Electrophoretic Pattern, Thermal Denaturation, and in Vitro Digestibility of Oxidized Myosin†

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Physicochemical changes and in vitro digestibility of chicken breast myosin oxidized with a nonenzymic free-radical-generating system ($FeCl_3/H_2O_2$/ascorbate) were studied by SDS−PAGE, differential scanning calorimetry, and *o*-phthaldialdehyde assay. Oxidation caused fragmentation and polymerization of myosin. Myosin polymers were cross-linked mainly through disulfide bonds. Hydroxyl radicals destabilized myosin, lowering its denaturation temperature by up to 4 °C. Oxidized myosin also produced a new thermal transition in the 60−80 °C temperature range, which could be attributed to the formation of disulfide-stabilized polymers. The proteolytic susceptibility of myosin to pepsin, trypsin, and chymotrypsin was increased by oxidation. Under nonreducing conditions, however, oxidized myosin showed decreased digestibility. The results may help explain variations in the functionality and nutritional quality of muscle foods in meat processing in which oxidation is involved.

Keywords: *Oxidation; myosin; electrophoresis; thermal denaturation; digestibility*

INTRODUCTION

Muscle proteins not only are responsible for textural properties of meat products but also serve as an important source of essential amino acids for humans. Recent studies have shown that muscle proteins, like other muscle components such as lipids and pigments, are vulnerable to oxidative attack during the processing and storage of muscle foods (Martinaud et al., 1997; Wang et al., 1997). Oxidative modifications often lead to alterations in muscle protein functionalities, including gelation, emulsification, viscosity, solubility, and water-holding capacity (Smith, 1987; Decker et al., 1993; Wan et al., 1993; Xiong et al., 1993; Kelleher et al., 1994; Srinivasan and Hultin, 1997; Wang and Xiong, 1998).

The susceptibility of muscle foods to oxidative processes stems from their relatively high concentrations of unsaturated lipids, heme pigments, metal catalysts, and various other oxidizing agents (Johns et al., 1989). Oxidation is also facilitated by processes such as comminuting and grinding, which introduce molecular oxygen and mix oxidants with susceptible meat components (Xiong and Decker, 1995). Oxidizing agents present in muscle tissues are capable of generating a number of reactive oxygen species (ROS), which include free radicals, such as the hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), and thiyl radical ($RS^{\bullet}$), as well as nonradical oxygen derivatives, such as H_2O_2, singlet oxygen, 4-hydroxy-2-nonenal, and ketoamines (Stadtman and Berlett, 1997). γ-Irradiation, a newly approved process for pasteurizing fresh meat and poultry, is notorious for its ability to produce $^{\bullet}OH$ and $O_2^{\bullet-}$ by γ-radiolysis of water (Swallow, 1960). Direct damage of proteins by ROS has been well established in numerous biomedical studies that involve living tissues under pathological or extraordinary physiological conditions, as discussed in several recent reviews (Stadtman, 1993; Dean et al., 1997; Stadtman and Berlett, 1997). Polypeptide backbone and many amino acid residue side chains are vulnerable to oxidative attack. Among the common changes in oxidized proteins are destruction of amino acids, unfolding, fragmentation, and cross-linkage of proteins, and formation of protein carbonyls. The oxidative modifications usually result in increased susceptibility of the proteins to digestive enzymes, which is important for preventing toxin buildup in living cells (Levine, 1989; Stadtman, 1990; Agarwal and Sohal, 1994).

Consistent with the observations in living tissues, similar oxidative changes have been noted in proteins from postmortem muscle tissues, that is, meat. Li and King (1996) reported considerable increases in the surface hydrophobicity of myosin oxidized by an Fe(II)/ascorbate system. Using gel electrophoresis, several studies showed degradation and polymerization of myofibrillar proteins incubated with different model oxidation systems closely resembling meat or processed meat conditions (Decker et al., 1993; Martinaud et al., 1997; Srinivasan and Hultin, 1997). Kamin-Belsky et al. (1996) demonstrated a decreased digestibility of H_2O_2/hemin-cross-linked myosin. In a more recent study, it was shown that oxidized myoglobin could form long-lived radicals that were capable of causing oxidation of many other proteins naturally present in the muscle cell (Irwin et al., 1999). Despite these published studies, many questions regarding the behavior of muscle proteins under oxidative stresses are yet to be answered. For instance, studies have shown that oxidative changes in muscle proteins result in loss of functional and nutritional quality of finished food products [for a review, see Xiong (1996)], but several recent investigations indicate that mild oxidative modification of myo-

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fibrillar proteins could facilitate protein gelation (Srinivasan and Xiong, 1996) and emulsification (Srinivasan and Hultin, 1997). The conflicting results suggest that the oxidative effect on muscle proteins may be extremely complex, subject to various enzymic and nonenzymic oxidative factors either inherent to muscle or introduced through meat processing. Clearly, more detailed and fundamental research must be conducted to elucidate the exact mechanism of muscle protein oxidation and its implications for the processing and nutritional quality of muscle foods. The objective of this study was to examine structural changes in oxidatively modified myosin, the most predominant myofibrillar component, by measuring its electrophoretic pattern, thermal denaturation properties, and in vitro digestibility after exposure to a free-radical-generating system.

