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Biochemical Susceptibility of Myosin in Chicken Myofibrils Subjected to Hydroxyl Radical Oxidizing Systems

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Biochemical changes of myosin in chicken myofibrils exposed to nonenzymatic, hydroxyl radical generation systems (HRGS) were investigated by means of cross-linking reaction, ATPase activity, salt solubility, and 40% saturated ammonium sulfate (AS) extractability. HRGS treatment of myofibrils caused cross-linking of myosin heavy chains (MHC) via disulfide bonding, an increase in Ca-ATPase activity, and a decrease in K-ATPase activity, suggesting that thiol groups of myosin including those at the active site were modified. The specific changes depended on the concentrations of H_2O_2 in HRGS as well as the weight ratio of H_2O_2 to myofibrils. On the other hand, the decrease in salt solubility or AS extractability of myosin in HRGS-treated samples proceeded slowly when compared with the cross-linking reaction of MHC, indicating that considerable amounts of myosin biopolymers remained hydrophilic in the ionic solutions. The results demonstrated that initial cross-linking of MHC occurred inside the myosin molecule, and this was followed by progressive aggregation of myosin molecules through intermolecular cross-linking. Oxidation under the current experimental condition decreased the gel-forming ability of myofibrillar proteins, which coincided with the progress of the intra- and intermolecular cross-linking reactions as well as with ATPase activity changes.

KEYWORDS: Oxidation; myofibrils; myosin; cross-linking; aggregation; ATPase activity; thiol groups

INTRODUCTION

Oxidative damage of proteins and enzymes by reactive oxygen species has been demonstrated in numerous biomedical studies on living tissue under pathological or extraordinary physiological conditions as discussed in several reviews (*1*−*3*). Consistent with the observation in living tissues, similar oxidative changes have been noted in proteins from post-mortem muscle tissues, that is, meat. Using gel electrophoresis, several studies showed degradation as well as polymerization of myofibrillar proteins incubated with different model oxidation systems that closely resemble meat or processed meat conditions (*4*−*6*). Oxidative modifications have been found to alter muscle protein functionalities, including gelation, emulsification, viscosity, solubility, and water-holding capacity (*6*−*10*).

Recent studies about oxidation-induced chemical changes and their effects on functionality of chicken myofibrillar proteins have revealed that oxidation decreased myosin conformational stability, increased protein carbonyls, and caused myosin heavy chains to cross-link, resulting in a decrease in gel-forming ability of myofibrillar proteins (*11*−*13*). Furthermore, Tunhun et al. (*14*) showed that the gel-forming ability of fish myofibrillar proteins was decreased with the progress of cross-linking reactions of myosin that occurred during washing of minced muscle tissue. These findings suggested that oxidation of myofibrillar proteins occurring in processing and storage may have a significant impact on texture and binding strength of muscle foods, such as reconstructed meats, luncheon meats, and surimi-based products. Despite these prior studies, the behavior of muscle proteins under oxidative stresses remains poorly understood. In particular, the mechanism of oxidation-induced functionality changes of myofibrillar proteins is yet to be defined.

Myosin is largely responsible for gel formation of myofibrillar proteins (*15*), and the aggregation process of myosin is believed to be a critical factor contributing to gel formation (*16*). Further research is needed to elucidate oxidative impact on the conformation and biochemical characteristics of myosin in relation to changes in gel-forming ability. Information on oxidation-induced myosin aggregation and the reaction kinetics are of particular importance to the revelation of the mechanism of functionality changes for oxidatively stressed myofibrillar proteins.

The objective of the study was to determine the oxidative impact on myosin ATPase activity as well as the aggregation pattern of myosin in a myofibrillar protein preparation that was exposed to nonenzymatic, hydroxyl radical generation systems. The relationship between the oxidation-induced biochemical

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changes of myosin and the subsequent alteration in gel-forming ability of myofibrillar proteins was also examined.

