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Concentration effects of hydroxyl radical oxidizing systems on biochemical properties of porcine muscle myofibrillar protein ☆

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Abstract

The objective of the study was to determine the dose-dependency of myofibrillar protein oxidation on oxidizing ferric ion. Pork myofibrillar protein isolates (MPI) were suspended in 15 mM piperazine-*N*,*N* bis(2-ethane sulfonic acid) (PIPES) buffer (pH 6.0) with 0.6 M NaCl, and incubated at 4 °C for 24 h with two levels of ferric ion (0.01 and 0.1 mM $FeCl_3$) at eight concentrations of hydrogen peroxide (0.00–10 mM H_2O_2). In both high and low [$FeCl_3$] oxidizing systems, the Ca-ATPase activity steadily increased with the H_2O_2 concentration. On the other hand, K-ATPase activity, protein carbonyl content, and 2-thiobarbituric acid-reactive substances increased with H_2O_2 up to 1.0 mM, and then gradually declined. Protein unfolding and loss of myosin heavy chain occurred continuously with increasing H_2O_2 concentrations. All changes, except for K-ATPase activity, were generally more rapid and extensive in the high [$FeCl_3$] oxidizing system. Overall, the biochemical changes in MPI exposed to ferric iron-oxidizing systems were more pronounced at high [$FeCl_3$] than at low [$FeCl_3$], but the pattern of the biochemical alterations appeared to be independent of the $FeCl_3$ concentration.

Keywords: Meat; Protein oxidation; Hydroxyl radicals; Myosin ATPase

1. Introduction

Sarcoplasmic, myofibrillar, and stromal proteins from red meat, poultry, and fish species are all susceptible to oxidative damage initiated by oxidizing lipids, metal ions, and other prooxidants indigenous to muscle or generated during meat processing (Xiong & Decker, 1995). Oxidative damage in muscle protein includes the alteration of protein structure, peptide chain scission, formation of amino acid derivatives and polymers, decreases in solubility, and changes in the functional properties of the affected proteins such as hydration, emulsification, and gelation (Srinivasan & Hultin, 1997; Xiong, Sirinivasan, & Liu, 1997). These oxidative changes are implicated in quality deterioration of oxidized meat products, including off-flavor, discoloration, destruction of nutrients, formation of toxic compounds, and reduced consumer acceptability (Kanner, 1994).

Susceptibility of muscle foods to oxidative reactions stems from the high-concentration oxidation catalysts present in the muscle tissue (e.g., iron and heme compounds) and the abundance of unsaturated lipids (Asghar, Gary, Buckley, Pearson, & Booren, 1988). In the process of myoglobin auto-oxidation, oxymyoglobin (OxyMb) further oxidizes into metmyoglobin (MetMb), H_2O_2, and peroxyl radicals ($RO_2^{\bullet}$). In the presence of iron, the peroxyl radical can be transformed into hydroxyl radical ($^{\bullet}OH$, a highly reactive oxygen species) by the Fenton reaction that can catalyze lipid oxidation (Harel & Kanner, 1985).

It is now understood that reactive oxygen species can oxidize proteins in living tissue as well as in food (Rezinick, Witt, Matsumoto, & Parker, 1992; Stadtman, 1990; Stadtman & Berlett, 1997; Xiong et al., 1997). In particular, hydroxyl radicals are known to be a major oxidizing agent that impact biochemical and functional properties

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of myofibrillar proteins in vitro. Martinaud et al. (1997) measured changes in bovine myofibrillar protein oxidized by three iron-containing solutions (2.5 mM Fe^{2+}/2.5 mM H_2O_2; 0.1 mM Fe^{3+}/25 mM ascorbate; and 4 mM Fe^{2+}/8 mM EDTA). The Fe^{2+}/H_2O_2 system generated the highest carbonyl content, while the Fe^{3+}/ascorbate system produced the lowest decrease in sulfhydryl content. In a recent report, radical-induced cross-linking of myosin, changes in myosin ATPase activity, as well as the reduction in solubility and rheology of chickin myofibrils, were found to greatly depend on the concentration of H_2O_2 and the weight ratio of H_2O_2 to myofibrils (Ooizumi & Xiong, 2004).

