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Oxidation-Induced Unfolding Facilitates Myosin Cross-Linking in Myofibrillar Protein by Microbial Transglutaminase

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ABSTRACT: Myofibrillar protein from pork *Longissimus* muscle was oxidatively stressed for 2 and 24 h at 4 °C with mixed 10 μM $FeCl_3$/100 μM ascorbic acid/1, 5, or 10 mM H_2O_2 (which produces hydroxyl radicals) and then treated with microbial transglutaminase (MTG) (E:S = 1:20) for 2 h at 4 °C. Oxidation induced significant protein structural changes ($P < 0.05$) as evidenced by suppressed K-ATPase activity, elevated Ca-ATPase activity, increased carbonyl and disulfide contents, and reduced conformational stability, all in a H_2O_2 dose-dependent manner. The structural alterations, notably with mild oxidation, led to stronger MTG catalysis. More substantial amine reductions (19.8−27.6%) at 1 mM H_2O_2 occurred as compared to 11.6% in nonoxidized samples ($P < 0.05$) after MTG treatment. This coincided with more pronounced losses of myosin in oxidized samples (up to 33.2%) as compared to 21.1% in nonoxidized ($P < 0.05$), which was attributed to glutamine−lysine cross-linking as suggested by sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

KEYWORDS: *myofibrillar protein, protein oxidation, transglutaminase, cross-linking*

INTRODUCTION

The successful production of a well-formed, uniformly textured, and physically and chemically stable meat product critically depends on the ability of meat proteins to form a cohesive gel upon heating that is capable of binding meat particles, stabilizing fat globules, and entrapping moisture via the reduction of interfacial tension in a protein matrix.[1,2] Under acidic conditions[3] or in the presence of transglutaminase,[4] cold set gels can also be formed by muscle proteins. However, to produce a viscoelstic gel with a high degree of rigidity, heating to a final temperature of about 70 °C is generally required.[5]

Transglutaminase (EC 2.3.2.13), derived from a variety of sources, including animal and microbial origins, has been introduced to promote the texture and gel formation in various restructured muscle foods by virtue of catalyzing an acyl transfer reaction between glutamine and lysine residues to form ε-(γ-Glu)-Lys isopeptide bonds between proteins.[6] Microbial transglutaminase (MTG) is a Ca^{2+}-independent enzyme and has an activity over a broad range of pH[4−9] and temperatures (0−70 °C),[7] making it particularly attractive for processed meats. The effectiveness of MTG depends on the availability of glutamine and lysine residues under specific processing conditions.[8] For example, pale, soft, and exudative (PSE) meat protein has a lower reactivity with MTG when compared to protein in normal meat.[9] It is likely that the originally available glutamine and lysine residues become inaccessible due to protein structure changes, that is, denaturation. Moreover, MTG-mediated cross-linking processes vary in reaction rate and end products between animal species probably due to different distribution patterns of glutamine and lysine residues in different muscle types.[10] Because one of the consequences of oxidative stress of proteins is structure unfolding or the exposure of certain amino acid side chain groups,[11,12] it is hypothesized that susceptibility of muscle protein to MTG could be influenced by the oxidative status of the protein.

Muscle proteins are readily oxidized by reactive oxygen species (ROS) during storage and processing,[13−15] and modification of amino acid residues is a direct manifestation of oxidation. It is noteworthy that lysine, one of the substrates of MTG-mediated cross-linking reaction, is susceptible to ROS.[16] Consequently, ROS-stressed proteins could have a reduced reactivity with MTG. Moreover, under a strong oxidizing condition, proteins have a tendency to form heterogeneous polymers linked primarily by S−S bonds,[17] and the aggregation would increase structural hindrance and limit the enzyme access to glutamine and lysine groups. Indeed, excessively oxidized proteins are insensitive to proteolytic enzymes due to the accumulation of polymerized aggregates.[18,19]

On the other hand, oxidation also subjects proteins to unfolding, which enhances the exposure of certain peptide segments and reactive amino acid residues recognizable by enzymes. It has been shown that increased structural unraveling by hydroxyl radical leads to a significantly improved degradation of proteins.[20,21] Glutamine residues in native muscle proteins are expected to be somewhat occluded in the tertiary structure but may become available for MTG-catalyzed cross-linking with lysine upon oxidation, thereby improving network structures required for gel formation.

Hence, when muscle proteins are subjected to oxidative stress, which is commonly encountered in meat processing, multiple changes that either favor or suppress the MTG action

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can occur simultaneously, and these factors are inseparable and coexist. The overall effect of MTG treatment is therefore a combined result of all factors. The objective of this study was to elucidate the structure-modifying effect of hydroxyl radical oxidation on the susceptibility of myofibrillar protein (MFP) to MTG at 0.6 M NaCl (equivalent to about 2−3% salt in processed meats). MFP was investigated because it is largely responsible for functional characteristics of processed meat products.[5]

MATERIALS AND METHODS

Materials. *Longissimus* muscle (pH 5.6−5.9) was collected from five pork carcasses (48 h postmortem). Individual samples (ca. 70 g) were vacuum packaged and kept in a −30 °C freezer until use in less than 6 months. MTG (Activa-TI, with an activity of 100U/g, 1% MTG blended in 99% maltodextrin) was donated by Ajinomoto Food Ingredients (Chicago, IL). All chemicals were obtained from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) and were at least reagent grade.

