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Variation in the Cross-Linking Pattern of Porcine Myofibrillar Protein Exposed to Three Oxidative Environments

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Myofibrillar protein (MP, 26 mg protein/mL in 0.6 M NaCl, pH 6.0) prepared from pork serratus ventralis muscle was incubated at 4 °C for 24 h with three oxidizing systems: (1) an iron-catalyzed oxidizing system (IOS: 10 μM $FeCl_3$, 0.1 mM ascorbic acid, and 0.05−5.0 mM H_2O_2), (2) a linoleic acid-oxidizing system (LOS: 0.05−5.0 mM linoleic acid and 3750 units of lipoxidase/mL), or (3) a H_2O_2-activated metmyoglobin-oxidizing system (MOS: 0.05−0.5 mM metmyoglobin/ H_2O_2). Oxidation in IOS and MOS promoted extensive, dose-dependent cross-linking and insolublization of MP, notably myosin, while the effect of LOS was minimal. Chymotrypsin digestion indicated that the rod (tail) subfragment of myosin was the preferred target of hydroxyl radicals and ferryl oxygen species, although the s-1 (head) region was also susceptible. Disulfide bonds were responsible for most of the cross-linking, and malonaldehyde appeared to contribute to the cross-linking as well. However, dityrosine was minimally involved. Overall, the systems that generate hydroxyl radicals and ferryl oxygen species were more potent than the system that produces peroxide in the cross-linking and aggregation of MP; such covalent links were implicated in the functionality changes of low-temperature-processed muscle foods.

KEYWORDS: Myofibrillar protein; protein oxidation; myosin; dityrosine; TBARS; SDS-PAGE

INTRODUCTION

A protein−protein interaction is one of the main physicochemical reactions that take place during processing and storage of muscle foods. The association and aggregation of muscle proteins through noncovalent forces or covalent links are intimately involved in the formation of viscoelastic gel matrix systems that play a crucial role immobilizing water and stabilizing fat particles in comminuted products, such as frankfurters and bologna (*1*). In cooked meat products, protein aggregation is generally accomplished by heating (cooking) that unfolds the structure of proteins and exposes the reactive amino acid side chain groups.

A number of studies have demonstrated that under oxidative conditions, which commonly exist in muscle foods during manufacturing and storage, the process of protein aggregation is modified by radicals and secondary products of lipid oxidation that are generated from compounds naturally present in meat (*2, 3*). For example, reactive oxygen species, such as hydroxyl radicals (*4, 5*) and H_2O_2-generated myoglobin radical (*6, 7*), have been shown to promote cross-linking of myosin. Among the possible agents involved in the cross-linking of oxidized proteins are the disulfide bonds, malonaldehyde (MDA), 4-hydroxy-2-nonenal (HNE), and dityrosin (*8, 9*). The modified protein−protein interaction is implicated in major shifts in the functional properties of myofibrilar proteins in processed muscle foods (*10*).

While extensive oxidation results in major losses in protein functionality, increased gelling and emulsifying properties were observed on mildly oxidized muscle proteins (*11−13*). These previous results led us to hypothesize that the type, not simply the extent, of myosin cross-linking was important in protein functionality. It is well-established that under nonoxidizing conditions, myosin aggregation at low temperatures starts through head−head (heavy meromyosin or the s-1 subfragment) association (*14*). However, it was not clear whether the tail portion (light meromyosin or the rod subfragment) was involved during the initial aggregation when oxidizing radicals were present. Indeed, a recent study conducted in our laboratory showed that an iron-catalyzed hydroxyl radical system heavily favored the cross-linking of chicken pectoralis myosin through the rod, and heating was not required (*5*).

In the present study, we investigated the effects of hydroxyl radicals, an oxidizing lipid (linoleic acid), and an oxidizing heme pigment (metmyoglobin), which are expected to coexist in

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processed muscle food products, on the aggregation of porcine myosin. Although our previous investigation indicated considerable variations in biochemical changes in myofibrillar protein (MP) that were exposed to these three oxidizing systems (*9*), the difference in protein cross-linking patterns remains unclear. Our objective was to elucidate the specific cross-linking site(s) in myosin when subjected to these three common oxidizing environments and the possible involvement of lipid-derived carbonyls and dityrosine.

MATERIALS AND METHODS

Materials. Fresh pork Boston shoulders (4 days postmortem) stored in vacuum packages were obtained from a commercial packing plant. Whole serratus ventralis muscle was removed from each shoulder, cut into approximately 15 g cubes, and hand-mixed to randomize. Aliquots of 250 g of these muscle cubes were 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 3−5 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.

Soybean lipoxidase (131000 units/mg), chymotrypsin (52 units/mg), horse heart metmyoglobin, L-tyrosine, bovine serum albumin (BSA), *N*-ethylmaleimide, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). Thiobarbituric acid (TBA) was obtained from ICN Biomedicals Inc. (Aurora, OH), and sodium dodecyl sulfate (SDS) was from Bio-Rad Laboratories (Hercules, CA). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ), unless specifically indicated, and were of reagent grade.

