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Protein Oxidation Enhances Hydration but Suppresses Water-Holding Capacity in Porcine Longissimus Muscle

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Pork longissimus muscle was oxidized at 4 °C by mixed 10 μM $FeCl_3$/100 μM ascorbate with 1, 5, 10, 20, 30, 40, or 50 mM H_2O_2 (pH 6.2). Oxidation with >1 mM H_2O_2 for 40 min significantly ($P < 0.05$) enhanced hydration of muscle samples, whereas oxidation with 40 and 50 mM H_2O_2 for 2 min or with 20 mM H_2O_2 for 40 min caused pronounced declines in water-holding capacity and product yield. The changes coincided with marked increases in the protein carbonyl content, TBARS formation, and cross-linking of both myofibrillar and sarcoplasmic proteins. Dye-tracing tests showed that the enhanced hydration at >1 mM H_2O_2 was due to facilitated water diffusion into muscle tissue. This result was strongly corroborated by microscopic images that illustrated enlargements of intercellular spacing, that is, gaps, in oxidized muscle tissue, which served as canals for water diffusion.

KEYWORDS: Protein oxidation; hydration; water-holding capacity; myofiber shrinkage

INTRODUCTION

Water accounts for approximately 75% of the weight of lean muscle tissue and imparts juiciness to cooked meat and meat products. The majority of the water present in fresh meat, whether endogenous or extraneous, is free or physically entrapped as bulk water rather than chemically bound. Bulk water is held within muscle fibers and between muscle fibers or muscle bundles. Using low-field NMR, Bertram et al. (*1*) demonstrated that water in muscle is mostly located in the inter- and intramyofilamental spaces via capillarity and that a small percentage is tightly associated with proteins through hydrogen bonds.

In marinated fresh meat, extraneous water is distributed in different locations inside muscle. The ability of muscle to hold this water as well as endogenous water during meat handling, storage, and cooking is influenced by pH, ionic strength, and phosphate (*2*−*4*). Marination with salt (NaCl or KCl) and phosphates (e.g., sodium pyrophosphate and tripolyphosphate) improves cooking yield of meat when compared with unmarinated meat (*5*, *6*). The salt and phosphate effect has been ascribed to their ability to depolymerize thick myofilament and weaken the actomyosin complex, predisposing myofibrils to swelling by which exogenous water is entrapped(*7*−*9*).

Recent studies have indicated that protein oxidation, which can readily occur in post-mortem muscle during processing and storage, is an important factor affecting the water-holding capacity of raw meat. Myofibrillar proteins are very susceptible to chemical modifications, such as amino acid destruction, peptide scission, protein cross-linking, and formation of protein−lipid complexes by common oxidants derived from inactive compounds or generated during processing (*10*, *11*). In whole-muscle tissue, oxidants such as hydrogen peroxide have been shown to reduce post-mortem protein fragmentation (proteolysis) and promote aggregation of myofibrillar proteins (*12*, *13*). These processes have been implicated in the observed increases in cooking loss of oxidized meat. Reduced water binding in pork packaged under oxygen-rich atmosphere correlates with protein oxidation (*14*). The relationship between the oxidative status of raw muscle and its water-holding capacity is further evidenced through animal feeding studies. It was reported that dietary vitamin E decreased lipid oxidation and reduced drip loss in post-mortem pork (*15*) and lamb (*16*).

Because the myofibril architecture is the primary apparatus for water immobilization in meat (*17*), isolated individual myofibrils offer a useful tool for the investigation of the mechanism of oxidation-induced changes in water binding and hydration in raw meat. In a recent study, we noted significant losses in the hydration capacity of myofibrils when they were exposed to hydroxyl radicals that caused myosin oxidation (*18*). Increased myosin cross-linking via disulfide bonds was identified as the main cause for reduced transverse expansion of myofibrils during salt irrigation. During whole-muscle marination, water must overcome physical barriers, for example, intracellular spaces and cell membrane, to arrive at the site of myofibrils through diffusion. It is not clear how the process of water diffusion, binding, and retention would be altered in oxidatively stressed muscle when compared with nonoxidized muscle samples.

The present study was conducted to answer two questions: (1) Does oxidation change the pattern of water diffusion into muscle tissue during marination? (2) If it does, will the oxidation affect water binding and, thus, its retention? A further objective was to investigate the structural and molecular mechanisms in an attempt to explain the oxidation-induced changes in the hydration properties of raw porcine meat.

MATERIALS AND METHODS

Muscle Sample Preparation. Loins (Longissimus lumborum) were excised from 24 h post-mortem pork carcasses processed at the University

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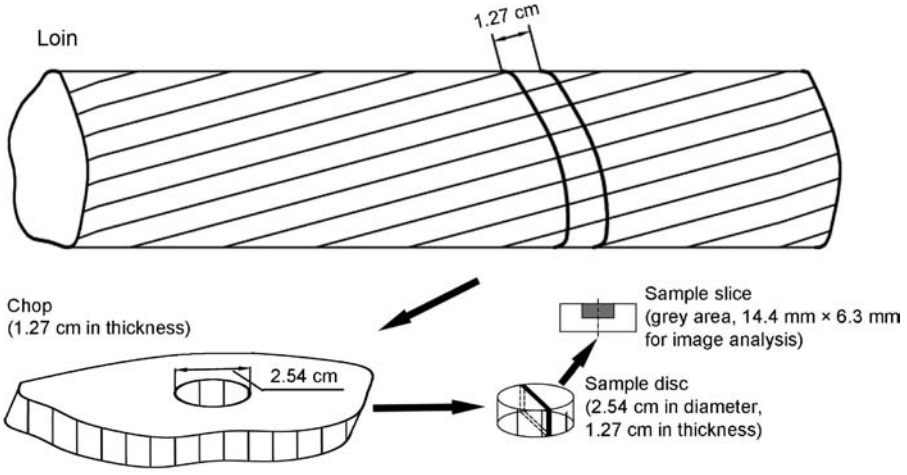

Figure 1. Schematic illustration of sample preparation.

of Kentucky Meat Laboratory, a USDA-approved facility, individually packaged with a vacuum seal, and stored in a −30 °C freezer. On the day of use, one frozen loin was tempered at 4 °C for 4 h, and slices (chops) approximately 1.27 cm thick were cut perpendicular to the fiber direction from the loins. A sample "round disk" (2.54 cm in diameter, 1.27 cm in thickness) was obtained with a sharp-edged metal corer (2.54 cm diameter) by carefully drilling parallel to the fiber direction through the pork chop as illustrated in **Figure 1**. Five such disks were obtained from each pork chop and used for muscle oxidation experiments.