Extraction of MFP. Frozen muscle samples were tempered at 4 °C for 4 h and then used for the MFP preparation according to Park et al.[22] using the isolation buffer of 10 mM sodium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EGTA at pH 7.0. The pH of MFP suspension at 0.1 M NaCl in the last wash was adjusted to 6.25 before centrifugation. The protein concentration was measured by the Biuret method[23] using bovine serum albumin as a standard.

Oxidation of MFP. The MFP pellet in 0.1 N NaCl was quantitatively diluted to a final protein concentration of 20 mg/mL in 15 mM piperazine-*N*,*N*-bis(2-ethanesulfonic acid) (PIPES) buffer containing 0.6 M NaCl (pH 6.25). This solution was oxidized for 2 or 24 h at 4 °C with hydroxyl radicals produced by a 10 μM $FeCl_3$/100 μM ascorbic acid solution with 1, 5, and 10 mM H_2O_2.[22] The oxidation reaction was terminated by propyl gallate/Trolox C/EDTA (1 mM each). The nonoxidized MFP solution containing propyl gallate/Trolox C/EDTA was used as the control.

Determination of Oxidative Changes of MFP. Chemical and structural changes in MFP induced by oxidation were analyzed by measurements of carbonyls, ATPase activity, sulfhydryls, disulfide bonds, surface hydrophobicity (S_o), and thermal stability using differential scanning calorimetry (DSC).

Carbonyls. The content of carbonyls was determined using the 2,4-dinitrophenylhydrazine (DNPH) colorimetric method of Levine et al.[24] as outlined by Liu et al.[25] Briefly, the DNPH-reacted MFP samples after 20% TCA precipitation were recovered by centrifugation and then washed with ethanol:ethyl acetate (1:1) solution three times. The final MFP pellets were dissolved in 6 M guanidine hydrochloride. The absorbance was read at 370 nm for carbonyl content and 280 nm for protein content. The carbonyl concentration was calculated using a molar extinction coefficient of 22000 M^{-1} cm^{-1}.

Total Sulfhydryls and Disulfide Bonds. Total free sulfhydryls and disulfide bonds were estimated using 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and 2-nitro-5-thiosulfoben-zoate (NTSB), respectively, as described by Liu et al.[25] and Thannhauser et al.[26] Blanks were run with 25 mM sodium phosphate buffer containing 0.6 M NaCl (pH 6.25). The molar extinction coefficient of 13600 M^{-1} cm^{-1} was used for calculation.

ATPase Assay. Ca-/K-ATPase activities of nonoxidized and oxidized MFP samples were determined with reaction solutions of 7.6 mM ATP, 15 mM CaCl2, 150 mM KCl, and 180 mM Tris-HCl, pH 7.4, for Ca-ATPase and 7.6 mM ATP, 300 mM KCl, 5.0 mM EDTA, and 180 mM Tris-HCl, pH 7.4, for K-ATPase, as described by Wells et al.[27] Results were expressed as μmol Pi/mg protein/10 min. A standard curve for inorganic phosphate release was constructed by the titration of a series of NaH_2PO_4 solutions (0−1.0 mM).

Surface Hydrophobicity (S_o). S_o was determined by assessing fluorescence intensity using the fluorescence probe 8-anilino-1-naphthalene sulfonate (ANS). A series of MFP solutions (0.2−3 mg/mL) were thoroughly mixed with 20 μL of 8.0 mM ANS, and the fluorescence intensity was measured after exactly 2 min using a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ). The excitation and emission wavelengths were 390 and 470 nm, respectively. The slope for the linear regression of fluorescence intensity against protein concentration was regarded as S_o.

DSC. Oxidized MFP samples (20 mg/mL in 0.6 M NaCl, 15 mM PIPES, pH 6.25) were diluted in 5 vol of 15 mM PIPES (pH 6.25), followed by centrifugation at 2000*g* for 15 min. The pellets were resuspended in 15 mM PIPES (pH 6.25) containing predetermined amounts of NaCl to obtain final MFP suspensions with 60 mg/mL protein and 0.6 M NaCl. Individual samples (16−18 mg), hermetically sealed in aluminum pans, were subjected to thermal scan (20−100 °C at 10 °C/min) using a model 2920 differential scanning calorimeter (TA Instruments, Inc., New Castle, DE). The temperature maxima (T_{max}) and enthalpy changes (ΔH) associated with protein transitions were measured using the Universal analysis software (Version 1.2 N) supplied by the DSC company. Because the transition peaks tended to overlap, the total area above the DSC curve in the 61−79 °C range for each MFP sample was integrated to calculate the total ΔH value.

Effects of Oxidation on MTG-Mediated Cross-Linking Reactions. Control (nonoxidized) and oxidized MFP samples (4 mg/mL) in a 25 mM sodium phosphate buffer (pH 6.25) containing 0.6 M NaCl were incubated with MTG (E:S = 1:20) at 4 °C for 2 h. The reaction was terminated by mixing with 0.1% *N*-ethylmaleimide. Free amines (lysine oxidation or cross-linking), SDS−PAGE (polymerization or cross-linkage), and solubility (physical property) were used to determine the effects of oxidation on MTG-catalyzed cross-linked products.