MATERIALS AND METHODS

Myosin Isolation. Myosin was isolated from two batches (replicates) of chicken pectoralis muscle immediately after sacrifice, according to the procedure described by Wang and Smith (1994). Ground muscle was extracted with Guba-Straub solution (0.3 M KCl, 0.1 M KH_2PO_4, 50 mM K_2HPO_4, 1 mM EDTA, 4 mM sodium pyrophosphate), followed by precipitation in 1 mM EDTA, 35%-48% $(NH_4)_2SO_4$ fractionation, and dialysis against 50 mM sodium phosphate buffer (pH 6.5) containing 0.6 M NaCl. Magnesium chloride and sodium pyrophosphate were used to dissociate actomyosin as described by Wang and Smith (1994). The resulting myosin solution was dialyzed against 25 mM sodium phosphate buffer (pH 6.5). The final myosin pellet was collected by centrifugation at 20000*g* for 30 min at 4 °C.

The protein concentration of the myosin pellet was measured according to the biuret method (Gornall et al., 1949) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as standard.

Myosin Oxidation. Purified myosin (40 mg/mL protein), after being treated with or without 10 mM *N*-ethylmaleimide (NEM; a thiol-blocking agent), was oxidized by incubation with 0.1 mM $FeCl_3$, 20 mM H_2O_2, and 1 mM ascorbate in 25 mM sodium phosphate buffer (pH 6.5) at 2 °C for up to 24 h.

Differential Scanning Calorimetry (DSC). Thermal denaturation of oxidized and nonoxidized myosin (40 mg/mL protein in 25 mM sodium phosphate buffer, pH 6.5) was measured by standard DSC. Myosin samples (14−17 mg) were accurately weighed into aluminum pans and hermetically sealed. DSC was performed on a model 2920 differential scanning calorimeter (TA Instruments, Inc., New Castle, DE). Samples were thermally scanned from 10 to 95 °C at 10 °C/min. An empty, hermetically sealed aluminum pan was used as reference. Myosin samples exhibited one or two thermal transitions, and the maximum transition temperature (T_m), which corresponds to 50% denaturation of the protein(s) involved in the transition, was recorded. Enthalpy of thermal transition (ΔH) was expressed in joules per gram of protein. All DSC scanning was performed on at least triplicate samples.

In Vitro Digestibility. Oxidized and nonoxidized myosin samples (2 mg/mL protein) were digested by gastric (10 units/mL pepsin at pH 1.8) and pancreatic (10 units/mL trypsin and 0.1 unit/mL chymotrypsin, both at pH 8.0) enzymes for 1 h at 37 °C. Myosin samples without added proteases (blanks) were incubated under the same condition. Propyl gallate (dissolved in absolute ethanol) with a final concentration of 0.01% was added to myosin samples prior to digestion to terminate oxidation by scavenging free radicals. Enzyme action was stopped by adjusting reaction mixtures to neutral pH for pepsin and by adding 0.2 mM phenylmethanesulfonyl fluoride (a protease inhibitor) for trypsin and chymotrypsin.

Quantitative determination of in vitro digestibility was conducted by reacting the digestion mixtures with *o*-phthaldialdehyde (OPA) according to the method of Church et al. (1983). The OPA reagent was freshly prepared (20 min prior to use) by mixing the following reagents and diluting to a final volume of 50 mL with distilled water: 25 mL of 100 mM sodium tetraborate (pH 9.0), 2.5 mL of 20% (w/w) sodium dodecyl sulfate (SDS), 40 mg of OPA (dissolved in 1 mL of methanol), and 100 μL of β-mercaptoethanol. A 50-μL aliquot of the digestion mixture was added to 1.0 mL of OPA reagent, and the assay solution was incubated for 2 min at room temperature. The absorbance at 340 nm was then measured. Glycine was used to construct the standard curve. The amount of α-amino groups (millimolar NH_2 per milligram of protein) generated from digestion was calculated by subtracting the NH_2 content of nondigested myosin sample (blank) from that of digested sample.

