Chemical, Physical, and Gel-forming Properties of Oxidized Myofibrils and Whey- and Soy-protein Isolates

G. LIU, Y.L. XIONG, AND D.A. BUTTERFIELD

ABSTRACT: Myofibrils, oxidized with $FeCl_3/H_2O_2$/ascorbate, exhibited an increase in carbonyls and amines, SH→SS conversion, peptide scission, myosin polymerization, and a decrease in thermal stability and gel-formation ability. Amino-acid side chains of whey-protein isolates (WPI) and soy-protein isolates (SPI) were also modified during oxidation, but the thermal stability of WPI or SPI was not significantly altered. Oxidation increased elasticity of SPI gel but not that of WPI gel. Similarly, oxidation promoted interactions of myofibrils with SPI but not with WPI, resulting in > 30% increases in elasticity of the myofibril/SPI composite gel over its nonoxidized control. Hence, in processed meats where oxidation occurs, the presence of soy proteins may enhance the functionality of myofibrillar proteins.

Key Words: myofibrils, whey, soy, protein oxidation

Introduction

WHEY AND SOY PROTEINS IN THE FORM of concentrates/isolates are widely used as low-cost protein additives in processed meats. The nature and extent of interaction between the nonmuscle proteins and muscle proteins are important for their functional performance (Lanier 1991). Meat products are usually cooked to a final internal temperature of 65 to 73 °C. Muscle proteins heated to these temperatures are essentially fully denatured, allowing the exposed reactive groups to impart desirable functionalities in processed meats. Whey and soy proteins, on the other hand, generally require higher temperatures to unfold than muscle proteins (German and others 1982; Imafidon and others 1991). Hence, these protein additives do not interact well with salt-soluble muscle proteins due to the lack of structural changes under normal meat-processing conditions (McCord and others 1998).

Our preliminary investigation showed that exposures to oxidizing agents can alter the interactions of β-lactoglobulin (the major whey protein) and 7S globulin (a major soy-protein component) with chicken myofibrillar proteins. And oxidation improved the rigidity of the myofibrils/7S globulin composite gel (Liu and Xiong 1998). However, the exact mechanism is unclear. Oxidation has been shown to induce a number of changes in proteins such as modification of amino-acid side chains (Uchida and others 1992; Butterfield and Stadtman 1997), formation of protein polymers (Srinivasan and Hultin 1997), and structural unfolding (Li and King 1996). These physicochemical changes may have contributed to the altered functionality of oxidized muscle and nonmuscle protein composite gels. Further research is warranted to examine the effect of oxidation on the chemical and functional properties of muscle and nonmuscle proteins. The objective of this study was to investigate changes in chemical, structural, and thermal gelation properties, as well as interactions of myofibrillar proteins, whey-protein isolates (WPI), and soy-protein isolates (SPI) in their individual and combined systems after treating with a free-radical-generating oxidant mixture.

Results and Discussion

Protein carbonyls

The formation of carbonyls is 1 of the most salient changes in oxidized proteins (Butterfield and Stadtman 1997; Stadtman and Berlett 1997). As shown in Fig. 1, incubation of proteins with the free-radical-generating system markedly increased ($P < 0.05$) the protein-carbonyl content for all samples. Nonoxidized myofibrils (control) contained about 1.2 nmole carbonyls/mg protein. After reacting with the oxidizing agents for 1 h, there was a 10-fold increase in carbonyl groups. On the other hand, WPI and SPI, which had higher initial carbonyl contents (2.0 and 6.4 nmole carbonyls/mg protein, respectively), exhibited approximately 3- and 2-fold increases in carbonyls, respectively, after 1 h of oxidation. While protein carbonyls can be generated by oxidative attack on protein side-chains or peptide

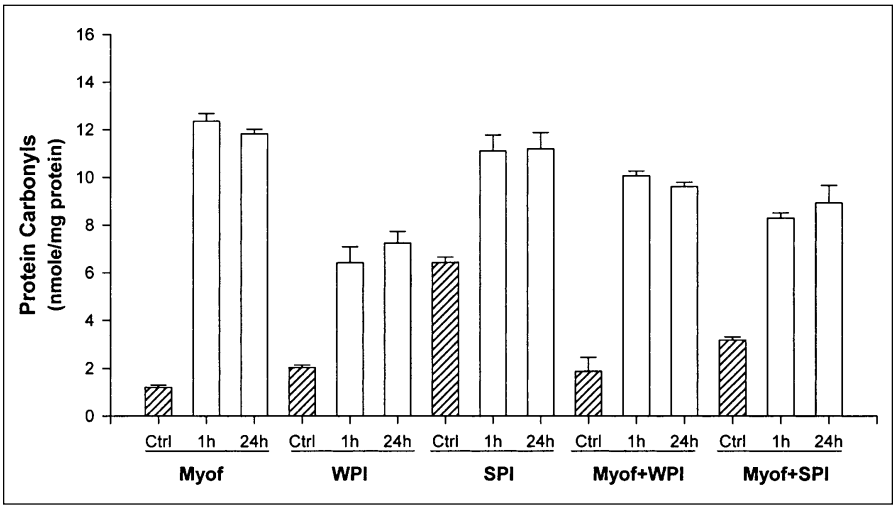

Fig. 1 — Protein carbonyls of myofibrils, WPI, and SPI, as well as their composites. Proteins were incubated with $FeCl_3/H_2O_2$/ascorbate for 1 and 24 h. Nonoxidized proteins were designated as control (Ctrl).

bonds, exogenous carbonyls may also be introduced into proteins by the reactions of lysine residues with oxidized ascorbate (dehydroascorbate) or by Michael-type addition of 4-hydroxy-2-nonenal (HNE), a product of lipid oxidation, to cysteine-sulfhydryl groups, lysine-amino groups, or histidine-imidazole moiety (Butterfield and Stadtman 1997). Since all 3 protein isolates contained a small amount of lipids, and ascorbate was used in the free-radical-generating system, contribution of the exogenous carbonyls to the increase in total carbonyl content was expected.

Similar to their individual protein components, myofibrils/WPI and myofibrils/SPI mixtures also showed a substantial increase ($P < 0.05$) in carbonyls upon oxidation. However, for the myofibrils/WPI mixture, the carbonyl content was essentially the average of the 2 constituting proteins when oxidized separately. For the myofibrils/SPI mixture, the carbonyl content was 20% to 30% less ($P < 0.05$) than the average value of the 2 individual components when oxidized separately, suggesting that there were oxidative reactions or interactions in the mixed protein system that diminished the detectable carbonyls. For example, carbonyls may form Schiff base adducts with NH_2 groups (Feeney and others 1975), leading to a loss in the concentration of carbonyls and free amines and possibly the formation of cross-linkage between proteins. Note that neither individual proteins nor their composites showed a significant difference ($P > 0.05$) in carbonyl content between 1 h and 24 h of oxidation. It is possible that most of the oxidative changes occurred within the first hour, and hence, no additional carbonyls were formed, or the extra carbonyls reacted with nucleophiles such as amines making them undetectable.

