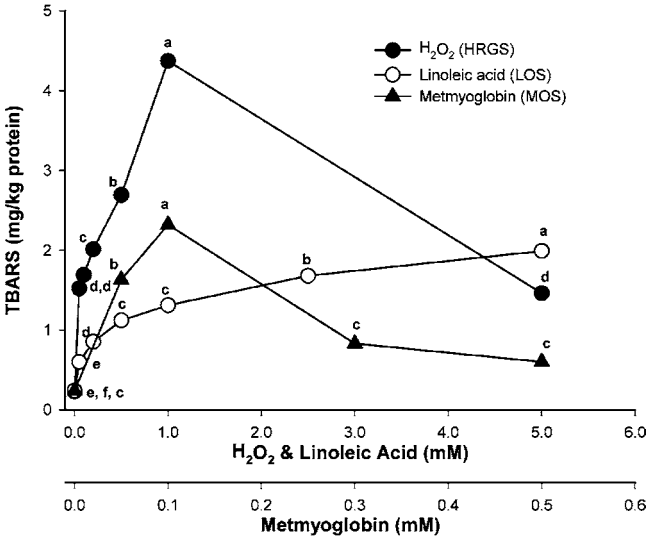

Figure 1. Formation of TBARS in pork MPI after 24 h of incubation in three oxidizing systems. For the letters a–f, means (three replications) without a common letter within the same oxidizing system differ significantly ($P < 0.05$).

ATPase Activities. The activities of both Ca-ATPase and K-ATPase exhibited oxidant dose-dependent changes and varied among the three oxidizing matrix systems (**Figure 2**). In HRGS, the Ca-ATPase activity gradually increased with increasing H_2O_2 concentrations, but in MOS, the enzyme activity was insensitive to the metmyoglobin concentration (**Figure 2A**). On the other hand, the Ca-ATPase activity in the LOS-treated samples increased markedly ($P < 0.05$) in the 0.25−1.0 mM linoleic concentration range and reached a plateau at higher linoleic concentrations. The Ca-ATPase activity in the LOS system with >1.0 mM linoleic acid was consistently higher ($P < 0.05$) than that in the other two oxidizing systems.

The response of K-ATPase to the three oxidizing matrices was more complex and differed from that of Ca-ATPase. The enzyme activity appeared to vary at low concentrations of oxidants in all three oxidizing systems, and no significant differences between the three oxidative treatments could be established (**Figure 2B**). However, while K-ATPase activity in the MOS-treated MPI remained largely stagnant across the oxidant concentrations, the enzyme activity in both HRGS and LOS systems continued to drop as the oxidant concentration was raised (>1.0 mM H_2O_2 for HRGS and >0.05 mM linoleic acid for LOS).

Protein Carbonyls. The content of protein or protein-bound carbonyls in MOS and LOS generally increased with increasing oxidant concentrations (**Figure 3**). However, the carbonyl content in HRGS abruptly increased with H_2O_2 up to 1.0 mM and then gradually decreased, probably due to interaction with available electron-dense groups (e.g., $-NH_2$) in the protein solution. The results indicated that the rate of HRGS-induced protein oxidation was higher and the reaction was more potent than in both MOS and LOS systems.

DSC. Figure 4 illustrates representative thermograms of myofibrillar proteins exposed to different oxidizing conditions. Nonoxidized MPI exhibited a main endothermic transition peak ($T_m = 66.5$ °C), which can be attributed mostly to myosin tail (light meromyosin or rod) denaturation (*30*), and a minor