Free Amines. MTG-reacted MFP samples were dissolved in 0.2125 M sodium phosphate buffer containing 1% SDS (pH 8.3) and then reacted with 2,4,6-trinitrobenzene sulfonic acid (TNBS) as described by Benjakul and Morrissey.[28] A standard curve was constructed using L-leucine in 1% SDS.

Electrophoresis. Protein polymerization induced by oxidation and MTG treatment was observed using SDS−PAGE with a 4% polyacrylamide stacking gel and a 12% resolving gel.[17] The diluted MFP solution (4 mg/mL) was mixed with an equal volume of sample buffer with or without 10% of β-mercaptoethanol (βME) and then boiled for 3 min. Each well was loaded with 50 μg of samples. Gels were stained with 1 mg/mL coomassie brilliant blue R250 in 50% methanol and 6.8% acetic acid and then destained with 5% methanol and 7.5% acetic acid. 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).[17]

Solubility. The MFP solution (2 mg/mL protein in 25 mM phosphate buffer containing 0.6 M NaCl, pH 6.25) was centrifuged at 5000*g* for 15 min at 4 °C to determine protein solubility. The protein concentrations of the supernatant and the original suspension were determined by the biuret method, and their ratio multiplied by 100 was expressed as the solubility (%).

Statistical Analysis. Data with three independent trials (n = 3) were subjected to the analysis of variance using the general linear model's procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL). Significant (P < 0.05) differences between means were identified by LSD all-pairwise multiple comparisons.

RESULTS

Hydroxyl radical formed by the reaction of Fe^{2+} with H_2O_2 in the presence of a reducing compound is a major ROS in meat products. It readily attacks MFP and can cause chemical and structural changes, including the generation of carbonyl derivatives, polymerization of myosin, structural unfolding, and loss of ATPase activity.[12,22] The extent of changes of these physicochemical attributes under a typical meat-processing condition (e.g., 0.6 M NaCl, pH 6.25) in different oxidant concentrations was assessed in the present study, and the

Table 1. Physicochemical Changes of MFP upon the Oxidation by 10 μM $FeCl_3$/100 μM Ascorbic Acid/1, 5, or 10 mM H_2O_2 at 4 °C for 2 or 24 h[a]

			sulfhydryls and disulfide bonds (nmol/mg protein)		ATPase activities (μmol Pi/mg protein/10 min)		
H_2O_2 (mM)	time (h)	carbonyls (μmol/g protein)	−SH	−S−S−	Ca-ATPase	K-ATPase	surface hydrophobicity
0	0	1.48 ± 0.04 e	79.57 ± 0.85 a	0.52 ± 0.13 d	0.48 ± 0.08 e	0.32 ± 0.03 a	56.58 ± 6.09 ab
1	2	2.80 ± 0.14 d	73.97 ± 1.52 b	4.20 ± 0.92 c	0.59 ± 0.10 de	0.27 ± 0.03 a	60.40 ± 6.85 a
	24	3.28 ± 0.32 bcd	72.79 ± 1.36 b	6.09 ± 0.51 abc	0.70 ± 0.08 cd	0.26 ± 0.05 a	46.46 ± 3.39 cd
5	2	3.18 ± 0.20 cd	66.78 ± 1.07 c	5.20 ± 0.20 bc	0.80 ± 0.11 bc	0.04 ± 0.01 b	52.27 ± 5.85 bc
	24	4.24 ± 0.21 a	61.01 ± 1.61 d	8.00 ± 1.69 a	0.93 ± 0.16 ab	0.04 ± 0.01 b	43.89 ± 4.98 de
10	2	3.63 ± 0.16 bc	61.35 ± 1.17 d	7.80 ± 0.66 ab	0.90 ± 0.18 abc	0.03 ± 0.01 b	47.83 ± 3.52 cd
	24	3.84 ± 0.20 ab	58.56 ± 1.05 e	7.11 ± 1.74 ab	1.08 ± 0.20 a	0.04 ± 0.00 b	38.41 ± 3.03 e

[a]Means (n = 3) within the same column sharing no common letters (a−e) differ significantly (P < 0.05).

impact of such changes on MTG catalysis of MFP cross-linking was subsequently evaluated.