Free amines

During oxidation, ϵ-NH_2 groups of lysine residues can be readily converted into carbonyls through a deamination process (Levine and others 1990). Moreover, as discussed above, oxidation-induced carbonyls may also react with NH_2 group and, hence, decrease the total amine concentration. In this study, however, oxidized myofibrils showed a 35% increase in amines after a 1-h incubation, compared to control (nonoxidized myofibrils) (Fig. 2). There was no further change ($P > 0.05$) in the amine content of oxidized myofibrils after 24 h Similar to our findings, a study on fish myofibrillar proteins also showed an increase in free amines after exposure to ·OH (Srinivasan and Hultin 1997). The increase in total amines in oxidized myofibrils could be attributed to fragmentation of protein molecules. Cleavage of peptide bonds, as evidenced by SDS-PAGE shown later, may have generated new α-NH_2 groups and thus compensated for the loss of ϵ-NH_2 caused by oxidation.

Oxidation did not cause significant changes ($P > 0.05$) in amines of WPI and SPI, indicating that whey and soy proteins are more resistant to oxidative attack than myofibrillar proteins. Similar to myofibrils, myofibrils/WPI and myofibrils/SPI mixtures exhibited a 17% and 14% increase, respectively, in amines after a 24 h oxidation. Myofibrillar proteins were probably responsible for much of the change in amine content for each composite system.

Sulfhydryls and disulfide bonds

Conversion of sulfhydryl groups into disulfides and other oxidized species is 1 of the earliest observable events during the radical-mediated oxidation of proteins (Dean and others 1997). Sulfhydryl content of myofibrils decreased drastically ($P < 0.05$) after the protein was oxidized for 1 h and continued to decrease ($P < 0.05$) after 24 h (Fig. 3). Simultaneously, the amount of disulfide bonds in myofibrils increased ($P < 0.05$) during oxidation. Similar changes in sulfhydryls and disulfide bonds were also observed in

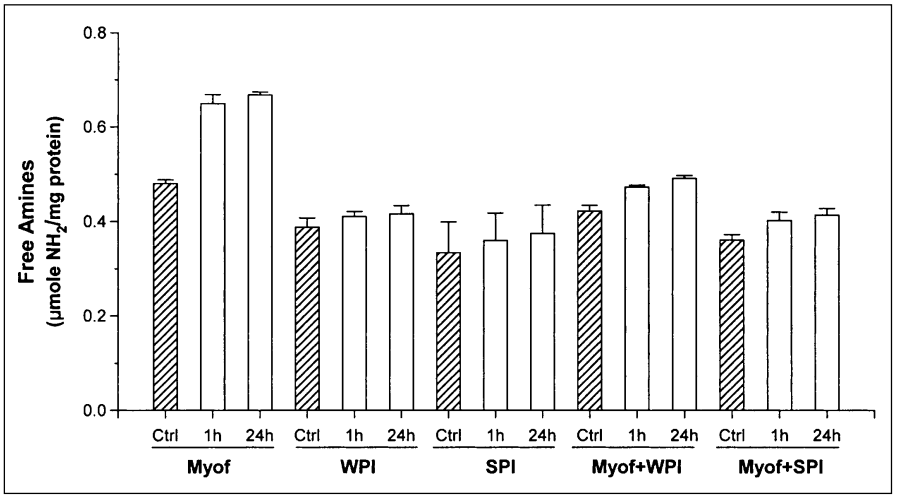

Fig. 2 — Free amines in myofibrils, WPI, and SPI, as well as their composites. Proteins were incubated with $FeCl_3/H_2O_2$/ascorbate for 1 and 24 h Nonoxidized proteins were designated as control (Ctrl).

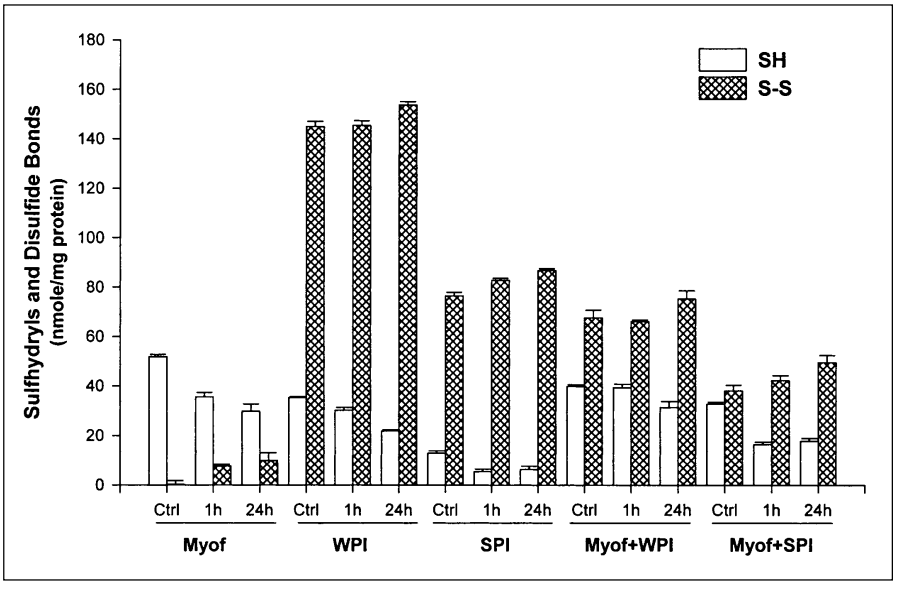

Fig. 3 — Free sulfhydryls and disulfide bonds in myofibrils, WPI, and SPI, as well as their composites. Proteins were incubated with $FeCl_3/H_2O_2$/ascorbate for 1 and 24 h Nonoxidized proteins were designated as control (Ctrl).

WPI and SPI. Myofibrils, WPI, and SPI exhibited a reduction of 42%, 38%, and 51%, respectively, in sulfhydryls after 24 h of oxidation. The sulfhydryl and disulfide contents of nonoxidized myofibrils/WPI and myofibrils/SPI composites were essentially the combination of those from the individual proteins and exhibited the same patterns of changes as the individual proteins during oxidation.