MATERIALS AND METHODS

Preparation of Myofibrils. Myofibrils were prepared from commercial post-rigor chicken breast muscle according to the method of Katoh et al. (*17*). The chicken carcasses (36−48 h post-mortem), obtained from Tyson Foods, Inc. (Springdale, AR), were unfrozen. Isolated myofibrils were suspended in a Tris buffer containing 0.1 M KCl and 20 mM Tris-HCl, pH 7.5, and the myofibril stock suspension stored at 0 °C was used within 10 days. Preliminary assays indicated negligible changes in the biochemical properties of myosin or in the gel-forming ability of the myofibril suspension within the storage time.

Oxidation of Myofibrils. Myofibrils were subjected to oxidation by nonenzymatic, hydroxyl radical generation systems (HRGS) consisting of 0.1 mM ascorbic acid, 0.01 mM $FeCl_3$, and various concentrations of H_2O_2 (0.05−10 mM) at 0 °C for 18 h. In the experiment designed to follow the time course of the biochemical changes, oxidation was carried out for up to 24 h and was terminated by adding 0.1 mM EDTA at appropriate time intervals. The biochemical changes of myosin subjected to oxidation by HRGS were irreversible, and the reduction by EDTA−iron complex was not observed (data not shown).

Cross-linking of Myosin Heavy Chains. Myofibrils exposed to HRGS were submitted to SDS-PAGE (*18*) using a 10% polyacrylamide gel in the presence or absence of 5% 2-mercaptoethanol (ME). When ME was not used, *N*-ethylmaleimide (0.5 mM) was added to the SDS-PAGE samples to prevent disulfide artifacts. To follow the progress of the cross-linking reaction of myosin heavy chains (MHC) by oxidation, the relative amounts of MHC on SDS-PAGE gels were determined by densitometric scan (Ultroscan XL, Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

ATPase Assay. Myosin ATPase was assayed in the medium of 25 mM Tris−maleate, pH 7.0, 1 mM ATP, and 0.2 mg/mL myofibrillar proteins in the presence of 0.5 M KCl and 5 mM $CaCl_2$ (for Ca-ATPase) or 1.0 M KCl and 5 mM EDTA (for K-ATPase) at 25 °C by calorimetrically measuring the liberated inorganic phosphate, as described by Katoh et al. (*17*).

Determination of the H_2O_2 to Myofibrils Ratio Effect. To identify whether the relationship between the amount of H_2O_2 and the extent of biochemical changes in myosin was influenced by the protein concentration, myofibril samples at different concentration levels were exposed to HRGS containing various concentrations of H_2O_2, and the oxidized samples were subsequently subjected to SDS-PAGE and K-ATPase assay. To establish a range of H_2O_2/myofibrils ratios, 3.4, 6.7, and 13.4 mg/mL of myofibrils and 0.1−0.4 mM H_2O_2 were used for SDS-PAGE and 3.0, 6.0, and 11.9 mg/mL of myofibrils and 0.5−5 mM H_2O_2 were used for K-ATPase. The weight ratios were estimated from both concentrations of H_2O_2 and myofibrils in the reaction mixture. The amounts of un-cross-linked myosin and K-ATPase activities of oxidized samples were analyzed in connection with H_2O_2/myofibrils weight ratio in the systems.

Salt Solubility and 40% Saturated Ammonium Sulfate Extractability of Myosin. The concentration of KCl in the myofibril stock suspension was raised to 0.5 M by adding 2.0 M KCl containing 20 mM Tris-HCl, pH 7.5, and the solution (1 mg/mL) was kept at 0 °C for 2 h. ATP-Mg (1 mM) was then added to the solution, which was immediately centrifuged at 5000*g* at 0 °C for 30 min to separate salt-soluble fractions (supernatants) from the insolubles. Ammonium sulfate fractionation of myofibrils was carried out in the presence of 0.5 M KCl, 1 mM ATP-Mg, and 40% saturated ammonium sulfate. The supernatants were also collected by centrifugation under the same condition. These fractions were submitted to SDS-PAGE with a 10% polyacrylamide gel. In the case of 40% saturated ammonium sulfate extractable fraction, protein was precipitated by 7.5% trichloroacetic acid. The precipitation was washed by acetone to remove ammonium sulfate and then dissolved into 8 M urea with 2% SDS and 2% ME before submission to SDS-PAGE. The bands of MHC on the SDS-PAGE gel were densitometrically scanned to establish the relative amounts.