Despite these previous studies, the iron-concentration dependency of H_2O_2 oxidative modifications of muscle proteins under meat processing conditions (pH ~ 6.0, NaCl ~ 0.6 M) has not been examined. Research in this area is needed as iron is a major prooxidant present in muscle foods. The objective of the present study was to investigate the effect of the intensity of H_2O_2/ascorbate-based hydroxyl radical-generating systems with a low (0.01 mM $FeCl_3$) and a high (0.1 mM $FeCl_3$) iron concentration on protein and lipid oxidation in porcine myofibrillar protein isolates.

2. Materials and methods

2.1. Materials

Ten Boston shoulders in five separate vacuum packages (4 d postmortem) 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-gram pieces. The muscle dices were mixed well by hand, and subsequently divided into 20 equal portions. Each portion, weighing approximately 200 g, was placed in Cryovac vacuum bags, vacuum-sealed, and stored in a −30 °C walk-in freezer for less than 2 months before use. Unless specified otherwise, all the data reported herein represent the means from 3 to 5 independent trials (i.e., replications). 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.

2.2. Preparation of myofibrils

Myofibril isolations (a total of five) were carried out as described previously (Liu & Xiong, 1996) with some modifications. Thawed and minced muscle was homogenized by blending 30 s in a Waring blendor with 4 vol (w/v) of an isolation buffer (10 mM sodium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EGTA, pH 7.0). The muscle homogenate was centrifuged at 2000*g* for 15 min, and the supernatant was discarded. The pellet was washed two more times with 4 vol of the same isolation buffer using the same blending and centrifugation condition as indicated above. The myofibril pellet was then washed three more times with 4 vol of 0.1 M NaCl under the same condition as above except that in the last wash, the myofibril suspension was filtered through four layers of cheese cloth to remove connective tissue and its pH was adjusted to 6.0 with 0.1 N HCl prior to centrifugation. The myofibrillar protein isolate (MPI) was stored in a tightly capped bottle, kept on ice, and utilized within 24 h. Protein concentration of the myofibril pellet was measured by the Biuret method (Gornall, Bardawill, & David, 1949) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as standard.

2.3. Crude fat content and fatty acid analysis

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.

The fatty acid composition of the crude fat was determined according to the modified Boron trifluoride method of the AOAC (1984) using a Perkin–Elmer AutoSystem Gas Chromatographer (Perkin–Elmer Inc., Wellesley, MA). The GC instrument was equipped with a DB-225 capillary column (30 m × 0.32 mm ID with a 0.25 μm film thickness, J& W Scientific, Folsom, CA) and a flame ionization detector, and the fatty acids in the sample were identified using methyl ester standard mixtures. Helium carrier gas flow rate was 1.0 mL per min, and the injector and detector in the machine were 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/min to 220 °C. The final temperature was held for 2.5 min. In the injector, a split valve was set at 1:60 ratio.

2.4. Oxidation of myofibrils

MPI was suspended in 15 mM piperazine-*N*,*N* bis(2-ethane sulfonic acid (PIPES) buffer containing 0.6 M NaCl (pH 6.0). The MPI suspension (40 mg protein/mL) was oxidized at 4 °C for 24 h with two hydroxyl radical-generating systems (HRGS): (a) low [Fe^{3+}], consisting of 0.01 mM $FeCl_3$, 0.1 mM ascorbic acid, and eight levels of H_2O_2 (0, 0.05, 0.1, 0.20, 0.50, 1, 5, and 10 mM) and (b) high [Fe^{3+}], consisting of 0.1 mM $FeCl_3$, 0.1 mM ascorbic acid, and the same eight levels of H_2O_2 as above. Oxidation was terminated by adding 1 mM propyl gallate/Trolox C/EDTA. The protein concentration in MPI after completion of oxidation treatment was adjusted to 30 mg/mL with the 15 mM PIPES buffer (pH 6.0) containing 0.6 M NaCl.

2.5. ATPase assay

Myosin ATPase activities in MPI samples were determined according to Wells, Werber, & Yount (1979) and Katoh, Uchiyama, Tsukamoto, & Arai (1977). Diluted

MPI samples (3.0 mg protein/mL) were reacted at 25 °C for 10 min with 7.6 mM ATP, 15 mM $CaCl_2$, and 0.15 M KCl in a 0.18 M Tris–HCl buffer (pH 7.4) for Ca-ATPase activity, and with 7.6 mM ATP, 0.3 M KCl, and 5.0 mM EDTA in a 0.18 M Tris–HCl buffer (pH 7.4) for K-ATPase activity. After treatment with 10% trichloroacetic acid to stop the reaction, the mixture was 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. A 0.5 mL aliquot 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 solution was read at 700 nm to determine the Ca-ATPase and K-ATPase activities. A series of NaH_2PO_4 solutions (0–1.0 mM) were used to prepare the standard curve for phosphate calculation.

