OXIDATION DESENSITIZES ACTOMYOSIN TO PYROPHOSPHATE-INDUCED DISSOCIATION BUT FACILITATES MYOSIN CROSS-LINKING BY MICROBIAL TRANSGLUTAMINASE

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Abstract – Structural changes of myosin are a major consequence of muscle protein oxidation. This study tested the hypothesis that oxidative structural modification of myosin in myofibrils by oxidants could change the dissociation of actomyosin by pyrophosphate (PP) and the aggregation of myosin by microbial transglutaminase (TG). Myofibrils or actomyosin prepared from pork *Longissimus lumborum* (LL) were exposed to hydroxyl radicals (•OH) generated under meat processing conditions at 4°C up to 12 h. Time-dependent protein oxidation was evidenced by the progressive formation of carbonyls, loss of sulfhydryls, increased surface hydrophobicity, and decreased myosin K^+-ATPase activity. The protein side-chain and conformational changes led to a weaker binding of PP to myosin and a slow response of the intrinsic viscosity of oxidized actomyosin to PP-Mg^{2+}, indicating suppressed dissociation of actomyosin due to oxidation. However, such structural modifications (unfolding) promoted TG-catalyzed myosin cross-linking which was indicated by SDS-PAGE and the amine content analysis. In conclusion, under mild oxidative conditions, •OH-modified actomyosin was less susceptible to PP dissociation, but the myosin moiety became more readily accessible to TG cross-linking. Such information may help meat processors decide appropriate ingredient formulations based on the meat source to maximize the product quality.

Key Words – myofibrillar proteins, actomyosin, phosphate, hydroxyl radical.

I. INTRODUCTION

Protein oxidation occurs ubiquitously in processed meats due to vigorous processing procedures involved (chopping, blending, etc.), the incorporation of high concentrations of salts, and the presence of endogenous prooxidants. Among different oxidation initiators, oxygen radicals (e.g., •OH) generated in processing are intimately involved in the oxidative changes in proteins, notably myosin or actomyosin [1,2]. Reactive oxygen species are capable of converting amino acid side chain groups to various derivatives, including carbonyls, dityrosine, and disulfides. Moreover, oxidatively stressed muscle proteins are generally less stable and exhibit partially unfolded structures [3]. Actomyosin is the main component in intact myofibrils at low ionic strengths (< 0.15 M NaCl) and in swollen or partially disintegrated myofibrils at high ionic strengths (>0.5 M NaCl). When muscle is subjected to radical attack, both the myofibril assembly and the constituting proteins (myosin, actomyosin, etc.) will lose structural integrity [4]. In particular, myosin conformational changes could affect the efficacy of PP, which dissociates actomyosin. It could also impact the action of TG, which catalyzes Lys-Gln cross-linking through acyl transfer reactions. If this hypothesis proves valid, then it can be expected that during meat processing in the presence of phosphate and TG, oxidation of muscle proteins could affect protein solubility, gelation, emulsification, water-binding, and other related properties. This study was conducted to test the above hypothesis, and the ultimate goal was to provide a knowledge base for appropriate product formulations of high-quality muscle foods.

II. MATERIALS AND METHODS

Oxidation

Myofibrils were isolated from pork *Longissimus lumborum* (LL) muscle (48 h postmortem) using a buffer containing 10 mM sodium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EGTA, pH 7.0. Actomyosin was extracted from LL with the Weber's solution (0.6 M KCl, 0.01 M Na_2CO_3, 0.04 M $NaHCO_3$). Myofibrils and actomyosin were oxidized for 2-24 h at 4°C with •OH produced by 10 μM $FeCl_3$/100 μM ascorbate with 1, 5, and 10 mM H_2O_2 at pH 6.2.

Microbial transglutaminase (TG) treatment

Myofibrils were suspended in a 25 mM sodium phosphate buffer containing 0.6 M NaCl (pH 6.2) and incubated with TG (E:S = 1:20) at 4°C for 2 h. The reaction was terminated by adding 0.1% *N*-ethylmaleimide.

Physicochemical analyses

Protein carbonyls, surface hydrophobicity (S_o), sulfhydryls, disulfide bonds, free amines, and myosin K^+-ATPase were measured with the respective procedures detailed by Park et al. [3] and Liu et al. [4] to elucidate oxidation-induced protein structural changes. Protein cross-linking was detected by SDS-PAGE with a 10% acrylamide resolving gel under both reducing and non-reducing conditions.

PP binding and actomyosin dissociation

Actomyosin was reacted with 0.01–10 mM PP (+2 mM $MgCl_2$) in 0.5 M NaCl at pH 6.2 and 24°C for 10 min. PP binding capacity was measured by the centrifugation method of Kiely and Martonosi [5]. Non-reacted PP and liberated Pi were quantified. Actomyosin dissociation was determined by measuring the intrinsic viscosity ($[\eta]$) of actomyosin using a Cannon-Fenske-Ostwald viscometer [6] with the equation:

$$[\eta] = \frac{2.3\log(\eta_{rel})}{c} = \frac{2.3\log(\frac{t\rho}{t_0\rho_0})}{c}$$

where η_{rel} represents relative viscosity (dimensionless); t and ρ represent outflow time (s) and density (g/mL) of actomyosin solution, respectively; t_0 and ρ_0 represent outflow time and density of solvent (g/mL), respectively; c represents the protein concentration in g/dL.