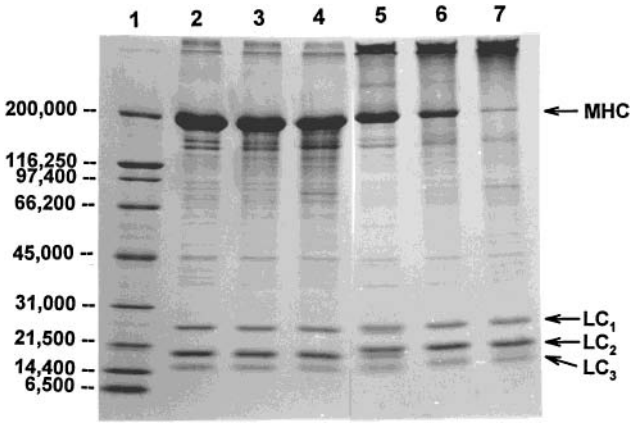

Figure 1. SDS−PAGE pattern of chicken breast myosin incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time. Electrophoresis of samples was performed on a 5−20% gradient gel in the presence (lanes 2−4) and absence (lanes 5−7) of 5% β-mercaptoethanol: (lane 1) molecular mass standard; (lanes 2 and 5) nonoxidized myosin; (lanes 3 and 6) myosin oxidized for 1 h; (lanes 4 and 7) myosin oxidized for 24 h. LC_1, LC_2, and LC_3 denote myosin light chains 1, 2, and 3, respectively.

Electrophoresis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) of oxidized and protease-digested myosin was run to qualitatively determine myosin polymerization, scission, and in vitro digestibility, using the method of Laemmli (1970) with modifications. A stacking gel with 3% acrylamide and a gradient resolving gel with 5−20% acrylamide were used. SDS−PAGE samples were prepared with and without 5% β-mercaptoethanol. For samples without β-mercaptoethanol, 1 mM NEM was added to prevent possible formation of disulfide cross-linkage during sample preparation.

Statistical Analysis. Data were analyzed using the general linear model procedure of the Statistix 3.5 software package (Statistix 3.5, Analytical Software Inc., St. Paul, MN). Analysis of variance (ANOVA) was conducted to determine the significance of main effect (oxidation). Significant ($P < 0.05$) differences between means were identified using the least significant difference procedure (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

SDS—PAGE of Oxidized Myosin. Oxidation altered the electrophoretic pattern of myosin. Compared to control (nonoxidized myosin), the oxidized samples in the presence of β-mercaptoethanol (a disulfide bond breaking agent) showed decreased band intensity of myosin heavy chain (MHC) (Figure 1, lanes 2−4). Simultaneously, new peptide bands with lower molecular masses, mostly in the range of 97−200 kDa, appeared, indicating that oxidation caused fragmentation of myosin. A number of studies have shown that reactive oxygen species (in this case, $^{\bullet}OH$ generated from $FeCl_3/H_2O_2$/ascorbate) can cause a wide variety of reactions on protein molecules, including modification of amino acids, fragmentation, and aggregation (Davies,

1987; Davies et al., 1987a; Uchida et al., 1992). According to Stadtman and Berlett (1997), fragmentation of protein is a consequence of direct attack by •OH on the polypeptide backbone or on the side chains of glutamyl or prolyl residues, whereas protein aggregates can be formed through disulfide cross-linkages, Schiff base adducts, or formation of carbon−carbon covalent bonds by the interaction of carbon-centered radicals in different protein molecules. It was clearly shown in this experiment that myosin molecule from post-mortem muscle was vulnerable to free radical attack, resulting in scission of peptide chains. Non-disulfide cross-linkages of myosin, which could be induced by oxidation, were not obvious in this study. Similar to our observations, the study by Martinaud et al. (1997) indicated formation of myosin degradation products in myofibrillar protein model systems subjected to different oxidative conditions. On the contrary, SDS−PAGE results of •OH-oxidized myofibrillar proteins reported by Srinivasan and Hultin (1997) did not show such degradation products.