2.6. Protein carbonyls

Protein carbonyl content in MPI samples was determined according to the method described by Levine et al. (1990). Diluted MPI samples (7.5 mg/mL protein) were incubated with 2,4-dinitrophenylhydrazine (DNPH) reagent for 30 min at room temperature, and 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 22,000 M^{-1} cm^{-1}. Results were expressed as nmol carbonyl/mg protein.

2.7. Measurement of lipid oxidation

The extent of lipid oxidation was assessed by measuring 2-thiobarbituric acid-reactive substances (TBARS) using a modified distillation procedure (Rhee, 1978). Quantitatively, 5.0 g of each 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, St. Louis, MO) were added to the sample flask, followed by mixing through swirling. The mixture (in the flask) was distilled at 100 °C. An aliquot of 3.0 mL distillate was mixed with 3.0 mL of 0.02 M TBA in a screw cap test tube, and the mixture was heated in boiling water (100 °C) for 35 min. Absorbance at 530 nm was read against an MPI-free blank, and the amount of TBARS was calculated from a malonaldehyde (MDA) standard curve. Results were expressed as mg MDA equivalent/kg sample.

2.8. Differential scanning calorimetry (DSC)

Changes in protein structural stability by oxidation were analyzed with a Model 2920 DSC instrument (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 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 individual protein denaturation 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 maxima (T_m) for protein thermal transitions were recorded using Universal analysis Version 1.2 N software as described in the user manual.

2.9. Protein cross-linking

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to detect oxidation-induced covalent aggregation of MPI proteins (Laemmli, 1970). Samples were prepared with or without 2-mercaptoethanol (5%) to determine the involvement of disulfide bonds, and electrophoresed with a 10% polyacrylamide resolving gel. Samples without 2-mercaptoethanol were treated with 0.5 mM *N*-ethylmaleimide to prevent disulfide artifacts.

2.10. Statistical analysis

Significance of the main effects (concentration of oxidizing reagents) and two-way ferric ion (Fe^{3+}) × hydrogen peroxide (H_2O_2) interactions were determined by analysis of variance (ANOVA) using SAS/STAT (SAS Institute Inc., Cary, NC). Differences between means were compared by Student–Newman–Kuels (SNK) multiple comparison using SAS at a significance level of 0.05.

3. Results

3.1. Protein and fat in MPI

A total of five MPI isolates were prepared and the mean protein content was 64.1 ± 3.7 mg/g (wet basis). Crude fat content in dry MPI was 0.49 ± 0.02%. The major fatty acids were palmitic acid (37.9 ± 1.5%), oleic acid (28.9 ± 2.1%), and stearic acid (20.8 ± 0.7%). Linoleic acid and linolenic acid (a main precursor of MDA) constituted, respectively, 5.5% and 0.7% of the total fatty acid content (results not shown).

3.2. ATPase activities

The Ca-ATPase activity of MPI exposed to the $FeCl_3$/ascorbate/H_2O_2 oxidizing system was significantly affected by the H_2O_2 concentration, but the changes were independent of the concentration of $FeCl_3$ (Fig. 1A). At both the high and low [$FeCl_3$] levels, the Ca-ATPase activity did not change at <1.0 mM H_2O_2, but increased with increasing peroxide concentration ($P < 0.05$).

In contrast, the K-ATPase activity in MPI was affected by both ferric ion and peroxide concentrations. At the high [$FeCl_3$], the enzyme activity decreased continuously ($P < 0.05$) with the addition of H_2O_2 (Fig. 1B). The K-ATPase activity in low [$FeCl_3$]-treated samples, on the other hand, increased initially (up to 1 mM H_2O_2) and then