Myosin, which makes up approximately 55% of myofibrillar proteins, has about 42 sulfhydryl groups and no disulfide bond, while actin (~20% of myofibrils) has around 12 sulfhydryl groups and no disulfide bond. Many of the sulfhydryls in myosin and some of those in actin are accessible to chemical reagents (Hofmann and Hamm 1978). Thus, it is not surprising that oxidized myofibrils showed substantial changes in sulfhydryls and disulfide bonds. On the other hand, among the 3 major protein components of WPI, only β-lactoglobulin (β-Lg) (4 S-S) and bovine-serum albumin (BSA) (17 S-S) have 1 free-thiol group. The other component, α-lactalbumin (α-La), contains 4 disulfide bonds and no free sulfhydryl (Kinsella and Whitehead 1989). The free sulfhydryl in native β-Lg is inaccessible to solvent at or below neutral pH (Papiz and others 1986). Thus, at the buffering condition used in our study (pH 6.0), the reactivity of sulfhydryls in β-Lg with ·OH would be low. BSA can be readily attacked by oxygen radicals, resulting in loss of sulfhydryls (Simplicio and others 1991), but its content in whey proteins is only about 5% (Kinsella and Whitehead 1989). This is probably why the overall changes in sulfhydryls and disulfide bonds in WPI were relatively small. The 7S and 11S globulins are the major constituents of SPI. Since 7S globulin has a very low sulfhydryl and disulfide content (Thanh and Shibasaki 1978; Coates and others 1985), 11S globulin, which contains 48 mole of half-cystine per mole of protein (Catsimpoolas and others 1969), must have contributed to most of the changes in sulfhydryl and disulfide groups in SPI.

SDS-PAGE

Electrophoretic analysis confirmed that myofibrillar proteins, especially myosin (the major component of myofibrils), were particularly sensitive to oxidation. Oxidized myofibrils in the presence of β-mercaptoethanol, a reducing agent, showed a decreased band intensity in myosin heavy chain (MHC) (Fig. 4, lanes 2 to 4). Concurrently, new peptide bands with lower molecular weights, mostly in the range of 97 to 195 kDa, appeared, indicating that oxidation caused fragmentation of MHC. Similar to our observations, the study by Martinaud and others (1997) also showed formation of myosin degradation products in oxidized myofibrillar protein. The fragmentation, caused by ·OH attack, probably contributed to the carbonyl formation and also the increase in total amines in oxidized myofibrils. Oxidation also caused a small reduction in the band intensity of actin (45 kDa) and troponin T/tropomyosin (42 kDa), which might be caused by the cleavage into small peptides or by cross-linking through nondisulfide covalent bonds to form polymers. As indicated by Stadtman and Berlett (1997), oxidation may induce formation of protein aggregates through Schiff base adducts or through formation of carbon-carbon covalent bonds by the interaction of carbon-centered radicals in different protein molecules.

Oxidized myofibrils without the β-mercaptoethanol treatment exhibited a much more drastic decrease in the band intensity of MHC, actin, and troponin T/tropomyosin than samples treated with β-mercaptoethanol (Fig. 4, lanes 5-7). After oxidation, the MHC and troponin T/tropomyosin band essentially disappeared, while numerous high-molecular-weight protein bands appeared as dark stains at or near the top of the separating gel. However, protein fragments, which were observed in oxidized myofibrils with β-mercaptoethanol (Fig. 4, lanes 3 and 4), were not evident in this electrophoretic pattern, suggesting that these polymers were probably formed from both intact myosin molecules and myosin fragments (probably actin and troponin T/tropomyosin as well) through disulfide-bond linkages. Additionally, a new peptide band (ca. 85 kDa) emerged in oxidized myofibrils without β-mercaptoethanol (Fig. 4, lanes 6 and 7). This peptide might be the disulfide-cross-linked dimer of troponin T/tropomyosin as judged from its molecular weight. Polymerization of myofibrillar proteins by disulfide cross-linkages was also reported by Srinivasan and Hultin (1997) and was consistent with the results of sulfhydryl- and disulfide-bond assays.

Electrophoretic pattern of oxidized WPI did not show any visible changes (Fig. 5), although a SH→SS conversion was revealed by chemical analysis. This may be because the change in thiol groups of β-Lg and BSA and the possible formation of disulfide-bond-linked oligomers/polymers from β-Lg and BSA, perhaps α-La also (through sulfhydryl/disulfide interchange), were too small to be within the sensitivity range of the SDS-PAGE method. On the other hand, oxidation slightly modified the electrophoretic

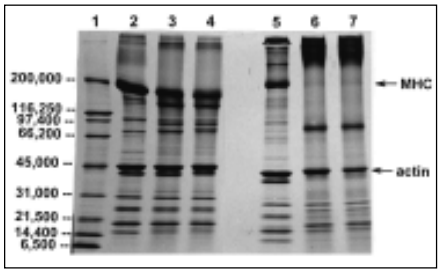

Fig. 4 — SDS-PAGE pattern of myofibrils incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time. Electrophoresis of myofibrils was performed on a 5% to 20% gradient gel in the presence (lanes 2 to 4) and absence (lanes 5 to 7) of 5% β-mercaptoethanol: lane 1, molecular-weight standard; lanes 2 and 5, nonoxidized myofibrils; lanes 3 and 6, myofibrils oxidized for 1 h; lanes 4 and 7, myofibrils oxidized for 24 h.

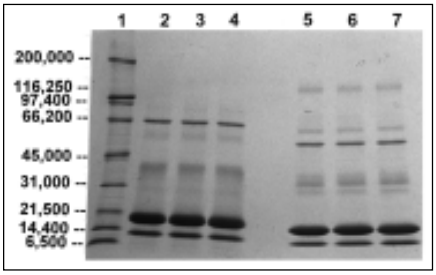

Fig. 5 — SDS-PAGE pattern of WPI incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time. Electrophoresis of WPI was performed on a 5% to 20% gradient gel in the presence (lanes 2 to 4) and absence (lanes 5 to 7) of 5% β-mercaptoethanol: lane 1, molecular-weight standard; lanes 2 and 5, nonoxidized WPI; lanes 3 and 6, WPI oxidized for 1 h; lanes 4 and 7, WPI oxidized for 24 h.

