Denaturation and Aggregation of Chicken Myosin Isoforms

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Heat-induced denaturation and aggregation of chicken myosins isolated from one white muscle (pectoralis) and two red muscles (iliotibialis and gastrocnemius) were investigated using differential scanning calorimetry and turbidity (OD_{350nm}) measurements. Peptide mapping showed that the three myosins had different heavy chain isoforms. Pectoralis and iliotibialis myosins had higher calorimetric enthalpies and a higher denaturation onset temperature than gastrocnemius myosin. Aggregation occurred for the pectoralis myosin at 45 °C, while the red muscle myosins required $T > 45$ °C for aggregation. The rates of aggregation were rapid at lower temperatures and then decreased as temperature increased. The red muscle myosins were more similar in peptide maps in comparison to the white muscle myosin; however, there were unique denaturation and aggregation properties for each type of myosin.

Keywords: *Myosin; aggregation; denaturation; muscle protein*

INTRODUCTION

Myosin functions in skeletal muscle contraction by interacting with actin and mechanically shortening the muscle (Rayment et al., 1993). In meat and meat products, myosin is responsible for binding together meat particles (MacFarlane et al., 1977) and is the major protein contributing to thermally induced gelation of muscle proteins (Samejima et al., 1969). Myosin has a molecular weight of approximately 520 000 daltons and is comprised of two heavy chains and four light chains (Starr and Offer, 1971). Both heavy chains and light chains have isoforms. The different myosin isoforms in skeletal muscle are associated with functional (fast and slow twitch) and developmental (embryonic, neonatal, and adult) programs (Emerson and Bernstein, 1987; Stockdale and Miller, 1987). There appears to be many myosin heavy-chain isoforms present in vertebrates (Moore et al., 1993). Robbins et al. (1982) found seven myosin heavy-chain genes and proposed that there might be 31 myosin heavy-chain genes in chickens. In a later study, Robbins et al. (1986) found the seven myosin heavy chains coded for white muscle (fast twitch) myosins in chicken. Hofmann et al. (1988) was able to identify six electrophoretically different myosin heavy-chain isoforms expressed during the development of chicken breast muscle.

Myosins isolated from a variety of skeletal muscle sources have different gelation properties. Differences in unfolding and aggregation reactions, along with rigidity of thermally induced gels, have been noted for myosins isolated from several species of fish (Chan et al., 1992; Gill et al., 1992). Chicken myosin gels formed from pH 6.0 dispersions have consistently shown that white muscle myosin gels are more rigid than red muscle myosin gels (Asghar et al., 1984; Morita et al., 1987). The pH optimums for gel rigidity of red and white myosin were found to be different between the previously cited studies. This may have been due to myosin isoform variation within each type of muscle (i.e., red and white). Asghar et al. (1984) used pectoralis profundus (white, breast muscle) and gastrocnemius (red, leg muscle) whereas Morita et al. (1987) used breast (white muscles) and leg (red muscles).

The reactions comprising thermally induced gelation are protein unfolding, aggregation, and formation of a gel network. The mechanical properties of gels (i.e., rigidity) are determined by the gel matrix and surrounding fluid. The rates of denaturation and aggregation have been associated with gel matrix properties. Assuming gelation is due to intermolecular interactions that act along the entire molecule, a rapid unfolding followed by a slow aggregation process would favor a fine-stranded gel structure (Ferry, 1948). Coarse gel matrices are produced by rapid aggregation (Ferry, 1948). It is therefore important to separate the processes of denaturation and aggregation to understand the mechanism(s) of matrix formation and mechanical properties.

Research to date has yet to explain the mechanism(s) of isoform-associated characteristics of gel rheological properties. Chicken muscles are excellent for these investigations because there are distinct white and red muscle types. Three chicken muscles were chosen for this study, one white (pectoralis) and two red (iliotibialis and gastrocnemius). The goal of this investigation was to determine isoform-associated differences in denaturation and aggregation. Isoform-associated differences in thermally induced gelation and rheological properties of gels will be addressed in a subsequent report.