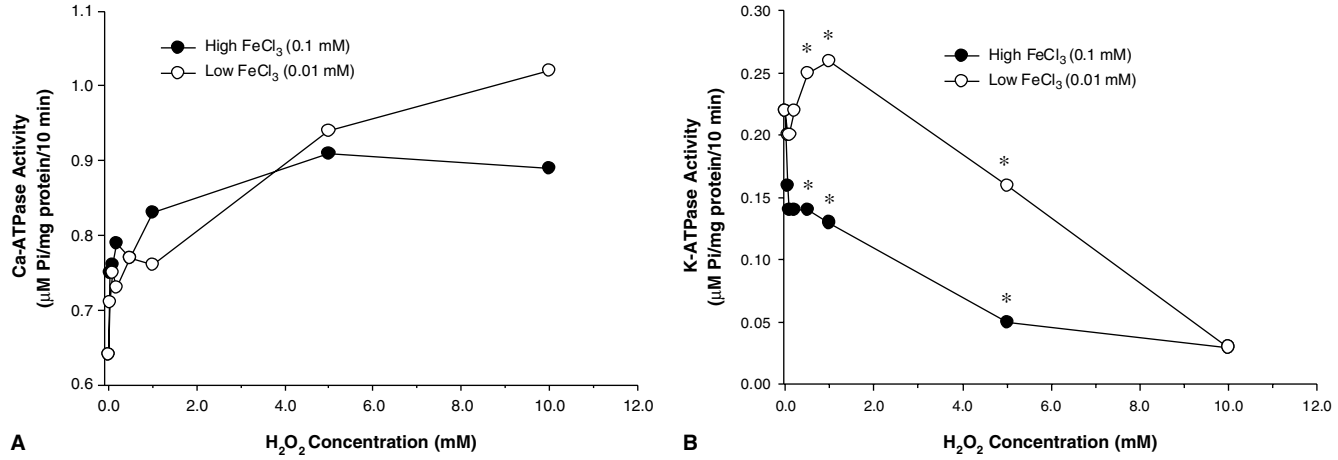

Fig. 1. Myosin ATPase activities in pork myofibrillar protein isolate exposed to hydroxyl radical-generating systems with low (0.01 mM) or high (0.1 mM) $FeCl_3$ concentrations at various levels of H_2O_2. The asterisks denote significant differences ($P < 0.05$) between high and low [$FeCl_3$] treatments.

decreased. The difference between the low and high [$FeCl_3$] treatments appeared to be H_2O_2-concentration dependent, i.e., the K-ATPase activity was higher ($P < 0.05$) at high [$FeCl_3$] than at low [$FeCl_3$] in the 0.5–5.0 mM H_2O_2 concentration range (Fig. 1B).

3.3. Protein carbonyls

In the high [$FeCl_3$] system, the carbonyl content increased markedly even with the lowest H_2O_2 addition (0.05 mM). The carbonyl level reached a maximum at 0.5 mM H_2O_2 before showing a small decline at higher H_2O_2 concentrations (Fig. 2). As seen in the high [$FeCl_3$] system, formation of protein carbonyls in the low [$FeCl_3$] system, albeit at a reduced level, was H_2O_2 dose-dependent. The carbonyl contents in MPI samples treated with high [$FeCl_3$] were approximately twofold higher ($P < 0.05$) than those treated with a low dose of $FeCl_3$ across the entire H_2O_2 concentration range. The results indicated that the concentration of ferric ion was a significant factor to the production of carbonyls in HRGS-oxidized muscle proteins.

3.4. Lipid oxidation

The production of TBA-reactive compounds in high [$FeCl_3$]-treated MPI samples surpassed that of low [$FeCl_3$] treatments across the H_2O_2 concentration range (Fig. 3), which seemed to parallel the generation of protein carbonyls. In both oxidizing systems, TBARS were formed most rapidly with the presence of low concentrations of H_2O_2 (<0.5 mM), and the TBARS level declined at increasing H_2O_2 concentrations.

3.5. Protein thermal stability

Thermal scan of nonoxidized MPI revealed a major endothermic transition with a temperature maximum (T_m) of 66.5 °C (Fig. 4; Table 1). Additionally, the DSC

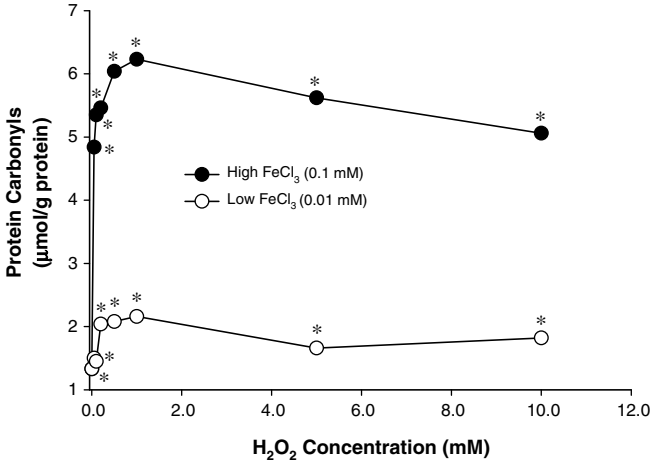

Fig. 2. Protein carbonyl content in pork myofibrillar protein isolate exposed to hydroxyl radical-generating systems with low (0.01 mM) or high (0.1 mM) $FeCl_3$ concentrations at various levels of H_2O_2. The asterisks denote significant differences ($P < 0.05$) between high and low [$FeCl_3$] treatments.