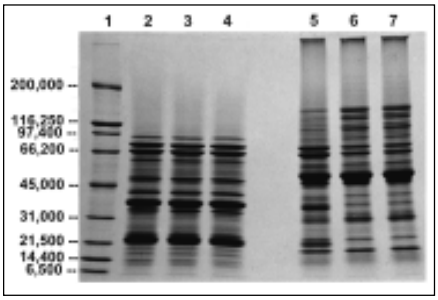

Fig. 6 — SDS-PAGE pattern of SPI incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time. Electrophoresis of SPI was performed on a 5% to 20% gradient gel in the presence (lanes 2 to 4) and absence (lanes 5 to 7) of 5% β-mercaptoethanol: lane 1, molecular-weight standard; lanes 2 and 5, nonoxidized SPI; lanes 3 and 6, SPI oxidized for 1 h; lanes 4 and 7, SPI oxidized for 24 h.

pattern of SPI (Fig. 6). In the presence of β-mercaptoethanol, nonoxidized SPI showed 5 major bands with molecular weights of 86, 66, 51, 36, and 22 kDa, which were tentatively identified as α′, α, and β subunits, and acidic and basic peptides, respectively (Fig. 6, lane 2) (Nagano and others 1996). The α′, α, and β subunits belong to 7S globulin, while the acidic and basic peptides are components of 11S globulin. The acidic and basic peptides are linked together by a disulfide bond (Staswick and others 1984), which occurs as a 55 kDa band in the electrophoretic pattern of SPI when β-mercaptoethanol is not used. During oxidation, there were no observable changes in the subunits/peptides of SPI (Fig 6, lanes 3 and 4). In the absence of β-mercaptoethanol, however, there was a small decrease in the band intensity of α′, and α subunits (Fig. 6, lanes 6 and 7). The acidic and basic peptides, which were not cross-linked into the 55-kDa peptide as shown in Fig. 6, lane 5, disappeared from oxidized samples. Furthermore, some larger peptides in the range of 97 to 150 kDa were formed. These subunits/peptides were apparently cross-linked by disulfide bonds.

Electrophoretic pattern of the myofibrils/WPI mixture appeared to be mainly a combination of those of its individual protein components, regardless of oxidation (Fig. 7). Myofibrils/SPI composite showed a similar result (Fig. 8). However, in both myofibrils/WPI and myofibrils/SPI composites, oxidation produced a minor new band (ca. 230 kDa), with or without the presence of β-mercaptoethanol, which was not shown in any of the 3 individual proteins. This band appeared to represent the polymer that was formed from myosin and certain components of WPI or SPI through nondisulfide-covalent bonds (nondissociable by β-mercaptoethanol). These cross-linkages could be formed through interaction of carbon-centered radicals in 2 different protein molecules or through reaction of protein or protein-bound carbonyls in 1 protein with amines in another protein (Butterfield and Stadtman 1997; Stadtman and Berlett 1997).

DSC

Nonoxidized myofibrils exhibited 2 endothermic transition peaks with T_m values of 59.9 °C and 67.8 °C (Fig. 9), which tentatively could be ascribed to myosin and actin (Stabursvik and Martens 1980). T_m of the 2 transition peaks decreased ($P < 0.05$) progressively during oxidation. After 24 h, the T_m values were lowered by 10 and 5 °C for myosin and actin, respectively. Also, enthalpy (ΔH) of the myosin peak also decreased by about 15% after a 24 h oxidation, while ΔH of actin did not change ($P > 0.05$) during oxidation. The results indicated that oxidation destabilized the native structures of myosin and actin. The decrease in thermal stability of myosin and actin was presumably the consequence of oxidative modifications in their primary structures, which were demonstrated in chemical and electrophoretic analysis.

Nonoxidized WPI showed a major endothermic peak with a T_m of 83.5 °C and a minor peak at 71.5 °C (Fig. 9), which could be assigned to β-Lg and α-La, respectively (Ruegg and others 1977). Oxidation induced a slight decrease ($P < 0.05$) in the T_m of β-Lg, by 1.2 °C, in 24 h, but did not change ($P > 0.05$) T_m and ΔH of α-La. Nonoxidized SPI formed a major endothermic peak (11S globulin) at 104.4 °C and a smaller peak (7S globulin) at 87.7 °C (Fig. 10) (Petruccelli and Añón 1996). Neither 7S globulin nor 11S globulin showed significant changes ($P > 0.05$) in T_m and ΔH after treatment with the oxidants. The changes in the thermal stability of whey proteins were much less than those in myofibrillar proteins. This is probably because oxidative modifications of amino-acid residues in whey proteins were not extensive enough to alter their secondary and tertiary structures, as opposed to the substantial changes in the primary structure of myofibrillar proteins. The lack of change in spatial structures of soy proteins could be due to the same reason.

Nonoxidized myofibrils/WPI composite showed 2 transitions, with 1 peak at 59.1 °C, which was apparently derived from myosin, and the other peak at 84.7 °C, which can be attributed to β-Lg (Fig. 9). Note that in this mixture, T_m of myosin decreased by about 1 °C, and T_m

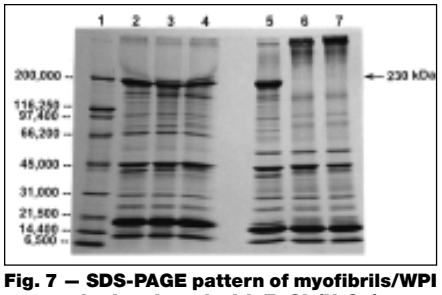

Fig. 7 — SDS-PAGE pattern of myofibrils/WPI composite incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time. Electrophoresis of the composite was performed on a 5% to 20% gradient gel in the presence (lanes 2 to 4) and absence (lanes 5 to 7) of 5% β-mercaptoethanol: lane 1, molecular-weight standard; lanes 2 and 5, nonoxidized composite; lanes 3 and 6, composite oxidized for 1 h; lanes 4 and 7, composite oxidized for 24 h.

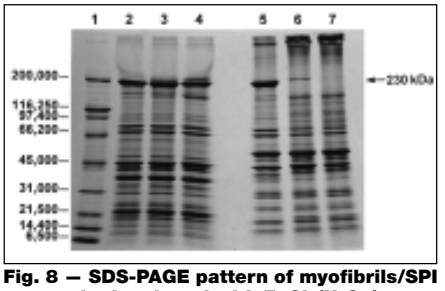

Fig. 8 — SDS-PAGE pattern of myofibrils/SPI composite incubated with $FeCl_3/H_2O_2$/ascorbate for different periods of time. Electrophoresis of the composite was performed on a 5% to 20% gradient gel in the presence (lanes 2 to 4) and absence (lanes 5 to 7) of 5% β-mercaptoethanol: lane 1, molecular-weight standard; lanes 2 and 5, nonoxidized composite; lanes 3 and 6, composite oxidized for 1 h; lanes 4 and 7, composite oxidized for 24 h.