Oxidation of Muscle. All freshly prepared muscle sample disks were equilibrated at 4 °C for 2 h to allow complete thaw and then gently blotted with a paper towel to remove any surface moisture. Muscle disks were immersed and incubated in cold (4 °C) hydroxyl radical-generating solutions (HRGS) with two levels of hydrogen peroxide in the buffer containing 15 mM piperazine−*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) and 0.1 M NaCl at pH 6.2. They were (a) low H_2O_2 with long-time incubations in 10 μM $FeCl_3$, 100 μM ascorbic acid, and four low concentrations of H_2O_2 (1, 5, 10, or 20 mM) for 40 min and (b) high H_2O_2 with short-time incubations in 10 μM $FeCl_3$, 100 μM ascorbic acid, and four higher concentrations of H_2O_2 (20, 30, 40, or 50 mM) for 2 min. The muscle/solution ratio was 1:4.5 (w/v), and the mixtures were gently agitated during oxidation. The control muscle samples were incubated in 1 mM propyl gallate instead of H_2O_2, and ascorbic acid and $FeCl_3$ were not added. After oxidation, which was terminated by adding 1 mM EDTA, muscle disks were gently blotted and immediately subjected to the analyses for protein oxidation, moisture pickup, water-holding capacity, and product yield. To determine the influence of oxidation on water penetration and muscle structural changes, a separate oxidation experiment the same as described above was run except that a dye was added to the oxidation solutions and different sampling times were used to monitor the migration of the incubation solution (see below).

Diffusion of Oxidizing Solutions and Image Acquisition. To the oxidation solutions described above, 1% (w/v, final concentration) FD&C Blue No. 1 was added. The time-dependent dye penetration, which indicated the diffusion of the oxidizing solutions, was investigated at different oxidation incubation times (2, 10, 20, 40, and 80 min) using the procedure described previously (*6*). A thin slice (2 mm thick) was cut off with a scalpel along the central axis (parallel to the fiber direction) of the muscle disk (**Figure 1**). The RGB image of the flat surface of the slice was digitally captured (678 × 282 pixels; 600 dpi) with a Scanjet 4200C scanner (Hewlett-Packard, Palo Alto, CA). A rectangular region (a 14.4 mm × 6.3 mm slab) was cropped to determine the characteristics of dye distribution. The images of the entire slab as well as those of dye-stained area were analyzed using ImageJ software (ver 1.41o, NIH, Bethesda, MD). The images were converted to grayscale (RGB stack) followed by a binary approach with an automatic threshold on the R layer to calculate the percentage of dye-covered area by pixels (*19*). The percent area of dye coverage was designated dye penetration.

Oxidation-Induced Chemical Changes. Chemical changes of proteins and lipids in oxidatively stressed muscle samples were examined by using the following analyses.

SDS−PAGE. Control (nonoxidized) and oxidatively stressed muscle samples were homogenized in nanopure water (1:100, w/v), and 1 mL of the homogenate was then mixed with an equal volume of a pH 6.8 buffer containing 4% SDS, 0.125 M Tris-HCl, and 20% glycerol, with or without 10% β-mercaptoethanol (βME), to obtain an approximately 1 mg/mL protein concentration. The mixture was boiled for 3 min to completely dissolve protein before being subjected to SDS−PAGE with a 3% acrylamide stacking gel and a 10% acrylamide resolving gel in a Mini-PROTEAN 3 Cell electrophoresis system (Bio-Rad Laboratories, Hercules, CA). Protein profiles were obtained with a Scanjet 4200C Hewlett-Packard scanner, and the intensity (pixels) of selected protein bands, which gave quantitative changes, was measured using ImageJ software (ver 1.41o, NIH, Bethesda, MD) in the digitizing mode.

Protein Carbonyls. The carbonyl contents of control and oxidized muscle samples were determined using the procedure of Levine et al. (*20*). Muscle samples were homogenized and diluted in 9 volumes (w/v) of nanopure water. Aliquots of 100 μL of the homogenates were transferred into microcentrifuge tubes and reacted with 500 μL of 2,4 dinitrophenylhydrazine (DNPH) in 2 N HCl at room temperature for 40 min. The absorbance of the derivative at 370 and 280 nm was recorded. Protein carbonyl contents were expressed as nanomoles per milligram of protein.

Lipid Oxidation Products. The extent of lipid oxidation in muscle samples was assessed by measuring the content of 2-thiobarbituric acid-reactive substances (TBARS) as described by Vyncke (*21*) with slight modifications. Aliquots of 10 mL of homogenized muscle in 9 volumes of nanopure water were precipitated by 7.5% trichloroacetic acid solution containing 0.1% propyl gallate and 0.1% EDTA (final concentration) and filtered with Whatman no. 1 filter paper. The TBA reaction was carried out by mixing 2.5 mL of the filtrates with an equal volume of 0.01 M TBA in test tubes with screw caps and heating in boiling water for 40 min. Absorbance of cooled samples at 530 nm was read against protein-free blank, and the amount of TBARS was calculated from a standard curve generated with malondialdehyde (MDA). Results were expressed as milligrams of MDA per kilogram of muscle.

Oxidation-Induced Muscle Structural Changes. Alterations in muscle morphology due to oxidation were observed under a scanning electron microscope. Small samples of 1 mm thickness, cut perpendicular to the fiber direction to expose the cross section (3 × 3 mm), were obtained 1 mm below the surface of original muscle samples with a razor blade. Specimens were fixed in 0.09 M cacodylate buffer containing 2.5% glutaraldehyde (pH 7.2) for 1 h and then dehydrated in a series of increasing concentrations of ethanol: once at 10, 40, and 70% for 5 min each and twice at 100% for 5 min, successively. Dehydrated specimens were placed in hexamethyldisilazane (HMDS) for 5 min and then air-dried at room temperature. In the last step, dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter Coater SPI-Module). The specimens were then examined with a model S-3400N Hitachi scanning electron microscope (Tokyo, Japan).

Oxidation-Induced Myofibril Structural Changes. Because myofibrils are largely responsible for water-holding capacity in fresh meat,

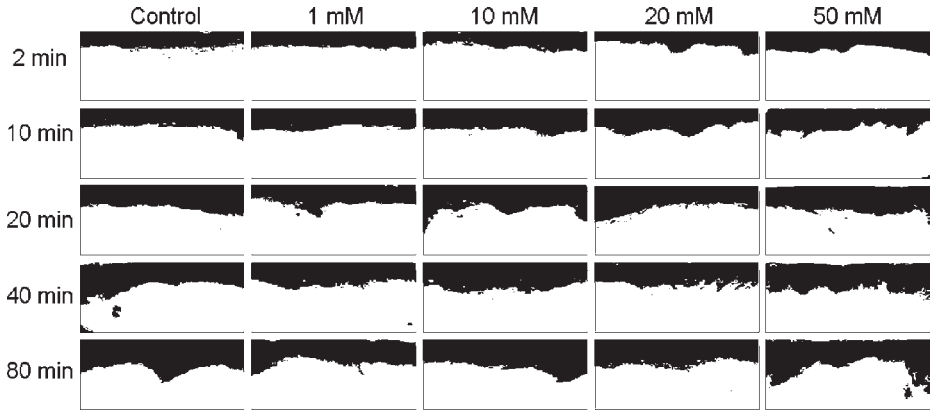

Figure 2. Binarized images showing the temporal changes of diffused dye into different oxidatively stressed muscles. Oxidation was conducted with 10 μM $FeCl_3$, 100 μM ascorbic acid, and various H_2O_2 concentrations (mM) in 0.1 M NaCl−15 mM PIPES buffer (pH 6.2) for different incubation times.

an additional experiment was performed to establish their structural response to oxidation. This was done by irrigation of isolated myofibrils with oxidizing solutions, and the resulting ultrastructural changes in myofilaments were observed by phase contrast microscopy. Specifically, myofibrils were isolated from nonoxidized fresh loin muscle with a rigor buffer containing 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 10 mM K_2HPO_4 (pH 7.0) as described by Xiong et al. (*22*). The pH of the final washing solution was adjusted to 6.0. Isolated myofibrils were irrigated with solutions consisting of graded H_2O_2 (0, 1, 5, 10, 20, 30, 40, or 50 mM) with 10 μM $FeCl_3$, 100 μM ascorbic acid, and 0.1 M NaCl in 15 mM PIPES buffer (pH 6.2) for 2 min using the procedure of Liu et al. (*18*). Morphological changes in the myofibril architechture were observed and photographed using a model MICROPHOT-FXA Nikon photomicroscope via a 100× oil immersion objective with a digital camera.