Statistical analysis

Three independent trials (n = 3) were conducted. Data were subjected to analysis of variance using the general linear model's procedure. When a treatment effect was found significant, Tukey's HSD all-pairwise multiple comparisons was performed to identify significant differences between individual means.

III. RESULTS AND DISCUSSION

Structural characteristics

Exposures of myofibrils to •OH produced with 1, 5 and 10 mM H_2O_2 for 2 h resulted in 0.9, 1.2, and 1.5-fold increases ($P < 0.05$), respectively, in the carbonyl content (Table 1). The carbonyl production was more pronounced for the longer reaction time (24 h). Because carbonyl derivatives are generated from oxidative modification of amino acid side chain groups and peptide bond cleavage [7], they are a good indicator of oxidation-induced protein conformational changes. Surface hydrophobicity (S_o), which measures the extent of exposure of nonpolar groups resulting from protein unfolding, increased under the mild oxidative condition (1 mM H_2O_2 for 2 h). However, S_o generally declined when myofibrils were more extensively oxidized probably due to protein aggregation. As expected, the content of sulfhydryls (SH) decreased ($P < 0.05$) under all oxidizing conditions largely due to the conversion to disulfides (data not shown). The K^+-ATPase activity, which is located at the globular head of myosin (heavy meromyosin), was significantly reduced by •OH in the presence of 1 mM H_2O_2, irrespective of oxidation time, and the activity reached an almost undetectable level when the H_2O_2 concentration was raised to 5 mM or above. These biochemical changes were strong evidence of structural modification of myofibrillar proteins.

Table 1. Structural parameters of myofibrillar protein as affected by •OH stress.*

H_2O_2 (mM)	Oxid time (h)	Carbonyls (μmol/g protein)	Surface hydrophob.	Sulfhydryls (nmol/mg protein)	K^+-ATPase (μmol Pi/mg Prot./10min)
0	0	1.48e	56.6ab	79.6a	0.32a
1	2	2.80d	60.4a	74.0b	0.27a
	24	3.28bcd	46.5d	72.8b	0.26a
5	2	3.18cd	52.3bc	66.8c	0.04b
	24	4.24a	43.9de	61.0d	0.04b
10	2	3.63bc	47.8cd	61.4d	0.03b
	24	3.84ab	38.4f	58.6e	0.04b

*The •OH was produced in 10 μM $FeCl_3$, 100 μM Ascorbic acid, and 1, 5, and 10 mM H_2O_2) at 4°C for 2 or 24 h. Means within the same column sharing no common letter differ significantly ($P < 0.05$).

Oxidation effect on actomyosin dissociation by PP

The dissociation of the actomyosin complex by PP is through the mediation of Mg^{2+} that forms the MgPP salt [8]. Hence, 2 mM was added. As presented in Fig. 1, the viscosity of nonoxidized

actomyosin solution decreased rapidly with increasing PP concentrations. Because the split of actomyosin into constituting myosin and actin leads to reduced hydrodynamic radii (hence, lower shear resistances), the drop in the viscosity was indicative of actomyosin dissociation induced by PP [6]. The unique ability of PP to extract myosin from myofibrils or actomyosin was widely reported [9,10]. In contrast, the viscosity of oxidized actomyosin even in the absence of PP was low (1.82 dL/g) when compared with non-oxidized samples (2.24 dL/g). This was due to the formation of protein aggregates that reduced the effective concentration of hydrodynamic actomyosin molecules [1]. Disulfide bonds formed intermolecularly were a main contributor to the aggregation of actomyosin (Table 1). Because of oxidation, actomyosin was no longer sensitive to PP, and there was no appreciable change in viscosity over the PP concentration range (Fig. 1).

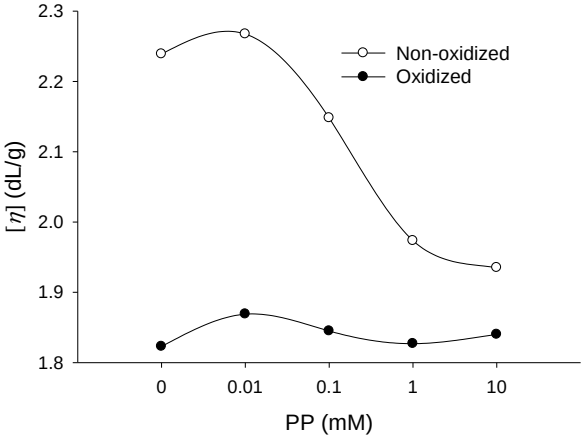

Fig. 1. Dissociation of non-oxidized and oxidized actomyosin (expressed as loss of intrinsic viscosity, [η]) at various concentrations of PP with 0.5 mM Mg^{2+}. Oxidation: 10 μM $FeCl_3$, 100 μM ascorbic acid, 1 mM H_2O_2, at pH 6.2 and 4°C for 6 h.