MP Preparation. The procedure described by Park et al. (*9*) was followed to extract MP from thawed muscle tissue in a 4 °C walk-in cooler, using an isolation buffer containing 0.1 M NaCl, 2 mM $MgCl_2$, 1 mM EGTA, and 10 mM sodium phosphate (pH 7.0). In the final washing, the extracted MP was suspended in 4 vol of 0.1 M NaCl solution, and the pH was adjusted to 6.0 (to closely simulate the pH condition in processed meats) with 0.1 N HCl prior to centrifugation. Collagen fragments, if any, would visibly stay at the bottom of the centrifuge bottle and could readily be removed. Final MP pellets were kept in tightly capped bottles and stored on ice before use (in 18 h). The protein concentration of the MP pellets was measured by the Biuret method using BSA as the standard.

Crude Fat Content. MP pellet samples were freeze-dried, and the total crude fat was determined by extracting 1.0 g of dry samples 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 (*9*).

Oxidation. MP pellets were suspended (26 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 MP was oxidized with (1) an iron-catalyzed oxidizing system (IOS: 10 μM $FeCl_3$, 0.1 mM ascorbic acid, and 0.1−10.0 mM H_2O_2), (2) a linoleic acid oxidizing system (LOS: 0.1−10.0 mM linoleic acid and 3750 units of lipoxidase/mL), or (3) a H_2O_2-activated metmyoglobin-oxidizing system (MOS: 0.05−0.5 mM metmyoglobin and 0.05−0.5 mM H_2O_2). The protein concentration in MP samples after the addition of oxidizing agents was adjusted to 20 mg/mL with the 15 mM PIPES buffer (pH 6.0) containing 0.6 M NaCl and then oxidized for 24 h at 4 °C. Oxidants were then removed by washing the MP samples with 5 volumes of 15 mM PIPES buffer (no NaCl) at pH 7.0, followed by centrifugation at 2000*g* for 15 min. The protein pellet was resuspended in 3 volumes of the 15 mM PIPES buffer containing 0.1 M NaCl (pH 7.0) and centrifuged at 2000*g* for 15 min. The washed MP pellets were immediately analyzed.

Protein-Bound MDA. In a distillation TBA procedure, heat in an acidic condition will liberate MDA from its bound state with protein (*15*). Accordingly, the amount of protein-bound MDA (expressed as 2-thiobarbituric acid-reactive substances, TBARS) in the MP pellets was determined with a modified distillation procedure (*16*). Briefly, oxidized and nonoxidized MP samples were individually mixed with 5 volumes of 15 mM PIPES buffer containing no NaCl (pH 7.0), and centrifuged at 2000*g* for 15 min. After the supernatant was discarded, the protein pellet was suspended again in 3 volumes of the PIPES buffer containing 0.1 M NaCl (pH 7.0) and centrifuged at 2000*g* for 15 min. The washed MP pellets, expected to contain negligible amounts of free MDA, were subsequently resuspended in a 15 mM PIPES buffer (pH 6.0) containing 0.6 M NaCl, and the protein content in the suspensions was adjusted to 20 mg/mL. The protein suspensions were then subjected to the distillation procedure to determine the protein-bound MDA (μg MDA equivalent/g sample protein).

Dityrosine. The measurement of dityrosine in oxidized and control MP samples entailed a dityrosine standard, which was not commercially available. Thus, dityrosine was synthesized by following the method described by Giulivi and Davies (*17*) and Stewart et al. (*18*) with modifications. Briefly, 1 g of L-tyrosine was dissolved in a solution of 605 mL of 0.289% (w/v) ammonium hydroxide, and the solution was titrated to pH 7.6 with 2.0 M formic acid. After reaction with 100 μL of 30% H_2O_2 for 1 h at 40 °C, 5 mL of horseradish extract was added. The horseradish extract was freshly prepared by extracting 2 g of a commercial horseradish powder with 10 mL of 10 mM sodium phosphate buffer (pH 7.0) for 10 min. The reaction mixture was then incubated for 10 h at 40 °C, during which 100 μL of H_2O_2 (30%) was added every 2 h until after a total of 500 μL had been added.

The reacted mixture, which contained dityrosine, was filtered through #1 Whatman filter paper, and the filtrate was dried in a rotary evaporator. The residue was dissolved in 100% ethanol and chromatographed on Whatman silica gel 60A thin layer plates (20 cm × 20 cm, 250 μm layer). After they were developed with *n*-butanol:water:acetic acid (4:1:2, v/v/v), the plates were exposed to NH_4OH vapor, and the blue fluorescent dityrosine band ($R_f = 0.26$) was excised and extracted with methanol. Crude dityrosine was obtained after the solvent was removed using a rotary evaporator and was rechromatographed to purify on the thin layer plates using the same solvent system as before. The dityrosine band ($R_f = 0.26$) was excised and extracted with methanol. The purified dityrosine was recovered by removing the solvent with N_2 gas. The white dityrosine powder was dissolved in aqueous acetonitrile (acetonitrile:water, 70:20) and used for constructing chromatographic standard curve.

