Lipid Oxidation and Myosin Denaturation in Dark Chicken Meat

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Hand-deboned samples of dark ground meat from 6-week-old broilers were treated with varying combinations of $FeSO_4$, ascorbic acid, and α-tocopherol. Mixtures were incubated at 37 °C for 0, 50, 100, and 200 min. Lipid oxidation was determined by measuring thiobarbituric acid reactive substances. Myosin was isolated from incubated samples, and denaturation was determined by measuring intrinsic tryptophan fluorescence, 1-anilino-8-naphthalenesulfonate binding, and Ca^{2+}-ATPase activity. Myosin denaturation during heating was positively correlated with lipid oxidation. Exogeneously added α-tocopherol reduced lipid oxidation and decreased myosin denaturation during heating.

Keywords: *α-Tocopherol; myosin; lipid oxidation; chicken meat*

INTRODUCTION

Myofibrillar proteins, such as myosin, may be altered due to their interaction with different types of lipid or byproducts of lipid oxidation during heating (Khayat and Schwall, 1983). Amino acid destruction, insolubilization, protein polymerization, cross-linking, scission, and formation of Schiff bases are known to occur from lipid−protein interactions (Buttkus, 1967; Logani and Davies, 1980; Funes and Karel, 1981; Nakhost and Karel, 1983, 1984; Riley and Harding, 1993). Smith (1987) examined several indices of protein denaturation for frozen, mechanically deboned, and hand-deboned turkey meat and concluded that thiobarbituric acid (TBA) values were not highly correlated with loss of protein solubility or decrease in ATPase activity. However, as Smith and others have observed, TBA values can not be used to measure lipid oxidation in meat subjected to long-term storage (Seo, 1976; Patel et al., 1988). Thus, correlation of TBA values with indices of protein denaturation for meat stored for longer than 2−3 months may not give meaningful results.

Accelerated lipid oxidation may provide a means to study the denaturation of protein by lipid products. Ascorbic acid/iron (Fe^{2+})-induced lipid oxidation and subsequent denaturation of protein can be identified by the ability of α-tocopherol and other antioxidants to retard the reactions (Sakurai et al., 1991). Thus, protein denaturation associated with lipid oxidation can be separated from site-specific metal (ferrous iron)-catalyzed protein oxidation (Devies and Goldberg, 1987; Decker et al., 1993). In Smith's study (1987), a combination of butylated hydroxyanisole, propyl gallate, and citric acid prevented some biochemical and physical changes in the meat as indicated by protein solubility and Ca^{2+}-ATPase activity tests. Possibly, due to a reduction in oxidation caused by additional antioxidants, lipid−protein interactions (myosin head region) did not occur; therefore, subsequent denaturation of myosin precipitated by unfolding of the myosin head did not occur when antioxidants were added (Wagner and Anon, 1986).

Smyth et al. (1995) indicated that chicken breast muscle myosin undergoes initial unfolding at 37 °C, with peaks at 46.7 and 54 °C and shoulders at 57.6 and 63.1 °C. Little research has been conducted on the lipid−protein interaction in chicken meat as myosin undergoes unfolding during heating. In our studies, whole chicken thigh meat was treated with varying combinations of ascorbic acid and ferrous sulfate and heated at 37 °C for varying lengths of time. Thiobarbituric acid values and indices of protein denaturation were measured for heated chicken meat slurries with and without prooxidants. Effects of the antioxidant α-tocopherol are also reported.

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MATERIALS AND METHODS

Sample Preparation. Thigh muscle from 6-week-old broilers was harvested immediately after humane slaughter and processing and then stored at −85 °C. Samples, which included the adductor, semitendinosus, sartorius, semimembranosus, and quadriceps femoris muscles, were homogeneously ground together. The thigh muscle contained red, intermediate, and white fiber types with red predominating. A 5 g sample was homogenized in an Omni mixer (Sorvall Inc., Norwalk, CT) with 45 mL of 0.154 M KCl for 2 min. Samples were divided and treated to reach final concentration as indicated: treatment I, no additions (control); treatment II, 1.138 mM $FeSO_4$ and 0.368 mM ascorbic acid; treatment III, 1.17 mM α-tocopherol; and treatment IV, 1.138 M $FeSO_4$, 0.368 mM ascorbic acid, and 1.17 mM α-tocopherol. To accelerate oxidation, the mixtures were vigorously shaken by hand and then incubated at 37 °C for 0, 50, 100, and 200 min (Sheehy, 1993). After incubation, the samples were stored immediately at −85 °C to stop the reaction. Samples were analyzed for levels of lipid oxidation and protein denaturation by the procedures delineated below.