Oxidation effect on myosin cross-linking by TG

Lysine is one of the two reactants in the TG-catalyzed reaction. However, it is also one of the most susceptible amino acid residues to oxidation. Hence, the total amine content in •OH-stressed myofibrils before and after reaction with TG was analyzed. As shown in Fig. 2, oxidation by •OH generated with 1-10 mM H_2O_2 caused time-dependent steady losses (P < 0.05) of amines. The TG treatment resulted in significant further amine reductions, which was more pronounced in samples oxidized with 1 mM H_2O_2 than non-oxidized (P < 0.05). At this oxidant concentration, the extra amine loss due to TG was up to 16% when compared with the non-oxidized sample. The result suggested that structural changes (Table 1) in mildly oxidized myofibrillar protein promoted Lys-Gln cross-linking presumably due to increased accessibility of substrates.

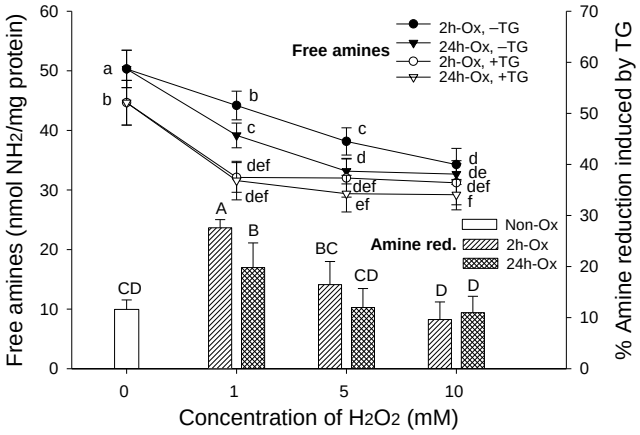

Fig. 2. Free amines in oxidized myofibrils before and after TG treatment, and the percent reduction attributed to the TG action. Oxidation: 10 μM $FeCl_3$, 100 μM ascorbic acid, 1-10 mM H_2O_2, at pH 6.2 and 4°C for 2 or 24 h. Means with different letters (a-f; A-D) differ significantly (P < 0.05).

SDS-PAGE was performed to offer physical evidence of cross-linking products. Of all myofibril constituents, myosin heavy chain (MHC) was most susceptible to •OH oxidation, showing 30% and 44% losses after 2 and 24 h oxidation, respectively (Fig. 3). This was mainly due to the oxidative conversion of sulfhydryls to disulfide bonds as the MHC band was almost completely recovered when treated with β-mercaptoethanol that cleaves the S-S bond (result not displayed). The results were consistent with the sulfhydryl analysis (Table 1) and in good agreement with previous reports [3,4]. The TG treatment caused further reductions in the MHC band from those by oxidation. While the TG-induced loss of MHC in non-oxidized sample was 8%, the total losses of the MHC due to combined TG treatment and oxidation for 2 and 24 h were 36 and 45%, respectively. Therefore, the •OH-initiated oxidation of myofibrils had a two-fold effect: it

caused damage to amines independently of TG, and it also unraveled the protein structure enabling an increased accessibility of TG to lysine and glutamine residues.

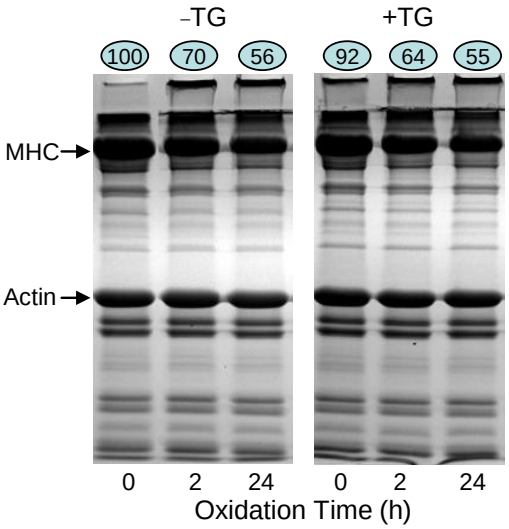

Fig. 3. SDS-PAGE of oxidized myofibrils before and after TG treatment under non-reducing conditions (no β-mercaptoethanol). The relative content of myosin heavy chain (MHC) is indicated by enclosed values. Oxidation: 10 μM $FeCl_3$, 100 μM ascorbic acid, 1 mM H_2O_2, at pH 6.2 and 4°C for 2 or 24 h.

IV. CONCLUSION

Mild hydroxyl radical oxidation under the condition similar to meat processing (4°C, pH 6.2) can modify amino acid side chains and alter the conformation of myofibrillar protein. Such structural changes decrease the efficacy of pyrophosphate to dissociate actomyosin but promote the catalysis of myosin cross-linking by TG. The results may explain why the efficacy of PP and TG often varies in meat processing.

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