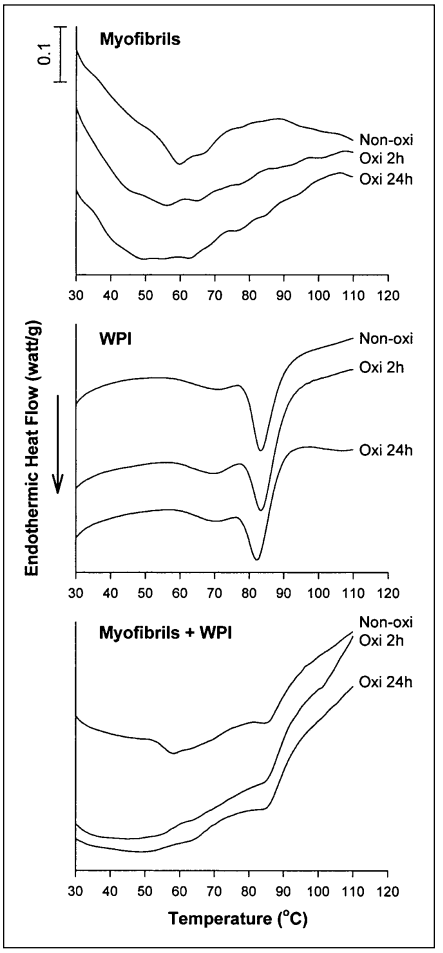

Fig. 9 — Differential scanning calorimetric endotherm of myofibrils, WPI, and their composite (20, 100, and 40 mg/mL protein, respectively, in 25 mM sodium-phosphate buffer, 0.6 M NaCl, pH 6.0) heated from 10 to 115 °C at 10 °C/min. Oxidized proteins were prepared by incubating with $FeCl_3/H_2O_2$/ascorbate for different periods of time.

of β-Lg increased by approximately 1 °C, compared to the corresponding individual proteins. Additionally, the actin peak occurring at 67.8 °C in the individual myofibril sample was absent in the DSC endotherm of the myofibrils/WPI composite. The results suggested that whey proteins interacted with both myosin and actin during heating. In the oxidized myofibril/WPI mixture, myosin and β-Lg showed essentially the same changes as those in the individually oxidized myofibrils and WPI samples. The nonoxidized myofibrils/SPI composite showed 3 transition peaks, which corresponded to the endothermic transitions of its individual constituents (Fig. 10). During oxidation, only the myosin peak was altered, while the 7S and 11S globulin peaks did not exhibit noticeable changes ($P > 0.05$) in T_m and ΔH.

Gelation

Nonoxidized myofibrils exhibited a complex rheological pattern during heating from 20 to 83 °C (Fig. 11). The G′ of myofibrils started to increase at about 42 °C and showed a peak with maximum G′ value around 49 °C. After reaching the peak maximum, the G′ decreased abruptly, reached a minimum at 53 °C, and then continuously increased towards the end of heating (83 °C). This viscoelastic pattern was essentially identical to a previous report on dynamic rheological properties of chicken myofibrillar proteins (Xiong and Blanchard 1994).

The viscoelastic pattern of myofibrils was changed after oxidation. The onset temperatures of G′ development for myofibrils were changed to 37 °C after 2 h of oxidation and 36 °C after 24 h of oxidation. The transition peak temperature was also lowered ($P < 0.05$) by 3 °C. Meanwhile, the magnitude of G′ for oxidized myofibrils at the peak (46 to 47 °C) and at the end of heating (83 °C) were reduced by about 55% and 42%, respectively, compared with the nonoxidized control. No significant difference ($P > 0.05$) in the final G′ value was found between myofibrils oxidized for 2 and 24 h.

The decreases in the onset temperature and the peak temperature of myofibril gelation after oxidation were in agreement with the DSC results, which showed a reduction in denaturation temperatures of myosin and actin in oxidized myofibrils (Fig. 9). The decrease in the G′ of oxidized myofibrils might result from alterations in the functional groups of myofibrillar proteins as shown in the chemical analysis. Moreover, it could be due to disulfide-bond formation. While formation of disulfide cross-links is beneficial for strengthening the gel network during thermal gelation (Smyth and others 1998), excessive cross-linkage of myofibrillar proteins before heating would generate large protein aggregates (Fig. 4, lanes 6 and 7), which might hinder ordered interactions of reactive functional groups and, hence, inhibit formation of a fine gel network.

Oxidation reduced the onset temperature of WPI gelation by about 1 °C (Fig. 11), which corresponded to the small decrease in the denaturation temperature of β-Lg (Fig. 9). After 24 h of oxidation, the final G′ value (at 83 °C) of oxidized WPI decreased by approximately 30%, compared to nonoxidized WPI. Under the experimental conditions, nonoxidized SPI did not form a strong gel (Fig. 12). Oxidation, however, induced gel formation and substantially increased the final G′ value of SPI in 24 h, even though its thermal stability was not significantly changed by the oxidants. These changes in viscoelastic properties of oxidized WPI and SPI may have resulted from alterations in surface properties of their protein components during oxidation, for example, the increase in protein carbonyls, which did not necessarily lead to a major shift in their thermal stability. In the case of soy proteins, the modification of surface functional groups was apparently conducive to protein-protein interactions, whereas for whey proteins, these changes may not be favorable for the interactions.

At temperatures below 78 °C, nonoxidized myofibrils/WPI composite exhibited the same rheological profile as did myofibrils alone (Fig. 12). However, unlike myofibrils, which showed a slow rate of increase in G′ at temperatures above 72 °C, the composite demonstrated a

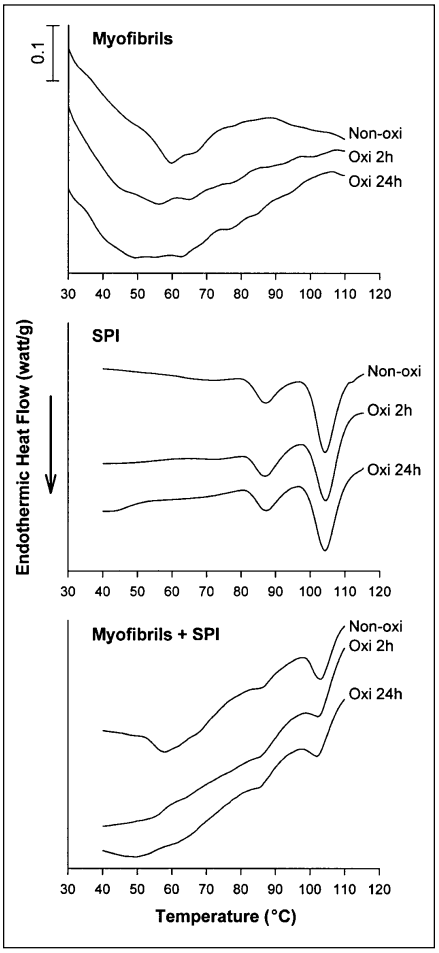

Fig. 10 — Differential scanning calorimetric endotherm of myofibrils, SPI, and their composite (20, 75, and 40 mg/mL protein, respectively, in 25 mM sodium-phosphate buffer, 0.6 M NaCl, pH 6.0) heated from 10 to 115 °C at 10 °C/min. Oxidized proteins were prepared by incubating with $FeCl_3/H_2O_2$/ascorbate for different periods of time.

