Structural Changes of Rabbit Myosin Subfragment 1 Altered by Malonaldehyde, a Byproduct of Lipid Oxidation

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Rabbit myosin subfragment 1 was allowed to react with malonaldehyde, a byproduct of lipid oxidation in a model system containing the subfragment 1 and malonaldehyde. The modified subfragment 1 was compared to the control, with use of sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), circular dichorism (CD), Fourier transform infrared spectroscopy (FT-IR), and free amino group measurements. Both the modified and control samples were cleaved with cyanogen bromide (BrCN) treatment, and the BrCN fragments were analyzed by SDS−PAGE with tricine gel. Results from SDS−PAGE suggest that malonaldehyde causes cross-linking or polymerization of the protein during incubation. Malonaldehyde also reduced α-helix content (CD), increased random structure (FT-IR), and eliminated some β-strand structure (FT-IR) in subfragment 1.

Keywords: *Rabbit myosin subfragment 1; malonaldehyde; secondary structure*

INTRODUCTION

It is well documented that lipid oxidation leads to modification of proteins in biological systems (Andrews et al., 1965; Tappel and Roubal, 1966; Kanner and Karel, 1976; Benedetti et al., 1979; Logani and Davies, 1980; Shimasaki et al., 1982; Riley and Harding, 1993). Fatty acids, other lipids, and oxidized lipids react with proteins to change the functional properties of meat (Sikorski, 1978; Funes et al., 1982; Smith, 1987; Decker et al., 1993; Xiong et al., 1993). Our previous results showed that pro-oxidants (ascorbic acid/Fe^{2+}) accelerated the denaturation of myosin during heating by increasing lipid oxidation in dark chicken meat. Myosin denaturation, measured as Ca^{2+}-ATPase activity, after heating was positively correlated with lipid oxidation. Exogenously added α-tocopherol reduced lipid oxidation and decreased myosin denaturation during heating (Li and King, 1996).

Specific byproducts of lipid oxidation, such as malonaldehyde (MDA), can interact with ϵ-amino groups of proteins to cause cross-linking (Esterbauer and Cheeseman, 1987). A more recent study has been conducted in our laboratory to further elucidate changes occurring when MDA associates with rabbit myosin subfragment 1 in a model system containing the subfragment 1 and MDA. Results showed from the study that MDA decreased Ca^{2+}-ATPase activity and caused conformational changes as measured by sulfhydryl content, protein-bound MDA, tryptophan fluorescence, and 1-anilino-8-naphthalenesulfonate fluorescence (King and Li, 1999).

Myosin is one of the most important muscle proteins. Several studies suggested that lipid oxidation could decrease functionality of myofibrillar protein in muscle foods (Sikorski, 1978; Logani and Davies, 1980; Funes et al., 1982). It is important to know how MDA changes the actual structure of the protein. Myosin subfragment 1, which contains two globular heads, has a low molecular mass (95000 Da) compared to myosin and is one of the major domains of myosin (King and Li, 1999). Thus, subfragment 1 is more practical for studying the modification and denaturation by MDA. Our objective is to determine if association of MDA with rabbit myosin subfragment 1 leads to cross-linking and changes in the secondary structure of the protein.

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MATERIALS AND METHODS

Materials. Myosin subfragment 1 from rabbit muscle, a mixture of five protein standards (hen egg white ovalbumin, bovine serum albumin, rabbit muscle phosphorylase *b*, *Escherichia coli* β-galactosidase, and rabbit skeletal muscle myosin), and other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Precasting tricine gel, its stains, destaining solutions, running buffer, and a mixture of 12 protein standards were obtained from Novex (San Diego, CA).

Preparation of MDA Solution. Preparation of MDA was the same as previously published (King and Li, 1999). Briefly, MDA was prepared according to the method of Riley and Harding (1993). MDA was obtained by acid hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) for 24 h at room temperature. The solution was adjusted to pH 6.2 with NaOH and buffered with 0.025 M potassium phosphate containing 0.5 M KCl. The buffer was identical to that of rabbit myosin subfragment 1 mentioned below. The final concentration of MDA (100 mM) was diluted to various concentrations ranging from 0 to 10 mM with the same buffer solution. MDA solutions were made fresh daily.