Oxidized myosin samples without the β-mercaptoethanol treatment exhibited a much more dramatic reduction in MHC band intensity than samples treated with β-mercaptoethanol (Figure 1, lanes 5−7). After incubation with the oxidants for 24 h, the MHC band essentially disappeared. On the other hand, oxidation increased the formation of numerous high molecular weight protein bands, which appeared as smears or dark stains at the top of the separating gel. However, formation of protein fragments was less evident than that observed in oxidized myosin samples with β-mercaptoethanol. Apparently, oxidation caused disulfide cross-linkage (dissociable by β-mercaptoethanol) of myosin to form polymers. The myosin molecule has ~42 sulfhydryl groups, many of which can be readily accessed by chemical reagents (Hofmann and Hamm, 1978). Upon exposure to hydroxyl radicals, the sulfhydryl groups of MHC would be oxidized to form intermolecular disulfide bonds. Thus, oxidation not only caused fragmentation of MHC but also cross-linking of MHC, producing oligomers or polymers. This would account for the observation of more MHC loss in the oxidized myosin without β-mercaptoethanol. The relative absence of myosin fragments immediately below 200 kDa in the samples without β-mercaptoethanol can be explained by disulfide cross-linking of the fragments to form high molecular mass (>200 kDa) polymers. Bhoite-Solomon et al. (1992) reported that H_2O_2 alone could also cause myosin to form disulfide-cross-linked aggregates but did not induce fragmentation of myosin. Therefore, in our study, oxidation-induced polymerization of myosin or its fragments could be due to the actions of both H_2O_2 and •OH, whereas degradation of myosin was probably caused primarily by •OH.

More supportive evidence of the major involvement of disulfide bonds in the oxidation came from myosin samples treated with 10 mM NEM prior to oxidation (Figure 2). In the presence of β-mercaptoethanol, oxidized NEM-treated myosin (Figure 2, lanes 2−4) showed loss of MHC and formation of fragments similar to myosin samples not treated with NEM (Figure 1, lanes 2−4). Note that there was a slight formation of myosin polymers cross-linked through non-disulfide covalent bonds, which was not shown in myosin samples not treated with NEM. The subtle discrepancy between the two observations could be due to the competitive nature

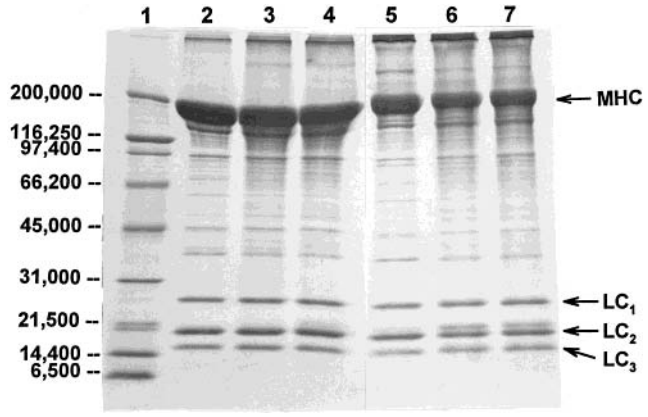

Figure 2. SDS−PAGE pattern of chicken breast myosin incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time. Myosin samples were treated with 10 mM NEM prior to oxidation. Electrophoresis of samples was performed on a 5−20% gradient gel in the presence (lanes 2−4) and absence (lanes 5−7) of 5% β-mercaptoethanol. Labels are the same as in Figure 1.

of different mechanisms of polymerization. Cysteine is perhaps the most susceptible amino acid residue and is usually one of the first to be oxidized (Dean et al., 1997). Therefore, for samples not treated with NEM, myosin polymers were formed mainly through disulfide bonds. When sulfhydryl groups were blocked by NEM, the tendency to form polymers through other covalent bonds became prevalent. One example would be dehydroascorbate-mediated cross-linking. Because ascorbate was used in the free-radical-generating system, its oxidized form, dehydroascorbate (which is a dialdehyde), could act as a bifunctional reagent to cross-link proteins through the formation of Schiff adducts with ϵ-NH_2 groups (Pischetsrieder, 1996; Butterfield and Stadtman, 1997). Even so, there were not many myosin polymers formed through non-disulfide mechanisms, suggesting these pathways were inherently more difficult to follow.

Previous studies using the H_2O_2/hemin or H_2O_2/myoglobin oxidizing system indicated that oxidized myosin formed a substantial amount of polymers cross-linked by non-disulfide covalent bonds (Bhoite-Solomon et al., 1992; Hanan and Shaklai, 1995). In the case of H_2O_2/hemin, myosin aggregates were suggested to result from pairing of myosin radicals formed by the H_2O_2-induced ferryl iron state in free hemin. As to H_2O_2/myoglobin, which contained tyrosine radicals on the globin, cross-linkage of myosin was probably due to intermolecular coupling of myosin tyrosine radicals generated through electron transfer between surface tyrosines of myosin and myoglobin. In our present study, in which •OH was generated from metal-catalyzed oxidation, it appeared that myosin was oxidized via a pathway different from these two.