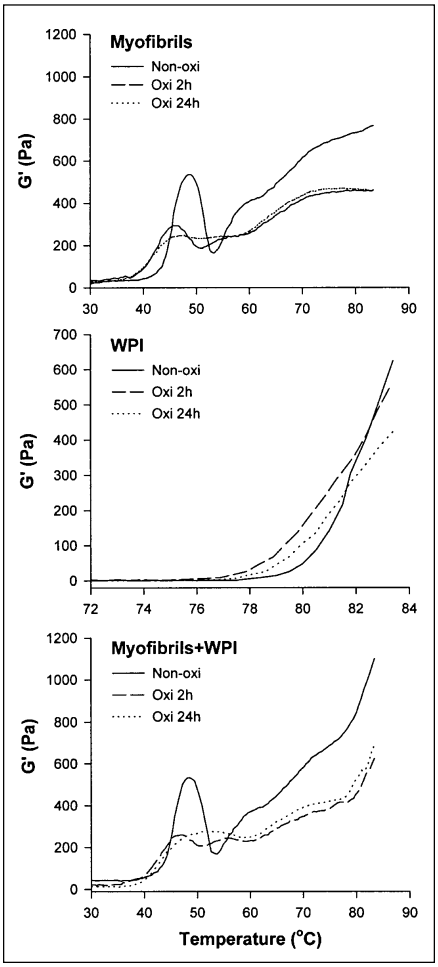

Fig. 11 — Changes in storage modulus (G′) of myofibrils, WPI, and their composite (20, 100, and 40 mg/mL protein, respectively, in 25 mM sodium-phosphate buffer, 0.6 M NaCl, pH 6.0) during thermal gelation. Oxidized proteins were prepared by incubating with $FeCl_3/H_2O_2$/ascorbate for different periods of time.

sharp increase starting from 78 °C. The final G′ value of the composite was 45% higher than that of myofibrils alone. This was apparently due to unfolding of whey proteins (mainly β-Lg) in the high-temperature range. The unfolded whey-protein molecules probably interacted with each other or with myofibrillar proteins that already formed a primary gel network ("backbone"), and thus caused formation of either a "filled" gel system, where whey protein gel filled the void space in the myofibril gel matrix, or a "complex" gel system (Ziegler and Foegeding 1990). The rigidity of myofibril gel would be improved in either situation. The effect of WPI on myofibril gelation diminished when the composite was oxidized. As discussed above, oxidation substantially reduced the gelling ability of myofibrillar proteins. Therefore, oxidation probably did not induce significant interactions between WPI and myofibrils to compensate for its negative effect on myofibril gelation. As a result, the final G′ values of the oxidized myofibrils/WPI composites were ~43% less than that of nonoxidized composite sample.

The nonoxidized myofibrils/SPI composite showed a viscoelastic pattern quite similar to that of nonoxidized myofibrils (Fig. 12). However, addition of SPI to myofibrils markedly lowered ($P < 0.05$) the G′ of myofibril gel. The G′ values of the composite at the peak (49 °C) and the end of heating (83 °C) were reduced by 72% and 60%, respectively, compared to myofibrils alone. This was also demonstrated in a previous study (Liu and Xiong 1998) where addition of β-Lg to myofibrils decreased the gel-forming ability. The effect of SPI on myofibril gelation was probably due to disruption of the gel network of myofibrillar proteins by physical interference from the soy-protein peptide chains. However, the G′ at peak and the final G′ of myofibrils/SPI composite progressively increased ($P < 0.05$) after prolonged incubation with oxidants. After 24 h of incubation with oxidants, the composite had a 90% and 33% increase in the G′ at peak and at the end of heating, respectively, compared to nonoxidized control. The higher G′ values were apparently due to oxidation-enhanced interactions among the proteins in the composite, which probably originated from oxidative modification of surface functional groups of the proteins in the mixture.

Conclusions

MYOFIBRILLAR PROTEINS WERE MORE sensitive to oxidative modifications than whey and soy proteins. Oxidation could alter the interactions between nonmuscle proteins and muscle proteins. The oxidation-induced alteration in interaction between WPI and myofibrils, however, did not result in favorable changes in the gelling ability of myofibrils/WPI mixture. On the other hand, while addition of SPI to myofibrils impaired gelation, oxidation could enhance the interactions between these 2 protein systems, resulting in formation of a stronger gel. Thus, oxidative modification may be considered as a potential means to improve functionality of muscle and soy-protein composite systems, or meat processed under oxidative conditions may have enhanced functionality by incorporating soy proteins into the product formulation.

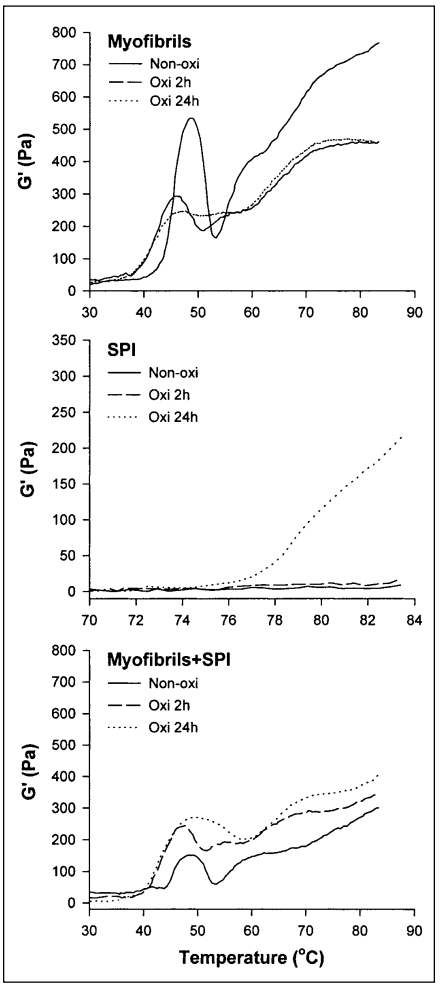

Fig. 12 — Changes in storage modulus (G′) of myofibrils, SPI, and their composite (20, 75, and 40 mg/mL protein, respectively, in 25 mM sodium-phosphate buffer, 0.6 M NaCl, pH 6.0) during thermal gelation. Oxidized proteins were prepared by incubating with $FeCl_3/H_2O_2$/ascorbate for different periods of time.

Materials and Methods

Myofibrils, WPI, and SPI

Myofibrils were isolated at 2 °C essentially as described by Xiong (1993). Ground pectoralis muscle from 6-wk-old commercially processed fresh broilers (obtained from a local retailer, stored on crushed ice, and used within 36 to 48 h after slaughter) was washed 4 times using an isolation buffer (pH 7.0) containing 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 10 mM potassium phosphate, followed by 2 washes using 0.1 M NaCl solution containing 1 mM NaN_3. Myofibril suspension was adjusted to pH 6.0 using 0.1 N HCl prior to the final centrifugation. The purified myofibril pellets were not dried but were stored on ice and used within 24 h of isolation. Protein concentration of the myofibril pellet was measured by the biuret method (Gornall and others 1949) using bovine-serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as standard.