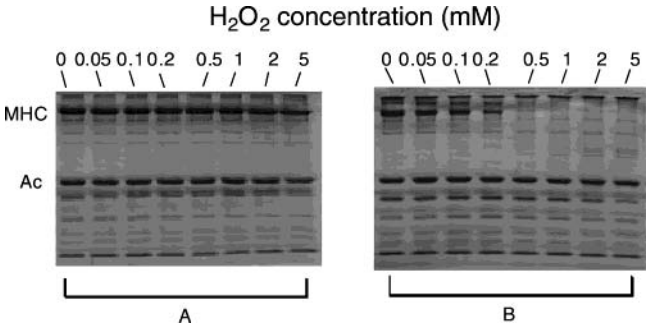

Figure 1. Changes in SDS-PAGE pattern of myofibrils due to oxidation by HRGS. Myofibrils treated with HRGS containing various concentrations of H_2O_2 were subjected to SDS-PAGE (10% polyacrylamide gel) in the presence (**A**) or absence (**B**) of 5% 2-mercaptoethanol. MHC, myosin heavy chains; Ac, actin.

Dynamic Rheological Measurements. Myofibrils exposed to HRGS were subjected to nondestructive, oscillatory measurements during gelation using a model VOR rheometer (Bohlin Instruments, East Brunswick, NJ) (*19*) after removal of HRGS by washing with 0.1 M NaCl/20 mM Tris−maleate, pH 6.0. Thermal gelation was induced by heating the protein sample (15.8 mg/mL in 0.5 M NaCl, 20 mM Tris−maleate, pH 6.0), which was placed between two parallel plates, from 20 to 83 °C at a 1 °C/min heating rate. During heating, the sample was simultaneously sheared at a fixed frequency of 100 mHz with a maximum strain of 0.02. Dynamic rheological properties of the samples were described in terms of shear storage modulus (G', the elastic component of the gel).

RESULTS AND DISCUSSION

Cross-linking Reaction of Myosin Heavy Chains. There was no apparent difference in SDS-PAGE pattern between oxidized myofibril samples in the presence of ME (**Figure 1A**). However, when the oxidized samples were not treated with the reducing agent, they showed a loss in MHC band, which was intensified at increasing H_2O_2 concentration, with a total diminishment of the MHC band at >0.5 mM H_2O_2 (**Figure 1B**). MHC migrated as a high molecular weight aggregate that did not penetrate the top of the gel under these conditions. These results were in accordance with a previous finding (*11*), confirming that MHC cross-linked through disulfide bonds by oxidation. However, fragmentation of myosin as reported previously (*11*) was not observed under the present oxidative condition. This may be due to the lower concentrations of H_2O_2 employed in our present study.

ATPase Activity Changes. Sekine and Yamaguchi (*20*) reported that two reactive thiol groups located at the active site of myosin were responsible for Ca- and K-ATPase and the blocking of them by an SH reagent such as *N*-ethylmaleimide caused ATPase activity changes. Therefore, oxidation of the thiol groups at the active site of myosin can be detected by monitoring Ca- and K-ATPase changes. The HRGS treatment of myofibrils markedly enhanced the Ca-ATPase activity. The activity reached a maximum at 0.5 mM H_2O_2; a further increase in H_2O_2 concentration caused a slight decrease of the activity (**Figure 2A**). On the other hand, K-ATPase activity was gradually lowered with increasing H_2O_2 concentrations (**Figure 2B**). These ATPase changes were strikingly similar to those observed when myosin was treated with *N*-ethylmaleimide (*20*−*22*), suggesting that the thiol groups at the myosin active site were modified by oxidation.