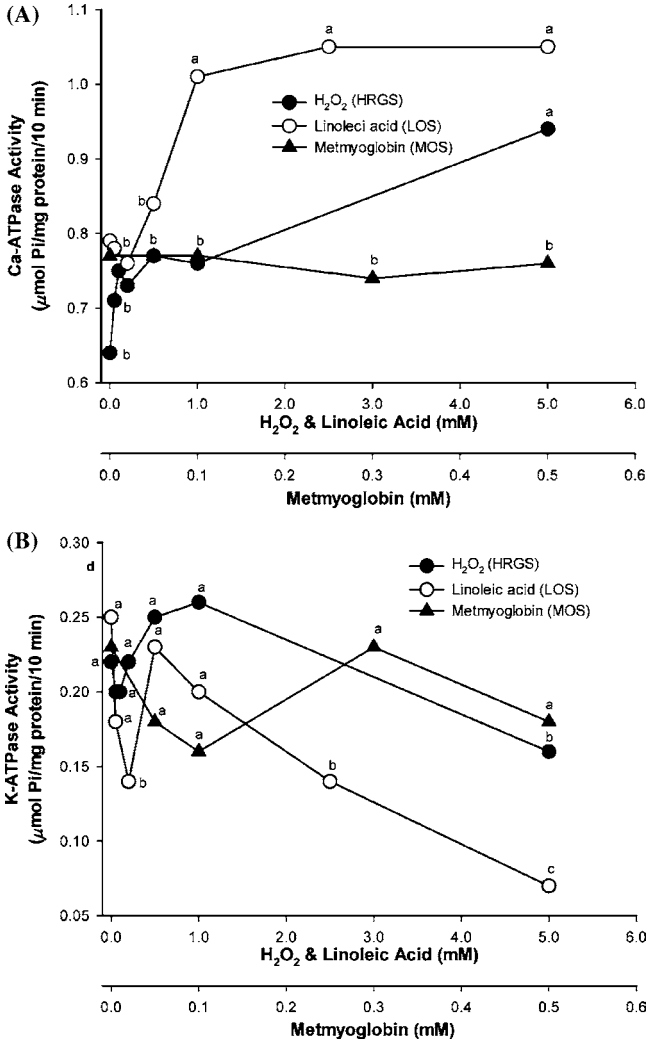

Figure 2. Myosin Ca-ATPase (**A**) and K-ATPase (**B**) activities in pork MPI after 24 h of incubation in three oxidizing systems. For the letters a–c, means (three replications) without a common letter within the same oxidizing system differ significantly ($P < 0.05$).

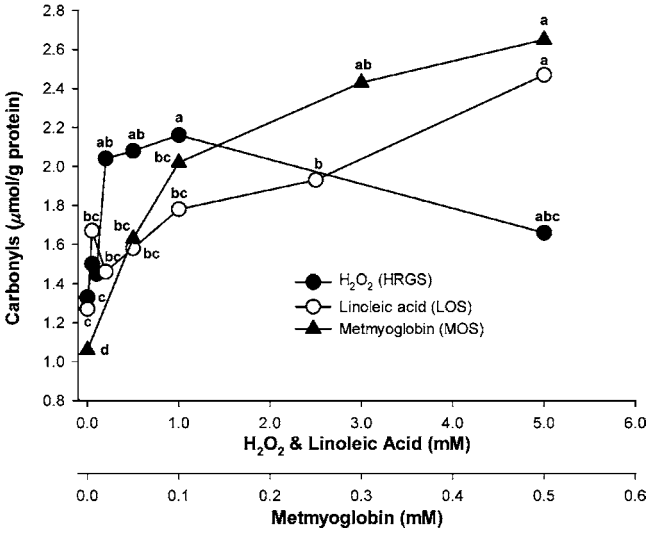

Figure 3. Concentration of protein carbonyls in pork MPI after 24 h of incubation in three oxidizing systems. For the letters a–c, means (three replications) without a common letter within the same oxidizing system differ significantly ($P < 0.05$).