Water-Holding Capacity (WHC) of Muscle. Control and oxidized muscle samples (~5 g) were centrifuged (2000*g*) at 4 °C for 20 min and then dried in a 105 °C oven (*23*) to evaluate WHC (*24*). WHC was defined as the percentage of meat juice released, which is expressed by the equation

$$\text{WHC } (\%) = \left(1 - \frac{W_0 - W_1}{W_0 - W_2}\right) \times 100\%$$

where W_0, W_1, and W_2 represent, respectively, weight of original meat (g), weight of centrifuged meat (g), and weight of dried (105 °C) meat (g).

Hydration and Moisture Retention Properties of Muscle. To investigate the impact of oxidation on the moisture absorption and retention properties, oxidized raw meat samples were subjected to salt−pyrophosphate marination. Moisture pickup and product yield were subsequently determined.

Hydration of Muscle Samples. Muscle disks of known weight (W_0) obtained from control and oxidized samples were placed in a 4 °C brine solution containing 0.6 M sodium chloride and 15 mM sodium pyrophosphate (pH 6.2) with gentle agitation for exactly 5 min. The marinated samples were drained for 5 min by suspension on a stainless steel rack, and the drained weight (W_3) was recorded. Hydration capacity (%) is defined using the following equation:

$$\text{hydration capacity } (\%) = \frac{W_3 - W_0}{W_0} \times 100$$

Cooking Loss and Product Yield. The drained marinated meat samples were immediately cooked on a George Foreman Grill (Salton Inc., Mt. Prospect, IL) preheated to 170 °C. Meat was cooked to an internal temperature of 71 °C (monitored with a thermocouple), and the cooked weight was recorded (W_4). Cooking loss and product yield were calculated as

$$\text{cooking loss } (\%) = \frac{W_3 - W_4}{W_3} \times 100$$

$$\text{product yield } (\%) = \frac{W_4}{W_0} \times 100$$

Statistical Analysis. Three independent trials ($n = 3$), unless specified otherwise, with triplicate or quadruplicate sample analyses were performed.

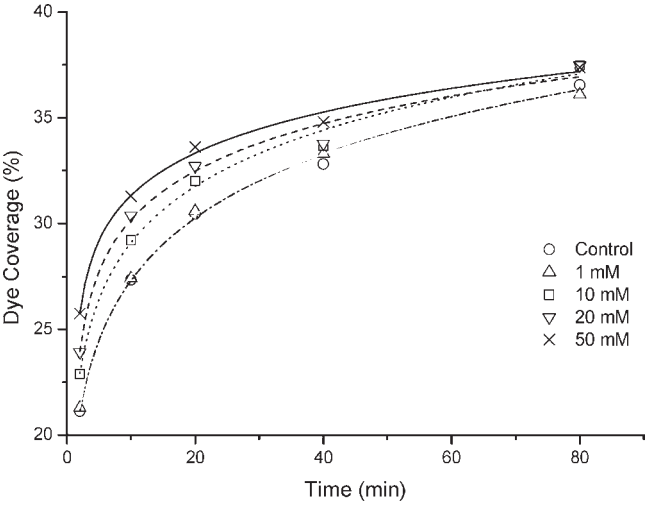

Figure 3. Influence of oxidation on dye penetration in pork muscle tissue as a function of oxidation time expressed as the percentage of area covered by dye ($n = 3$). Oxidation conditions: 10 μM $FeCl_3$, 100 μM ascorbic acid, and various H_2O_2 concentrations (mM, indicated in the graph) in 0.1 M NaCl−15 mM PIPES buffer, pH 6.2.

Data were analyzed using the general linear model procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL). Analysis of variance (ANOVA) was conducted to determine the significance of main effect (e.g., oxidation). Significant differences ($P < 0.05$) between individual means were identified by the LSD all-pairwise multiple comparisons.

RESULTS

Diffusion of Oxidizing Solutions in Muscle. On the longitudinal section, the relative area of dye coverage was used to estimate the influence of oxidation on oxidant transfer in muscle. The binarized images of a set of typical stained samples corresponding to different oxidation times in the HRGS that generated $^{\bullet}OH$ at the specific H_2O_2 concentrations are presented in **Figure 2** to illustrate the dynamic process of the diffusion of the oxidant solutions. The control (nonoxidized) muscle had a less stained area than samples treated with H_2O_2, notably at high concentrations (≥10 mM), indicating a lower degree of solution migration into the muscle tissue. For all muscle samples, regardless of oxidation, the penetration of the incubation solution (0.1 M NaCl) into the muscle tissue increased with time, apparently driven by the osmotic pressure gradient because the ionic strength of intact muscle tissue is expected to be about 0.16. The calculated percentage of the stained area for each sample is depicted in **Figure 3**. All curves