Modification of Rabbit Myosin Subfragment 1 with MDA. Modification of subfragment 1 has been previously published (King and Li, 1999). Briefly, rabbit myosin subfragment 1 was dialyzed against 0.5 M KCl, 0.025 M phosphate buffer (pH 6.2) in a cold room. Each 0.50 mL of rabbit myosin subfragment 1 (2.5 mg/mL) was incubated with 0.50 mL of 0, 2, 4, 8, and 10 mM MDA solutions at 37 °C in a shaking water bath for 2 h. In another set of experiments, each 0.50 mL of myosin subfragment 1 (2.0 mg/mL) was incubated with 4 mM MDA at 37 °C for 0, 1, 2, 3, and 4 h. The range of MDA concentrations between 0 and 10 mM was used on the basis of TBARS values and myosin concentrations in chicken slurry from our previous study (Li and King, 1996). After incubation,

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samples were immediately transferred into small tubes, kept at 0 °C. Cold acetone (0.5 mL) was added to the reaction mixture to precipitate the modified protein; samples were centrifuged at 11400*g* for 5 min in a cold room. The supernatant was discarded, and the same volume of cold acetone was added into the pellet to remove any residue of MDA. After centrifugation, the pellets were redissolved in phosphate buffer. The samples were stored at −85 °C until further analysis.

Determination of Contents of Free Amino Groups. For each sample treated with 4 mM MDA for 0, 1, and 4 h, the content of free amino groups was determined as described by Kakade and Liener (1969). Protein concentration was measured according to the procedure of Lowry et al. (1951).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was used to monitor the polymerization of the modified proteins. The samples of myosin subfragment 1 were incubated with 4 mM MDA for 0, 1, 2, 3, and 4 h before application to the gel. Samples were then treated with SDS treatment buffer containing β-mercaptoethanol. Protein standards ranging from 45000 to 200000 Da were used. In another experiment, each 0.25 mL of rabbit myosin subfragment 1 (2.0 mg/mL) was incubated with 0.25 mL of 0,2, 4, 8, and 10 mM MDA solutions and applied into each gel lane. Protein standards ranging from 36000 to 205000 Da were used. SDS−PAGE was performed on 4% stacking gels and 12% running gels (Laemmli, 1970) using an SE 250 Might Small II slab gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA), stained with Coomassie blue R-250.

Cyanogen Bromide (BrCN) Cleavage Fragments. About 1.0 mg of myosin subfragment 1 incubated with 4 mM MDA for 4 h samples and the control (without MDA) samples were dialyzed against deionized−distilled water to remove the substances of small molecular weight. Samples were lyophilized and treated with 1.0 mL 0.2 N BrCN in 70% formic acid to cleave the polypeptides into small fragments. After 24 h of incubation at room temperature, the samples containing BrCN and formic acid were evaporated by nitrogen under the hood, dissolved into deionized water, lyophilized, and redissolved into 1 mL of deionized water. An aliquot of each sample was run on SDS−PAGE with 10−20% precasting tricine gel (Novex) to separate BrCN fragments. A mixture of 12 protein standards from Novex with molecular masses ranging between 2500 and 200000 Da was used.

Circular Dichroism (CD) Measurement. The ellipticity of proteins was measured by using a JASCO J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with a scanning wavelength from 200 to 260 nm. Prior to the experiment, samples of myosin subfragment 1 incubated with 4 mM MDA for 0, 1, and 4 h were dialyzed against 0.1 M KCl and 10 mM phosphate buffer (pH 7.2). After dialysis, samples were centrifuged at 1600*g* for 10 min to remove insoluble substances. Protein concentrations in supernatants were measured at 280 nm. Because the CD instrument cannot make accurate measurements on samples with an absorbance >1.0, the chosen concentration and path length were kept at an absorbance <1.0. The contents of α-helix were expressed as $\Delta\epsilon$ M^{-1} cm^{-1} at 222 nm.

Fourier Transform-Infrared Spectroscopy (FT-IR). Samples of myosin subfragment 1 incubated with 4 mM MDA for 0, 1, and 4 h were subjected to FT-IR analysis. Samples in 0.5 M KCl/0.025 M phosphate buffer (pH 6.2) were lyophilized until dry and dissolved into the same volume of D_2O solution. Control was prepared by lyophilizing the phosphate buffer and redissolved into D_2O solution.