As expected, in the absence of β-mercaptoethanol, NEM-treated myosin (Figure 2, lanes 5−7) exhibited less MHC loss than its counterpart (Figure 1, lanes 5−7) because most (if not all) of the sulfhydryl groups were already tied up with NEM. Also, more peptide fragments were observed in NEM-treated myosin samples compared to nontreated samples. Obviously, in the latter case, at least some of the myosin fragments were cross-linked via the disulfide bonds to form heterogeneous polymers that differed from those derived from the intact myosin.

It is noteworthy that under all treatment conditions, myosin light chains showed little or no change during

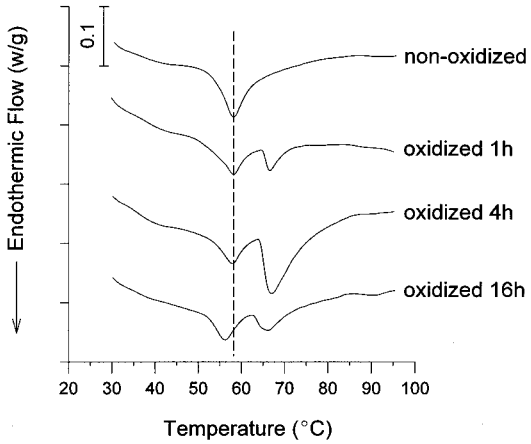

Figure 3. DSC endotherm of chicken breast myosin samples (40 mg/mL protein in 25 mM sodium phosphate buffer, pH 6.5) incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time, heated from 10 to 95 °C at 10 °C/min.

Table 1. Temperature Maxima (T_m) and Enthalpy (ΔH) of Thermal Transition of Chicken Breast Myosin Incubated with $FeCl_3/H_2O_2$/Ascorbate for Different Periods of Time As Analyzed by DSC[a]

	T_m (°C)	ΔH (J/g)
nonoxidized	58.3[a]	493[a]
oxidized for 1 h	57.9[b]	127[b]
oxidized for 6 h	56.5[c]	145[b]
oxidized for 24 h	54.4[d]	152[b]

[a] Data are means of two replicated experiments. Means within the same column bearing different letters differ significantly ($P < 0.05$).

oxidation, indicating that they probably were more resistant to oxidants than MHC. However, it is still possible that a small amount of myosin light chains, which could not be detected by the electrophoretic method, participated in polymer formation.

Differential Scanning Calorimetry (DSC). Nonoxidized myosin exhibited a single endothermic transition peak with a temperature maximum (T_m) at 58.3 °C (Figure 3).The hydroxyl radical-generating system markedly lowered the T_m of myosin by up to 4 °C in 24 h. As shown in Figure 3 and Table 1, the T_m of myosin decreased ($P < 0.05$) progressively during incubation with the oxidizing agents, indicating that oxidation caused destabilization of the myosin native structure. Enthalpy of the transition (ΔH) of myosin, which is correlated with the content of ordered secondary structure of the protein (Koshiyama et al., 1981), also decreased ($P < 0.05$) (Figure 3 and Table 1). The changes in secondary and tertiary structure of myosin presumably resulted from modification of its primary structure by oxidation. The hydroxyl radical can modify essentially any aspect of protein primary structure (Davies et al., 1987a). Some changes in the primary structure of myosin were already demonstrated by the SDS−PAGE. In addition to causing protein fragmentation and polymerization, •OH is also capable of modifying side-chain groups of many amino acid residues. Certain charged amino acid residues, for example, histidine, lysine, and arginine, are particularly sensitive to •OH oxidation (Amici et al., 1989). Such modifications could affect the overall electrical charge and the distribution of the electrical charges of proteins as seen in the example of bovine serum albumin (Davies et al., 1987a), causing proteins to become less stable. Davies and Delsignore (1987) have demonstrated that alterations to primary structure by •OH result in distortions of secondary and tertiary structure, as indicated by the decrease in solubility of bovine serum albumin.

Sharpness as well as magnitude of the transition peak showed a tendency to decrease after myosin was oxidized, suggesting that the cooperative nature of myosin denaturation was changed. The cooperativity of protein denaturation is usually estimated using another transition peak parameter, $\Delta T_{1/2}$, or the peak width at half-peak height. In this study, however, this quantitative assessment could not be accomplished because ΔH or the endothermic peak size decreased significantly during myosin oxidation. Nevertheless, the seeming loss in the cooperativity of myosin denaturation could be explained by the fact that a variety of oxidatively modified myosin molecules might have contributed to the endothermic peak. These different individual myosin molecules probably varied in the extent of alteration in their primary, secondary, and tertiary structures. For example, they might differ in the degree of fragmentation or they could have had various amounts and different kinds of modified amino acid residues. Conceivably, denaturation temperatures of these myosin molecules would be diversified and spread over a broader range, resulting in a flattened transition peak.