Effect of H_2O_2 to Myofibril Weight Ratio. The amounts of un-cross-linked MHC decreased with an increase in the weight ratio of H_2O_2 to myofibrils (**Figure 3A**). However, within

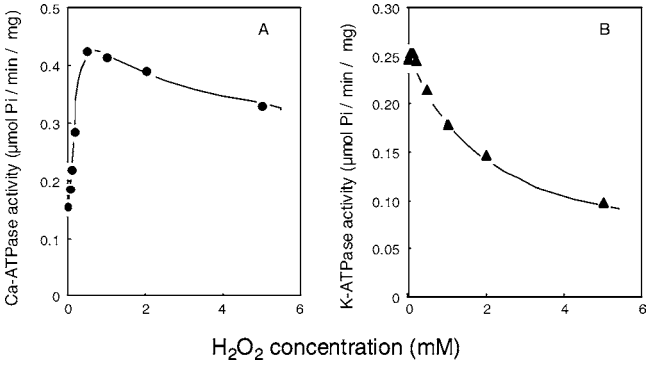

Figure 2. Changes in ATPase activity due to oxidation of myofibrils by HRGS. Ca-ATPase (**A**) and K-ATPase (**B**) activities of myofibrils oxidized under the same conditions as in **Figure 1** were assayed.

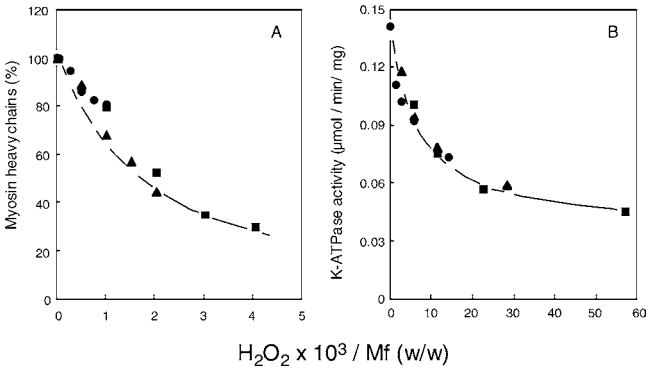

Figure 3. Effect of weight ratio of H_2O_2 in HRGS to myofibrils on cross-linking of myosin heavy chain (**A**) and on myosin K-ATPase decay (**B**). The oxidizing systems for myosin cross-linking included 13.4 mg/mL (●), 6.7 mg/mL (▲), and 3.4 mg/mL (■) myofibrils (Mf) and 0.1–0.4 mM H_2O_2, whereas those for K-ATPase included 11.9 mg/mL (●), 6.0 mg/mL (▲), and 3.0 mg/mL (■) myofibrils and 0.5–5 mM H_2O_2.

the three different protein concentrations (3.4, 6.7, and 13.4 mg/mL), the amounts MHC remaining un-cross-linked were essentially the same at equal weight ratios of H_2O_2 to myofibrils. Similarly, identical ($P < 0.05$) K-ATPase activities were obtained at the same weight ratios of H_2O_2 to myofibrils within the three samples (3.0, 6.0, and 11.9 mg/mL protein) (**Figure 3B**). These results indicate that the biochemical changes involved in the myosin head (subfragment 1) depended on the H_2O_2/myofibrils weight ratio. The amounts of H_2O_2 required to induce cross-linking reaction of MHC were ~10 times smaller than those for K-ATPase decay.

Time Course of Biochemical Changes. In the experiments described above, myofibrils were oxidized by HRGS for a fixed time (18 h). The time dependency of myosin oxidation was subsequently followed by using HRGS containing 1 mM H_2O_2. Cross-linking reaction and ATPase changes of myosin by oxidation were strongly suppressed by dilution of H_2O_2 to <0.2 mM and the addition of 0.1 mM EDTA (data not shown). Accordingly, oxidation of myofibrils was quenched by dilution of H_2O_2 concentration and adding 0.1 mM EDTA at appropriate time intervals. As depicted in **Figure 4**, the cross-linking reaction of MHC via disulfide bond (**Figure 4A**), the elevation in Ca-ATPase activity (**Figure 4B**), and the loss of K-ATPase activity (**Figure 4C**) were all enhanced with oxidation time, confirming that the effect of hydroxyl radical was time dependent. The cross-linking reaction of MHC and the Ca-ATPase changes proceeded more rapidly than the K-ATPase decay.

Salt Solubility and 40% Saturated Ammonium Sulfate Extractability. The aggregation process of myosin is closely related to thermal gelation of myofibrillar proteins (*16*). Hence, we investigated the cross-linking process of myosin by oxidation in connection with myosin aggregation by means of its salt solubility and AS extractability. The SDS-PAGE analysis revealed an abrupt loss of the MHC band in both the whole myofibrils and the salt-soluble fraction when exposed to 0.5 mM H_2O_2, which was recovered to a large extent after treatment with 5% ME (result not shown). Similarly, the AS extract of oxidized myofibrils, which was relatively homogeneous in myosin bands and was devoid of actin, exhibited a diminishment of MHC with increasing H_2O_2 concentrations (result not shown).