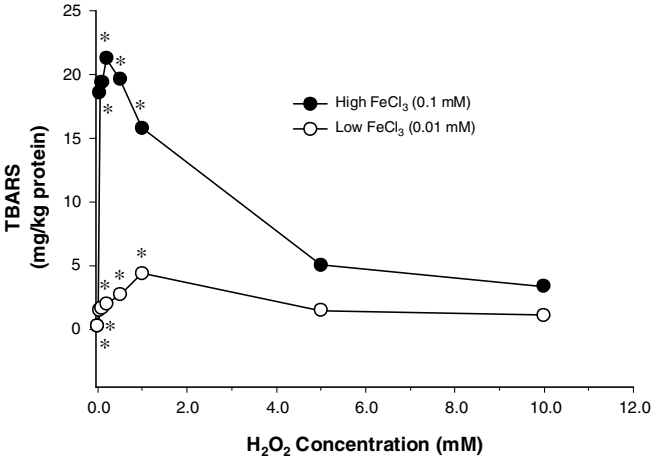

Fig. 3. TBARS content in pork myofibrillar protein isolate exposed to hydroxyl radical-generating systems with low (0.01 mM) or high (0.1 mM) $FeCl_3$ concentrations at various levels of H_2O_2. The asterisks denote significant differences ($P < 0.05$) between high and low [$FeCl_3$] treatments.

scan produced two minor transitions (54–66 °C; 73–83 °C) that were not well resolved and hence, were not further analyzed. The major transition, which can be ascribed to light meromyosin (LMM) or myosin rod (Liu & Xiong, 2000; Wright & Wilding, 1984), was influenced by the oxidizing agents and changed with the concentration of both H_2O_2 and $FeCl_3$ (Table 1). Specifically, while the T_m of LMM in the low [$FeCl_3$] system appeared to be constant across the H_2O_2 concentrations, the enthalpy of denaturation (ΔH, 68.0 J/g) tended to decrease with the same H_2O_2 treatments. The influence of H_2O_2 on both T_m and ΔH was accentuated by high [$FeCl_3$] treatment, i.e., it resulted in a greater reduction in both thermal values at elevated H_2O_2 concentrations ($P < 0.05$).

Table 1
Temperature maxima (T_m) and enthalpy (ΔH) of thermal transition of myosin rod in pork myofibrillar protein isolate exposed to hydroxyl radical-generating systems with two levels of $FeCl_3$

Treatment		T_m (°C)	ΔH (J/g)
[$FeCl_3$]	[H_2O_2] (mM)		
0.01 mM (Low)	0	$66.5^{a} \pm 0.75$	$68.0^{a} \pm 5.3$
	0.1	$67.1^{a} \pm 0.03$	$49.6^{ab} \pm 17.29$
	1.0	$67.9^{a} \pm 0.84$	$58.1^{ab} \pm 2.5$
	10.0	$66.6^{a} \pm 0.45$	$44.4^{ab} \pm 19.6$
0.1 mM (High)	0	$66.5^{a} \pm 0.75$	$68.0^{a} \pm 5.3$
	0.1	$67.6^{a} \pm 0.32$	$61.3^{a} \pm 8.11$
	1.0	$67.4^{a} \pm 0.23$	$36.6^{b} \pm 5.57$
	10.0	$64.6^{b} \pm 0.41$	$33.5^{b} \pm 3.54$

[a,b] Means (±standard errors) in the same column without a common superscript letter differ significantly ($P < 0.05$).