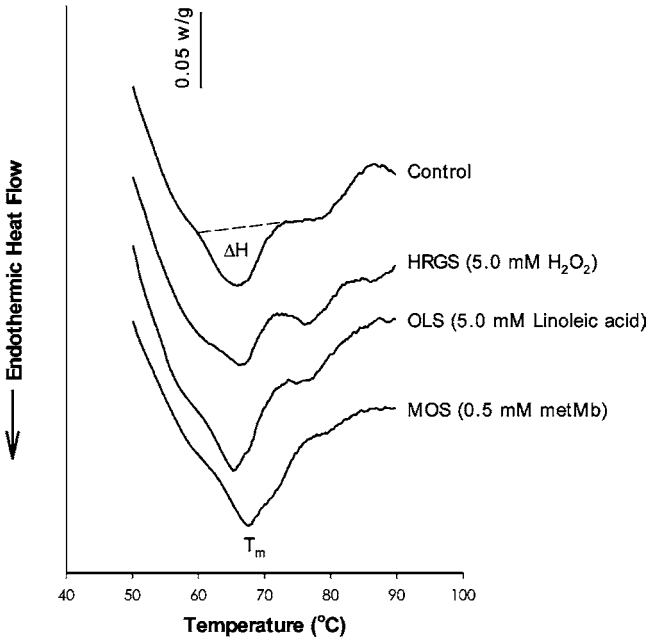

Figure 4. Representative differential scanning calorimetric curves of pork MPI after 24 h of incubation in three oxidizing systems.

transition at about 78 °C, which may be assigned to actin (*31*). The endortherm of myosin head (heavy meromyosin), which appeared in the 54−60 °C temperature zone, was not well-resolved. Low concentrations of HRGS and MOS tended to enhance the protein stability (increase in T_m) while LOS tended to destabilize it (**Table 2**). The treatment with 0.1 mM metmyoglobin resulted in the greatest T_m value (68.3 °C) while that with 1.0 mM linoleic acid gave rise to the least stable protein ($T_m = 64.9$ °C; $P < 0.05$).

The ΔH results varied considerably, and no clear oxidant effect could be established. Although nonsignificant ($P > 0.05$), the apparent reductions in the heat of transition for HRGS-treated MPI samples appeared to be greater than those for the LOS or MOS treatments.

Cross-Linking of Myosin Heavy Chains. One of the main consequences of protein oxidation is the formation of protein aggregates and, in some cases, also protein fragments. The electrophoretic patterns of the MPI samples with the reducing compound 2-mercaptoethanol manifested no loss in myosin heavy chain band in all three oxidizing matrices (results not shown). However, the band intensity of myosin heavy chain in MPI samples without the reducing compound decreased at high concentrations of oxidants, which was most noticeable for the HRGS treatment (**Figure 5**). Concomitantly, some high molecular weight polymers (protein aggregates), which stacked on the top of the resolving gel, were produced. Although peptide bond cleavage can occur in oxidatively stressed proteins, there were no detectable protein fragments in all three oxidizing systems. The results indicated that oxidation caused cross-linking in MHC through disulfide bonds, but the extent of such cross-linking was oxidant dose-dependent and specific to each oxidizing system.

By comparing the changes in carbonyls and the SDS-PAGE patterns of MPI exposed to the three oxidizing systems, it was noticed that the concentrations of H_2O_2 (0.5−1.0 mM) in HRGS, around which the carbonyl content started to decline, coincided with those for the MHC band loss. In both LOS- and MOS-treated samples, however, the carbonyl content did not decrease over the respective oxidant concentration ranges and the MHC loss was also less pronounced. It appeared that carbonyls were involved in protein−protein interactions; that is, the loss of

Table 2. Temperature Maxima (T_m) and Enthalpy Changes (ΔH) for the Denaturation of Myosin Tail in MPI Exposed to Three Oxidizing Systems[a]

treatment	concentration	T_m (°C)	ΔH (J/g)
control		66.5 ± 0.8 ab	68.0 ± 5.3
HRGS	0.1 mM H_2O_2	67.1 ± 0.0 ab	49.6 ± 20.9
	1.0 mM H_2O_2	67.9 ± 0.8 ab	58.1 ± 17.3
	5.0 mM H_2O_2	66.3 ± 0.6 ab	43.9 ± 18.1
LOS	0.1 mM linoleic acid	65.1 ± 0.9 ab	71.9 ± 31.5
	1.0 mM linoleci acid	64.9 ± 0.2 b	69.1 ± 19.9
	5.0 mM linoleic acid	65.4 ± 0.3 ab	55.8 ± 25.0
MOS	0.05 mM metmyoglobin	66.8 ± 1.3 ab	59.5 ± 19.4
	0.1 mM metmyoglobin	68.3 ± 1.4 a	60.3 ± 30.5
	0.5 mM metmyoglobin	67.8 ± 0.2 ab	55.0 ± 11.4

[a] Means (± standard errors) without a common letter differ significantly ($P < 0.05$).

myosin would not occur until protein carbonyl concentration/protein oxidation reached a certain high level.