MATERIALS AND METHODS

Myosin Extraction. The muscles chosen represent the largest muscle mass in the areas that they occur. The pectoralis comprises most of the breast muscles, the iliotibialis is the largest muscle in the thigh, and the gastrocnemius is the largest muscle in the lower leg. Myosin was prepared from adult Hubbard-type chickens (spent hens) obtained from New Hope Feeds Co., Goldsboro, NC. Chickens were anesthetized

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S0021-8561(95)00342-6 CCC: $12.00

by injection of 4−10 mg/kg of ketamine hydrochloride and xylazine (1:1) in a principal vein under the wing and euthanized by cervical dislocation. Three muscles (free of skin and fat), pectoralis, gastrocnemius, and iliotibialis, were removed prerigor. Three hundred grams of each muscle were separately ground through a meat grinder (washed with 10 mM EDTA) and placed in extraction solution (Foegeding et al., 1986) within 20 min postmortem. Myosin from each muscle was separately extracted at 4 °C using the method described by Foegeding et al. (1986). The myosin pellet obtained was dissolved in and dialyzed against "myosin buffer" (0.6 M NaCl, 10 mM sodium phosphate, and 0.02% sodium azide, pH 6.0) for 16 h, with one change in dialyzing solution after 8 h. Myosin was kept at 4 °C and all measurements were obtained within 1 week after extraction, with the exception of the differential scanning calorimetry (DSC) experiments where the myosin was mixed 1:1 with glycerol, frozen, and stored at −20 °C.

Myosin Concentration Determination and Sample Preparation. The myosin concentration (mg/mL) was calculated on the basis of A_{280} readings (UV-240 recording spectrophotometer, Shimadzu Corp., Tokyo, Japan), using an extinction coefficient of 0.52 cm^2/mg (Quass and Briskey, 1968). Three stock solutions (0.7 mg/mL) were prepared for each myosin for use in turbidity measurements to provide three observations per replication. Stock solutions were degassed under vacuum for 30 min to remove dissolved gases.

Electrophoresis and Peptide Mapping. The purity of myosin preparations was evaluated using sodium dodecyl sulfate polyacrylamide gel (SDS-PAG) electrophoresis by the method of Laemmli (1970), with a 4% acrylamide stacking gel and 10% acrylamide separating gel. Proteins were electrophoresed at 15 mA until the tracking dye reached the separating gel and then at 25 mA until the tracking dye reached the bottom of the gel. Polyacrylamide gels were held at 10 °C during electrophoresis. Gels were stained overnight in a solution containing 1 mg/mL of Coomassie Blue R-250, 50% methanol, and 6.8% glacial acetic acid. Stained gels were diffusion destained in a 7.5% glacial acetic acid and 5% methanol solution. Staining density was determined using a Molecular Dynamics 300A computing densitometer (Sunnyvale, CA). The amount of myosin (a sum of the heavy and light chains) was determined as a percentage of the total amount of proteins present in individual lanes of gel containing 15, 20, 25, 30, 35, 40, and 45 mg of protein/lane of myosin. Myosin was 80−90% pure on the basis of equivalent staining density among proteins.

The method of Cleveland et al. (1977) was used for peptide mapping. A 10 ng amount of myosin was electrophoresed, using the Laemmli (1970) method described above, to isolate the myosin heavy chain. After staining of the gel for 25 min using Coomassie Blue and destaining for 45 min, the myosin heavy chains were cut from the gel and allowed to equilibrate with "gel slices buffer" (0.125 M Tris buffer, 1 mM EDTA, and 0.1% SDS at pH 6.8) for 30 min. Gel slices in gel slices buffer were frozen overnight at −20 °C. Gel slices and buffer were thawed and placed into a stacking gel of 3.5% acrylamide (with 1 mM EDTA), 10 μL of tracking dye was added to all wells, and 10 μL of *Staphylococcus aureus* V8 protease (25 μg/mL) in a protease buffer (0.125 M Tris buffer, 1 mM EDTA, 0.1% SDS, 10% glycerol at pH 6.8) was layered over the gel slices. Electrophoresis was run (about 2 h) at 10 °C and 15 mA until the tracking dye was almost to the separating gel (13% acrylamide with 1 mM EDTA). The system was turned off for 30 min for proteolysis to occur. Electrophoresis was then continued at 10 °C and 25 mA until the tracking dye reached the bottom of the gel. Gels were stained with silver stain (Oakley et al., 1980).