Measurement of Lipid Oxidation. Samples were taken from the −85 °C freezer and thawed at room temperature for approximately 4 h. The samples were measured at 525 nm using a spectrophotometer (Model UV160U, Shimadzu Corp., Tokyo, Japan) and expressed as thiobarbituric acid reactive substances (TBARS) or mmol of malonaldehyde/kg of meat. TBARS values were determined on triplicate samples per treatment for duplicate runs of the experiment by the method of Raharjo et al. (1993).

Ca^{2+}-ATPase Activity. For each treatment and incubation period, each mixture containing 5 g of chicken meat slurry with 50.5 mL total volume was centrifuged at 5860*g* for 10 min at −4 °C. The supernatant was discarded, and the pellet was resuspended in low-salt buffer (0.1 M NaCl, 0.05 M sodium phosphate, pH 6.5). Myofibrillar proteins were isolated and prepared as described by Wang et al. (1990) with the following modification. The final pellet was solubilized in 0.6 M NaCl,

S0021-8561(96)00021-0 CCC: $12.00

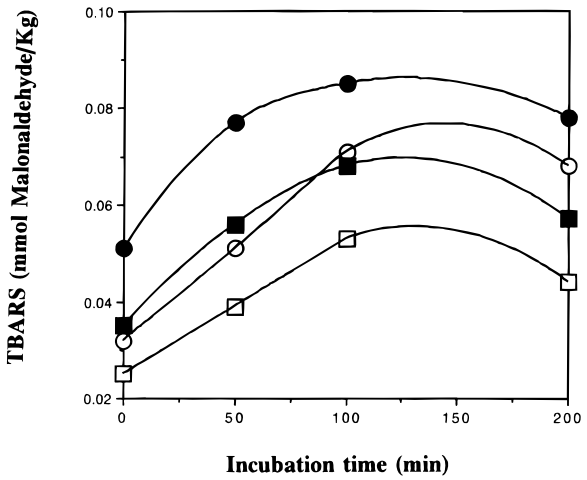

Figure 1. TBARS values ($n = 6$) of chicken slurries incubated at 37 °C with various treatments having the following final concentrations: (○) treatment I, no additions (control); (●) treatment II, 1.138 mM $FeSO_4$ + 0.368 mM ascorbic acid (final concentrations in the mixture); (□) treatment III, 1.17 mM α-tocopherol (final concentration in the mixture); and (■) treatment IV, 1.138 mM $FeSO_4$ + 0.368 mM ascorbic acid + 1.17 mM α-tocopherol.

0.03 M Tris-HCl (pH 7.6) buffer. The Ca^{2+}-ATPase activity of the myofibril preparations was assayed as described by Wells et al. (1979). Protein concentration was measured according to procedures of Lowry et al. (1951). Ca^{2+}-ATPase activity was determined on triplicate samples per treatment for duplicate runs of the experiment.

Preparation of Myosin. For each treatment and incubation period, myosin was extracted according to the procedure of Bandman et al. (1982). Samples were stored at −85 °C until further analysis of tryptophan fluorescence and 1-anilino-8-naphthalenesulfonate fluorescence.

Tryptophan Fluorescence. The intrinsic fluorescence of myosin (10 μg/mL) was measured spectrophotometrically (Luminescence Spectrometer LS 50B, Perkin Elmer, San Jose, CA). Excitation wavelength was 290 nm, and the scanned emission wavelength was from 260 to 470 nm (Bertazzon et al., 1990). Tryptophan fluorescence was measured for duplicate samples at each incubation time per treatment for duplicate runs of the experiment.

1-Anilino-8-naphthalenesulfonate (ANS) Fluorescence. For ANS fluorescence, the excitation and emission wavelengths were 400 and 470 nm, respectively. A 2.90 mL myosin sample was added into a series of tubes containing 0−100 μL of 0.10% ANS solution. Control samples were prepared by adding myosin buffer (80 mM sodium pyrophospate, 2 mM $MgCl_2$, 2 mM EGTA, pH 9.5) into the ANS solution. Final volumes were made up to 3.0 mL with myosin buffer. ANS was allowed to react with myosin for 10 min for each data point. The fraction of ANS bound to myosin was calculated from the method described by Paoletti et al. (1977). ANS binding was measured for duplicate samples at each incubation time per treatment for duplicate runs of the experiment.