Oxidative Changes of MFP. *Carbonyls.* The carbonyl content of nonoxidized MFP was 1.48 μmol/g (Table 1), which was close to that reported previously.[22,25] Exposures of MFP to oxidizing agents with 1−10 mM H_2O_2 resulted in a significant rise in the carbonyl content, showing a net increase from 1.32 to 2.76 μmol/g when compared with the control (P < 0.05, Table 1). The carbonyl production was more pronounced at longer reaction time except in samples oxidized with 10 mM H_2O_2 for 24 h. It is assumed that some carbonyls produced by oxidation can attack nucleophiles (such as ε-NH_2 of lysine) and, hence, contributed to the loss of total carbonyls.[12] This trend was consistent with previous reports.[22,25]

Total Sulfhydryls and Disulfide Bonds. MFP is abundant in SH groups, which are susceptible to hydroxyl radical and are readily converted to intra- and intermolecular S−S linkages.[17] Hydroxyl radicals generated in the presence of 1−10 mM H_2O_2 were responsible for the loss of sulfhydryls (7−26%; P < 0.05) as shown in Table 1. On the other hand, the disulfide bond content in MFP increased sharply (7−14-fold; P < 0.05) at all H_2O_2 concentrations from nonoxidized control MFP (Table 1). However, at 5 and 10 mM H_2O_2, the loss (pronounced) of SH did not seem to correspond to the generation (minor) of S−S bonds, suggesting that some SH groups under the oxidizing conditions were converted to other sulfur derivatives as well, such as cysteic acid.[12]

ATPase Activity. In MFP, the ATPase activity is ascribed to myosin. There are two reactive sulfhydryl groups (SH_1 and SH_2) in close proximity to the myosin head's catalytic site that are involved in the ATPase activity.[27] SH_1 is responsible for the Ca-ATPase activity, while both SH_1 and SH_2 are associated to the K-ATPase activity.[29] Blocking SH_1 causes an increase in Ca-ATPase activity, while blocking either SH_1 or SH_2 causes a decline in K-ATPase activity. The Ca-ATPase activity showed a significant time- and H_2O_2 concentration-dependent increase (Table 1); in samples oxidized for 24 h at 5 or 10 mM H_2O_2, the activity doubled that of the nonoxidized (P < 0.05). Oxidation by hydroxyl radical generated with 1 mM H_2O_2 only caused a slightly decrease (16−19%) of K-ATPase activity (Table 1). However, the K-ATPase activity of samples at 5 and 10 mM H_2O_2 lost by 88−91% (P < 0.05). The results indicated significant structural changes in the S1 head portion of myosin as a result of oxidation.

Surface Hydrophobicity (S_o). A marker of protein unfolding, S_o slightly increased (P < 0.05) under mild oxidative conditions (e.g., at 1 mM H_2O_2 for 2 h) as compared to the nonoxidized (Table 1). However, S_o declined remarkably when MFP was more extensively oxidized with high H_2O_2 concentrations. This may be explained because protein aggregation partially shielded the effect of unfolding in strongly oxidized MFP samples.

DSC. Nonoxidized MFP in 0.6 M NaCl solution resulted in two clear endothermic peaks with T_{max} at 66.4 and 77.2 °C (Figure 1); the first can be attributed to myosin and the second

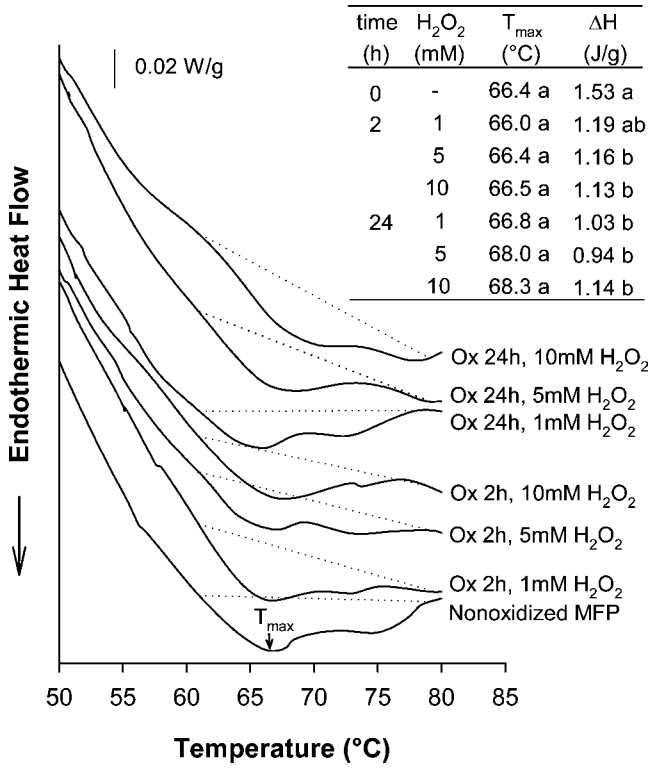

Figure 1. Representative DSC thermal curves of control (nonoxidized) and oxidized MFP samples. Oxidation was conducted with 10 μM $FeCl_3$/100 μM ascorbic acid/1, 5, or 10 mM H_2O_2 at 4 °C for 2 and 24 h. Inset: temperature maxima (T_{max}) for myosin transition and the total enthalpy changes (ΔH, calculated in the dotted line-defined areas, 61−79 °C). Values in the inset without a common letter (a, b) differ significantly (P < 0.05).

to actin.[30] Exposures to oxidants at 1−10 mM H_2O_2 tended to diminish the second peak. The first peak remained salient; hence, its T_{max} was recorded. The T_{max} of this transition was slightly decreased to 66.0 °C after treatment at 1 mM H_2O_2 for 2 h and then gradually increased by up to 2 °C with increasing H_2O_2/oxidizing time. However, the total heat of denaturation (ΔH) showed a remarkable reduction (P < 0.05) upon

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oxidation although this was insensitive to the H_2O_2 concentration or oxidation time. The result indicated that the tertiary structure of MFP was destabilized by hydroxyl radical, which could indicate unfolding due to oxidative modification. The formation of high-energy intra- and intermolecular S−S bonds seemed to have counteracted the stability change, thereby offsetting the decrease in both T_{max} and ΔH as shown at increasing H_2O_2 concentrations/oxidizing time.