Differential Scanning Calorimetry (DSC). Myosin was mixed 1:1 with glycerol and frozen (−20 °C) at North Carolina State University and then sent to Michigan State University for analysis. Frozen myosin was thawed, diluted with 10 volumes of cold water, and then centrifuged at 10000*g* for 1 h. The pellet was suspended in myosin buffer and dialyzed against the same buffer overnight. Protein concentration was determined using an extinction coefficient of 0.52 cm^2/mg (Quass and Briskey, 1968). No adjustments were made to the concentrations before DSC measurements. For each replication (extraction), duplicate samples were analyzed.

Denaturation of myosin was measured using a differential scanning microcalorimeter (MC-2, Microcal Inc., Amherst, MA), by heating from 20 to 90 °C at 1 °C/min. Myosin concentrations used for pectoralis, iliotibialis, and gastrocnemius myosin ranged from 3.6 to 8.3 mg/mL in 0.6 M NaCl, 0.05 M Na phosphate buffer, pH 6.0. Cell capacity was 1.24 mL. A base line obtained by heating buffer in both cells was subtracted from the endotherm before analysis. Heat capacity profiles (C_p vs temperature) were defined by a calorimetric enthalpy (ΔH_{cal}) and melting (denaturation) temperatures (T_m) at which domains were 50% denatured (Privalov and Potekhin, 1986; Tsong et al., 1970). Calorimetric enthalpies were calculated from endotherms (for transitions without an exothermic peak) using the software provided by the manufacturer (DA-2 Data Acquisition and Analysis System), and melting temperatures were the temperature corresponding to the highest point (maximum heat capacity) of the transition peak. Duplicate samples from each of two extractions were tested for a total of 4 observations.

Turbidity Measurements. A spectrophotometer (UV-240, Shimadzu) equipped with a water-jacketed cell holder connected to a programmable circulating bath (Neslab Instruments, Inc., Newington, NH) was used in all experiments. The optical density was measured at a wavelength of 350 nm for 25 min at constant temperatures of 45, 50, or 55 °C. Readings were taken every 0.5 minute for the first 13 min and then every 1 min thereafter. In some cases the myosin aggregates began to precipitate. This was indicated by a sudden decrease in optical density. Optical density readings were discontinued if there was a steady decrease in readings and the change was greater than 0.100.

Turbidity changes were sigmoidal with respect to time; therefore, the data were fit with a nonlinear regression equation (Giltinan and Davidian, 1994) using the NLIN procedure in SAS (SAS Institute Inc., Cary, NC). The equation used was

$$y = \beta_1 + (\beta_2 - \beta_1)/[1 + \exp\{\beta_4(\log x - \beta_3)\}] \qquad (1)$$

where y is the optical density, x is time, β_1 is the optical density at infinite time, β_2 is the optical density at zero time, β_3 is the location of the inflection point, and β_4 is the slope or rate of the reaction. These parameters can be used as the descriptors of the reaction, and statistics can be performed on the basis of the work done by Matthews et al. (1990).

Statistical Analysis. Each myosin isolation represented the pooled muscles from four birds. Two isolations of myosin were analyzed by differential scanning calorimetry and three isolations were used in turbidity and electrophoresis experiments. Analysis of variance was performed using PROC VARCOMP (SAS Institute Inc., 1990) with the replication as a random variable and muscle myosin as a fixed variable. For the treatments that had significant differences, means were separated using the Duncan test (Steel and Torrie, 1980).