Statistical Analysis. Statistical analyses were performed on duplicate sets of data for TBARS and ATPase activity. Data were analyzed by the SAS program (*SAS/STAT User's Guide*) for standard analysis of variance (ANOVA) to compare treatments and replications since the residual variance was constant across treatments. Tukey's studentized range test ($p < 0.05$) was used to determine differences among treatments and incubation time within treatment. Regression analysis of ATPase activity on TBARS was conducted using the Cricket Graphy program (Cricket Software, version 1.31, Alvern, PA) and SigmaPlot (Jandel Scientific Software, San Rafael, CA). No statistical analyses were performed on data for ANS binding and fluorescence intensity. Typical chromatograms are presented in the Results and Discussion.

RESULTS AND DISCUSSION

TBARS values of treated chicken samples incubated for up to 200 min are shown in Figure 1. TBARS values for all treatments increased with increasing incubation time up to 100 min at 37 °C. TBARS values were reduced at 200 min incubation time. However, prevailing thought (Kuusi et al., 1975; Seo, 1976; Davies and Goldberg, 1987; Patel et al., 1988) suggested that under conditions of accelerated oxidation at 200 min, byproducts of lipid oxidation are tightly bound to protein and can not be quantified using the procedure of Raharjo et al. (1993). TBARS values were measureable for all treatments at 0 min incubation time due to the time (4 h) needed to allow some thawing of samples at room temperature with no light. The TBARS value for treatment II was significantly higher compared to the control (treatment I) at zero incubation time, possibly due to an increase in oxidation caused by the added reagents.

For treatment II with $FeSO_4$ and ascorbic acid, TBARS values for all the incubation periods were higher than the corresponding points in all other treatments. With the addition of α-tocopherol in treatment III, TBARS values were lower for each incubation time when compared to values for all other treatments (Figure 1). Results for treatments I (control) and IV were intermediate to those of treatments II and III. Kornbrust and Mavis (1980) showed that ascorbate/Fe^{2+} could induce lipid peroxidation in microsomes from various tissue of rabbit. Furthermore, Davies and Goldberg (1987) showed that ascorbic acid and iron generate free radicals which can induce lipid peroxidation. Our results indicated that α-tocopherol was very effective in inhibiting lipid oxidation accelerated by ascorbate/Fe^{2+} in chicken slurry. This finding strongly suggested that ascorbic acid and iron accelerated lipid oxidation in heated chicken meat slurries.

The two globular heads in myosin are responsible for ATPase activity (Hultin, 1985). A decrease of ATPase activity is indicative of denaturation of the myosin heads. Results from Ca^{2+}-ATPase activity of variously treated myosin are shown in Table 1. Ca^{2+}-ATPase activity was regressed against incubation times for each

Table 1. Changes in the Ca^{2+}-ATPase Activity of Myofibrillar Protein

	ATPase activity (μmol of P_i/min/mg of protein) for treatment[a]			
incubation time (min)	I	II	III	IV
0	19.41 ± 1.16^{C}	19.88 ± 1.14^{BC}	20.73 ± 0.89^{AB}	21.33 ± 0.43^{A}
50	18.06 ± 0.79^{A}	16.11 ± 0.95^{B}	17.39 ± 0.91^{A}	17.18 ± 0.47^{A}
100	17.43 ± 1.18^{A}	13.80 ± 1.02^{B}	17.29 ± 0.97^{A}	17.20 ± 0.29^{A}
200	15.58 ± 0.30^{B}	12.98 ± 0.73^{C}	16.31 ± 0.59^{A}	16.36 ± 0.44^{A}

[a] Final concentrations were treatment I, no additions (control); treatment II, 1.138 mM $FeSO_4$ + 0.368 mM ascorbic acid; treatment III, 1.17 mM α-tocopherol; and treatment IV, 1.138 mM $FeSO_4$ + 0.368 mM ascorbic acid + 1.17 mM α-tocopherol. $n = 6$, means within rows with different superscripts differ significantly ($p < 0.05$).