DISCUSSION

Lipid-derived reactive oxygen species, such as peroxyl radicals (•OOR), are potential initiators of protein oxidation. Although the peroxide value (PV) in oxidized MPI could not be accurately quantified in our preliminary trials due to the extremely low lipid content in the samples and limited sensitivity of the PV testing method, the TBARS level was nevertheless readily determined to indicate the potential role of lipid-derived reactive species. The relatively low-level production of secondary products (e.g., MDA) in LOS-treated MPIs than in HRGS-treated MPIs suggested that the enzyme lipoxidase was probably inefficient in producing hydroperoxides under low-temperature meat-processing conditions. On the other hand, the nonenzymatic HRGS and MOS oxidative processes were less temperature-dependent in the production of reactive oxygen species from residual lipid in MPI.

The MPI samples in this study were not prepared from a constant storage time although all muscle samples were vacuum-packaged prior to freezing. Hence, there was a possibility that some endogenous protein oxidation might have occurred prior to the MPI analysis, contributing to the small variations in the measures of control samples. For example, the means and the standard deviations of the values in control samples were 0.75 ± 0.15 μmol phosphate/mg protein/10 min (Ca-ATPase), 0.24 ± 0.04 μmol phosphate/mg protein/10 min (K-ATPase), 1.23 ± 0.24 μmol/g protein (protein carbonyl), and 0.26 ± 0.07 mg/kg protein (TBARS). A more accurate assessment of the changes induced by the oxidants would have been made with prerigor muscle samples.

Most of the TBARS, measured with the distillation method, were assumed to originate from the small amounts of lipids (linoleic and linolenic acids) contained in the MPI samples. However, it is recognized that the specificity of the TBA test is somewhat low, and a variety of TBARS could be generated from other chemical compounds in oxidatively stressed biological samples including proteins. Thus, the TBARS determined in this study may not entirely be from lipid oxidation in the myofibril pellet.

The Ca- and K-ATPase activities are affected by modification of two active sulfhydryl groups located at the active site in the myosin head (*32*); hence, both could be used as indexes of oxidation and for the monitoring of myosin structural changes leading to denaturation (*33*). In our previous study (*12*), the Ca-ATPase activity in hydroxyl radical-oxidized chicken myofibrils increased sharply at low H_2O_2 concentrations, followed by gradual reductions. However, the K-ATPase activity continued to decrease with increasing oxidant concentrations. Studies with carp muscle also showed that the addition of low concentrations of photooxidized methyl oleate (*34*) or peroxidized triacylglycerols (*35*) increased myofibrillar Ca-ATPase activity. However, the K-ATPase activity was reduced steadily by both oxidants over a broad concentration range. Wang et al. (*20*) reported that myosin Ca-ATPase activity in isolated fresh beef heart myofibrils was closely related to the sulfhydryl content, but in frozen myofibril samples, it was closely related to the exposure of hydrophobic amino acid residues.

Our present results were in general agreement with these previous findings and demonstrated a complex structural susceptibility of the enzymic myosin head to the three oxidizing matrices investigated. While there was no clear pattern in oxidant specificity, most of the protein structural alterations appeared to occur when MPI was exposed to low concentrations of oxidants that produced active oxygen species, e.g., •OH in HRGS and •OOC in oxidized linoleic acid. Under in situ conditions, all three oxidizing matrices could contribute to the overall Ca-ATPase and K-ATPase changes.

The increase in protein carbonyls is one of the key biochemical changes that occur during protein oxidation. Protein-bound carbonyls can be derived from direct oxidative attack on amino acid side chains, fragmentation of the peptide backbone, and oxidized ascorbate and lipids (*36*). An enhanced rate of protein oxidation by the presence of ascorbic acid is due to ascorbate-driven redox cycling of metal ions (Fe^{2+}, Cu^{2+}, etc.). Metal-catalyzed formation of active oxygen species (i.e., •OH) in the presence of ascorbate was responsible for the conversion of some amino acid residues to carbonyl derivatives (*28*). Levine et al. (*28*) reported that carbonyl content in iron-oxidized proteins increased in the presence of ascorbic acid. Beef cardiac muscle myofibrillar protein prepared in the presence of ascorbate has been shown to markedly increase protein-bound carbonyls (*37*). A similar finding with bovine skeletal muscle was also reported (*10*).