RESULTS

Peptide Mapping. Myosin heavy-chain peptide maps for the three muscles are shown in Figure 1. The pectoralis myosin heavy-chain had a larger number of peptides in the middle region (arrow A) of the fragments in comparison to the iliotibialis and gastrocnemius myosins. In general, the two red muscle myosin maps were similar, and different from the pectoralis myosin, supporting previous work showing different isoforms for red and white myosins (Emerson and Bernstein, 1987). There were positional and concentration differences between the red myosin maps (arrows B−D). This could be due to different myosin isoforms, or different concentrations of two or more isoforms present in both muscles. While the number and concentration of specific myosin isoforms in each preparation could not be quantitatively established, peptide maps showed that

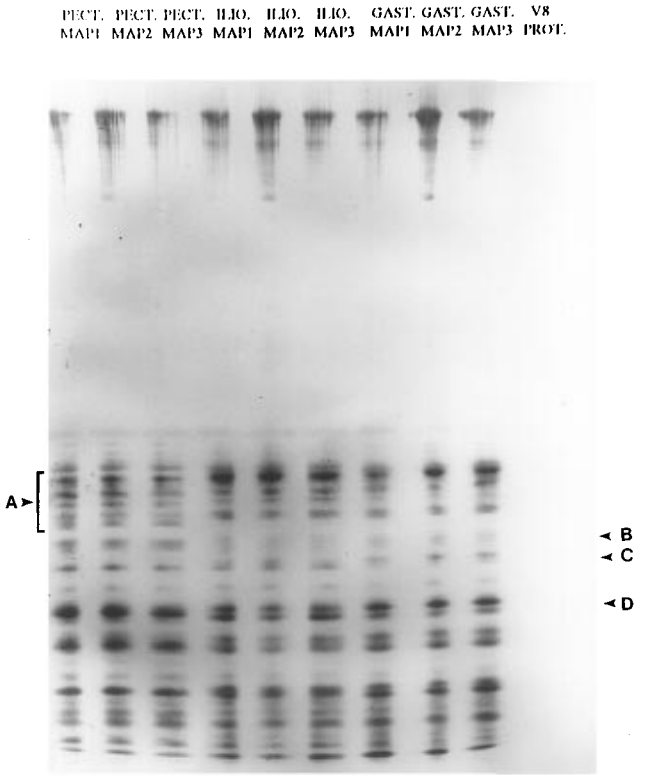


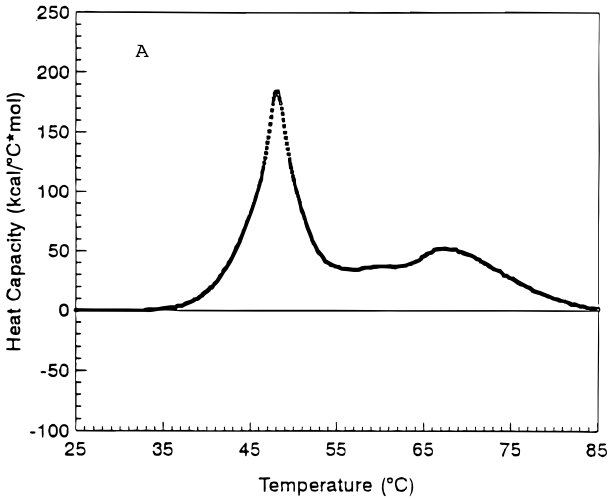
Figure 1. Peptide map of myosin heavy chains from pectoralis, iliotibialis, and gastrocnemius muscles. Arrow A shows differences among white (pectoralis) and red (iliotibialis and gastrocnemius) muscle myosins. Differences among the red muscle myosins can be seen at arrows B−D.

myosin extracted from each muscle could be considered "unique" in composition.