Table 2. Correlation Coefficients between TBARS Values and Ca^{2+}-ATPase Activity

incubation time (min)	treatment[a]			
	I	II	III	IV
0−100	−0.640	−0.869	−0.686	−0.907
0−200	−0.643	−0.814	−0.691	−0.846

[a] Final concentrations were treatment i, no additions (control); treatment II, 1.138 mM $FeSO_4$ + 0.368 mM ascorbic acid; treatment III, 1.17 mM α-tocopherol; and treatment IV, 1.138 mM $FeSO_4$ + 0.368 mM ascorbic acid + 1.17 mM α-tocopherol. $n = 6$.

treatment. Comparisons of slopes of the lines from linear regression of results shown in Table 1 indicated that the enzyme activity of treatment II decreased most rapidly. Slopes of lines for treatments I−IV were −0.0184 ($r = -0.791$), −0.0324 ($r = -0.852$), −0.019 ($r = -0.761$), and −0.021 ($r = -0.778$), respectively.

Smith (1987) noted that previously frozen hand-deboned and mechanically deboned turkey meat treated with Tenox 2 as an antioxidant had significantly higher Ca^{2+}-ATPase activity when compared to a control. Our results for Ca^{2+}-ATPase activity also supported Smith;s findings about additional antioxidants. When comparing treatment I to III at 200 min incubation, α-tocopherol was effective in increasing Ca^{2+}-ATPase activity. When comparing treatment II to IV, α-tocopherol increased ATPase activity after heating for 100 and 200 min (Table 1).

Ca^{2+}-ATPase activity was regressed against TBARS values for each incubation time (in min) per treatment. With increasing oxidation up to 100 min (Figure 1), Ca^{2+}-ATPase activity decreased (Table 1). The correlation coefficients between TBARS values and Ca^{2+}-ATPase activity for treatments I−IV (for 0−100 and 0−200 min, respectively) are shown in Table 2. TBARS values were correlated with Ca^{2+}-ATPase activity for treatments I−IV.

The fluorescent probe, ANS, was used to monitor conformational changes in myosin. ANS fluorescence increases in intensity when bound to hydrophobic regions of the protein (Bertazzon et al., 1990). Figure 2 is typical of binding curves obtained and shows the differences in ANS binding after the unfolding of myosin and its exposure to byproducts of lipid oxidation for 0−200 min incubation under conditions of treatments I−IV. It seems that more ANS was bound to myosin without α-tocopherol (Figure 2a,b), when compared to the samples with α-tocopherol (Figure 2c,d). The amount of ANS bound to myosin (M/M) seemed greatest at 200 min incubation, under conditions where heating and addition of $FeSO_4$ and ascorbic acid occurred (treatment II, Figure 2b). Possibly, continued heating at 37 °C and increased lipid oxidation at 200 min incubation resulted in greater myosin denaturation, although Figure 1 does not verify this. Treatments I, III, and IV (Figure 2a,c,d, respectively) probably did not show maximum ANS binding at 200 min incubation because no pro-oxidant was added to treatment I (Figure 2a) and α-tocopherol was added to treatments III and IV (Figure 2c,d). It is not clear why ANS binding at 0 min incubation was decreased with the addition of ferrous sulfate and ascorbic acid (treatment II) in Figure 2b as compared to the control in Figure 2a.