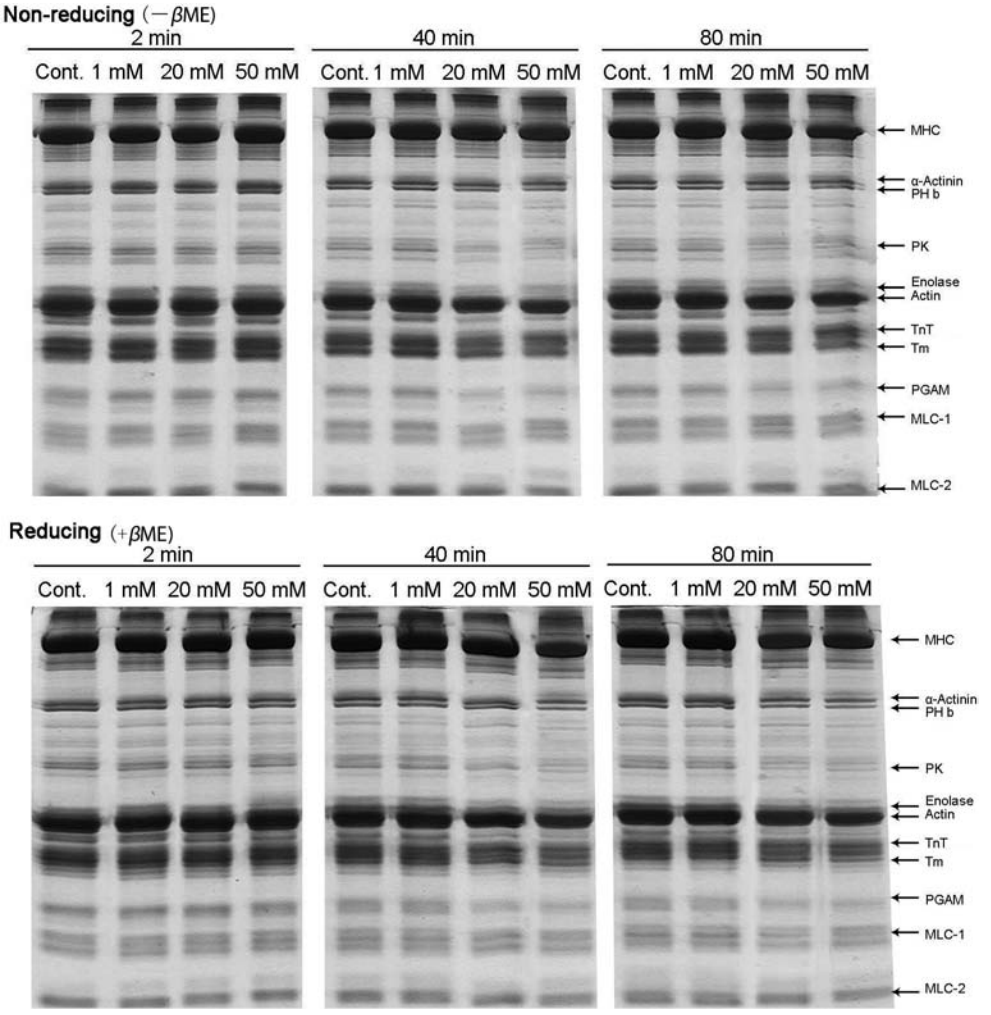

Figure 4. SDS−PAGE patterns of nonoxidized (control) and oxidized whole muscle tissue homogenates in the absence (nonreducing, upper panel) or presence (reducing, lower panel) of 5% β-mercaptoethanol (βME). The oxidation was conducted with 10 μM $FeCl_3$, 100 μM ascorbic acid, and various H_2O_2 concentrations (mM, indicated in the graph) in 0.1 M NaCl−15 mM PIPES buffer (pH 6.2) for different incubation times. MHC, myosin heavy chain; PH b, phosphorylase *b*; PK, pyruvate kinase; TnT, troponin T; Tm, tropomyosin; PGAM, phosphoglycerate mutase; MLC-1, myosin light chain-1; MLC-2, myosin light chain-2.

were fitted (r^2 = 0.97−1.00) with the three-parameter logarithm regression equation, yielding an excellent dye penetration versus incubation time relationship. Pronounced differences between control and oxidized samples were observed at the initial stage of oxidation (2−20 min); such differences tended to diminish at longer oxidation times and became unnoticeable by 80 min. The 1 mM H_2O_2 treatment and the control had a similar solution penetration.

Oxidation-Induced Chemical Changes in Muscle. To identify how the degree of oxidative stress induced by HRGS would affect muscle proteins, homogenized muscle samples were subjected to SDS−PAGE (**Figure 4**). Comparison of the intensity of protein bands in nonreducing (−βME) and reducing (+βME) gels indicates that a portion MHC and actin was lost following the treatments with 50 mM H_2O_2 for 40 and 80 min. Digitization of main protein bands in nonreducing gels revealed approximately 21 and 30% losses in MHC and 33 and 35% losses in actin after muscle samples were oxidized at 50 mM H_2O_2 for 40 and 80 min, respectively. Most of the lost proteins were only partially recovered by βME, suggesting covalent bonds other than disulfide linkages were also involved. A similar, albeit less conspicuous, oxidation-induced protein loss was found by the treatment with 20 mM H_2O_2 for 80 min. The SDS−PAGE without βME also revealed a substantial attenuation in the band intensity of the 97, 57, 47, and 27 kDa polypeptides that presumably were phosphorylase *b*, pyruvate kinase, enolase, and phosphoglycerate mutase, respectively, on the basis their relative mobility against published literature (*25*). To avoid severe oxidation, the "intermediate" oxidation conditions established above, that is, 2 and 40 min oxidation times for high (20−50 mM) and low (1−20 mM) H_2O_2 concentration treatments, respectively, were selected for subsequent chemical analyses and muscle hydration studies.

The DNPH−protein adduct assay on oxidized and nonoxidized (control) muscle samples yielded quantitative information about the production of protein carbonyls due to reaction with H_2O_2-generated $^{\bullet}OH$. As displayed in **Figure 5A**, the generation of protein carbonyls was greatly influenced by the high-oxidant (20−50 mM)−short-time (2 min) treatments with the amount formed increasing with the H_2O_2 dosage ($P < 0.05$). The formation of protein carbonyls for long oxidation time (40 min) at lower H_2O_2 concentrations (1−20 mM) was less but was still moderately promoted by increasing the H_2O_2 concentration. Comparison of the samples treated with 20 mM H_2O_2 for 2 and 40 min (noted by arrows) indicated a nearly 1-fold increase in the carbonyl content by the longer oxidation time.

The H_2O_2-based HRGS oxidation systems also caused lipid oxidation in the muscle tissue. This is manifested by the substantial

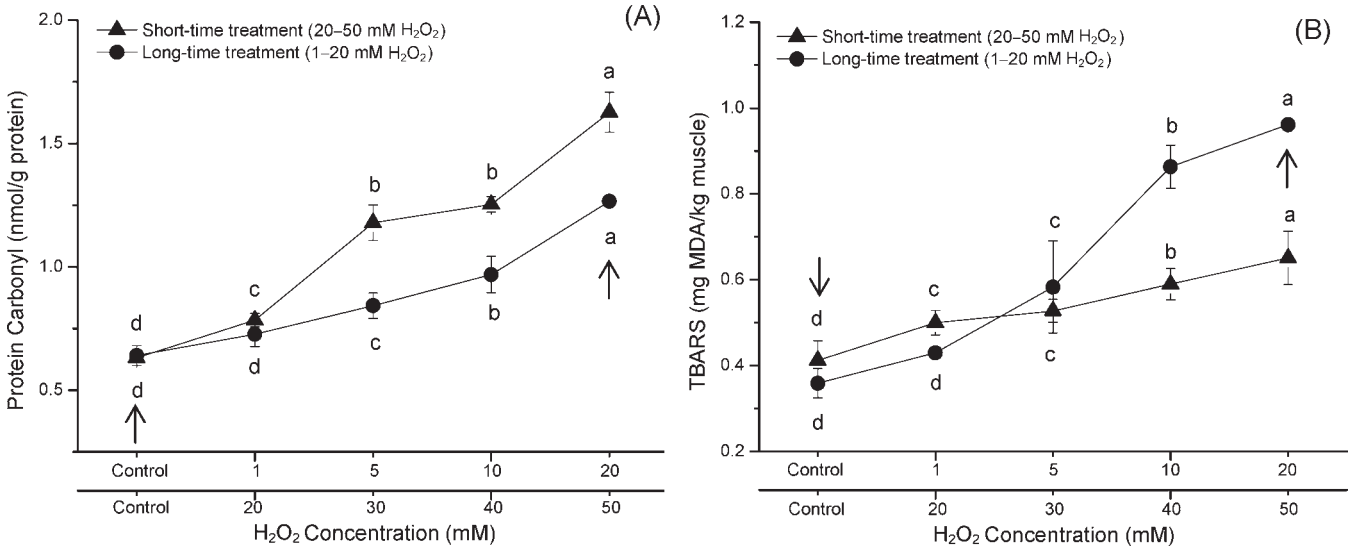

Figure 5. Protein carbonyl (**A**) and TBARS (**B**) contents in nonoxidized (control) and oxidized pork loin muscle samples. Oxidation was conducted by short-time (2 min) incubation in solutions containing 10 μM $FeCl_3$ and 100 μM ascorbic acid in 0.1 M NaCl−15 mM PIPES buffer (pH 6.2) with 20, 30, 40, or 50 mM H_2O_2 or by long-time (40 min) incubation in the same solutions but with 1, 5, 10, or 20 mM H_2O_2. Means from three replications (n = 3) within the same treatment with different letters differ significantly ($P < 0.05$).