Differential Scanning Calorimetry (DSC). Myosin has several folding domains that are seen as multiple transitions in DSC (Figures 2−4). Multiple unfolding transitions in rabbit skeletal myosin have been observed by calorimetric (Potekhin et al., 1979) and spectroscopic (King and Lehrer, 1989) analyses. Pectoralis and iliotibialis myosin ΔH_{cal} were not significantly different but were significantly higher than gastrocnemius myosin (Table 1). The denaturation ΔH_{cal} of pectoralis myosin (2140 kcal/mol) was close to the ΔH_{cal} of chicken breast muscle myosin (2216 kcal/mol) reported by Wang and Smith (1994). The pectoralis muscle comprises the majority of breast muscle mass, so such similarity in enthalpies was expected. Bertazzon and Tsong (1989) reported a multi-stage unfolding of rabbit myosin with a ΔH_{cal} of 1715 kcal/mol; thus our range of 1580−2140 kcal/mol is in agreement with other studies.

The endotherms of all three myosins showed multiple transitions (Table 1 and Figures 2−4). Gastrocnemius myosin had a significantly lower denaturation onset temperature (T_o = 33.1 °C) than pectoralis and iliotibialis myosins which were not significantly different. No significant difference was found among the myosins for the first transition temperature (T_{m1}, 48.1−49.3 °C). A broad peak at 65.3−67 °C (T_{m3}) was observed in all three myosins (Figures 2−4) and was not significantly different among the myosins. The second transition temperatures (T_{m2}) for each of the myosins were significantly different.

The endotherm of pectoralis myosin varied with myosin concentration (Figure 2). The second peak at 49.9 °C, a shoulder (about 47 °C), and a rapid decrease



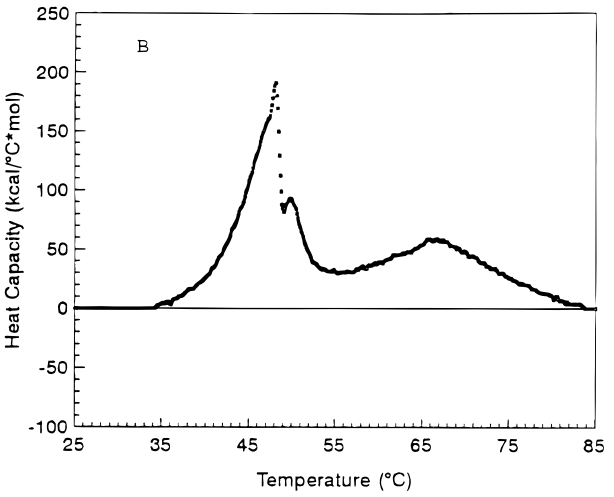


Figure 2. Heat capacity profile of pectoralis myosin in 0.6 M NaCl, 0.05 M sodium phosphate buffer, pH 6. The scan rate was 1 °C/min. Myosin concentrations were 8.30 mg/mL (A) and 3.79 mg/mL (B).

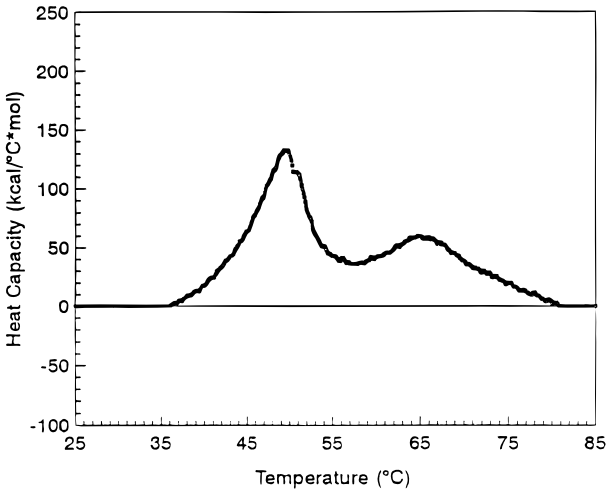


Figure 3. Heat capacity profile of iliotibialis myosin in 0.6 M NaCl, 0.05 M Na phosphate buffer, pH 6. The scan rate was 1 °C/min. Myosin concentration was 5.5 mg/mL.