Concomitantly with the change in endothermic peak, a salient, new transition in the 60−80 °C temperature range emerged in the oxidized myosin. T_m and ΔH of the new transition varied frequently among samples and with oxidation time, although Figure 3 represents a general pattern of the changes. With the information revealed from the SDS−PAGE analysis, we hypothesize that the new transition could be derived from myosin monomers which formed intramolecular disulfide bond(s) during oxidation or polymers of these oxidized myosin molecules. Much effort in the introduction of new disulfide bonds into proteins to improve thermal stability and understanding of the essential role of disulfide linkages has been made in the past decade using site-directed mutagenesis techniques. The effects of engineered disulfide bonds on protein stability depend on the exact bonding geometry of the disulfides. Under some circumstances, the denaturation temperature of proteins could be increased by as much as 11 °C with the newly introduced disulfide bonds, whereas, in other cases, no beneficial effect or even negative impact on protein stability by adding disulfide bonds was observed (Kristjansson and Kinsella, 1991). Therefore, it is reasonable to speculate that the formation of intra- and intermolecular disulfide bonds in myosin, under certain conditions (i.e., with the proper configuration), could actually improve myosin stability. The disulfide bonds might stabilize the native structure of protein by providing a physical constraint to unfolding or by reducing the conformational entropy of the unfolded state (Kristjansson and Kinsella, 1991). To further test this theory, we treated myosin with 10 mM NEM prior to oxidation. The 60−80 °C thermal transition was notably absent in NEM-treated myosin samples (Figure 4), whereas the endothermic peak at ~54−58 °C showed a pattern of change similar to that of myosin samples without the NEM treatment. This result strongly supports the hypothesis of the active involvement of disulfide bonds with the appearance of the new thermal transition. The dynamic nature of the new peak could be attributed to the randomness of disulfide bond formation. Moreover, it could also have resulted from

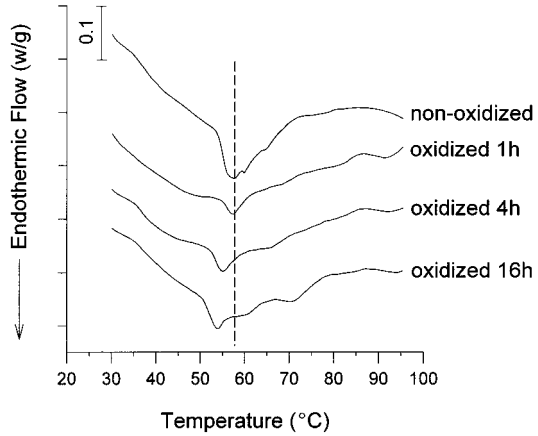

Figure 4. DSC endotherm of chicken breast myosin samples (40 mg/mL protein in 25 mM sodium phosphate buffer, pH 6.5) incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time, heated from 10 to 95 °C at 10 °C/min. Myosin samples were treated with 10 mM NEM prior to oxidation.

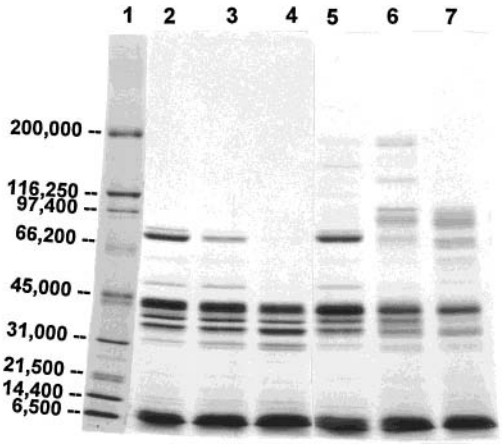

Figure 5. SDS−PAGE pattern of pepsin-digested chicken breast myosin. Myosin samples were incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time and then treated with 10 units/mL pepsin at 37 °C for 1 h. Electrophoresis of samples was performed on a 5−20% gradient gel in the presence (lanes 2−4) and absence (lanes 5−7) of 5% β-mercaptoethanol: (lane 1) molecular mass standard; (lanes 2 and 5) nonoxidized myosin; (lanes 3 and 6) myosin oxidized for 1 h; (lanes 4 and 7) myosin oxidized for 24 h.

the counterbalancing effect of other ever-changing oxidative modifications, such as fragmentation, or even excessive disulfide cross-linking.