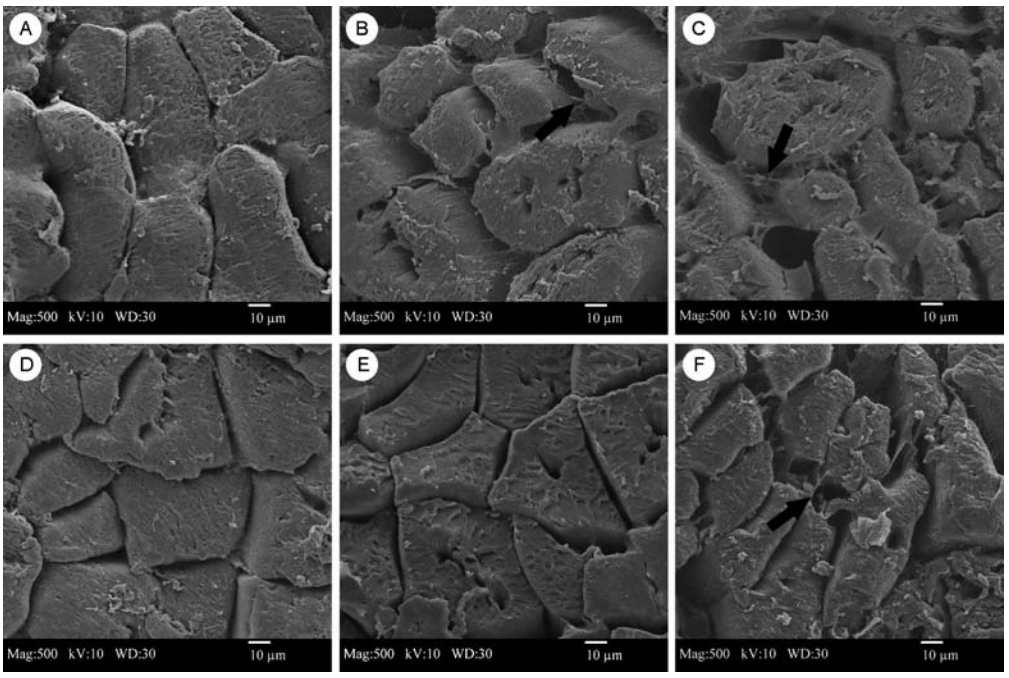

Figure 6. Scanning electron micrographs of fiber cross sections of nonoxidized (**A**, **D**) and oxidized pork muscle samples. Oxidation was conducted by short-time (2 min, upper panel) incubation in solutions containing 10 μM $FeCl_3$ and 100 μM ascorbic acid in 0.1 M NaCl−15 mM PIPES buffer (pH 6.2) with (**B**) 20 mM H_2O_2 or (**C**) 50 mM H_2O_2 or by long-time (40 min, lower panel) incubation in the same solutions but with (**E**) 1 mM H_2O_2 or (**F**) 20 mM H_2O_2.

increase ($P < 0.05$) in the MDA content (TBARS) in muscle samples treated with H_2O_2 at concentrations greater than 5 mM in long-time (40 min) or with 20 mM in short-time (2 min) when compared with the nonoxidized sample (**Figure 5B**). Similar to the oxidation time effect on protein carbonyl production, the sample treated with 20 mM H_2O_2 for long time (40 min) showed twice as much TBARS as that for short time (2 min) (see arrows).

Oxidation-Induced Ultrastructural Changes in Muscle. The general appearances of SEM micrographs of control and HRGS-oxidized muscle samples are shown in **Figure 6**. The cross section of the fibers in nonoxidized muscle samples (**A**, **D**) had a regular pattern with limited intercellular spacing. Treatment with 20 mM H_2O_2 for 2 min (short time) (**B**) produced evident increases in the spacing between fibers. Raising the H_2O_2 concentration to 50 mM while maintaining the incubation time at 2 min (**C**) resulted in significant disruption of muscle fibers and an enlargement of some of the intercellular spaces. On the other hand, oxidation at 40 min (long time) with 1 mM H_2O_2 (**E**) did not have a remarkable effect on the muscle fiber structure when compared with the control (**D**). However, when the concentration of H_2O_2 was increased to 20 mM, the 40 min incubation time (**F**) created substantial gaps between muscle fibers as well as notable rupture of some fibers. The creation of extra intercellular spaces under all of the oxidation conditions tested was accompanied by the

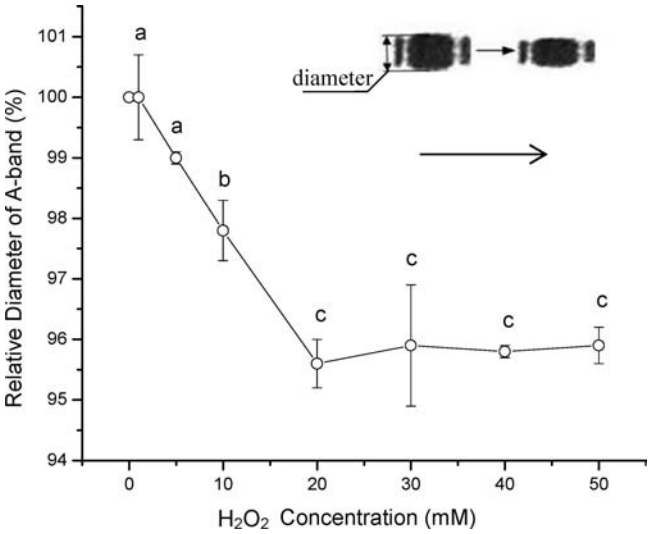

Figure 7. Relative diameter of A-band in myofibrils after sequential irrigation (2 min) with oxidizing solutions containing 10 μM $FeCl_3$ and 100 μM ascorbic acid with various concentrations of H_2O_2 in 0.1 M NaCl−15 mM PIPES buffer (pH 6.2). The arrow indicates the sequence of graded H_2O_2 solutions for the myofibril irrigation. (Inset) Myofibril shrinkage. Means from six replications (n = 6) with different letters differ significantly ($P < 0.05$).