The IR window consisted of calcium fluoride (CaF_2) with a 25 μm path length (Wilmad, Buena, NJ). Infrared spectra of the control and modified myosin subfragment 1 were recorded with a 16PC FT-IR spectrometer (Perkin-Elmer, Norwalk, CT). Nitrogen gas was continuously purged into the IR chamber to eliminate moisture. Each sample was scanned 512 times at 2.0 cm^{-1} resolution. The spectrum of each sample was obtained by subtracting the control buffer in D_2O. The second derivatives of spectra were analyzed using a bandwidth at 2 cm^{-1}.

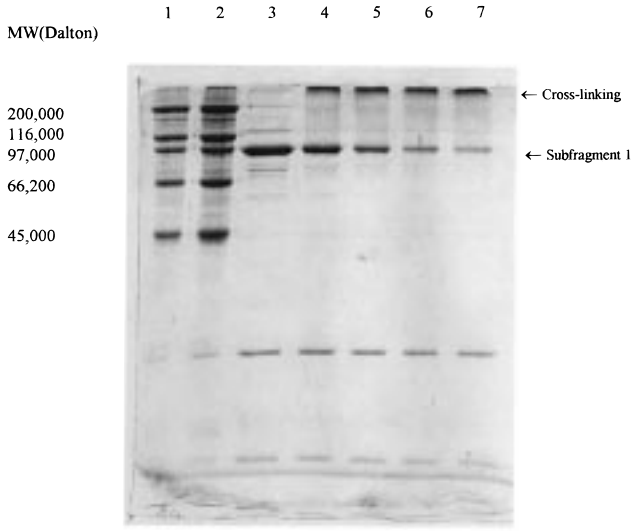

Figure 1. Typical SDS−PAGE chromatogram of rabbit muscle myosin subfragment 1 (0.25 mL, 2.0 mg/mL) treated with 0.25 mL of 4 mM MDA and incubated at 37 °C from 0 to 4 h: (lanes 1 and 2) molecular weight standards; (lane 3) 0 h of incubation; (lane 4) 1 h of incubation; (lane 5) 2 h of incubation; (lane 6) 3 h of incubation; (lane 7) 4 h of incubation. Each lane contained 5 μg of protein except lane 2 (10 μg). Bands of myosin subfragment 1 (95000 Da) are shown in lanes 3−7.

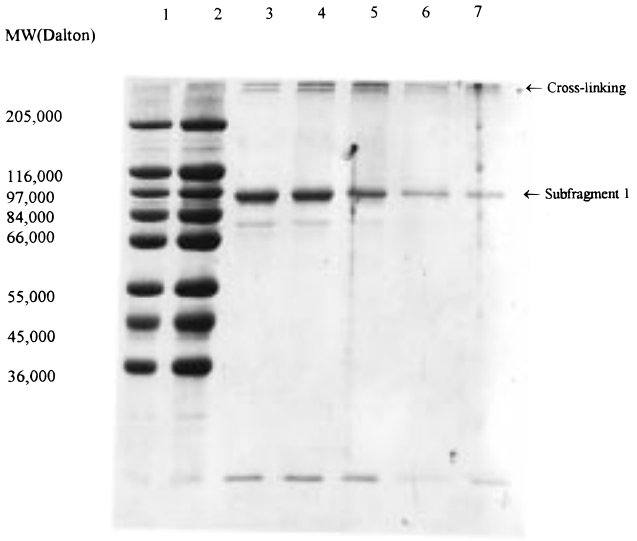

Figure 2. Typical SDS−PAGE chromatogram of rabbit muscle myosin subfragment 1 (0.25 mL, 2.5 mg/mL) treated with 0.25 mL of 0, 2, 4, 8, and 10 mM MDA solutions and incubated at 37 °C for 2 h: (lanes 1 and 2) molecular weight standards; (lane 3) 0 mM MDA; (lane 4) 2 mM MDA; (lane 5) 4 mM MDA; (lane 6) 8 mM MDA; (lane 7) 10 mM MDA. Each lane contained 5 μg of protein. Bands of myosin subfragment 1 (95000 Da) are shown in lanes 3−7.

Statistical Analysis. Data for free amino groups were analyzed for a single factor with analysis of variance (ANOVA) to compare various treatments. Tukey's Studentized range test ($p < 0.05$) was used to determine differences between treatments. All determinations of SDS−PAGE, CD, and FT-IR experiments were repeated at least twice.