in heat capacity between 48 and 49 °C were observed only in myosin from one replication (extraction). This preparation contained a lower myosin concentration (3.79 mg/mL) (Figure 2B) when compared to the second replicate (Figure 2A). Iliotibialis myosin had one minor peak at 51.1 °C (Figure 3). Gastrocnemius myosin had a sharp decrease in heat capacity at 51.8 °C and a peak

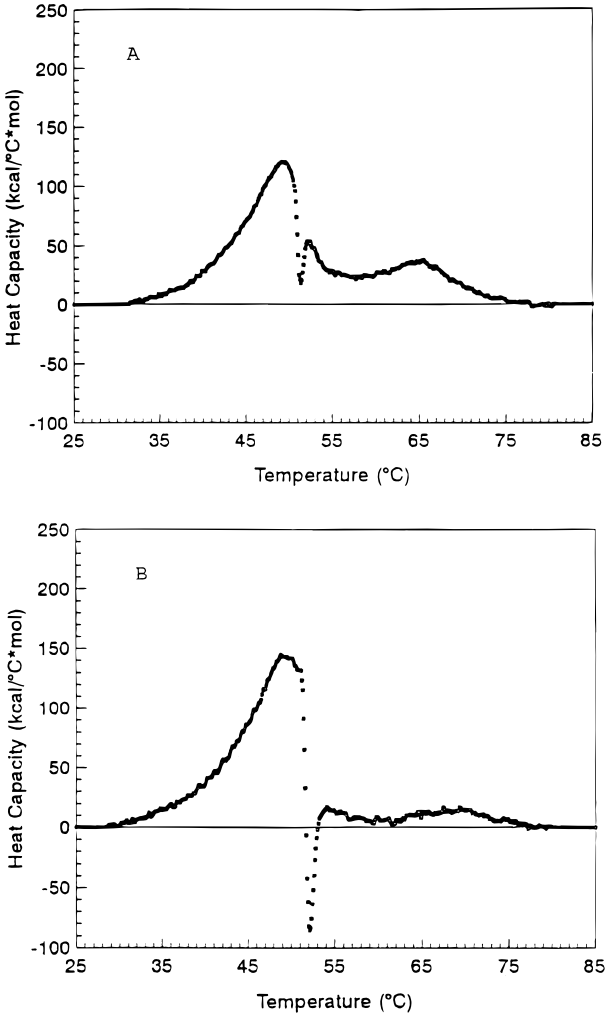


Figure 4. Heat capacity profile of gastrocnemius myosin in 0.6 M NaCl, 0.05 M sodium phosphate buffer, pH 6. The scan rate was 1 °C/min. Myosin concentrations were 5.23 mg/mL (A) and 3.55 mg/mL (B).

Table 1. Calorimetric (ΔH_{cal}) Enthalpies and Transition Temperatures of Chicken Myosin Endotherms[d]

muscle source	ΔH_{cal} (kcal/mol)	transition temp (°C)			
		T_0	T_{m1}	T_{m2}	T_{m3}
pectoralis	2140[a]	35.3[a]	48.1[a]	49.9[c,e]	67.0[a]
iliotibialis	1965[a]	35.8[a]	49.5[a]	51.1[b]	65.3[a]
gastrocnemius	1588[b]	33.1[b]	49.3[a]	52.3[a,f]	67.0[a]

[a–c] Means within a column with different superscripts are significantly different ($P < 0.05$). [d] Mean of four measurements. [e] This peak occurred only in one myosin extract. [f] Mean of two measurements in the same extraction.