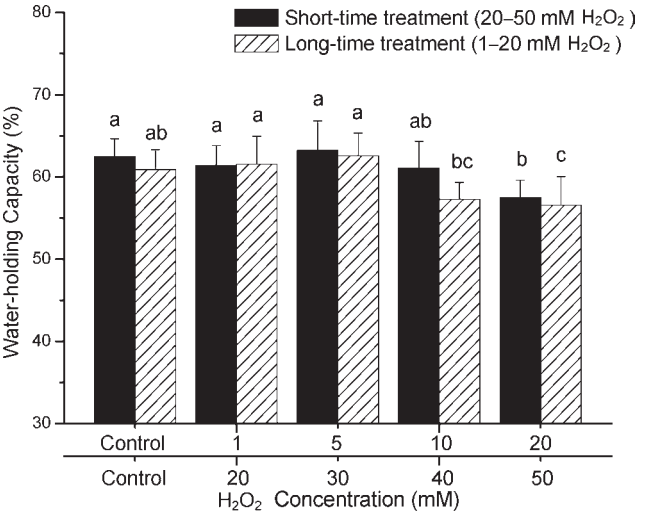

Figure 8. Water-holding capacity of nonoxidized (control) and oxidized pork muscle. Oxidation was conducted by short-time (2 min) incubation in solutions containing 10 μM $FeCl_3$ and 100 μM ascorbic acid with 20, 30, 40, and 50 mM H_2O_2 in 0.1 M NaCl−15 mM PIPES buffer (pH 6.2) or by long-time (40 min) incubation in the same solutions but with 1, 5, 10, and 20 mM H_2O_2. Means from three replications (n = 3) within the same treatment with different letters differ significantly ($P < 0.05$).

conspicuous detachment and stretching of some sheath or thread-like structures (see arrows), which could include cell membrane and the endomysium connective tissue.

Because an increased extracellular space caused by oxidation can result from myofibril shrinkage, myofibrils were isolated from nonoxidized muscle and subsequently treated with graded H_2O_2 oxidation solutions (0−50 mM). Microscopic examination with a phase contrast mode revealed substantial, linear reduction in A-band diameter until 20 mM H_2O_2 (**Figure 7**). No further myofibril shrinkage was seen at higher H_2O_2 concentrations. The length of the A-band as well as that of sarcomere did not show appreciable change by the oxidation treatments (data not shown).

Hydration and Moisture Retention Properties of Muscle. The oxidative effect on WHC is shown in **Figure 8**. Distinct weight losses were found by oxidation in HRGS with 50 mM H_2O_2 for 2 min and with 20 mM H_2O_2 for 40 min ($P < 0.05$), indicating reduced ability of muscle to retain endogenous water.

In meat processing, salt (NaCl) in combination with polyphosphates is commonly used to enhance the hydration of raw muscle and, hence, the juiciness of cooked products, through the ion−dipole interaction of myofibrillar proteins and water. **Figure 9** presents the hydration, cooking loss, and product yield of NaCl-marinated control and oxidatively stressed muscle. The weight gain after marination, which indicated the amount of water absorbed or the extent of hydration, was surprisingly improved when the muscle was oxidized. With a short oxidation time (2 min), the hydration capacity was insensitive to the concentration of H_2O_2 (20−50 mM) (**A**), but with a long oxidation time (40 min), oxidation with 5 mM H_2O_2 produced the greatest hydration capacity of all samples (1−20 mM H_2O_2) (**B**). Some of the weight gain was lost as the H_2O_2 concentration was increased to 20 mM. Comparison between the short-time and long-time oxidation samples showed that the former had an overall higher hydration capacity. This was likely due to the fact that the short-time (2 min) oxidized and control samples prior to the NaCl marination had not already absorbed as much extraneous moisture from the oxidizing solution as the long-time (40 min) oxidized and control samples (**Figures 2** and **3**).

The results from the cooking loss measurement showed a clear trend in moisture loss upon cooking from both short-time and long-time oxidized muscle samples, some of which can be attributed to the absorbed water during marination. However, the cooking loss was significantly greater ($P < 0.05$) in long-time oxidized (and control) samples than in short-time oxidized (and control) samples (38.0 vs 29.7%), probably due to osmotic diffusion of water as indicated previously (**Figures 2** and **3**). Consistent with the cooking loss data, there was a trend of progressive reduction in the overall product yield, more so for long-time oxidized samples, as the degree of oxidation increased in both oxidation systems.

DISCUSSION

When muscle tissue is exposed to $^{\bullet}$OH-oxidizing environments, which are commonplace in fresh and processed muscle foods (*26*), chemical modifications on the side-chain groups or protein peptide bonds are triggered (*11*). In our present study, the loss of myosin and actin after the muscle samples were exposed to HRGS, shown by SDS−PAGE, was not completely recoverable by βME. This suggested that non-disulfide cross-linkages occurred, which could include the formation of Schiff bases between carbonyl and amino groups (*27*). Carbonyl compounds produced from lipid oxidation, such as TBARS, can attach to proteins (*28*, *29*), thereby contributing to total protein-bound carbonyls detected in oxidized muscle. Prochniweicz et al. also reported unreducible oxidation in 50 mM H_2O_2-oxidized muscle fibers, but not in 5 mM H_2O_2-treated samples (*30*). Both reducible and unreducible protein cross-linking can influence water-binding properties of muscle. In addition, denatured sarcoplasmic proteins, which were shown to precipitate on myofibrils, negatively affected the water-holding capacity of myofibrils (*31*, *32*). The concomitant disappearance (insolubilization) of sarcoplasmic proteins and the main contractile proteins (myosin and actin) at high H_2O_2 concentrations supported the hypothesis that interactions between these proteins led to the change in muscle water-binding properties.

As a consequence of oxidation, a larger extracellular space was created between adjacent fibers due to fiber shrinkage. This space enlargement between fibers can be attributed to increased association

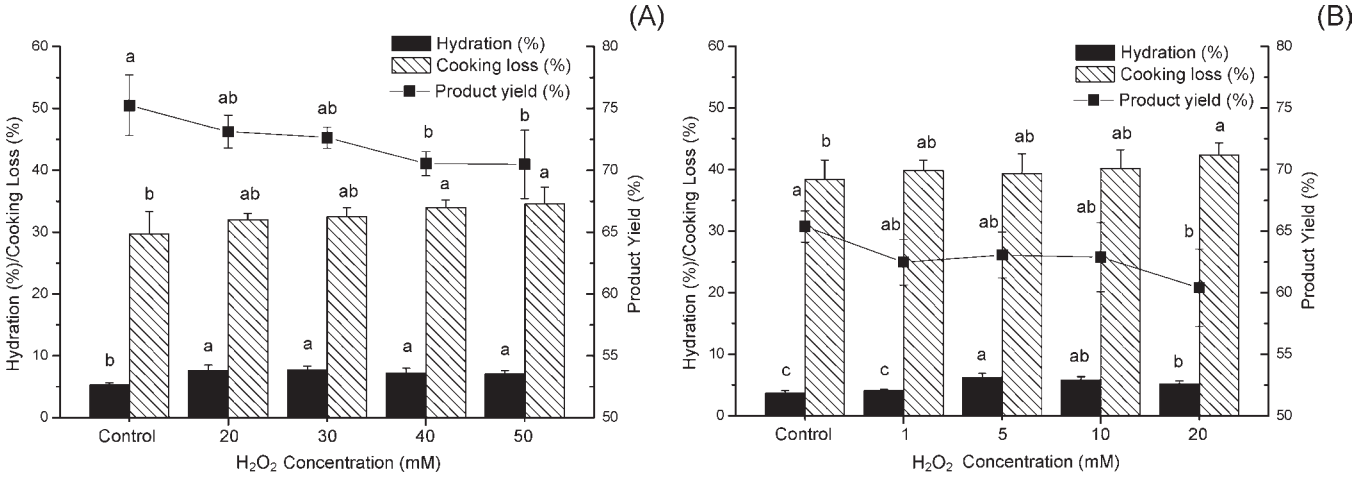

Figure 9. Hydration (%), cooking loss (%), and product yield (%) of nonoxidized (control) and oxidized pork muscle. Oxidation was conducted by short-time (2 min) incubation in solutions containing 10 μM $FeCl_3$ and 100 μM ascorbic acid in 0.1 M NaCl−15 mM PIPES buffer (pH 6.2) with 20, 30, 40, and 50 mM H_2O_2 (**A**) or by long-time (40 min) incubation in the same solutions but with 1, 5, 10, and 20 mM H_2O_2 (**B**). Means from three replications ($n = 3$) within the same quality parameter with different letters differ significantly ($P < 0.05$).