To quantify dityrosine in MP samples, aliquots of 3.0 g for diluted control and oxidized MP (20 mg/mL) were weighed into 15 mm × 100 mm screw cap test tubes. A 3 mL aliquot of 12 N Optima HCl (Seastar Chemical Inc., Pittsburgh, PA) was slowly added to the sample tube by gently mixing with a vortex. The headspace of the sample tubes was then flushed with N_2 gas for 15 s. The sample tubes were tightly capped and placed in a 110 °C oven (Lipshaw Electric Laboratory Incubator-Oven, Lipshaw MFG. Co., Detroit, MI) for 24 h to allow complete hydrolysis. The cooled MP digests were quantitatively transferred into 100 mL volumetric flasks, and the solution was then brought to volume with nanopure water. Approximately 4 mL of each of the sample solutions was filtered through a 0.45 μm MCE sterile syringe filter (Fisher Scientific, Hampton, NH). After approximately 1 mL of the initial filtrate was disposed, 1 mL of filtered solution was collected into a microcentrifuge tube.

Quantitation of dityrosine was done by measuring flourescence at 325 (excitation) and 400 nm (emission) using a Flouromax-3 spectroflourometer (HORBA Jobin Yuon, Edison, NJ), which was controlled by DataMax software version 2.20 (Instruments S.A. Inc., Edison, NJ). Photon counts were converted to μg dityrosine per g samples by a standard curve built with a range of dityrosine standard solutions (1−200 μM). The assay for the standards yielded the following equation: μg dityrosine/mL aqueous solution = (0.00315 × photon counts − 0.76172). The quantity of dityrosine was expressed as mg dityrosine per g protein in MP.

Chymotryptic Digestion. Control and oxidized MP samples were suspended (10 mg protein/mL) in a 15 mM PIPES buffer (pH 8.0) containing 0.12 M NaCl and 1 mM EDTA and digested with chymotrypsin for 60 min at 21 °C to produce s-1 and rod fragments from myosin (*19, 20*). The enzyme to substrate ratio was 1:500. The digestion was terminated by adding 0.5 mM PMSF.

Table 1. Quantity of Dityrosine and Protein-Bound MDA in MP Isolate Exposed to Three Oxidizing Systems at 4 °C for 24 h[a]

oxidizing system	oxidant	protein-bound MDA (μg/g protein)	tyrosine (mg/g protein)[b]	dityrosineb (mg/g protein)
control	0	0.79 ± 0.27 f	38.4 ab	0.37 ± 0.04 b
IOS	0.1 mM H_2O_2	6.97 ± 0.52 a	49.1 a	0.69 ± 0.11 a
	0.2	5.20 ± 0.80 bc	–	0.50 ± 0.03 ab
	0.5	4.59 ± 0.75 bcd	–	0.46 ± 0.02 ab
	1.0	4.58 ± 0.31 bcd	40.5 ab	0.31 ± 0.01 b
	5.0	4.53 ± 0.51 bcd	–	0.30 ± 0.00 b
	10.0	3.19 ± 0.28 cde	25.5 c	0.53 ± 0.11 ab
LOS	0.1 mM linoleic acid	2.17 ± 0.17 e	42.6 ab	0.43 ± 0.04 ab
	0.2	3.21 ± 0.67 cde	–	0.60 ± 0.21 ab
	0.5	2.92 ± 0.23 de	–	0.52 ± 0.09 ab
	1.0	2.89 ± 0.05 de	41.2 ab	0.39 ± 0.04 b
	5.0	2.89 ± 0.29 de	–	0.50 ± 0.06 ab
	10.0	3.80 ± 0.34 cde	43.5 ab	0.53 ± 0.12 ab
MOS	0.05 mM MetMb	6.17 ± 0.13 ab	39.9 ab	0.45 ± 0.07 ab
	0.1	7.16 ± 0.16 a	34.2 b	0.56 ± 0.06 ab
	0.3	6.43 ± 0.87 ab	–	0.59 ± 0.11 ab
	0.5	4.94 ± 0.57 bcd	36.4 ab	0.49 ± 0.05 ab

[a] Means (±SD) in the same column without a common letter differ significantly ($P < 0.05$). [b] From Park and Xiong (*28*). The dash line indicates a skipped measurement.

Gel Electrophoresis. Control and oxidized MP samples, before and after chymotrypsin digestion, were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) to determine covalent links and the site of cross-linking in myosin (s-1 or rod) (*20*). Prior to electrophoresis, MP samples were dissolved in the SDS-PAGE sample buffer (4% SDS, 20% glycerol, and 0.125 M Tris, pH 6.8), with or without 5% of β-mercaptoethanol (βME), to obtain a final protein concentration of 1.0 mg/mL. For samples without βME, 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 protein) of samples was loaded to each well in the 3% polyacrylamide stacking gel, and individual proteins were separated in the 10% resolving gel.

To estimate the percentages of individual proteins or protein fragments in MP samples, images of destained gels were captured with a digital camera, and protein bands were quantitatively analyzed using the UN-SCAN-IT Gel digitizing software (Ver. 6.1, Silk Scientific Corp., Orem, UT) as described elsewhere (*21*). The number of pixels in each whole band was recorded and used to calculate the percent weight of the corresponding protein in the MP sample, assuming that the amount of pixels was equivalent to the mass of the protein.