RESULTS AND DISCUSSION

SDS−PAGE patterns indicated cross-linking or polymerization of myosin subfragment 1 reacted with MDA at 37 °C for 0, 1, 2, 3, and 4 h (Figure 1). The intensity of the subfragment 1 band (95000 Da) de-

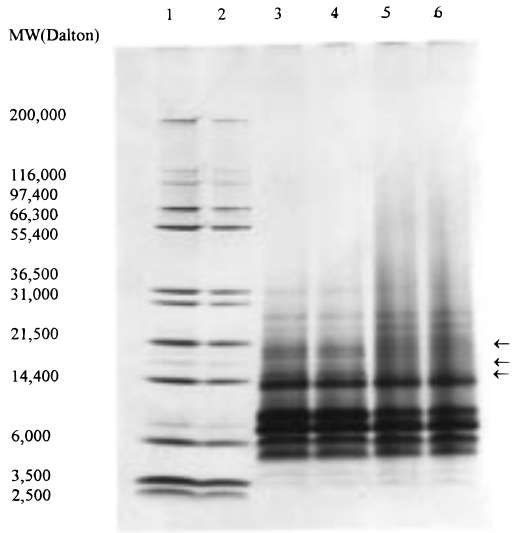

Figure 3. SDS−PAGE with tricine gel pattern of BrCN cleavage fragments from rabbit myosin subfragment 1 treated with 4 mM MDA incubated for 4 h and the control (without MDA treatment) samples: (lanes 1 and 2) molecular weight standards; (lanes 3 and 4) BrCN cleavage fragments from MDA-treated sample; (lanes 5 and 6) BrCN cleavage fragments from the control sample.

creased with increasing incubation time from 0 to 4 h, and the cross-linking protein band appeared on top of the gel after 1 h of incubation (Figure 1). Prior to loading of the protein samples into gel, β-mercaptoethanol was added to the sample to break the disulfide bonds in the protein. Therefore, the bands on top of the gel were attributed to the cross-linking of subfragment 1 via nondisulfide covalent linkage. Riley and Harding (1993) reported that lens protein showed increased intensity of the cross-linking band after 1 h of incubation with MDA and suggested that MDA causes covalent cross-linking of protein. The effect of various MDA concentrations on subfragment 1 is shown in Figure 2. The intensity of the subfragment 1 band decreased with increasing concentrations of MDA; the cross-linking band appears on lanes 4 and 5 (2 and 4 mM MDA, respectively), but not for 8 and 10 mM MDA, probably because high concentrations of MDA could cause the polypeptide chain scission. Our previous result showed that increased MDA concentration during incubation possibly led to destruction of the active sites of the Ca^{2+}-ATPase in the globular heads and caused conformational changes of the protein. Ca^{2+}-ATPase activity and free sulfhydryl content of myosin subfragment 1 decreased with either increasing incubation time or concentration when treated with MDA (King and Li, 1999). The data shown here together with those from our previous results suggest that MDA could cause cross-linking, decreased myosin ATPase activity, and conformational changes of subfragment 1. Buttkus (1967) reported that the reaction of myosin with MDA modified 50−60% of the ϵ-amino groups of lysines in the protein. Kuusi et al. (1975) also reported that MDA reacted with the free amino groups of fish proteins during frozen storage. MDA reacts with τ-crystallin to cause nondisulfide covalent cross-linking (Riley and Harding, 1993).

To further understand how MDA causes the cross-linking or polymerization of the protein, cleaved peptides from the polymerized protein were separated by SDS−PAGE and tricine gel and compared with the control. Basically, the chemical reagent BrCN reacts specifically with methionine residues to cleave the peptide chain and produce C-terminal fragments. Because the protein contains a relatively low amount of methionine residues, only a few fragments could be generated by BrCN cleavage. SDS−PAGE showed fragments resulting from BrCN cleavage of cross-linking protein previously incubated (4 h) with MDA as compared to a control sample (Figure 3). Extra bands (lanes 3 and 4) as shown in Figure 3 were found from BrCN cleavage fragments of the polymerized protein compared to the control samples (lanes 5 and 6). The molecular masses of these extra BrCN fragments ranged between 14000 and 21500 Da (Figure 3). These extra bands could be fragments of cross-linking protein by MDA, which could resist BrCN cleavage. The results further confirmed that MDA causes cross-linking or polymerization