In Vitro Digestibility. Myosin, hydrolyzed by different proteases, exhibited distinctive band patterns on SDS−PAGE, and these patterns were also affected by oxidative conditions (Figures 5−7). In the presence of β-mercaptoethanol, pepsin-digested oxidized myosin showed more peptides of low molecular mass and fewer peptides of high molecular mass than pepsin-digested nonoxidized myosin, as judged from the intensity of the peptide bands (Figure 5, lanes 2−4). Differences in the digestion products between nonoxidized and oxidized myosin were more conspicuous when myosin became more oxidized. The most prominent change was the largest peptide (~82 kDa) produced from pepsin hydrolysis, which totally disappeared from the digestion mixture of myosin oxidized for 24 h. The results indicate enhanced proteolytic susceptibility of myosin once oxidized. Increased proteolysis upon oxidation has been established for nonmuscle proteins and many common proteases and thus appears to be a generalized phenomenon (Davies and Goldberg, 1987; Davies et al., 1987b; Grune et al., 1995). Davies et al. (1987b) attributed the increased enzymatic hydrolysis to free-radical-induced protein denaturation, which exposes previously shielded peptide bonds to proteolytic attack. In the DSC results, we have demonstrated denaturation of myosin by hydroxyl radicals. Thus, the alteration of myosin's native structure is consistent with the observed changes in proteolytic susceptibility of oxidized myosin.

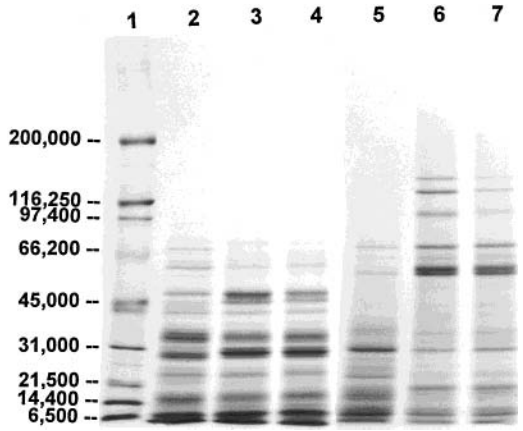

Figure 6. SDS−PAGE pattern of typsin-digested chicken breast myosin. Myosin samples were incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time and then treated with 10 units/mL trypsin at 37 °C for 1 h. Other conditions were the same as described in Figure 5.

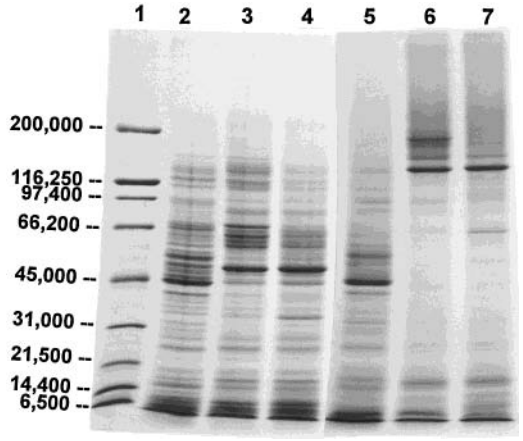

Figure 7. SDS−PAGE pattern of chymotrypsin-digested chicken breast myosin. Myosin samples were incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time and then treated with 0.1 unit/mL chymotrypsin at 37 °C for 1 h. Other conditions were the same as described in Figure 5.

In the absence of β-mercaptoethanol, however, pepsin-digested oxidized myosin showed peptide bands of higher molecular mass than those from nonoxidized myosin (Figure 5, lanes 5−7), whereas a digestion mixture of nonoxidized myosin displayed essentially the same band pattern as that shown in the presence of β-mercaptoethanol (Figure 5, lane 2). Thus, some of the small peptides derived from myosin due to proteolytic attack were actually disulfide-cross-linked into larger peptides. Therefore, it is reasonable to conclude that oxidation decreased the digestibility of myosin by proteases under nonreducing conditions. Decreased digestibility was also observed on H_2O_2/hemin-oxidized myosin (Kamin-Belsky et al., 1996), in which myosin cross-linkages were formed through non-disulfide covalent bonds.