Statistical Analysis. A randomized complete block design (with replication as the block) with repeated measures was used. 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 from 3–5 replicated experiments were compared by Student–Newman–Kuels (SNK) multiple comparison using SAS at a significance level of 0.05.

RESULTS

Protein-Bound MDA. The MP pellets obtained by the extensive washing procedure still contained 0.49% fat (dry basis). These residual lipids could be readily oxidized, yielding secondary products such as MDA after the MP samples were incubated for 24 h at 4 °C with IOS, MOS, and LOS (*9*). In muscle or muscle protein samples, MDA can bind to proteins through carbonyl–amine condensation reactions (*3, 15, 22*).

The protein isolation process produced a small but measurable amount of protein-bound MDA (0.79 μg/g protein) (**Table 1**). The concentration of bound MDA rose sharply ($P < 0.05$) to 6.97 μg/g protein at 0.1 mM H_2O_2 in IOS and to a similar level (6.17 μg/g protein) at 0.05 mM MetMb in MOS. The amount of protein-bound MDA in IOS tended to drop at increasing H_2O_2 concentrations and reached the lowest level ($P < 0.05$) at 10 mM H_2O_2. In MOS, protein-bound MDA remained constant within 0.05–0.3 mM MetMb but declined to a lower level ($P < 0.05$) at 0.5 mM MetMb from its peak value at 0.1 mM MetMb. On the other hand, relatively low amounts (2.17–3.80 μg/g protein) of protein-bound MDA were detected in the LOS system, independent of the linoleic acid concentration. The low MDA production in LOS may be due to reduced activity of lipoxidase at the incubation temperature (4 °C). The activity assay temperature of the enzyme indicated by the enzyme supplier was 25 °C.

Dityrosine. A common consequence of protein oxidation is the formation of dityrosine, for example, cross-linking of proteins through two tyrosine residues. Dityrosine was detected in the control MP samples. When MP was exposed to 0.1 mM H_2O_2 in IOS, the dityrosine concentration increased by 86% (**Table 1**). At higher levels of H_2O_2 in IOS and at all LOS or MOS oxidant dosages tested, numerical increases in dityrosine were observed, but statistically, the changes were nonsignificant ($P > 0.05$). The dityrosine concentrations in oxidized MP samples represented roughly 1 or 2% of the tyrosine in control MP (**Table 1**), that is, 38.4 mg/g (or 3.84 g/100 g) protein in the control MP. The tyrosine content in the control sample was in agreement with levels reported in the literature, for example, 3.88 g/100 g protein for market weight pigs (*23*).

SDS-PAGE. The electrophoresis of polyacrylamide gel with SDS offers a powerful means through which to visualize the individual MP proteins and their cross-linked products. The exposure of MP to IOS resulted in a gradual and eventual complete disappearance of myosin and a concomitant production of polymers that barely entered the resolving gel with increasing H_2O_2 concentrations (**Figure 1A**). A significant amount of actin was also lost. The lost proteins were mostly (but not entirely) recovered when the oxidized MP samples were treated with βME, a reducing agent (**Figure 1B**). Still, at 10 mM H_2O_2, approximately 30% of myosin heavy chain (MHC) was not recovered (as determined by band intensity scanning). The results indicated that (1) disulfide links (i.e., from cysteine oxidation) were largely responsible for myosin loss and (2) other covalent bonds were involved as well. The presence of