Effects of Oxidation on MTG Catalysis. *Free Amines.* As shown in Figure 2, free amine content in nonoxidized MFP (50.3 nmol/mg) decreased ($P < 0.05$) after 2 h when oxidized with increasing H_2O_2 dosages and continued to decrease ($P < 0.05$) in 24 h. As expected, with the MTG treatment, nonoxidized MFP lost a significant amount of amines (about 12%) because lysine is a reactant. In fact, as one of the substrates for MTG, the change of lysine content (usually measured as free amines) is commonly used to express the degree of cross-linking by MTG.[31,32] However, the MTG-responsible amine losses in oxidized MFP samples were faster and more extensive (net change shown as line chart; percent further change shown as bar diagram). In the comparative plots (bar diagram), the relative amine reductions ranging from 9.6 to 27.6% ($P < 0.05$) due to MTG are displayed (Figure 2). The most notable loss associated with MTG treatment was with the MFP sample oxidized by 1 mM H_2O_2 for 2 h (27.6% loss) when compared with nonoxidized sample (11.6%) ($P < 0.05$). However, the effects of MTG treatment on free amines in the oxidized MFP with 5 or 10 mM H_2O_2 were not significant as compared to the nonoxidized probably due to protein aggregation that resulted in reduced reactivity of amines.

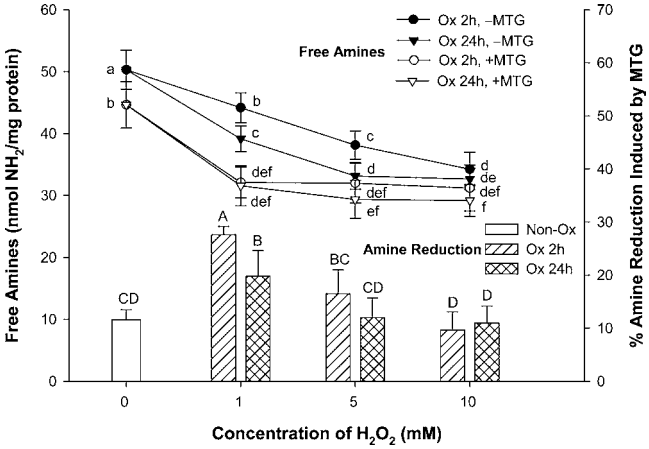

Figure 2. Free amine content of MFP oxidized by 10 μM $FeCl_3$/100 μM ascorbic acid/1, 5, or 10 mM H_2O_2 at 4 °C for 2 and 24 h and then treated with (+MTG) or without (−MTG) transglutaminase at 4 °C for 2 h. Nonoxidized sample: 0 mM H_2O_2. The bar chart indicates the relative reduction of free amines due to MTG treatment over nontreated samples. Means ($n = 3$) without a common lowercase letter (a−f, in line chart) or an uppercase letter (A−D, in bar chart) differ significantly ($P < 0.05$).

Electrophoresis. The exposure of MFP to $^{\bullet}OH$-generating oxidants caused a gradual and eventual disappearance of myosin with increasing H_2O_2 concentrations (Figure 3a). As compared to nonoxidized MFP, oxidized samples with 1 mM H_2O_2 had a small reduction of myosin heavy chain (MHC). However, for MFP oxidized at 5 and 10 mM H_2O_2, especially at 24 h, MHC almost completely vanished. For nonoxidized samples, there were almost no detectable polymers at the top of the stacking gel, but some high molecular weight (MW) bands appeared near or at the top of the separating gel (Figure 3a). These bands may contain titin and nebulin as well as their proteolytic fragments since they can readily pass through 5% acrylamide gel.[33] It appeared that some high MW polymers were produced as artifacts during the MFP extraction.