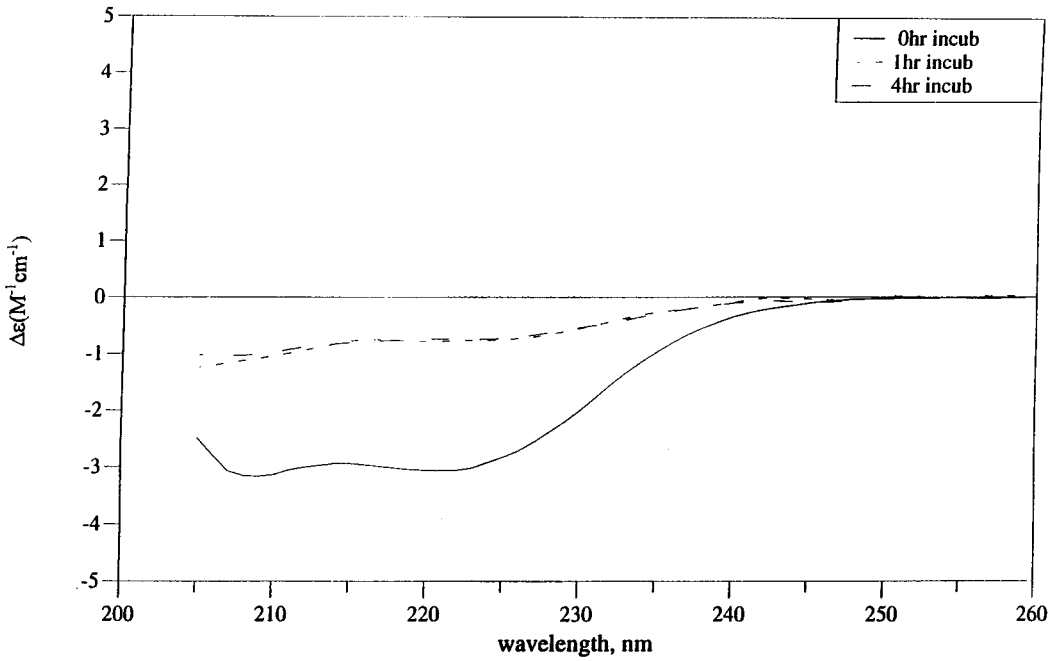

Figure 4. CD spectrum of rabbit myosin subfragment 1 incubated with 4 mM MDA for 0, 1, and 4 h.