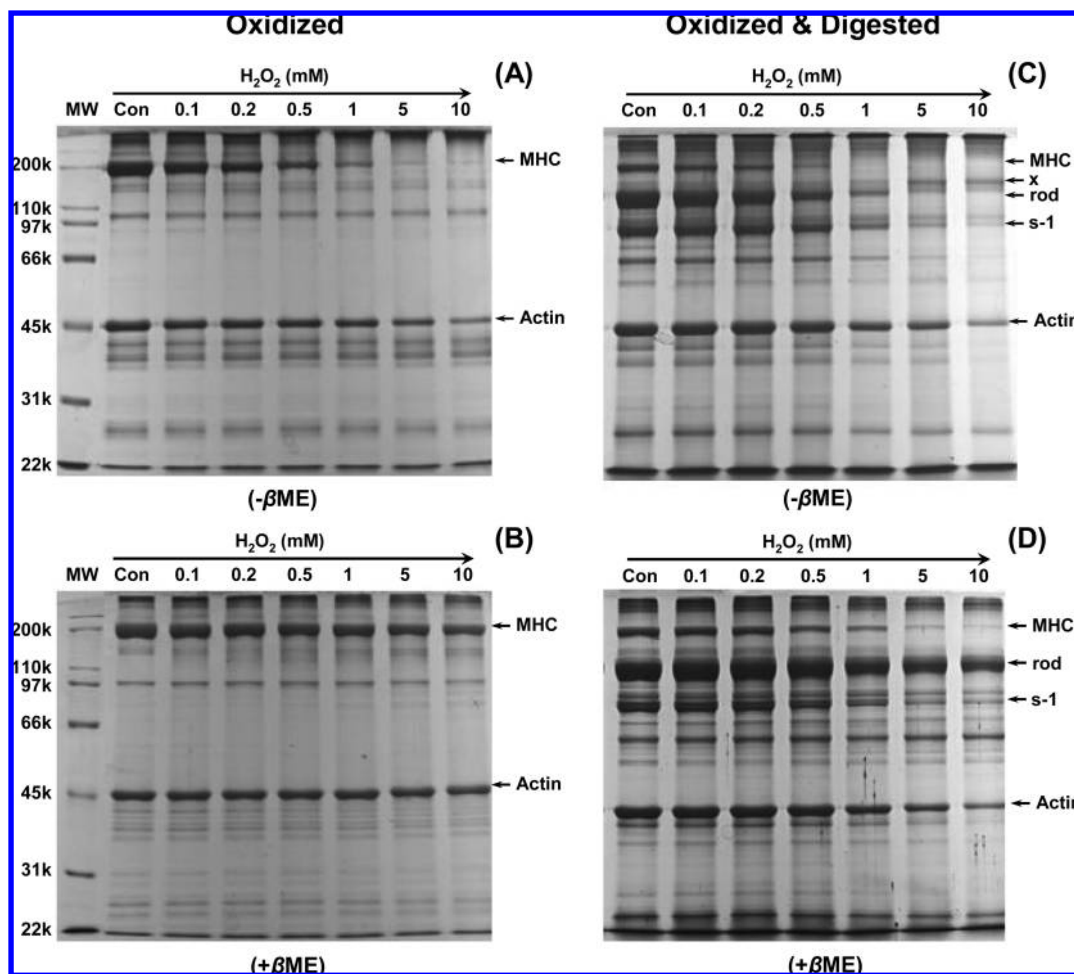

Figure 1. SDS-PAGE patterns of oxidized (4 °C, 24 h) (**A**, **B**) and oxidized/chymotrypsin-digested (**C**, **D**) MP by an IOS with various H_2O_2 concentrations. The samples were prepared without ($-\beta$ME) or with ($+\beta$ME) 5% βME. The MW marker, MHC, and its two subfragments (rod and s-1) are indicated. Con, nonoxidation control.

extremely large molecular weight (MW) bands at the top of the resolving gel in βME-treated samples (including the nonoxidized control) supports the above postulation. It was noted that in βME-treated samples, the change in the intensity of the MHC band over the H_2O_2 concentration range was unrelated to the content of dityrosine, which was essentially unaffected by H_2O_2 (**Table 1**). Thus, dityrosine cross-linking appeared to be minimally, if at all, involved with myosin loss in the IOS samples.

Because myosin is the predominant component of MP, its oxidative changes were further analyzed. To establish the sites of cross-linking in myosin, IOS-oxidized MP was subjected to limited chymotrypsin digestion under 0.12 M NaCl and 1 mM EDTA at pH 8. This ionic condition allows chymotrypsin to sever MHC at the kink region into s-1 and rod (*19*). Therefore, when a subfragment disappears from the SDS-PAGE, one could assume that it was covalently linked to form large, insoluble polymers. As shown in **Figure 1C**, MHC in the control MP sample was split into distinctive rod and s-1 bands. However, the recovery of both fragments was rapidly reduced at increasing H_2O_2 concentrations, and the polymers appearing at the top of the resolving gel remained salient and indigestible. At 5 mM H_2O_2, the rod totally vanished, while approximately 10% of s-1 remained present. Accompanying the loss of the rod and s-1 was the emergence of a band immediately above the rod (marked as “x”). This x-protein had an estimated MW of 173000 based on the log[MW] vs protein migration rate regression line and may be the dimer of s-1 (MW $\sim$ 90000).

The densitometry scan provided quantitative assessment of the protein changes. This analysis demonstrated a more rapid disappearance of the rod when compared with the s-1 subfragment in the IOS system at increasing H_2O_2 concentrations (**Figure 2**). The result was consistent with our previous finding on chicken MP that was oxidized with a similar hydroxyl radical-generating system (*20*). Yet, for chicken myosin, the rod diminished much sooner than the s-1 when compared to pork myosin. These results proved that in IOS, the myosin tail (rod) was more susceptible than its globular head (s-1) to hydroxyl radical attack and, thus, was a favorable site where cross-linking occurred. When oxidized samples were treated with βME prior to electrophoresis, a significant amount of rods and the s-1 bands were recovered (**Figure 1D**). However, even in the presence of the reducing agent, s-1 and actin at high H_2O_2 doses were mostly unrecovered. Thus, the loss of myosin from the cross-linking of its two subfragments, and that of actin, were due to disulfide linkages as well as other types of covalent bonds.

In contrast to the electrophoretic patterns of IOS-treated MP, the PAGE profiles of LOS-treated MP did not demonstrate remarkable effects of linoleic acid on myosin and actin (**Figure 3A**,**B**). Myosin rods and s-1 fragments that were produced by chymotrypsin digestion largely remained even in samples without the ME treatment (**Figure 3C**). Overall, less than 20% of rod and 12% of s-1 were cross-linked into high-MW, indigestible polymers (**Figure 2**).