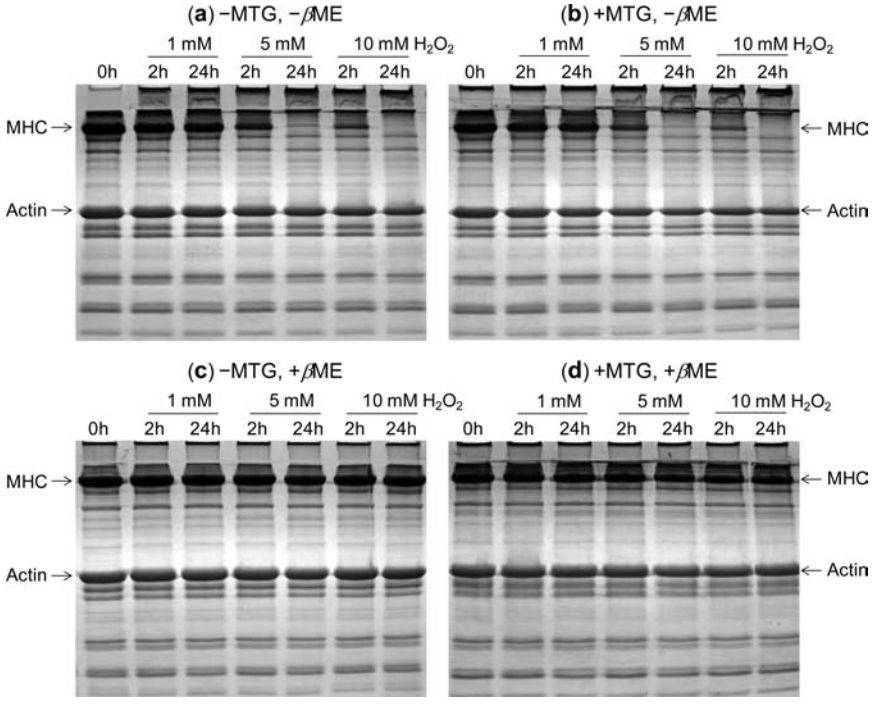

Figure 3. Representative SDS−PAGE patterns of MFP oxidized by 10 μM $FeCl_3$/100 μM ascorbic acid/1, 5, or 10 mM H_2O_2 at 4 °C for 2 and 24 h and then treated with (+MTG) or without (−MTG) transglutaminase at 4 °C for 2 h; 0 h, nonoxidized protein. Samples were prepared in the presence (+βME) or absence (−βME) of 10% βME.

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Upon oxidation, high MW polymers, which barely entered the stacking gel, became accentuated (Figure 3a). These polymers were largely derived from myosin, as the amount of MHC (especially at 5 and 10 mM H_2O_2) drastically decreased. At the same time, polypeptides and protein polymers at the top of the separating gel and also actin showed decreases after oxidation, which may also contribute to the increased band intensity at the top of the stacking gel. Because myosin and actin were mostly recovered when oxidized samples were treated with βME (Figure 3c), it is believed that these polymers were formed largely by disulfide bonds. Nevertheless, a small portion of MHC was not recovered, which may be due to the formation of other covalent bonds, such as Tyr−Tyr and active carbonyl−NH_2 interactions.[12,17] As compared to samples without βME (Figure 3a), all of the samples after the βME treatment (Figure 3c) exhibited a rather intense single band at the top of the stacking gel, suggesting that covalent polymers containing no S−S bonds but previously linked together by S−S (therefore too large to enter the stacking gel) were present in all protein samples.

After the MTG treatment, the MHC band in all samples attenuated conspicuously (Figure 3b) when compared with samples without MTG treatment (Figure 3a). The SDS−PAGE pattern without MTG but treated with βME (Figure 3c) was compared to that with MTG (Figure 3d) to separate the role of S−S bond from the role of isopeptides. The reduction of MHC caused by MTG in the 2 h-oxidized samples at 1 mM H_2O_2 was noticeably more extensive than that in nonoxidized or other oxidized MFP (5−10 mM H_2O_2). To more clearly delineate the specific changes, the relative reduction of MHC attributed to MTG was established by digitizing the band in stained gels, which confirmed that the MFP sample subjected to oxidative for 2 h at 1 mM H_2O_2 was most sensitive to MTG cross-linking reaction (Figure 4). Overall, the relative reduction pattern of MHC was extremely similar to that of free amines, substantiating that the change of free amines was a good indicator for cross-linking degree when treated with MTG.

Solubility. The solubility of MFP decreased ($P < 0.05$) at all H_2O_2 concentrations even for mildly oxidized samples. The exposure of hydrophobic patches and individual groups, aggregation, and polymerization through disulfide bonds are all contributing factors to the solubility reduction. It seemed that oxidizing time did not influence the solubility. When subjected to MTG treatment, the solubility of all oxidized samples showed a further but nonsignificant reduction (Figure 5). The highest solubility reduction induced by MTG treatment was only approximately 4% in the present study.

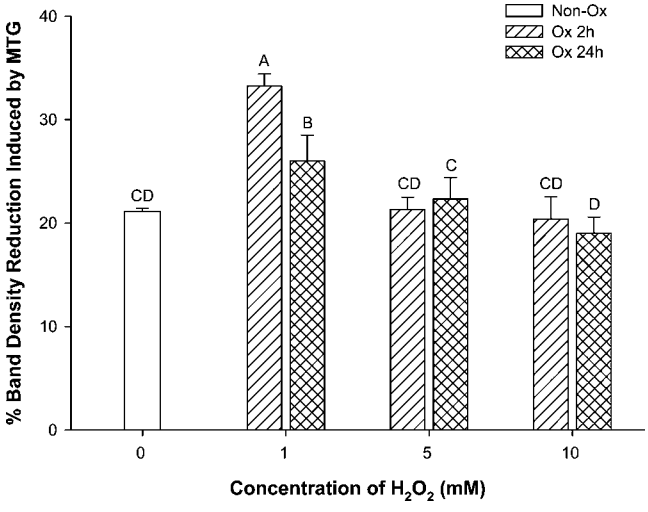

Figure 4. Percent reduction (%) of MHC in MFP due to transglutaminase treatment over nontreated samples. The reductions were calculated based on lanes in Figure 3d over the corresponding lanes in Figure 3c. Means ($n = 3$) without a common letter (A−D) differ significantly ($P < 0.05$).