The role of oxidized lipids in the initiation of protein oxidation in muscle foods has been subjected to considerable debate. Stadtman and Oliver (*8*) showed that site-specific modification of proteins, a common consequence of metal-catalyzed reaction in cells, was independent of lipids. However, oxidation of beef muscle protein was found to coincide with lipid oxidation (*37*). Li and King (*38*) postulated that myosin denaturation in dark chicken muscle was influenced mainly by lipid oxidation rather than by direct metal-catalyzed site-specific oxidation. On the other hand, the experimental evidence presented by Liu and Xiong (*39*) suggested that carbonyl contents in oxidized chicken breast and leg myofibrillar proteins were minimally affected by lipid oxidation. Consistent with this observation, Davies and Goldberg (*40*) demonstrated that protein degradation and lipid peroxidation in rabbit erythrocytes exposed to different radical-generating systems at 37 °C were independent events, noting that protein degradation occurred approximately 2 h earlier as compared to lipid peroxidation. Therefore, it appears that the environment (muscle types, animal species, etc.) within which protein and lipid oxidation occur has a major influence on the "coupling" of the two oxidative processes.

Results from the present study further demonstrated that lipid and protein oxidation may be related in one oxidizing system but not in another. In particular, the amounts of TBARS and of protein carbonyls were apparently related in HRGS and LOS but not in MOS. In HRGS, TBARS and protein carbonyls were

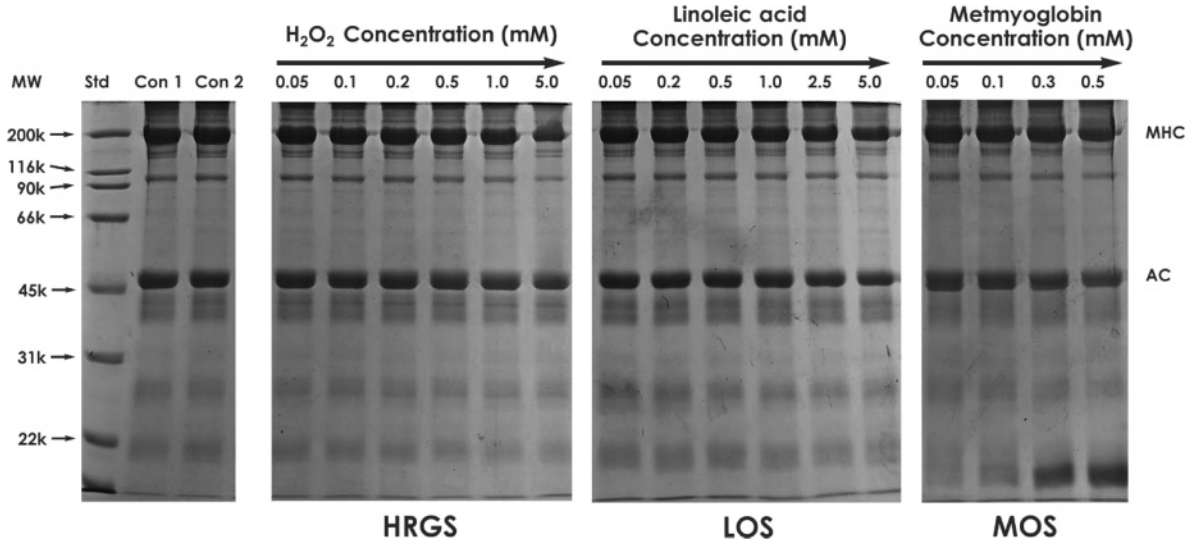

Figure 5. SDS–PAGE of pork MPI after 24 h of incubation in three oxidizing systems. MHC, myosin heavy chain; and AC, actin. Lane Std is the molecular weight (MW) standard; lane Con 1 and lane Con 2 are, respectively, 0 and 24 h MPIs not exposed to any of the oxidizing agents.