On the other hand, incubation of MP with MOS resulted in marked loss of myosin and actin bands, with major reductions

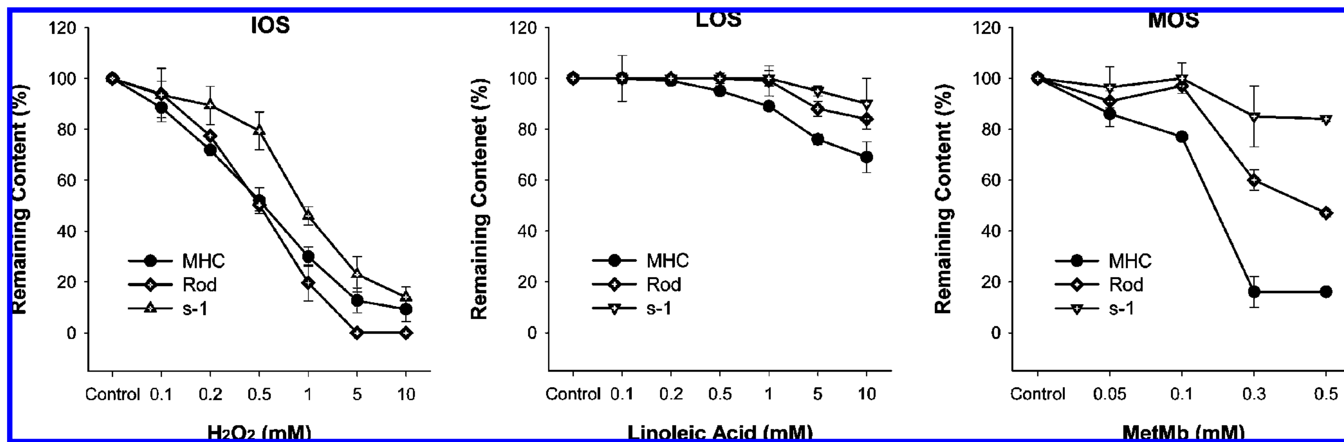

Figure 2. Changes in the content (%) of MHC and its subfragments (rod and s-1) in MP isolate as a result of oxidation by an IOS, a LOS, and a MOS at 4 °C for 24 h.