WPI were obtained from a commercial supplier (New Zealand Milk Products, Inc., Santa Rosa, Calif., U.S.A.) and used without further purification.

SPI were prepared from defatted soy flake (Archer Daniels Midland Co., Decatur, Ill., U.S.A.) according to the procedure described by Puppo and others (1995). Soy flour was first made from defatted soy flake, using a Waring blender at 4 °C and then dispersed in distilled water (1:10 w/w). The dispersion was adjusted to pH 8.0 with 2 N NaOH, stirred at room temperature for 2 h, and centrifuged at 8280 × g for 40 min at room temperature. The supernatant was then adjusted to pH 4.5 with 2 N HCl and centrifuged at 3300 × g for 20 min. The pellet was resuspended in 5 volumes of distilled water, adjusted to pH 7.0 with 2 N NaOH, and lyophilized.

Protein oxidation

WPI and SPI were dissolved in 25 mM sodium-phosphate buffer (pH 6.0) containing 0.6 M NaCl. This buffering condition was used because it closely simulates the in situ conditions in processed-meat products. Protein concentrations (20 to 100 mg/mL) were measured using the biuret method. Purified myofibrils were then mixed with WPI or SPI solution (1:1 ratio; v/v) with a final protein concentration of 20 mg/mL for each component. Individual proteins (that is, myofibrils, WPI, and SPI with a protein concentration of 20, 100, and 75

mg/mL, respectively) as well as their mixtures were oxidized by incubation with 0.1 mM $FeCl_3$, 20 mM H_2O_2, and 1 mM ascorbate at 2 °C for up to 24 h according to Srinivasan and Hultin (1997).

Protein carbonyls

Protein carbonyls, an index of protein oxidation (Butterfield and Stadtman 1997), were measured essentially according to the method of Levine and others (1990) with some modifications as outlined by Srinivasan and Hultin (1995). A 50-µL aliquot of protein samples (20 to 40 mg protein/mL) in triplicate was reacted with 2.0 mL 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.0 N HCl for 1 h at room temperature; another 50 µL of sample was added with 2.0 mL 2.0 N HCl (control). After incubation, 2.0 mL of 20% trichloroacetic acid was added to precipitate the protein. The precipitate was washed twice with 4.0 mL of an ethanol:ethyl acetate (1:1; v/v) mixture to remove unreacted DNPH, blow-dried, and dissolved in 1.5 mL 6.0 M guanidine hydrochloride, 20 mM potassium phosphate (pH 2.3). Absorbance of the protein solution was measured at 370 nm. An aliquot of the protein solution was reacted with Coomassie protein assay reagent to measure its protein concentration, according to the method of Bradford (1976). A molar absorptivity of 22,400 M^{-1} cm^{-1} was used to calculate protein-carbonyl content.

Free amines

Free amines in proteins were measured as described by Snyder and Sobocinski (1975) with modifications. Protein samples were solubilized and diluted with 1% sodium dodecyl sulfate (SDS) in 0.1 M sodium tetraborate (pH 9.3) to 0.2 mg protein/mL. A 1.0-mL aliquot of the diluted sample in triplicate was then incubated with 50 µL of 0.03 M 2,4,6-trinitrobenzenesulfonic acid (TNBS) for 60 min at 37 °C. The test tubes and the water bath were covered with aluminum foil to avoid accelerating the blank reaction by exposure to light (Adler-Nissen 1979). One mL of 0.24 M HCl, which lowered pH of the reaction mixture to 3.5 to 4.0, was added to terminate the reaction. Absorbances at 420 nm were then measured. Free-amine content was calculated as µmole free amines/mg protein using the standard curve constructed from glycine.

Sulfhydryls and disulfide bonds

Total free-sulfhydryl groups were determined by reacting with 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB) based on the procedure of Srinivasan and Hultin (1997). Protein samples were diluted to 2 mg/mL protein with 0.1 M phosphate buffer (pH 7.4). A 0.5-mL aliquot of diluted sample in triplicate was dissolved in 2.0 mL urea-SDS solution (8.0 M urea, 3% SDS, 0.1 M phosphate, pH 7.4). After incubation with 0.5 mL DTNB reagent (10 mM DTNB in 0.1 M phosphate buffer, pH 7.4) at room temperature for 15 min, absorbance at 412 nm was read. Sample blank was run with 0.5 mL phosphate buffer without DTNB; reagent blank was run with water only. Sulfhydryl content was calculated using a molar absorptivity of 11,400 M^{-1} cm^{-1}.

Disulfide bonds in proteins were determined by reacting with disodium 2-nitro-5-thiosulfobenzoate (NTSB) as described by Damodaran (1985). Protein samples were diluted to 5 mg/mL protein with 25 mM phosphate buffer (pH 6.0) containing 0.6 M NaCl. A 100-µL aliquot of diluted protein solution in triplicate was mixed with 1.5 mL NTSB assay solution (freshly made) and incubated in the dark at room temperature for 25 min. Absorbance at 412 nm was then measured. A molar absorptivity of 13600 M^{-1} cm^{-1} was used for calculation. Because NTSB reagent forms chromophoric derivative with both free-sulfhydryl groups and disulfide bonds, the numbers calculated from the absorbance readings represented the total concentrations of the sulfhydryl and disulfide groups in the samples. Disulfide content was estimated by subtracting the free sulfhydryls (obtained from sulfhydryl assay) from the total content of the sulfhydryl and disulfide groups.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of oxidized and nonoxidized protein samples was performed using the method of Laemmli (1970) with modifications. A stacking gel with 3% acrylamide and a gradient resolving gel with 5% to 20% acrylamide were used. SDS-PAGE samples were prepared with and without 5% β-mercaptoethanol. For samples without β-mercaptoethanol, 1 mM N-ethylmaleimide (a thiol-blocking agent) was added before heating to prevent possible formation of disulfide cross-linkage during sample preparation.

Differential scanning calorimetry (DSC)

Thermal denaturation of oxidized and nonoxidized protein samples was measured by standard DSC. Samples (14 to 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, Del., U.S.A.). Samples were thermally scanned from 10 to 115 °C at 10 °C/min. An empty, hermetically sealed aluminum pan was used as reference. Samples exhibited 1 or 2 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 measurements were performed with at least triplicate samples.

Dynamic rheological measurements

Nondestructive, oscillatory measurements of the protein samples during gelation were carried out using a Bohlin VOR rheometer (Bohlin Instruments, Inc., Cranbury, N.J., U.S.A.) as described by Xiong (1993). Thermal gelation was induced by heating protein samples from 20 to 83 °C at 1 °C/min and was monitored by shearing the sample placed between 2 parallel plates (upper plate: 30 mm dia) 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).