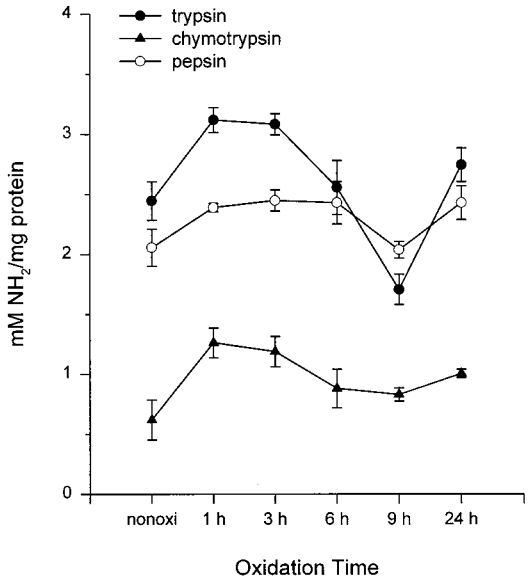

Figure 8. Amount of α-amino groups produced from chicken breast myosin by different proteases as measured by *o*-phthaldialdehyde assay. Myosin samples were incubated with $FeCl_3/H_2O_2$/ascorbate for 1, 3, 6, 9, and 24 h before digestion. The proteases used include pepsin (10 units/mL), trypsin (10 units/mL), and chymotrypsin (0.1 unit/mL).

Similar results were obtained for trypsin-digested myosin samples, which showed increased proteolytic susceptibility with β-mercaptoethanol (Figure 6, lanes 2−4) and decreased digestibility without β-mercaptoethanol (Figure 6, lanes 5−7). It appears, however, that the same conclusion could not be drawn for chymotrypsin-digested samples. As shown in Figure 7, lanes 2−4, oxidized myosin in the presence of β-mercaptoethanol produced more large peptides than nonoxidized samples, although these large peptides gradually diminished upon further oxidation. Without β-mercaptoethanol treatment, chymotrypsin-digested myosin (Figure 7, lanes 5−7) also formed disulfide-cross-linked peptides like those shown in pepsin- and trypsin-digested samples. The individual behaviors of the three proteases were associated with their different specificities. Trypsin recognizes the amino acid sequences containing lysine and arginine, whereas chymotrypsin mainly attacks the peptide bond adjacent to tyrosine, phenylalanine, and tryptophan. These amino acids are among the list of sensitive targets for free radicals (Stadtman, 1993). Hence, the available attack sites on myosin for trypsin and chymotrypsin could be limited by oxidative modification. As a result, oxidized myosin would generate peptide patterns different from those of nonoxidized myosin, as seen in the case of chymotrypsin. Pepsin, on the other hand, has a broader specificity. It works on a number of nonpolar amino acid residues. Therefore, pepsin probably was less affected by this limiting factor and, hence, the results were more dependent on myosin denaturation caused by oxidation.

Although SDS−PAGE does provide valuable information on the proteolytic susceptibility of oxidized myosin, it cannot detect amino acids and very short peptides (e.g., <5 kDa) generated from digestion. To assess the extent of proteolytic degradation of myosin, a quantitative measurement was performed. Figure 8 shows the amount of α-amino groups produced from myosin samples by different proteases as an indication of the quantity of peptide bonds that had been cleaved during proteolysis. After incubation with oxidants for 1 h, oxidized myosin showed increased ($P < 0.05$) peptide bond cleavage by all proteases compared to nonoxidized myosin. After 9 h, however, oxidized myosin exhibited a drop in proteolytic susceptibility to all enzymes. This may be explained by the increase in myosin cross-linking, which would naturally limit the access of proteases to available sites on myosin. The result was consistent with that of SDS−PAGE analysis, which clearly demonstrated the formation of large disulfide-cross-linked peptides in all digested samples without β-mercaptoethanol. Meanwhile, as myosin became further oxidized, fragmentation of myosin also increased, which would counteract the effect of cross-linkage formation on proteolysis. This is probably the reason formation of amino groups increased again at 24 h of oxidation. Oxidation-induced changes in myosin, that is, denaturation, amino acid destruction, fragmentation, and polymerization, all affect the vulnerability of myosin to proteolytic attack. Therefore, variations in digestibility of oxidized myosin were the result of the overall effects of these factors.

Conclusion. The $FeCl_3/H_2O_2$/ascorbate-induced oxidation caused fragmentation and polymerization of myosin. The cross-linking of myosin was mediated mainly by disulfide bonds. Oxidative modification of myosin's primary structure led to decreased thermal stability of myosin. However, it either increased or decreased myosin digestibility depending on the reducing condition of the digestion media. Because many meat products, including surimi, are processed under oxidative conditions, reactive oxygen species can induce structural changes in their muscle protein components, resulting in alterations of protein functionality and nutritional quality of muscle foods.

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