at 52.3 °C (Figure 4). In the extract with a lower myosin concentration (3.55 mg/mL), the decrease in heat capacity went to the point that it was exothermic (Figure 4B). Shriver and Kamath (1990) observed an exothermic peak in purified rabbit myosin head (subfragment 1) at 48 °C in 50 mM TRIS buffer (pH 8.0), 0.6 M KCl, as well as in heavy meromyosin at 65 °C in 0.1 M KCl. The authors suggested the exotherm resulted from aggregation and precipitation of the unfolded proteins. Wang (1993) also reported an exothermic peak at 57.5 °C in 1 mg/mL of chicken breast myosin. The exothermic process disappeared as myosin concentration was increased to ≥4 mg/mL. In this study, the rapid decrease in heat capacity of pectoralis myosin, or exothermic peak in gastrocnemius myosin, only occurred at myosin concentrations <4 mg/mL. For gastrocnemius myosin, the rapid decrease in heat capacity was still present at a concentration of 5.23 mg/mL, which may relate to the propensity toward aggregation/precipitation of gastrocnemius myosin.

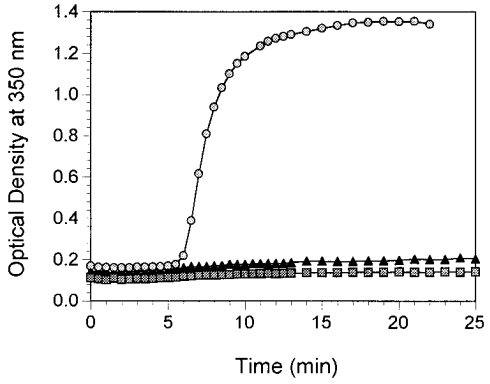


Figure 5. Effect of isothermal heating at 45 °C on optical density for pectoralis (circles), iliotibialis (squares), and gastrocnemius (triangles) muscle myosins.

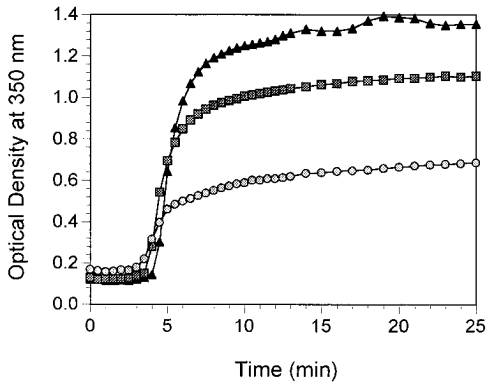


Figure 6. Effect of isothermal heating at 50 °C on optical density for pectoralis (circles), iliotibialis (squares), and gastrocnemius (triangles) muscle myosins.

Protein Aggregation (Turbidity). Figures 5–7 show experimental data from the replication which was closest to the mean values obtained with statistical analysis. All data had similar curves as those shown; however, small variability in the initial turbidity transposed the curves relative to the turbidity axis. Pectoralis myosin aggregated at all three temperatures (45, 50, and 55 °C). The iliotibialis and gastrocnemius myosins showed significant aggregation only at 50 and 55 °C; there was very little change in turbidity at 45 °C (Figure 5).

With the protein concentration and solutions conditions used in this investigation, all turbidity curves showed a sigmoidal shape. Analysis of these curves by fitting to a sigmoidal model by nonlinear regression determined four parameters that described the of aggregation pattern: the initial turbidity (β_2), inflection point (β_3), slope of rapid turbidity increase (β_4), and turbidity at the end of heating (β_1). This method was effective in describing all the aggregation profiles with the exception of the iliotibialis and gastrocnemius myosins at 45 °C, where there was no significant increase in optical density (Figure 5 and Table 2); thus, β_3 and β_4 were not calculated for these treatments.