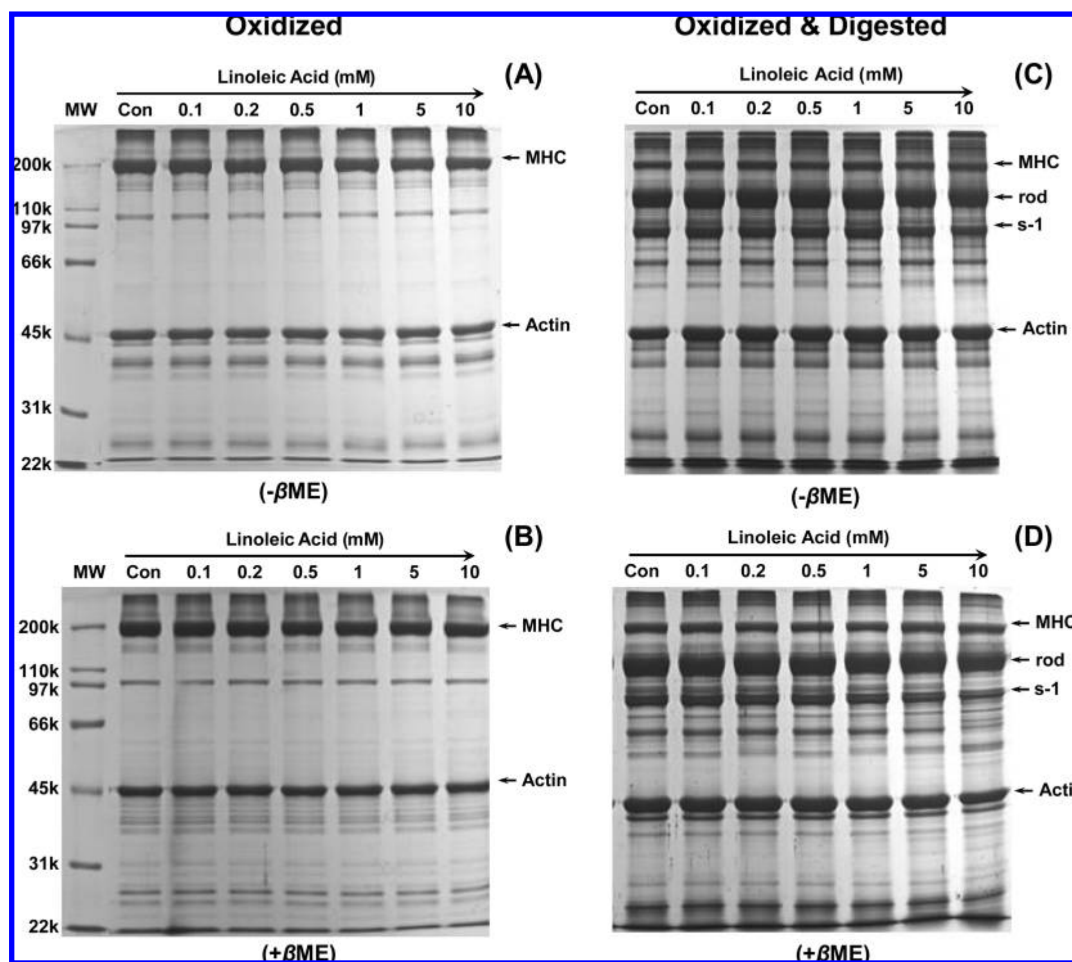

Figure 3. SDS-PAGE patterns of oxidized (4 °C, 24 h) and oxidized/chymotrypsin-digested MP by a LOS with various linoleic acid concentrations. The samples were prepared without (−βME) or with (+βME) 5% βME. The MW marker, MHC, and its two subfragments (rod and s-1) are indicated. Con, nonoxidation control.

occurring when the MetMb concentration was raised from 0.1 to 0.3 mM (**Figure 4A**). Treatment with ME restored only about 13−17% of the bands (**Figure 4B**). Unlike samples oxidized by IOS, the polymers generated in MOS were insoluble and formed precipitates, which, therefore, did not appear on the top of the PAGE resolving gel. Some insoluble aggregates (at 0.3 and 0.5 mM MetMb) were digested by chymotrypsin, which produced intense rod and s-1 bands (**Figure 4C**). Similar to the digestion results for the IOS treatments, more s-1 was recovered by the enzyme digestion than the rod, for example, 46% at 0.5 mm MetMb ($P < 0.05$) (**Figure 2**). This indicates that in MOS, cross-linking of myosin rod was more extensive than that of s-1. When digested samples were treated with βME, both the rod and the s-1 subfragments were mostly regenerated (**Figure 4D**).

DISCUSSION

The main active oxygen species produced by IOS and MOS are hydroxyl radicals (•OH) and ferryl [iron(IV)-oxy] species,

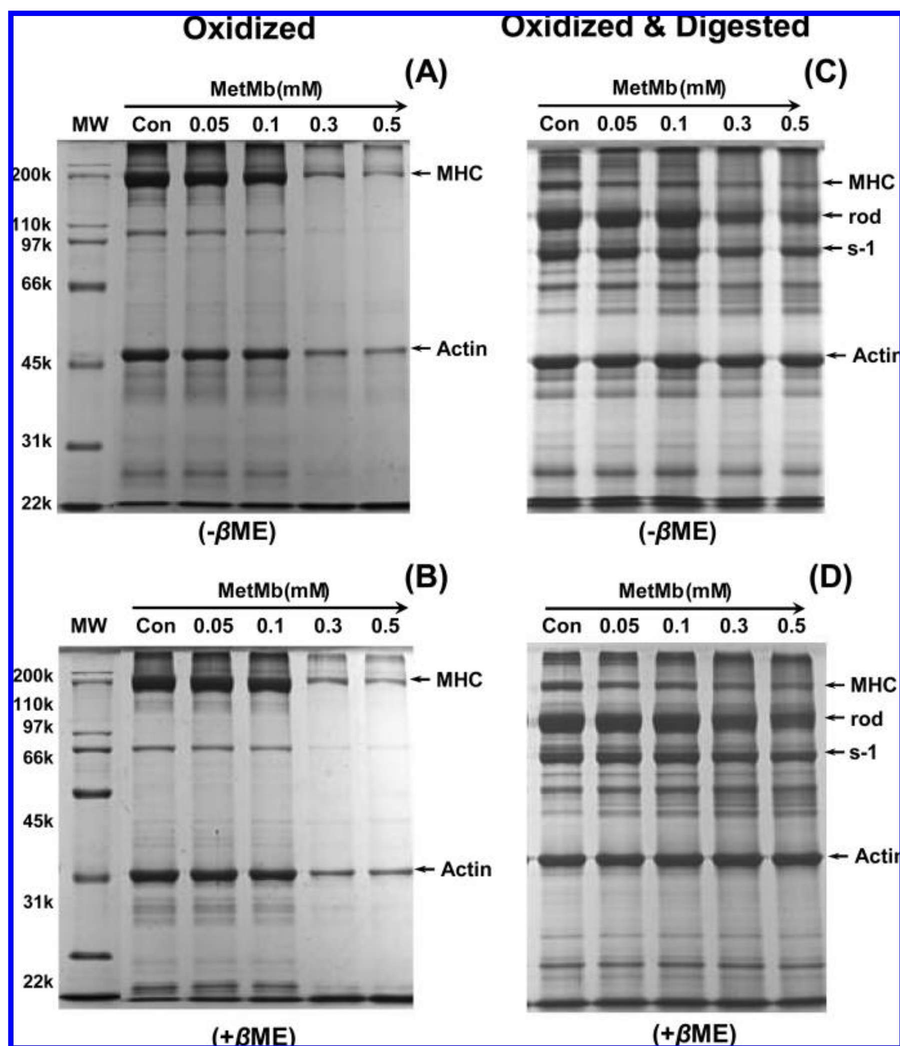

Figure 4. SDS-PAGE patterns of oxidized (4 °C, 24 h) and oxidized/chymotrypsin-digested MP by a metmyoglobin (MetMb)-oxidizing system (MOS) with various MetMb concentrations. The samples were prepared without (−βME) or with (+βME) 5% βME. The MW marker, MHC, and its two subfragments (rod and s-1) are indicated. Con, nonoxidation control.