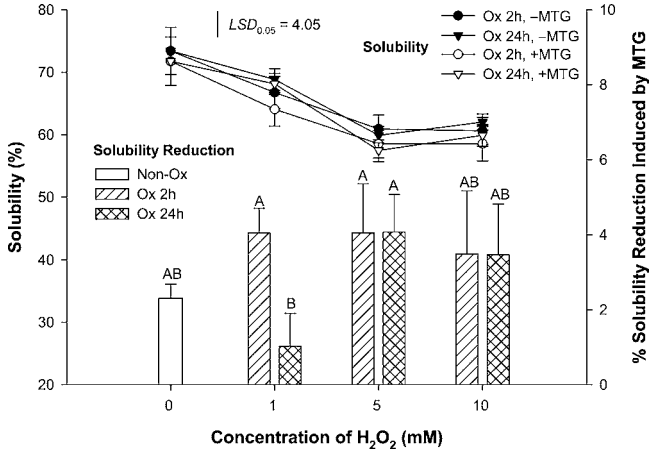

Figure 5. Solubility of MFP oxidized by 10 μM $FeCl_3$/100 μM ascorbic acid/1, 5, or 10 mM H_2O_2 at 4 °C for 2 and 24 h and then treated with (+MTG) or without (−MTG) transglutaminase at 4 °C for 2 h. Nonoxidized sample: 0 mM H_2O_2. The bar chart indicates the relative reduction of solubility due to transglutaminase treatment (+MTG) over nontreated samples (−MTG). Means ($n = 3$) without a common letter (A, B) differ significantly ($P < 0.05$).

DISCUSSION

Structure changes in proteins induced by radicals are a complex phenomenon, which involve multiple pathways and physicochemical reactions often manifested by carbonylation of amino acid side chains, cleavage of peptide backbones, and formation of intermolecular cross-linking with S−S bonds being a primary mechanism.[11,34] More direct evidence of structural changes, elucidated by the calorimetric scanning, surface hydrophobicity tests, and myosin ATPase assays, suggested that the amount of accessible lysine and glutamine residues was varied. As a consequence, MTG catalysis was altered.

However, the analysis of physicochemical parameters as related to the protein structure, and that of protein aggregation by SDS−PAGE and the solubility test, indicated the coexistence of protein unfolding and aggregation under oxidative conditions. The effectiveness of MTG was apparently determined by the preponderance of the specific processes. Where protein unfolding was the dominant event, as in the case of relatively mild oxidation with 1 mM H_2O_2 for a short period (2 h), MTG-induced myosin cross-linking was favored. Conversely, when myosin unfolding led to significant polymerization and aggregation, as in the case of oxidation with high concentrations of H_2O_2 (5 and 10 mM), the MTG reaction became limited. The latter situation appeared to be aggravated when disulfide formation became extensive and deamination (carbonylation of lysine) occurred significantly. This was noted in all strongly oxidized MFP samples.

Evidence supporting the hypothesis that oxidative unfolding facilitates MTG-dependent protein cross-linking can be found

in a number of studies. For example, α-lactalbumin and β-lactoglobulin in whey proteins were resistant to MTG because the glutamine and lysine residues were somewhat occluded but became enzymatically susceptible after their intramolecular S−S bonds, which stabilize the protein structure, were cleaved by a reducing agent.[35] Likewise, brief heating[8] or adsorbing at the oil/water interface,[36] which also caused proteins to unfold, promoted MTG-mediated cross-linking reactions. Mild oxidation would allow initially buried glutamine and lysine residues to be transferred to the protein surface, thereby increasing their probability of contact with MTG.

Cysteine residues, which are extremely susceptible to hydroxyl radicals, might affect the accessibility of glutamine and lysine by MTG. The oxidative conversion of SH groups to S−S bonds is a principal reaction in the dimerization and polymerization of myosin.[17] If intermolecular S−S linkages were limited to one or a few, such linkages could facilitate the MTG reaction because glutamine and lysine residues could be brought into an immediate proximity. There was evidence suggesting this possibility in MTG-catalyzed whey protein gelation.[37] However, if the S−S linkages were excessive, large protein polymers would form that would either shield the reactive amino acid residues or make it difficult for MTG to gain access due to steric hindrances.

The formation of carbonyl derivatives is a reliable marker of protein oxidation.[16] Lysine, threonine, arginine, and proline residues have been reported to be precursors of carbonyls. The cleavage of peptide backbones and covalent attachments of secondary lipid oxidation products, such as malondialdehyde and 4-hydroxy-2-nonenal, can also contribute to carbonyl formation in MFP.[12,34] Because of the high reactivity of carbonyls with free amines, particularly ε-NH_2 of lysine, oxidation-induced carbonyl production was probably an important cause for the reduced MTG cross-linking in MFP exposed to high concentrations of H_2O_2. Both the production and the consumption of carbonyls appeared to be at the expense of lysine and affected the MTG catalysis.