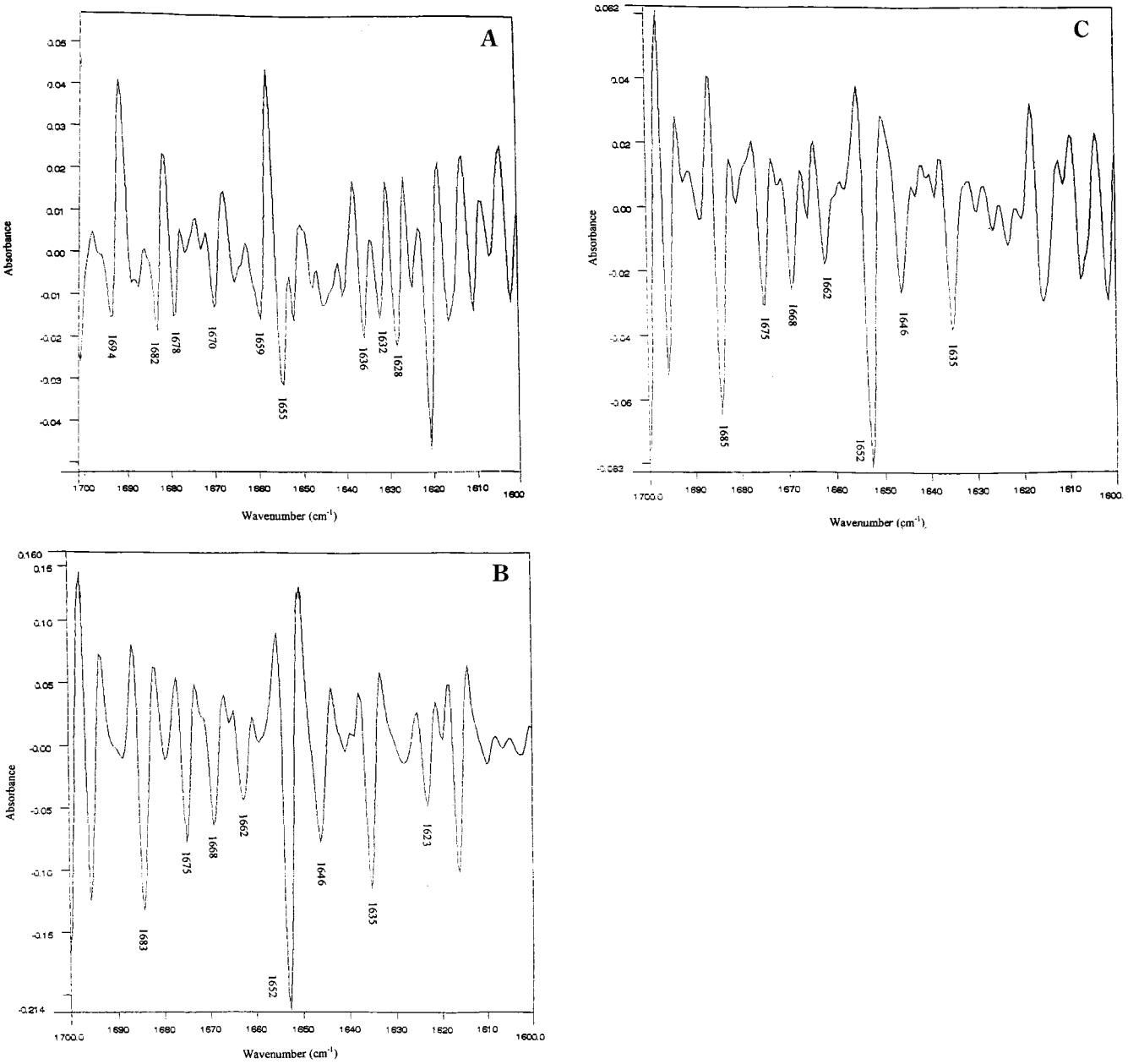

Figure 5. (A) Second derivative of FT-IR spectrum of rabbit myosin subfragment 1 (control sample); (B) second derivative of FT-IR spectrum of rabbit myosin subfragment 1 incubated with 4 mM MDA for 1 h; (C) second derivative of FT-IR spectrum of rabbit myosin subfragment 1 incubated with 4 mM MDA for 4 h.

of the protein to form a large molecule in the system. No further BrCN fragment was observed for either sample with a molecular mass >31000 Da, indicating that the polypeptides were cleaved into relatively small fragments (MW < 31000 Da). In future studies, these BrCN fragments can be further cleaved with enzymes; sequence analysis and mass determination of these cross-linking fragments will allow us to pinpoint modified amino acids and perhaps the nature of modification involved in the process of cross-linking of the molecule.

It is well-known that ϵ-NH_2 groups of lysine are very susceptible to MDA modification (Buttkus, 1967; Esterbauer et al., 1991). Results in Table 1 show a significant decrease in free amino groups of subfragment 1 treated with MDA with increasing incubation time up to 4 h ($p < 0.05$). The data shown here and in our previous study (King and Li, 1999) confirm that MDA could react with specific amino acids, such as free amino and sulfhydryl groups, and lead to cross-linking via formation of covalent bonds. Results from our previous study indicated that association of MDA with rabbit myosin subfragment 1 led to denaturation of the protein in vitro, as measured by Ca^{2+}-ATPase activity, tryptophan fluorescence, and 1-anilino-8-naphthalenesulfonate fluorescence (King and Li, 1999). Most denatured proteins are not completely unfolded, and some ordered structures may exist even after heating (Clark and Lee-Tuffnell, 1986). We wanted to know how association of

Table 1. Content of Free Amino Group of MDA-Treated and Control Samples

incubation with MDA (h)	free amino group[a] (nmol/mg of protein)
0	0.307 ± 0.01^a
1	0.289 ± 0.003^b
4	0.238 ± 0.01^c

[a] $N = 4$; means within columns with different superscripts differ significantly ($p < 0.05$).

MDA with subfragment 1 caused a change in the secondary structure of subfragment 1. Thus, CD was run to determine the change of α-helix content. Wavelengths below 200 nm could not be used due to high amounts of KCl present in the samples, which reduces transparency. Therefore, information about β-sheet and β-turn contents is not available from the CD study.