To obtain quantitative information about the specific changes in myosin in the three sample fractions, the relative intensity of the MHC band from the SDS-PAGE patterns of each fraction was determined by densitometric scan, and the results were plotted against the H_2O_2 concentration. As illustrated in **Figure 5**, the susceptibility of myosin to oxidation is H_2O_2 dose-dependent. For example, when myofibrils were treated with HRGS containing 10 mM H_2O_2, essentially all myosin molecules were cross-linked, mostly via disulfide bonds (90% recovery with ME) (open and solid circles). These myosin oligomers or aggregates, along with myosin monomers, made up ~78% of the total myosin that remained soluble in salt solution after oxidation with 10 mM H_2O_2 (solid triangles). On the other hand, the 40% AS extract retained 45% myosin after oxidation with 10 mM H_2O_2 (solid squares). Salt solubility reflects the extent of myosin aggregation (*23*). Furthermore, Kato et al. (*24*) reported that only monomeric myosin was extracted with AS. Hence, the results would indicate that intramolecular cross-linking of MHC occurred initially, and this was probably followed by intermolecular cross-linking, which led to progressive aggregation of myosin molecules.

Dynamic Rheological Properties. To establish a relationship between the extent of myosin oxidative modification and gel-forming ability of myofibrillar proteins, myofibril samples oxidized by HRGS were subjected to dynamic rheological measurement. As reported previously (*19*), G', a measure of the elasticity of a gel network, of nonoxidized myofibrils reached a peak at ~50 °C and then declined temporarily, reflecting structural changes in myosin that led to a shift in intra- and intermolecular forces (*25*) (**Figure 6**). A further elevation in the heating temperature markedly increased G'. Oxidation of myofibrils by HRGS decreased both the peak G' value at ~50 °C (G'_{50}) and the final one at 83 °C (G'_{83}), and the effect was H_2O_2 concentration-dependent.

The plots of both storage moduli against the H_2O_2 concentration showed that, in myofibrils treated with HRGS containing 2 mM H_2O_2, the G'_{50} value dropped to ~20% of that of nonoxidized myofibrils (**Figure 7A**). This change coincided with the process of cross-linking reaction of MHC as well as with the Ca-ATPase activity change, suggesting that intramolecular modification of thiol groups was involved in the decrease in G'_{50}. On the other hand, G'_{83} dropped to ~50% of that of nonoxidized myofibrils (**Figure 7B**), which was in concert with the decrease in AS extractability of myosin (i.e., conversion of myosin monomers to polymers), indicating that intermolecular aggregation of myosin played a role in the reduction of G'_{83}. Interactions between myosin and actin in heating processes were postulated to be a main contributing factor to G'_{50} because it was less pronounced in the rheogram of purified myosin gels (*12*, *19*). Therefore, oxidation of myosin might modify the actin−myosin interaction in the heating process. However,