continuously generated ($P < 0.05$), but beyond a certain oxidant concentration level, both declined. The reduction (or depletion) corresponded also to the loss of the myosin heavy chain, suggesting the possibility that protein carbonyls and protein-bound carbonyls (from MDA, dehydroascorbate, etc.) might have reacted with free amino groups leading to cross-linked myosin polymers and protein aggregates (*36*, *38*, *41*). In LOS, both protein carbonyls and TBARS were continuously generated upon the fatty acid addition ($P < 0.05$), and in MOS, the continuous production of protein carbonyls was accompanied by the depletion of TBARS. Consistent with the carbonyl and TBARS production, the extent of ΔH reduction in HRGS-treated MPI tended to be greater (albeit nonsignificant) than those in LOS and MOS. This may be because in HRGS, active oxygen species could be readily generated and transferred to proteins via the Fe^{2+}−protein complex. Presumably, generation and dispersion of active oxygen species in HRGS were faster because of the complete system for ascorbate-driven redox cycling, whereas without ascorbate and H_2O_2 (for MOS and LOS) or with a low incubation temperature (for LOS), the production and dispersion of active oxygen species were slow.

It is worth noting that many of the protein oxidation studies were conducted at ambient or higher temperatures; therefore, the results may be of little relevance to meat processing. The current study demonstrating different potencies of three oxidizing systems was conducted at refrigerator temperatures and the immediate oxidizing compounds were generated in situ. In a number of previous studies that reported the effect of oxidized lipids on ATPase activity and cross-linking of myosin in fish myofibrillar protein, reactive oxygen species from lipid oxidation were prepared by incubating fatty acids or fish oil at elevated temperatures (up to 37 °C) or a long period (up to 112 h) and then mixing the oxidized lipids with myofibrillar protein to initiate protein oxidation (*34*, *35*). Kanner and Harel (*42*, *43*) and Gatellier et al. (*44*) reported that H_2O_2-activated metmyoglobin, which may involve a ferryl [iron (IV)-oxo] species and protein radicals, induced lipid oxidation. However, the enhanced rate of the lipid peroxidation was observed by incubation of lipid/metmyoglobin/H_2O_2 at 37 °C.

In conclusion, the results from this study demonstrated that biochemical changes in oxidized MPI were dependent upon the specific oxidizing systems. The changes were nonlinear and abrupt at low concentrations of oxidants but became more or less linear at higher concentrations, indicating a saturation status. Among the three oxidizing systems investigated, the impact of a HRGS appeared to be the most potent. This research was a further contribution to the understanding of the mechanisms of protein oxidation occurring in the complex muscle food systems.