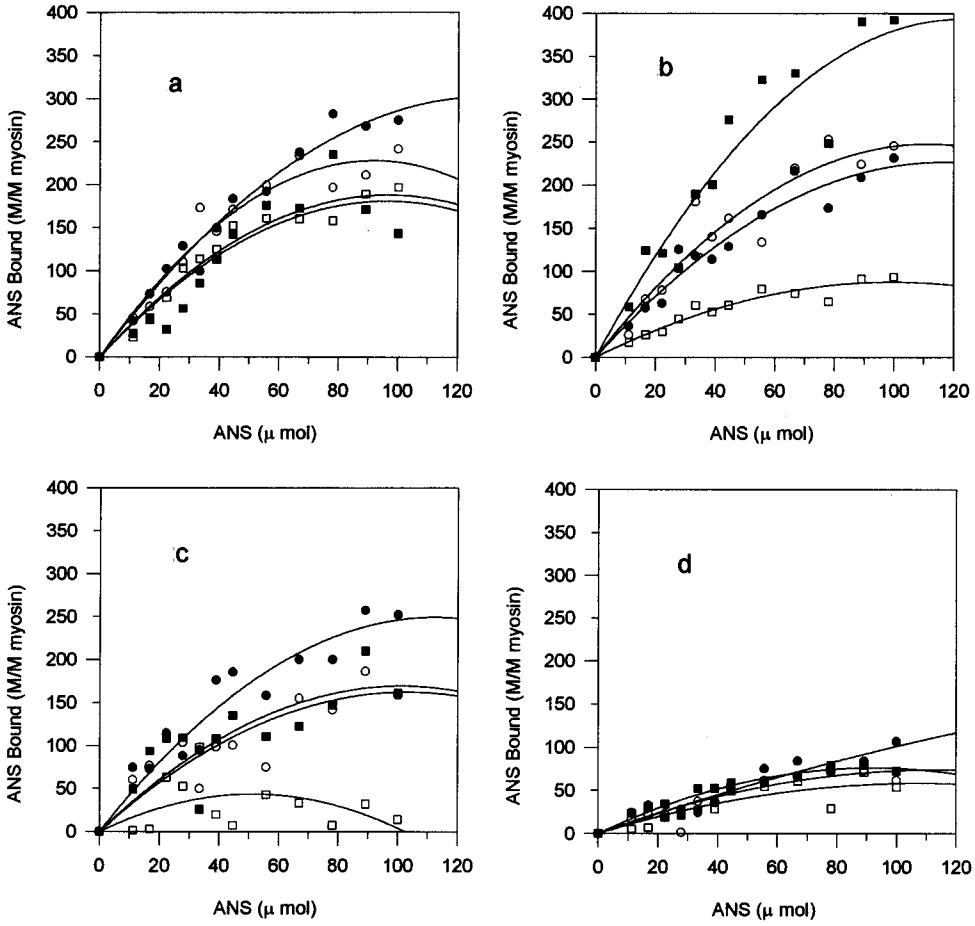

Figure 2. Typical chromatogram of ANS bound to myosin: (□) 0, (●) 50, (○) 100, and (■) 200 min. Final concentrations were (a) treatment I, no additions (control); (b) treatment II, 1.138 mM $FeSO_4$ + 0.368 mM ascorbic acid; (c) treatment III, 1.17 mM α-tocopherol; and (d) treatment IV, 1.138 mM $FeSO_4$ + 0.368 mM ascorbic acid + 1.17 mM α-tocopherol.

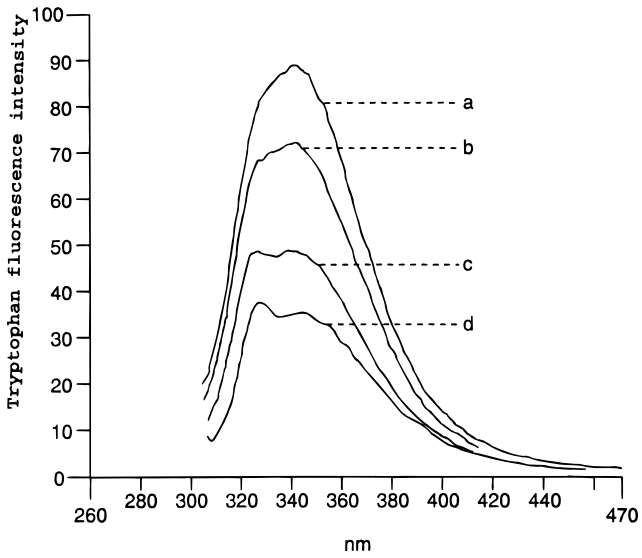

Figure 3. Typical tryptophan fluorescence intensity of chicken myosin in treatment II (1.138 mM $FeSO_4$ + 0.368 mM ascorbic acid). Incubation times were (a) 0, (b) 50, (c) 100, and (d) 200 min.