Typical CD spectra of the control (0 h of incubation) and cross-linking samples (1 and 4 h of incubation) are shown in Figure 4. The control contained ~32% α-helix content on the basis of the measurement at 222 nm, whereas 1 and 4 h samples had about 8 and 7%, respectively, indicating that cross-linking of the protein by MDA significantly reduced the amount of α-helix content. Johnson et al. (1991) reported that α-helix content of myosin subfragment 1 from the back muscle of rabbit was 40%. The difference between their results and those reported here was probably due to various sources of muscle and methods of isolation. We have reported that more hydrophobic exposure of subfragment 1 was associated with MDA at 1 and 4 h (King and Li, 1999). The decrease in α-helix content in 1 and 4 h samples coincided with the result from ANS fluorescence, suggesting that MDA caused the more hydrophobic exposure and loss of α-helix structure in the protein. The structure of α-helix is stabilized by hydrogen bonds between −CO and NH− of a polypeptide chain (Sano et al., 1994). Modification of the free amino group by MDA may decrease hydrogen bonds by reducing the amount of proton donation.

The FT-IR method has been well used to study the secondary structures of proteins (Carrier et al., 1990; Fabian et al., 1992; Haris and Chapman, 1992; Surewicz et al., 1993; Boye et al., 1997). A protein containing different types of secondary structures gives a different amide 1 maxima. By using Fourier deconvolution and Fourier derivation technique, it is possible to distinguish between the individual component of the intrinsically broad and overlapping individual amide 1 band contours (Fabian et al., 1992). The secondary derivative of the FT-IR spectrum of samples with 0, 1, and 4 h of incubation is shown in Figure 5. The FT-IR spectra of subfragment 1 samples with 0 h of incubation (Figure 5A) showed major bands at about 1628 (β-strand), 1632, 1636 (β-sheet), 1655 (α-helix), 1659, 1670 (β-turn), 1678, 1682 (β-turn/sheet), and 1694 cm^{-1} (β-type structure) (Boye et al., 1997; Fabian et al., 1992). The band at 1646 cm^{-1} (random structure) is not obvious for the 0 h sample. After 1 h of incubation, the band at 1646 cm^{-1} appeared; other bands at 1623, 1635 (β-sheet), 1652 (α-helix), 1662, 1668(β-turn), and 1675 and 1683 cm^{-1} (β-turn/β-sheet) are shown in Figure 5B. After 4 h of incubation, the band at 1628 (β-strand) disappeared; other main bands at 1635 (β-sheet), 1652 (α-helix), 1662, 1668 (β-turn), 1675, and 1685 (β-sheet/turn) are shown in Figure 5C. It becomes obvious that random structure appeared after 1 h of incubation with MDA. In addition, β-strand structure was also affected because the band at 1628 cm^{-1} disappeared for 1 and 4 h samples in the FT-IR spectrum. It seems that neither β-sheet nor β-turn structure was significantly affected during incubation with MDA, because all samples showed major bands at 1635−1636 (β-sheet), 1668−1670, and 1675−1685 cm^{-1} (β-turn) (Figure 5). However, we used the FT-IR technique to do only qualitative measurement of the secondary structure of the myosin subfragment 1 altered by MDA, not quantitative analysis. It is concluded that α-helix and β-strand contents of subfragment 1 significantly decreased during incubation with MDA and increased the random structure on the basis of the results from CD and FT-IR. MDA may form hydrogen bonds with the peptide bond, destabilizing the secondary structure of the protein. Moreover, the reaction of MDA with free amino groups could reduce the amount of hydrogen bonding and then destabilize the secondary structure of protein.

As indicated before, rabbit myosin subfragment 1 was substituted for myosin in the model study. MDA, a major byproduct of lipid oxidation, was also chosen for the model system, which simulated the lipid oxidation and muscle protein interaction hypothesized in muscle food. Lipid oxidation and protein interactions can result in myosin denaturation, destruction, and quality changes of muscle foods. However, little information is available concerning how specific changes in myosin structure are altered by MDA, probably because of practical difficulties in working with molecules of high molecular weight. We have reported that this active compound can lead to denaturation of myosin subfragment 1 in a model system (King and Li, 1999). A similar mechanism could occur in a muscle food system because of the presence of high amounts of polyunsaturated fatty acids. The results presented here provide more specific information on how the compound causes cross-linking and loss of secondary structure of the purified subfragment 1. It is important to note that changes of the secondary structure altered by lipid oxidation could affect protein functionality in muscle food systems; cross-linking or modification of protein side chains, such as ε-amino groups of lysine, may alter protein solubility, digestibility, gelation, etc. Ultimately, future studies will help us understand how myosin structure and functionality are altered due to oxidative damage caused by lipid deterioration.

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