3.6. Protein covalent cross-linking

In the presence of 2-mercaptoethanol (a disulfide bond breaking agent), the SDS-PAGE patterns of low [$FeCl_3$]-oxidized MPI exhibited no detectable loss of myosin heavy chain (MHC) or any other myofibrillar components (result not shown). However, MPI samples that were oxidized by high [$FeCl_3$] had a slight decrease in MHC band intensity at >1.0 mM H_2O_2 (result not shown). The SDS-PAGE in the absence of 2-mercaptoethanol revealed substantial MHC losses, which were more pronounced with high [$FeCl_3$] than with low [$FeCl_3$], i.e., at >1.0 mM for low [$FeCl_3$] and at >0.2 mM for high [$FeCl_3$] (Fig. 5). Oxidation at both [$FeCl_3$] levels also resulted in a reduced band intensity of actin, although the change was less than that of MHC. The results indicated that myosin cross-linking in the high [$FeCl_3$] system originated largely from disulfide bonds, and that other types of chemical bonds, such as dityrosine and ditryptophan bonds, may also be involved in myosin cross-linking at high H_2O_2 concentrations.

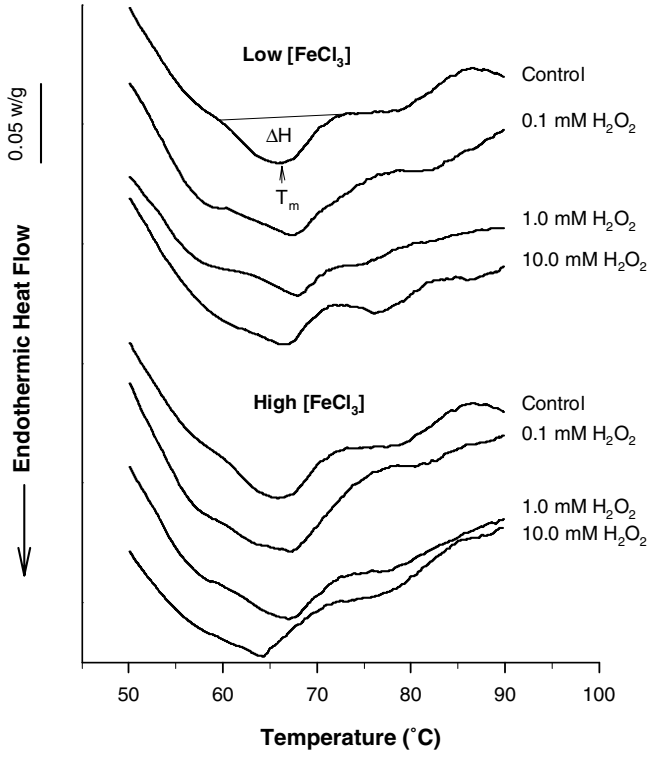

Fig. 4. Differential scanning calorimetry of pork myofibrillar protein isolate exposed to hydroxyl radical-generating systems with low (0.01 mM) or high (0.1 mM) $FeCl_3$ concentrations at various levels of H_2O_2.

4. Discussion

In the process of metal-catalyzed protein oxidation, activated oxygen species could be generated in situ when the protein-Fe^{2+} complex reacts with H_2O_2 (Stadtman & Oliver, 1991). The activated oxygen species subsequently react with the side chains of amino acid residues at the metal-binding site(s), with some amino acid residues being converted to carbonyl derivatives (Amici, Levine, Tsai, & Stadtman, 1989; Levine, 1983).

In muscle foods, free or heme-bound iron and copper represent some of the main prooxidants. Protein oxidation catalyzed by these metal ions has been shown to be site-specific (Stadtman & Oliver, 1991). In the present oxidizing system with high [$FeCl_3$], a larger amount of Fe^{2+} would be generated through the ascorbate-driven redox cycling of Fe^{3+} than in the low [$FeCl_3$] oxidizing system, allowing more protein-Fe^{2+} complexes to form. The ensuing generation of activated oxygen species would occur at a faster rate, and the biochemical changes in myofirillar protein, in turn, would proceed faster and to a greater extent in the high [$FeCl_3$] system.