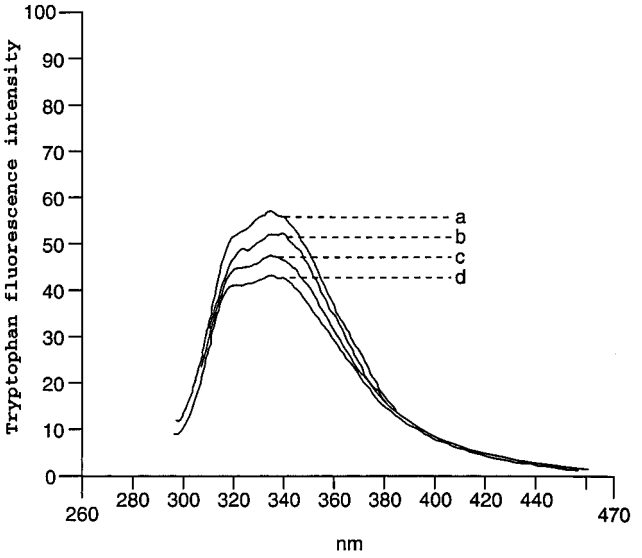

Figure 4. Typical tryptophan fluorescence intensity of chicken myosin in treatment I (control) with the following incubation times: (a) 0, (b) 50, (c) 100, and (d) 200 min.

Few studies have been conducted on denaturation of protein by products of lipid oxidation during heating (Nakai and Li-Chan, 1988). These investigators noted that, when heated, the denaturation of fish protein was accelerated by the presence of lipids. Bertazzon et al. (1990) reported that native actin had a lower ANS accessibility compared to heated denatured actin. In our work, we have shown that possible denaturation with the addition of lipid oxidation byproducts increased the amounts of ANS bound to heated myosin.

Two tryptophan residues which are located in the light meromyosin region contribute to the fluorescence of the entire myosin molecule. The unfolding of myosin affects the tryptophan environment which can be monitored by the relative fluorescence quantum yield (King and Lehrer, 1989). Increasing lipid oxidation by incubation for 200 min greatly decreased the fluorescence intensity for treatment II (Figure 3). Assuming 100% fluorescence for 0 min incubation in treatment II, the percent reduction of maximum tryptophan intensity for 50, 100, and 200 min incubation was 19.14%, 45.38%, and 59.03%, respectively. The decrease in tryptophan fluorescence is a reflection of the denaturation of the myosin and the exposure of the indole residues to an aqueous environment. The rate of fluorescence intensity over time was not as depressed for the control (treatment I), where low oxidation occurred (Figure 4). When α-tocopherol was added (treatment III and IV), there was no change in tryptophan intensity over time (data not shown).

It is important to distinguish protein denaturation caused by lipid deterioration from that induced by the Fenton reaction. It is well documented that the hydroxyl radical produced by ascorbate/Fe^{2+} in the presence of hydrogen peroxide (Fenton reaction) can directly catalyze protein oxidation (Davies and Goldberg, 1987; Stadtman and Oliver, 1991; Decker et al., 1993). Free radical scavengers (antioxidants, like α-tocopherol) can not inhibit the metal-catalyzed oxidation of protein because hydroxyl radical reacts preferentially with functional groups at the metal binding sites (Stadtman and Oliver, 1991). Davies and Goldberg (1987) indicated that protein degradation induced by the Fenton reaction occurred 2 h earlier compared to lipid peroxidation in rabbit erythrocytes incubated at 37 °C. They concluded that protein degradation and lipid peroxidation are independent events. Our results showed that both lipid oxidation and myosin denaturation occurred simultaneously in the system and that α-tocopherol was effective in inhibiting the accelerated lipid oxidation and myosin denaturation. This finding strongly supported the idea that myosin denaturation was mainly affected by lipid oxidation rather than by direct metal-catalyzed protein oxidation in our system. Srinivansan et al. (1996) recently reported that α-tocopherol (0.2%) inhibited both lipid oxidation and protein oxidation in beef heart surimi pellet; they concluded that protein oxidation was probably coupled with the lipid oxidation in the surimi pellet. Furthermore, only the side chains of histidine, arginine, lysine, and proline were found to be sensitive to oxidation by the metal-catalyzed oxidation systems; the content of tryptophan was not affected (Levine, 1983; Amici et al., 1989). A decrease in intensity of tryptophan fluorescence with increasing lipid oxidation (Figure 3) suggested that myosin denaturation was caused by accelerated lipid oxidation via ascorbate/Fe^{2+} in chicken muscle slurry.

Our results showed that pro-oxidants accelerated the denaturation of myosin by increasing lipid oxidation beyond that caused by heating. It is important to know how denaturation of myosin due to addition of lipid byproducts changes the structure of the protein beyond that caused by heating.

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Received for review January 17, 1996. Revised manuscript received July 1, 1996. Accepted July 26, 1996.[⊗]

JF9600216

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1996.