LITERATURE CITED

(1) Butterfield, D. A.; Stadtman, E. R. Protein oxidation processes in aging brain. *Adv. Cell Aging Gerontol.* **1997**, *2*, 161−191.
(2) Stadtman, E. R.; Berlett, B. S. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem. Res. Toxicol.* **1997**, *10*, 485−494.
(3) Judge, A. R.; Dodd, S. L. Xanthine oxidase and activated neutrophils cause oxidative damage to skeletal muscle after contractile claudication. *Am. J. Physiol. Heart Circ. Physiol.* **2004**, *286*, H252−H256.
(4) Frankel, E. N. *Lipid Oxidation*; The Oily Press: Dundee, Scotland, 2004.
(5) Irwin, J. A.; Ostdal, H.; Davies, M. J. Myoglobin-induced oxidative damage. Evidence for radical transfer from oxidized myoglobin to other proteins and antioxidants. *Arch. Biochem. Biophys.* **1999**, *362*, 94−104.
(6) Phillips, A. L.; Faustman, L. C.; Liebler, D. C.; Hill, D. W. The use of proteomics tools to understand the effects of lipid derived aldehydes on myoglobin redox stability. *Proc. Recip. Meat Conf.* **2002**, *55*, 109−117.
(7) Alderton, A. L.; Faustman, C.; Liebler, D. C.; Hill, D. W. Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. *Biochemistry* **2003**, *42*, 4398−4405.
(8) Stadtman, E. R.; Oliver, C. N. Metal-catalyzed oxidation of proteins *J. Biol. Chem.* **1991**, *266*, 2005−2008.
(9) Decker, E. A.; Xiong, Y. L.; Clavert, J. T.; Crum, A. D.; Blanchard, S. P. Chemical, physical and functional properties of oxidized turkey white muscle myofibrillar proteins. *J. Agric. Food Chem.* **1993**, *41*, 186−189.
(10) Martinaud, A.; Mercier, Y.; Marinova, P.; Tassy, C.; Gatellier, P.; Renerre, M. Comparison of oxidative processes on myofibrillar proteins from beef during maturation and by different model oxidation systems. *J. Agric. Food Chem.* **1997**, *45*, 2481−2487.
(11) Liu, G.; Xiong, Y. L.; Butterfield, D. A. Chemical, physical and gel-formaing properties of oxidized myofibrils and whey- and soy-protein isolates. *J. Food Sci.* **2000**, *65*, 811−817.
(12) Ooizumi, T.; Xiong, Y. L. Biochemical susceptibility of myosin in chicken myofibrils subjected to hydroxyl radical oxidizing systems. *J. Agric. Food Chem.* **2004**, *52*, 4303−4307.