Common sites of oxidative modification on proteins are side chain groups of alkaline and sulfur-containing amino acids, such as histidine, lysine, methionine, and cysteine, as well as proline (Butterfield & Stadtman, 1997; Ramirez, Gomez-Mejiba, & Mason, 2005; Roubal & Tappel, 1966; Srinivasan & Xiong, 1997). These amino acid residues were presumably involved in the biochemical changes in oxidized myosin, which were evidenced by the alterations of the myosin ATPase activities and the formation of protein

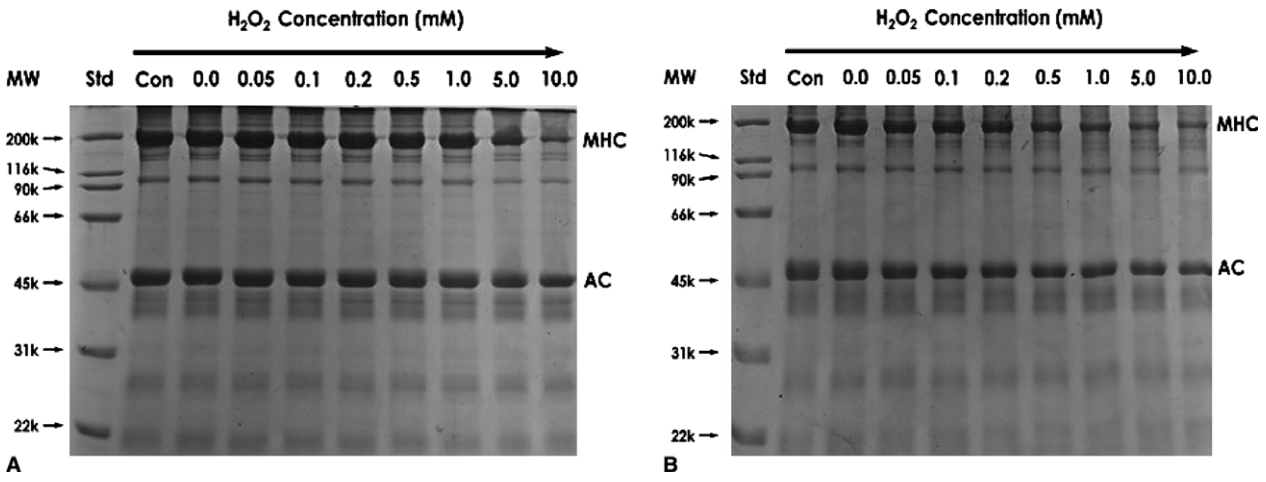

Fig. 5. SDS-PAGE patterns of pork myofibrillar protein isolate exposed to hydroxyl radical-generating systems with low (0.01 mM; A) or high (0.1 mM; B) $FeCl_3$ concentrations at various levels of H_2O_2. Std, molecular weight (MW) standard; Con, control (not exposed to oxidizing agents); MHC, myosin heavy chain; AC, actin.

carbonyls. Cysteine appeared to be a particularly susceptible amino acid. Two active sulfhydryl groups are located at the catalytic center of the myosin head, which are responsible for the myosin's Ca- and K-ATPase activities (Sekine & Yamaguchi, 1963). The high sensitivity of both ATPase forms to low concentrations of H_2O_2 at both low and high levels of $FeCl_3$ suggested that the cysteine residues at the enzyme active site were readily modified. The fact that the Ca-ATPase activity was unaffected by $FeCl_3$ concentration change but the K-ATPase activity dropped significantly when [$FeCl_3$] was raised suggested that iron concentration was a rate-limiting factor only in the oxidation of the sulfhydryl group present at the K-ATPase active site. Thus, while the oxidation-induced activation/inactivation of both enzyme forms was indicative of modification of both cysteine residues, the exact oxidative changes would depend on the concentration of H_2O_2.

Carbonyl content is one of the most reliable measures of the extent of protein oxidation (Levine et al., 1990). Active oxygen species (i.e., hydroxyl radicals) generated from metal catalysis are responsible for the conversion of some amino acid residues to carbonyl derivatives (Butterfield & Stadtman, 1997). The marked increase in the protein carbonyl content when MPI was exposed to a high [$FeCl_3$] environment with low [H_2O_2] demonstrated iron dependency for such oxidative conversions. Furthermore, the apparent relationship between protein carbonyls and TBARS indicated a strong likelihood that some dicarbonyl compounds derived from lipid oxidation, notably malondialdehyde, formed complexes with proteins (Li & King, 1996; Xiong, 2000). Carbonyls could also result from oxidative peptide scission (Xiong et al., 1997). However, this production source seemed negligible because no fragments from myosin or from any other myofibrillar components could be visualized from the SDS-PAGE patterns of oxidized MPI samples.