respectively; for LOS, hydroperoxide or peroxyl radicals could be the main reactive species (*24*). However, the refrigeration condition (4 °C), which is typical of meat processing, appeared to be inefficient in producing peroxides from linoleic acid by lipoxidase. Otherwise, more pronounced myosin aggregation would have been observed (*25*). Although ferric myoglobin (MetMb) is widely reported to initiate lipid and protein oxidation in muscle foods, the exact mechanism is not entirely understood. However, it is known that in the presence H_2O_2, MetMb (III) is converted to highly reactive quadrivalent ferryl(IV) species (*26*). The strong oxidizing capacity of ferryl species is attributed to the oxoferryl moiety as well as the myoglobin radical (Mb$^\bullet$).

Disulfide bridges were the main covalent force involved in the aggregation of oxidized myosin and other myofibrillar components. Yet, even when disulfide bonds were cleaved, as much as 30% myosin in the $^\bullet$OH-generating system (IOS) remained unrecoverable. Thus, other covalent linkages or reactive groups were involved, which could include protein carbonyls, dityrosine, and lipid-derived bifunctional compounds such as MDA and HNE (*8, 27, 28*). In particular, MDA appeared to be an important contributor to the association of MP exposed to IOS. MDA is a bifunctional compound (i.e., having two free carbonyl groups); therefore, protein-bound MDA could form Schiff's base with free amines, thus producing protein aggregates (*22*). This premise is supported by the observation that under reducing conditions (with βME), the decrease in the amount of protein-bound MDA, at increasing H_2O_2 dosages, in IOS coincided with the major loss of myosin.

Although the exposure of MP to the H_2O_2-activated MetMb oxidizing system (MOS) resulted in a greater apparent loss of myosin and actin (up to 86%), most of the lost proteins were recovered when the oxidized samples were treated with chymotrypsin. Structural hindrances due to extensive protein aggregation and agglomeration may be attributed, in part, to MDA cross-linking. This probably prevented the dissociation of large, insoluble protein aggregates by βME. Namely, the failure of βME to completely resolubilize MOS-oxidized MP, unless the oxidized proteins were first treated with chymotrypsin, suggested that most disulfides formed were occluded in protein aggregates and agglomerates and were not readily accessible to ME. The removal of external cross-linked polypeptides by chymotrypsin probably exposed the inner cryptic disulfide bonds, thereby increasing their accessibility. On the other hand, the rod and s-1 junction (the "kink" region), where a key proline residue is present (*29*), probably remained flexible and accessible by chymotrypsin. Notwithstanding, protein structural changes by themselves and their ensuing aggregation can affect the ultimate chymotryptic digestibility under oxidative conditions.

The role of dityrosine in the cross-linking of oxidized MP seemed minimal. Dityrosine in oxidized MP was completely

recovered by the strong acid hydrolysis (12 N HCl, heated at 110 °C) during the sample preparation. Despite some small but inconsistent increases in oxidized MP, dityrosine concentration stayed fairly constant. The result indicated that dityrosine was not a main cross-linking agent for oxidation-induced aggregation of MP in all three oxidizing systems (IOS, MOS, or LOS). The present finding differed from several previous reports that H_2O_2-activated MetMb and •OH promoted dityrosine production (*6, 8*). However, in these studies, dityrosine was not purified from oxidized sample extracts, which could contain other aromatic compounds, and the estimation was made without a dityrosine standard. Therefore, MDA and possibly other secondary lipid oxidation products such as HNE (*27*), protein carbonyls (*28, 30*), and even protein radicals themselves were more likely involved in the cross-linking of oxidized MP.

The cross-linking patterns in both IOS and MOS indicated a preference for the tail portion of myosin over its globular head as the initial target. As compared with the well-folded structure of the globular head (s-1) (*29, 31*), the helical coiled-coil myosin tail (rod) is elongated with amino acid residue side chain groups more exposed and accessible. Therefore, sufhydryl groups, which are mostly distributed throughout the rod, would readily be subjected to reactive oxygen species attack. The polarity of the reactants was a plausible factor as well. Because the main reactive oxygen species produced in both IOS and MOS systems are hydrophilic compounds, they could have a greater affinity for myosin rods, which are more polar than myosin heads. The more rapid and extensive loss of myosin in MOS when compared to that in IOS demonstrated that myosin was more sensitive to ferryl oxygen species than to •OH at the specific dosage levels tested. The greater recovery rate of the constituting myosin subfragments (rod, s-1) when MOS-oxidized MP was treated with βME following chymotrypsin cleavage, as compared with IOS-oxidized samples, indicated a greater susceptibility of sulfhydryls to ferryl myoglobin species than hydroxyl radicals.

LITERATURE CITED

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