On the basis of the experimental evidence and the above analysis, a model that explains the MTG-catalyzed cross-linking of oxidatively modified MFP is proposed (Figure 6). This model, which emphasizes the availability (accessibility) of glutamine and lysine, encompasses three possible situations: (1) nonoxidized MFP, (2) mildly oxidized MFP, and (3) strongly oxidized MFP. Myosin is the focal point in this mechanism because it is the predominant protein in MFP responsible for the overall functionality of meat products, such as gelation, emulsification, and water-binding, and it is the general target for improvement by MTG.[6,8]

For nonoxidized MFP (Figure 6a), myosin assumes a three-dimensional rigid structure where most lysine residues and a small proportion of glutamine residues are located on or near the surface. When subjected to MTG, cross-linking reactions take place between these residues producing high MW polymers via isopeptide bonds (Figure 6b). Under mild oxidation conditions, such as 1 mM H_2O_2, myosin exists in a partially unfolded conformation (Figure 6c) where some initially occluded glutamine residues become exposed. Although a small portion of lysine residues is oxidized, the total amount of accessible lysine and glutamine by MTG is increased as compared to nonoxidized MFP. Therefore, the overall MTG cross-linking reaction is promoted. In the case of strong oxidation (e.g., 5 and 10 mM H_2O_2), most myosin is already aggregated mostly via disulfide bonds (Figure 6e),

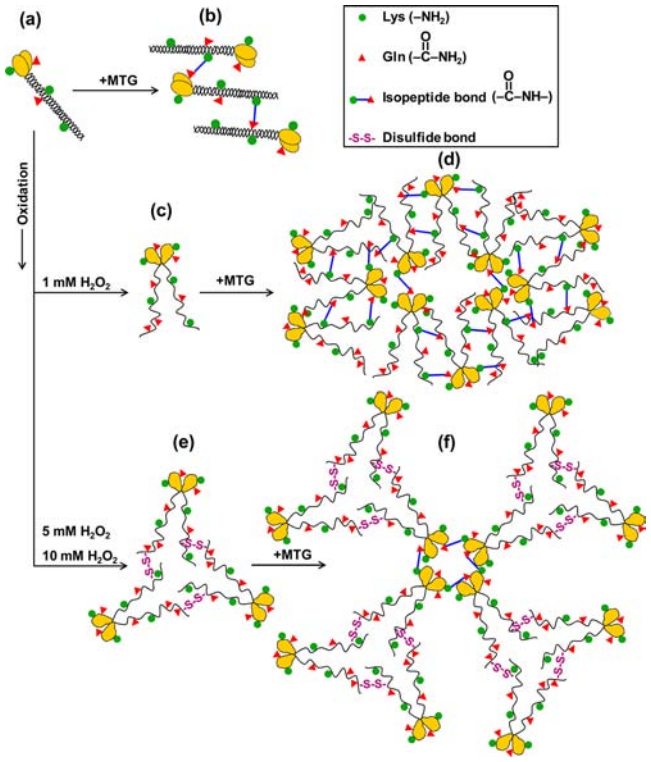

Figure 6. Proposed mechanism of MTG-mediated cross-linking of myosin in MFP as affected by oxidation. (a) Nonoxidized myosin, (b) nonoxidized myosin treated with MTG, (c) oxidized myosin at 1 mM H_2O_2, (d) oxidized myosin at 1 mM H_2O_2 treated with MTG, (e) oxidized myosin at 5 or 10 mM H_2O_2, and (f) oxidized myosin at 5 or 10 mM H_2O_2 treated with MTG.

presumably through tail−tail interactions.[17] Glutamine and lysine residues are reburied, becoming difficult to access because of steric hindrance, or damaged due to radical attack. Considering this tail−tail cross-linked structure as well as the greater distribution of glutamine and lysine residues (4.7 and 6.6%, respectively) in the rod subfragment than in the head (1.8 and 4.0%, respectively),[38] MTG cross-linking would only occur to the remaining glutamine and lysine residues that are limited in quantity and located principally at or near the head region of myosin (Figure 6f). It is noteworthy that according to the results from the free amine analysis, there was no appreciable difference in the cross-linking degree between the nonoxidized and the "highly" oxidized (10 mM H_2O_2) MFP samples (Figure 2), although the tendency did indicate more amine losses in the oxidized samples. Therefore, further studies that use more severe oxidation condition are desirable to validate the above hypothesis.

In conclusion, oxidation could change the susceptibility of MFP to MTG. Mild oxidation promoted MTG-mediated cross-linking reactions as compared to nonoxidation, while strong oxidation hampered the MTG effect. Understanding the efficacy of MTG in different oxidative status of the protein substrate would help material and ingredient selection for producing high-quality meat products. Raw meat that has been subjected to mild oxidizing conditions (storage, chopping, blending with salt, etc.) seems desirable as the ingredient for restructured products processed with MTG. However, significantly oxidized raw meat (long-time frozen storage, aerobic packaging, etc.) is obviously an undesirable candidate for MTG-processed products. Although MFP was most

responsible for the functionality of meat products, the oxidation on MTG treatment based on whole meat warrants further investigation since there are other proteins existing in the muscle, namely, sarcoplasmic and connective tissue proteins.

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Notes

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