(13) Ostdal, H.; Bjerrum, M. J.; Pedersen, J. A.; Andersen, H. J. Lactoperoxidase-induced protein oxidation in milk. *J. Agric. Food Chem.* **2000**, *48*, 3939−3944.
(14) Schaich, K. M. Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation. III, Free radical transfer from oxidizing lipids. *CRC Crit. Rev. Food Sci. Nutr.* **1980**, *13*, 189−244.
(15) Xiong, Y. L.; Decker, E. A. Alterations in muscle protein functionality by oxidative and antioxidative processes. *J. Muscle Foods* **1995**, *6*, 139−160.
(16) Wan, L.; Xiong, Y. L.; Decker, E. A. Inhibition of oxidation during washing improves the functionality of bovine cardiac myofibrillar protein. *J. Agric. Food Chem.* **1993**, *41*, 2267−2271.
(17) Xiong, Y. L.; Decker, E. A.; Robe, G. H.; Moody, W. G. Gelation of crude myofibrillar protein isolated from beef heart under antioxidative conditions. *J. Food Sci.* **1993**, *58*, 1241−1244.
(18) Kelleher, S. D.; Hultin, H. O.; Wilhelm, K. A. Stability of mackerel surimi prepared under lipid-stabilizing processing conditions. *J. Food Sci.* **1994**, *59*, 269−271.
(19) Jaranback, L.; Lijemark, A. Ultrastructure changes during frozen storage of cod. III, Effect of linoleic acid and linolenic acid hydroperoxides on myofibrillar proteins. *J. Food Technol.* **1975**, *10*, 437−452.
(20) Wang, B.; Xiong, Y. L.; Srinivasan, S. Chemical stability of antioxidant-washed beef heart surimi during frozen storage. *J. Food Sci.* **1997**, *62*, 939−945, 991.
(21) Bhoite-Solomon, V.; Kessler-Icekson, G.; Shaklai, N. Peroxidative cross-linking of myosins. *Biochem. Int.* **1992**, *26*, 181−189.
(22) Hanan, T.; Shaklai, N. The role of H_2O_2-generated myoglobin radical in cross-linking of myosin. *Free Radical Res.* **1995**, *22*, 215−217.
(23) Gornall, A. G.; Bardawill, C. J.; David, M. M. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **1949**, *177*, 751−766.
(24) AOAC. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Washington, DC, 1984.
(25) Rhee, K. S. Minimization of further lipid peroxidation in the distillation 2-thiobarbituric acid test of fish and meat. *J. Food Sci.* **1978**, *43*, 1776−1778, 1781.
(26) Wells, J. A.; Werber, M. M.; Yount, R. G. Inactivation of myosin subfragment one by cobalt(II)/cobalt(III) phenanthroline complexes. 2. Cobalt chelation of two critical SH groups. *Biochemistry* **1979**, *18*, 4800−4805.
(27) Katoh, N.; Uchiyama, H.; Tsukamoto, S.; Arai, K. A biochemical study on fish myofibrillar ATPase. *Nippon Suisan Gakkaishi* **1977**, *43*, 857−867.
(28) Levine, R. L.; Garland, D.; Oliver, C. N.; Amici, A.; Climent, I.; Lenz, A. G.; Ahn, B. W.; Shaltiel, S.; Stadtman, E. R. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* **1990**, *186*, 464−477.
(29) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(30) Wright, D. J.; Wilding, P. Differential scanning calorimetric study of muscle and its proteins: Myosin and its subfragments. *J. Sci. Food Agric.* **1984**, *35*, 357−372.
(31) Xiong, Y. L.; Brekke, C. J.; Leung, H. K. Thermal denaturation of muscle proteins from different species and muscle types as studied by differential scanning calorimetry. *Can. Inst. Food Sci. Technol. J.* **1987**, *20*, 357−362.
(32) Sekine, T.; Yamaguchi, M. Effect of ATP on the binding of *N*-ethylmaleimide to SH groups in the active site of myosin ATPase. *J. Biochem.* **1963**, *54*, 196−198.
(33) Hultin, H. O. Characteristics of muscle tissue. In *Food Chemistry*; Fennema, O. R., Ed.; Marcel Dekker Inc.: New York, 1985; pp 725−789.
(34) Kawasaki, K.; Ooizumi, T. Effect of hydroperoxides produced by photosensitized oxidation of methyl oleate on carp myofibrillar protein. *Fish. Sci.* **1996**, *62*, 69−72.
(35) Ooizumi, T.; Kawasaki, K. Comparison of reactivity of several fish myofibrils with peroxidized lipid. *Fish. Sci.* **1997**, *63*, 615−618.
(36) Xiong, Y. L. Protein oxidation and implications for muscle food quality. In *Antioxiants in Muscle Foods*; Decker, E. A., Faustman, C. L., Lopez-Bote, C. J., Eds.; John Wiley & Sons Inc.: New York, 2000; pp 85−111.
(37) Srinivasan, S.; Xiong, Y. L.; Decker, E. A. Inhibition of protein and lipid oxidation in beef heart surimi-like material by antioxidants and combinations of pH, NaCl, and buffer type in the washing media. *J. Agric. Food Chem.* **1996**, *44*, 119−125.
(38) Li, S. J.; King, A. J. Lipid oxidation and myosin denaturation in dark chicken meat. *J. Agric. Food Chem.* **1996**, *44*, 3080−3084.
(39) Liu, G.; Xiong, Y. L. Contribution of lipid and protein oxidation to rheological differences between chicken white and red muscle myofibrillar proteins. *J. Agric. Food Chem.* **1996**, *44*, 779−784.
(40) Davies, K. J.; Goldberg, A. L. Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J. Biol. Chem.* **1987**, *262*, 8220−8226.
(41) Buttkus, H. The reaction of myosin with malonaldehyde. *J. Food Sci.* **1967**, *32*, 432−434.
(42) Kanner J.; Harel, S. Initiation of membranal lipid peroxidation by activated metmyoglobin and methemoglobin. *Arch. Biochem. Biophys.* **1985a**, *237*, 314−321.
(43) Kanner, J.; Harel, S. Lipid peroxidation and oxidation of several compounds by H_2O_2-activated metmyoglobin. *Lipids* **1985b**, *20*, 625−628.
(44) Gatellier, P.; Anton, M.; Renerre, M. Lipid peroxidation induced by H_2O_2-activated metmyoglobin and detection of a myoglobin-derived radical. *J. Agric. Food Chem.* **1995**, *43*, 651−656.

Received for review December 20, 2005. Revised manuscript received April 4, 2006. Accepted April 20, 2006. Approved for publication as journal article number 05-07-133 by the Director of the Kentucky Agricultural Experiment Station. This research was supported by a CSREES/USDA NRI grant, under Agreement Grant 2004-35503-14122.

JF0531813