of myofilaments due to hydrophobic and covalent interactions and should be correlated with decreased water-holding capacity of fresh muscle. Cross-linkages through dityrosine (*33*), disulfide (*34*), and carbonyl−amine (*27*) bonds have been implicated in oxidized myosin, and both myosin head (heavy meromyosin) and tail (light meromyosin) have been shown to be susceptible (*27*, *35*). As presented above, the exposure of isolated myofibrils to $^{\bullet}OH$ resulted in the coagulation of thick (myosin) and thin (actin) filaments. This process apparently involved myosin intra- and intermolecular cross-linking (*36*) as well as cross-linking within and between myofilaments, leading to the observed shrinkage of interfilamental spaces. Cumulatively, the aggregation of the individual myofibrils and myofilaments resulted in increased gapping between muscle fibers, as seen in the present study. Similar morphological changes in oxidation-stressed muscle have been reported by other researchers (*37*, *38*).

The enhanced moisture absorption in marinated muscle tissue after oxidation was consistent with the augmented moisture diffusion and can be explained by the extra space created between muscle fibers. However, the diffusion of the exogenous liquid seemed to be a passive process, and the marinade retained was temporary. Given the significant size of the extracellular spaces created, the accumulated transient fluid was mostly loose and unbound and can be readily expelled by centrifugation or during cooking. These void spaces differ from the spaces in the myofibril matrices, where the majority of endogenous water is tightly withheld (*7*, *17*). The slight disruption of cell membrane in oxidatively stress muscle would also promote the exudation of endogenous water rather than the absorption of marinade due to osmotic pressure, further contributing to the intercellular gapping.

Overall, the degree of marination-induced fiber swelling, when compared with that of isolated myofibrils, in both control and oxidized muscle samples was limited. This can be ascribed to mechanical constraints by the endomysial collagenous sheath. Although marination will drive the diffusion of the salt solution into the interior of muscle cells due to osmotic gradients, the internal pressure buildup, similar to the turgor pressure in plant cells, will inhibit further migration of liquid into the cell. Nevertheless, upon marination with NaCl and phosphates, muscle will still exhibit moderate swelling (*39*, *40*) and a simultaneous reduction in the extracellular space (*41*, *42*).

Although oxidation favored hydration in brined muscle, it had a negative effect on the cooked product, as much of the absorbed endogenous water was lost after the cooked meat was cooled to room temperature. The salt and phosphate marinade migrating into the pockets outside the cells would extract myofibrillar proteins and form a water-binding gel upon cooking. However, the gelling process was likely impaired by oxidation due to excessive protein aggregation (*35*). Moreover, both cross-linking (*43*, *44*) and fragmentation (*37*, *45*, *46*) have been observed in oxidized collagen. However, there was no substantial disintegration of endomysial network in our present study. This does not preclude the strong likelihood that oxidation decreased collagen solubility; the damage to collagen fibrils would further aggregate cooking loss in oxidatively stressed muscle.

In conclusion, exposures of fresh porcine muscle tissue to $^{\bullet}OH$-oxidizing systems resulted in oxidant-dose-dependent changes in muscle proteins. In a strong oxidizing environment, oxidation promoted hydration (moisture pickup) in salt-marinated muscle but weakened its water-holding capacity and water-binding potential upon cooking. Evidence from microstructural examination pointed the oxidation-induced changes to the expansion of extracellular spaces that facilitated marinade diffusion into the muscle tissue but reduced the capability of myofibrils to withhold absorbed water. The results revealed an altered mechanism of muscle cell−moisture interaction by oxidation and indicated the necessity to incorporate proper ingredients in the processing of oxidatively stressed pork so as to ensure that absorbed moisture from marination is effectively retained.

LITERATURE CITED

(1) Bertram, H. C.; Karlsson, A. H.; Rasmussen, M.; Pedersen, O. D.; Donstrup, S.; Andersen, H. J. Origin of multiexponential T2 relaxation in muscle myowater. *J. Agric. Food Chem.* **2001**, *49*, 3092−3100.

(2) Bendall, J. R.; Swatland, H. J. A review of the relationships of pH with physical aspects of pork quality. *Meat Sci.* **1988**, *24*, 85−126.

(3) Knight, P.; Parsons, N. Action of NaCl and polyphosphates in meat processing: responses of myofibrils to concentrated salt solutions. *Meat Sci.* **1988**, *24*, 275−300.

(4) Sheard, P. R.; Nute, G. R.; Richardson, R. I.; Wood, J. D. Effects of breed and marination on the sensory attributes of pork from Large White and Hampshire-sired pigs. *Meat Sci.* **2005**, *70*, 699−707.

(5) Babji, A. S.; Froning, G. W.; Ngoka, D. A. The effect of short-term tumbling and salting on the quality of turkey breast muscle. *Poult. Sci.* **1982**, *61*, 300−303.

(6) Xiong, Y. L.; Kupski, D. R. Time-dependent marinade absorption and retention, cooking yield, and palatability of chicken filets