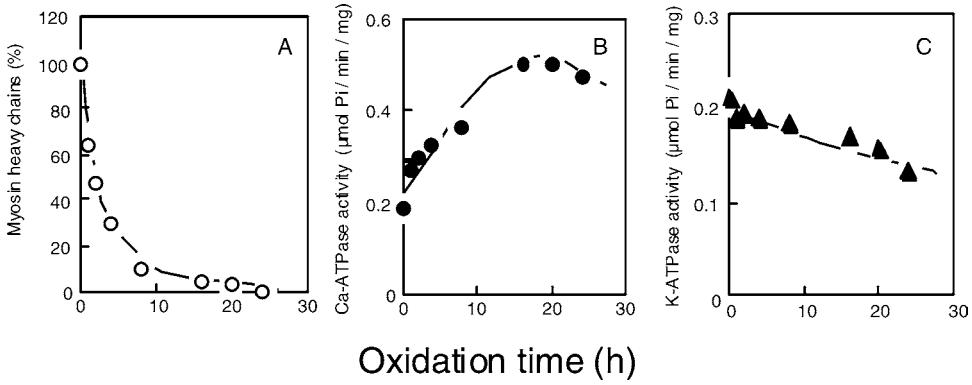

Figure 4. Time course of oxidation of myosin in myofibrils exposed to HRGS. Myofibrils were exposed to HRGS containing 1 mM H_2O_2. At appropriate time intervals, oxidation was terminated by dilution of H_2O_2 concentration and addition of 0.1 mM EDTA. The amount of un-cross-linked MHC (**A**) and the Ca-ATPase (**B**) and K-ATPase (C) activities were subsequently measured.

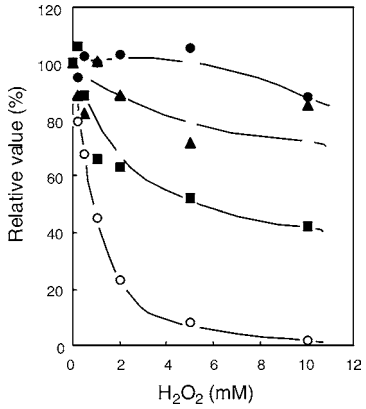

Figure 5. Changes in amounts of salt-soluble and 40% saturated ammonium sulfate extractable myosin due to oxidation of myofibrils by HRGS. The salt-soluble myosin (▲), 40% saturated ammonium sulfate extractable myosin (■), and whole myosin detected in the presence (●) or absence (○) of ME are plotted.

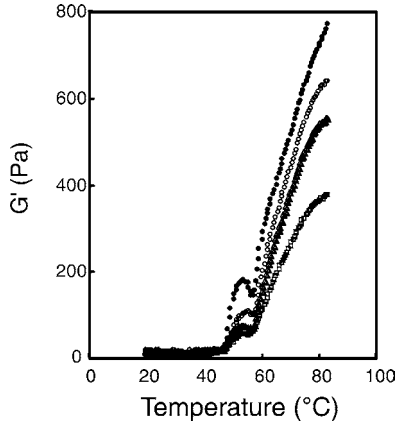

Figure 6. Oxidatively induced changes in thermal gelling properties (expressed as storage modulus, G') of myofibrillar protein. Myofibrils oxidized by HRGS containing 0.25 mM (○), 0.5 mM (▲), or 2 mM (□) of H_2O_2 were subjected to dynamic rheological measurement after removal of HRGS by washing with 0.1 M KCl and 20 mM Tris–maleate, pH 6.0. Dynamic viscoelastic properties of nonoxidized (control) myofibrils (●) are also presented.

further study is needed to investigate the heat-induced denaturation process of oxidized myosin in the presence or absence of actin.

Conclusion. Results from this study suggested that the initial oxidation-induced changes in myosin included intramolecular

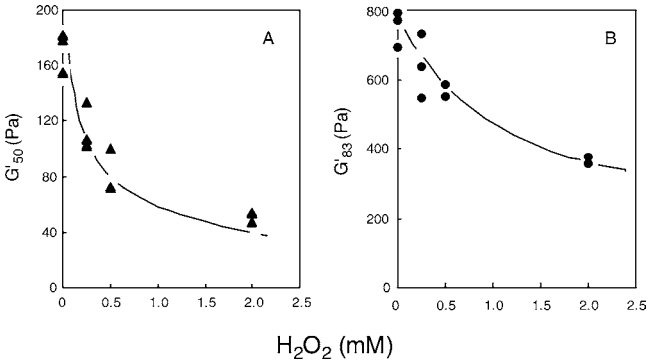

Figure 7. Plots of peak shear storage moduli of oxidized myofibrils at 50 °C (G'_{50}, **A**) and at the final heating temperature, 83 °C (G'_{83}, **B**) as a function of H_2O_2 concentrations in HRGS.

cross-linking of MHC and modifications of thiol groups at the myosin ATPase active site. These reactions were followed by intermolecular cross-linking leading to progressive aggregation of myosin molecules. The results also demonstrated that these chemical processes were involved in functionality changes in oxidatively stressed myofibrillar proteins, which may be implicated in texture characteristics and binding strength of processed muscle foods.

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