On the basis of H_2O_2 requirement, the oxidation-induced changes in myosin ATPase activity and in protein carbonyls (mostly at <0.5 mM H_2O_2) preceded myosin structural destabilization (DSC) and disulfide cross-linking (SDS-PAGE) (mostly at >1.0 mM H_2O_2), especially at the high [$FeCl_3$] level. Therefore, we postulate that modifications of susceptible amino acid side chain groups via radical attack led to the destabilization of MPI molecules, particularly myosin rod. A partial structural unfolding, in turn, enabled myosin to polymerize predominantly through the disulfide linkages. The initial modification of the sulfhydryl groups in myosin head (ATPase) and the likely exposure of those located in the rod, appeared to be essential to the aggregation of myosin. By converting to cystine (S-S) through reaction with radical (mainly $^{\bullet}OH$), the cysteine side chain groups played a critical role in initiating cross-linking of oxidized myofibrillar proteins. Hydrophobic interactions, which are undetectable by SDS-PAGE, would also be expected to be involved in the overall aggregation and insolubilization of MPI in the hydroxyl radical-generating system.

In the present study, active radicals would be generated through an ascorbic acid-driven redox cycle of ferrous ions, one-electron reduction of O_2 and H_2O_2 and through oxygen–iron metal complexes (Monahan, 2000). Although the exact concentrations of ascorbic acid and hydrogen peroxide in meat may vary with animal species, muscle types, degree of exsanguination, and the presence of different meat ingredients, the concentration of free iron in fresh chicken meat was estimated to be around 0.2–2.5 μg/g (Kanner, Hazan, & Doll, 1988). The two levels of [$FeCl_3$] examined in this study (0.01 and 0.1 mM) were equivalent to approximately 0.4–4 μg/g of quantities in meat (assuming a 75% moisture content), which were largely within the in situ range. The dependency of the Fe^{3+} concentration effect on the level of H_2O_2 present in the oxidizing system indicated a complex role of free Fe^{3+} in initiating biochemical changes in myofibrillar proteins.

Data from the present study, as well as from that of Ooizumi & Xiong (2004), showed that most of the

biochemical changes (except myosin destabilization and aggregation) in the •OH-generating system occurred with the presence of less than 1 mM of added hydrogen peroxide. Srinivasan & Hultin (1997) reported that an iron-catalyzed •OH-generating oxidizing solution was saturated with about 1 mM of H_2O_2. In the absence of added H_2O_2, negligible amounts of •OH were produced. However, the •OH concentration increased drastically with H_2O_2 up to about 1 mM, and no further generation was observed even when 10 mM of H_2O_2 was added. In the present study, significant losses in protein bands occurred with H_2O_2 concentrations above 5.0 mM in a low [$FeCl_3$] system and above 0.5 mM in a high [$FeCl_3$] system. If the oxidizing system was indeed saturated with 1.0 mM H_2O_2, then the role of the extra H_2O_2 became obscure.

It is plausible that the excess H_2O_2 may be involved in other chemical reactions as well, e.g., generation of other types of radicals such as superoxide radical, ferryl radical and alkoxyl radical (Frankel, 2004; Monahan, 2000), production of dehydroascorbate, and direct attack of the lipid double bonds, thereby contributing to the overall changes in proteins. Alkoxyl radicals can be produced by decomposition of lipid hydroperoxides (Frankel, 2004). Therefore, it is postulated that most of the biochemical changes in MPI at H_2O_2 concentrations below 1.0 mM resulted from oxidative attack by hydroxyl radicals generated via Fenton reaction, but at higher H_2O_2 concentrations, other radicals and nonradical species such as aldehydes generated via lipid oxidation were also responsible for the observed protein changes. Further research is required to investigate the effect of excess H_2O_2 in the protein oxidizing system, and to identify the specific radicals involved in protein and lipid oxidation.

5. Conclusion

This study demonstrated that biochemical changes in MPI exposed to iron/ascorbate/hydrogen peroxide oxidizing systems were dependent upon the chemical constituents that comprised the oxidizing system, and were generally more pronounced in a high-$FeCl_3$ (0.1 mM) than in a low-$FeCl_3$ (0.01 mM) system. The greater susceptibility of the biochemical properties that mainly involved amino acid side chain groups (myosin ATPase, carbonyls) than those that pertained to protein structure and cross-linking to the presence of H_2O_2 indicated a sequential order of the specific protein oxidative processes in the hydroxyl radical-generating systems. Additional research is needed to investigate the specific interaction of the various prooxidant initiators in the mixed oxidizing solution.

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