Statistical analysis

All experiments were replicated twice, and all measurements except for rheological test were performed at least in triplicate. Data were analyzed using the general linear-model procedure of the Statistix 3.5 software package (Statistix 3.5, Analytical Software Inc., St. Paul, Minn., U.S.A.). 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).

References are on next page.

References

Adler-Nissen J. 1979. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. J Agri Food Chem 27:1256-1262.

Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal BioChem 72:248-254.

Butterfield DA, Stadtman ER. 1997. Protein oxidation processes in aging brain. Adv Cell Aging Gerontol 2:161-191.

Catsimpoolas N, Campbell TG, Meyer EW. 1969. Association-dissociation phenomena in glycinin. Arch BioChem Biophys 131:577-586.

Coates JB, Medeiros JS, Thanh VH, Nielsen NC. 1985. Characterization of the subunits of _-conglycinin. Arch BioChem Biophys 243:184-194.

Damodaran S. 1985. Estimation of disulfide bonds using 2-nitro-5-thiosulfobenzoic acid: limitations. Anal BioChem 145:200-204.

Dean RT, Fu S, Stocker R, Davies MJ. 1997. Biochemistry and pathology of radical-mediated protein oxidation. BioChem J 324:1-18.

Feeney RE, Blankenhorn G, Dixon BF. 1975. Carbonyl-amine reactions in protein chemistry. Adv Protein Chem 29:135-203.

German B, Damodaran S, Kinsella JE. 1982. Thermal dissociation and association behavior of soy proteins. J Agri Food Chem 30:807-811.

Gornall AG, Bardawill CJ, David MM. 1949. Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751-766.

Hofmann K, Hamm R. 1978. Sulfhydryl and disulfide groups in meats. Adv Food Res 24:1-111.

Imafidon GI, Ng-Kwai-Hang KF, Harwalkar VR, Ma CY. 1991. Differential scanning calorimetric study of different genetic variants of β-lactoglobulin. J Dairy Sci 74:2416-2422.

Kinsella JE, Whitehead DM. 1989. Proteins in whey: chemical, physical, and functional properties. Adv Food Nutr Res 33:343-439.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacterio-phage T4. Nature 227:680-685.

Lanier TC. 1991. Interactions of muscle and nonmuscle proteins affecting heat-set gel rheology. In: Parris N, Barford R, editors. Interactions of food proteins. Washington, D.C.: American Chemical Society. ACS Symposium Series. p 268-284.

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz A-G, Ahn B-W, Shalitel S, Stadtman ER. 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 186:464-478.

Li SJ, King AJ. 1996. Lipid oxidation and myosin denaturation in dark chicken meat. J Agri Food Chem 44:3080-3084.

Liu G, Xiong YL. 1998. Rheological properties of oxidatively modified muscle, whey and soy protein composite systems [abstract]. In: IFT Annual Meeting Book of Abstracts; 1996 June 20-24; Atlanta, Ga. Chicago, Ill.: Institute of Food Technologists. p 107. Abstract nr 46C-1.

Martinaud A, Mercier Y, Marinova P, Tassy C, Gatellier P, Renerre M. 1997. Comparison of oxidative processes on myofibrillar proteins from beef during maturation and by different model oxidation systems. J Agri Food Chem 45:2481-2487.

McCord A, Smyth AB, O'Neill EE. 1998. Heat-induced gelation properties of salt-soluble muscle proteins as affected by non-meat proteins. J Food Sci 63:580-583.

Nagano T, Fukuda Y, Akasaka T. 1996. Dynamic viscoelastic study on the gelation properties of _-conglycinin-rich and glycinin-rich soybean protein isolates. J Agri Food Chem 44:3484-3488.

Papiz MZ, Sawyer L, Eliopoulos EE, North ACT, Findlay JBC, Slivaprasadarao R, Jones TA, Newcomer ME, Kraulis PJ. 1986. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. Nature 324:383-385.

Petruccelli S, Añón MC. 1996. pH-induced modifications in the thermal stability of soybean protein isolates. J Agri Food Chem 44:3005-3009.

Puppo MC, Lupano CE, Añón MC. 1995. Gelation of soy protein isolates in acidic conditions. Effect of pH and protein concentration. J Agri Food Chem 43:2356-2361.

Ruegg M, Moor U, Blanc B. 1977. A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. J Dairy Res 44:509-520.

Simplicio PD, Cheeseman KH, Slater TF. 1991. The reactivity of the SH group of bovine serum albumin with free radicals. Free Rad Res Comms 14:253-262.

Smyth AB, Smith DM, O'Neill E. 1998. Disulfide bonds influence the heat-induced gel properties of chicken breast muscle myosin. J Food Sci 63:584-588.

Snedecor GW, Cochran WG. 1989. Statistical methods. 8th ed. Ames, Iowa: Iowa State University Press. 503 p.

Snyder SL, Sobocinski PZ. 1975. An improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines. Anal BioChem 64:284-288.

Srinivasan S, Hultin HO. 1995. Hydroxyl radical modification of fish muscle proteins. J Food BioChem 18:405-425.

Srinivasan S, Hultin HO. 1997. Chemical, physical, and functional properties of cod proteins modified by a nonenzymic free-radical-generating system. J Agri Food Chem 45:310-320.

Stabursvik E, Martens H. 1980. Thermal denaturation of proteins in post rigor muscle tissue as studied by differential scanning calorimetry. J Sci Food Agri 31:1034-1042.

Stadtman ER, Berlett BS. 1997. Reactive oxygen-mediated protein oxidation in aging and disease. Chem Res Toxicol 10:485-494.

Staswick PE, Hermodson MA, Nielsen NC. 1984. Identification of the cysteines which link the acidic and basic components of the glycinin subunits. J Bio Chem 259:13431-13435.

Thanh VH, Shibasaki K. 1978. Major proteins of soybean seeds. Subunit structure of β-conglycinin. J Agri Food Chem 26:692-695.

Uchida K, Kato Y, Kawakishi S. 1992. Metal-catalyzed oxidative degradation of collagen. J Agri Food Chem 40:9-12.

Xiong YL. 1993. A comparison of the rheological characteristics of different fractions of chicken myofibrillar proteins. J Food BioChem 16:217-227.

Xiong YL, Blanchard SP. 1994. Dynamic gelling properties of myofibrillar protein from skeletal muscles of different chicken parts. J Agri Food Chem 42:670-674.

Ziegler GR, Foegeding EA. 1990. The gelation of proteins. Adv Food Nutr Res 34:203-298.

MS 19991012 received 10/5/99; revised 2/29/00; accepted 4/10/00.

Authors Liu and Xiong are with the Food Science Section, Department of Animal Sciences and Center of Membrane Sciences, University of Kentucky, Lexington, KY 40546. Author Butterfield is with the Department of Chemistry and Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506. Direct correspondence to Youling L. Xiong (E-mail: ylxiong@pop.uky.edu).