marinated in various phosphate solutions. *Poult. Sci.* **1999**, *78*, 1053−1059.
(7) Offer, G.; Trinick, J. On the mechanism of water holding in meat: the swelling and shrinking of myofibrils. *Meat Sci.* **1983**, *8*, 245−281.
(8) Parsons, N.; Knight, P. Origin of variable extraction of myosin from myofibrils treated with salt and pyrophosphate. *J. Sci. Food Agric.* **1990**, *51*, 71−90.
(9) Shen, Q. W.; Swartz, D. R. Influence of salt and pyrophosphate on bovine fast and slow myosin S1 dissociation from actin. *Meat Sci.* **2010**, *84*, 364−370.
(10) Harel, S.; Kanner, J. Hydrogen peroxide generation in ground muscle tissues. *J. Agric. Food Chem.* **2002**, *33*, 1186−1188.
(11) Xiong, Y. L. Protein oxidation and implications for muscle food quality. In *Antioxidants in Muscle Foods*; Decker, E., Faustman, C., Lopez-Bote, C. J., Eds.; Wiley: Chichester, U.K., 2000; pp 85−111.
(12) Huff-Lonergan, E.; Lonergan, S. M. Mechanisms of water-holding capacity of meat: the role of postmortem biochemical and structural changes. *Meat Sci.* **2005**, *71*, 194−204.
(13) Rowe, L. J.; Maddock, K. R.; Lonergan, S. M.; Huff-Lonergan, E. Influence of early postmortem protein oxidation on beef quality. *J. Anim. Sci.* **2004**, *82*, 785−793.
(14) Lund, M. N.; Lametsch, R.; Hviid, M. S.; Jensen, O. N.; Skibsted, L. H. High-oxygen packaging atmosphere influences protein oxidation and tenderness of porcine longissimus dorsi during chill storage. *Meat Sci.* **2007**, *77*, 295−303.
(15) Guo, Q.; Richert, B. T.; Burgess, J. R.; Webel, D. M.; Orr, D. E.; Blair, M.; Grant, A. L.; Gerrard, D. E. Effect of dietary vitamin E supplementation and feeding period on pork quality. *J. Anim. Sci.* **2006**, *84*, 3071−3078.
(16) Macit, M.; Aksakal, V.; Emsen, E.; Esenbuga, N.; Irfan Aksu, M. Effects of vitamin E supplementation on fattening performance, non-carcass components and retail cut percentages, and meat quality traits of Awassi lambs. *Meat Sci.* **2003**, *64*, 1−6.
(17) Hamm, R. Functional properties of the myofibrillar system and their measurements. In *Muscle as Food*; Bechtel, P. J., Ed.; Academic Press: San Diego, CA, 1986; pp 135−199.
(18) Liu, Z.; Xiong, Y. L.; Chen, J. Identification of restricting factors that inhibit swelling of oxidized myofibrils during brine irrigation. *J. Agric. Food Chem.* **2009**, *57*, 10999−11007.
(19) Papadopulos, F.; Spinelli, M.; Valente, S.; Foroni, L.; Orrico, C.; Alviano, F.; Pasquinelli, G. Common tasks in microscopic and ultrastructural image analysis using ImageJ. *Ultrastruct. Pathol.* **2007**, *31*, 401−407.
(20) Levine, R. L.; Williams, J. A.; Stadtman, E. R.; Shacter, E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* **1994**, *233*, 346−357.
(21) Vyncke, W. Evaluation of the direct thiobarbituric acid extraction method for determining oxidative rancidity in mackerel (*Scomber scombrus* L.). *Fette, Seifen, Anstrichm.* **1975**, *77*, 239−240.
(22) Xiong, Y. L.; Lou, X.; Harmon, R. J.; Wang, C.; Moody, W. G. Salt- and pyrophosphate-induced structural changes in myofibrils from chicken red and white muscles. *J. Sci. Food Agric.* **2000**, *80*, 1176−1182.
(23) AOAC. *Official Methods of Analysis*;Association of Official Analytical Chemists: Washington, DC, 1984.
(24) Gerelt, B.; Ikeuchi, Y.; Nishiumi, T.; Suzuki, A. Meat tenderization by calcium chloride after osmotic dehydration. *Meat Sci.* **2002**, *60*, 237−244.
(25) McCormick, R. J.; Reeck, G. R.; Kropf, D. H. Separation and identification of porcine sarcoplasmic proteins by reversed-phase high-performance liquid chromatography and polyacrylamide gel electrophoresis. *J. Agric. Food Chem.* **1988**, *36*, 1193−1196.
(26) Kanner, J. Oxidative processes in meat and meat products: quality implications. *Meat Sci.* **1994**, *36*, 169−189.
(27) Xiong, Y. L.; Park, D.; Ooizumi, T. Variation in the cross-linking pattern of porcine myofibrillar protein exposed to three oxidative environments. *J. Agric. Food Chem.* **2009**, *57*, 153−159.
(28) Li, S. J.; King, A. J. Lipid oxidation and myosin denaturation in dark chicken meat. *J. Agric. Food Chem.* **1996**, *44*, 3080−3084.
(29) Love, J.; Pearson, A. Lipid oxidation in meat and meat products − a review. *J. Am. Oil Chem. Soc.* **1971**, *48*, 547−549.
(30) Prochniewicz, E.; Lowe, D. A.; Spakowicz, D. J.; Higgins, L.; O'Conor, K.; Thompson, L. V.; Ferrington, D. A.; Thomas, D. D. Functional, structural, and chemical changes in myosin associated with hydrogen peroxide treatment of skeletal muscle fibers. *Am. J. Physiol. Cell Physiol.* **2008**, *294*, C613−626.
(31) Fernandez, X.; Forslid, A.; Tornberg, E. The effect of high post-mortem temperature on the development of pale, soft and exudative pork: interaction with ultimate pH. *Meat Sci.* **1994**, *37*, 133−147.
(32) Wilson, G. G.; van Laack, R. L. J. M. Sarcoplasmic proteins influence water-holding capacity of pork myofibrils. *J. Sci. Food Agric.* **1999**, *79*, 1939−1942.
(33) Lund, M. N.; Luxford, C.; Skibsted, L. H.; Davies, M. J. Oxidation of myosin by haem proteins generates myosin radicals and protein cross-links. *Biochem. J.* **2008**, *410*, 565−574.
(34) Frederiksen, A. M.; Lund, M. N.; Andersen, M. L.; Skibsted, L. H. Oxidation of porcine myosin by hpervalent myoglobin: the role of thiol groups. *J. Agric. Food Chem.* **2008**, *56*, 3297−3304.
(35) Ooizumi, T.; Xiong, Y. L. Identification of cross-linking site(s) of myosin heavy chains in oxidatively stressed chicken myofibrils. *J. Food Sci.* **2006**, *71*, 196−199.
(36) 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.
(37) Astruc, T.; Marinova, P.; Labas, R.; Gatellier, P.; Sante-Lhoutellier, V. Detection and localization of oxidized proteins in muscle cells by fluorescence microscopy. *J. Agric. Food Chem.* **2007**, *55*, 9554−9558.
(38) Bertram, H. C.; Kristensen, M.; Ostdal, H.; Baron, C. P.; Young, J. F.; Andersen, H. J. Does oxidation affect the water functionality of myofibrillar proteins? *J. Agric. Food Chem.* **2007**, *55*, 2342−2348.
(39) Offer, G.; Knight, P. The structural basis of water-holding in meat. Part 1. General principles and water uptake in meat processing. In *Developments in Meat Science*; Lawrie, R. A., Ed.; Elsevier Applied Science: New York, 1988; pp 112−115.
(40) Wilding, P.; Hedges, N.; Lillford, P. J. Salt-induced swelling of meat: the effect of storage time, pH, ion-type and concentration. *Meat Sci.* **1986**, *18*, 55−75.
(41) Bocker, U.; Kohler, A.; Aursand, I. G.; Ofstad, R. Effects of brine salting with regard to raw material variation of atlantic salmon (*Salmo salar*) muscle investigated by fourier transform infrared microspectroscopy. *J. Agric. Food Chem.* **2008**, *56*, 5129−5137.
(42) Pinotti, A.; Graiver, N.; Califano, A.; Zaritzky, N. Diffusion of nitrite and nitrate salts in pork tissue in the presence of sodium chloride. *J. Food Sci.* **2002**, *67*, 2165−2171.
(43) Chace, K. V.; Carubelli, R.; Nordquist, R. E.; Rowsey, J. J. Effect of oxygen free radicals on corneal collagen. *Free Radical Res.* **1991**, *13*, 591−594.
(44) Kano, Y.; Sakano, Y.; Fujjimoto, D. Cross-linking of collagen by ascorbate−copper ion systems. *J. Biochem.* **1987**, *102*, 839−842.
(45) Hawkins, C. L.; Davies, M. J. Oxidative damage to collagen and related substrates by metal ion/hydrogen peroxide systems: random attack or site-specific damage? *Biochim. Biophys. Acta* **1997**, *1360*, 84−96.
(46) Uchida, K.; Kato, Y.; Kawakishi, S. Metal-catalyzed oxidative degradation of collagen. *J. Agric. Food Chem.* **1992**, *40*, 9−12.

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