Turbidity measurements are based on the principle that light scattering properties (optical density) reflect the size and/or amount of particles present. All myosin dispersions had similar initial light scattering properties, as shown by no significant differences in β_2 values (Table 2). Since turbidity is a composite measure of

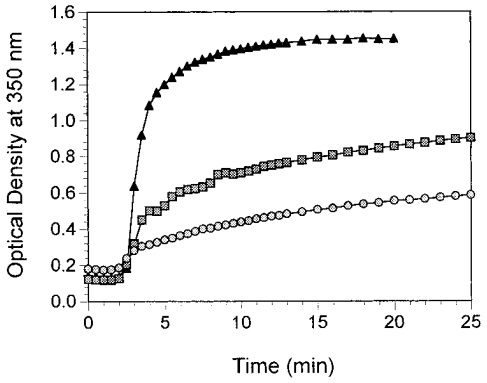


Figure 7. Effect of isothermal heating at 55 °C on optical density for pectoralis (circles), iliotibialis (squares), and gastrocnemius (triangles) muscle myosins.

number and shape of particles, similar values do not preclude differences in distributions in myosin aggregate sizes and shapes. The only significant difference in myosin aggregation at 50 °C was that pectoralis myosin formed a less turbid dispersion (lower β_1) after heating (Table 2). Since there were no significant differences in aggregation rate, this suggests that the types of aggregates formed by pectoralis myosin are different from red muscle myosins. At 55 °C the red muscle myosins reached the inflection point faster that pectoralis myosin, and gastrocnemius myosin had a significantly higher aggregation rate (β_4) (Table 2).

DISCUSSION

The muscles used in this study were chosen because peptide maps showed the myosin heavy-chain isoform composition to be different. Myosin was 80−90% pure, and the weight ratio of myosin to actin was 45. On the basis of the findings of Ishiorshi et al. (1980) and Yasui et al. (1982), the effects of the actin present should be negligible.

Stabursvik and Martens (1980) found greater differences in DSC profiles between red and white muscles than within type among species. However, in this study there was no clear separation between white and red muscle myosins in DSC profiles. The gastrocnemius myosin had significantly lower calorimetric enthalpies and denaturation onset temperatures than the pectoralis and iliotibialis myosins which were not significantly different from each other. Each muscle myosin had it own unique denaturation process due to differences in T_{m2}.

The gastrocnemius and pectoralis myosins exhibited rapid drops in heat capacity that are thought to be associated with aggregation in DSC experiments (Figures 2B and 4). This did not occur with the iliotibialis myosin. This aggregation reaction has only been reported with microcalorimeter experiments where dilute solutions <5 mg/mL were analyzed (Shriver and Kamath, 1990; Wang, 1993). Less sensitive differential scanning calorimeters require more concentrated dispersions (>20 mg/mL) and exothermic peaks are generally not observed (Samejima et al., 1983; Wright and Wilding, 1984). Thus, with larger concentrations of myosin there may be a gel-like structure formed that prevents the molecules from precipitating.

Thermally induced protein gelation starts with the reversible process of denaturation. Denaturation of a protein becomes irreversible once it aggregates with other protein molecules (Lepock et al., 1992). In this study, isoform differences were found in denaturation and aggregation. The temperature range between T_o and T_{m1}, shown by DSC results, indicates the first unfolding transition that has the "potential" to increase intermolecular interactions. Although all myosins showed endothermic transitions at 36−45 °C (Figures 2−4), a significant aggregation reaction was only observed in this temperature range with pectoralis myosin (Figure 5). This difference in aggregation may be associated with sequence differences between fast and slow isoforms.

Table 2. Effect of Temperature on Nonlinear Regression Parameters Derived from Turbidity Transitions

		nonlinear regression parameters[d]			
temp (°C)	myosin source	β_1 (OD_{350})	β_2 (OD_{350})	β_3 (min)	β_4 (1/min)
45	pectoralis	1.230[a]	0.073[a]	8.0	5.45
	iliotibialis	0.147[b]	0.101[a]		
	gastrocnemius	0.147[b]	0.113[a]		
50	pectoralis	0.498[b]	0.079[a]	5.4[a]	2.32[a]
	iliotibialis	0.982[a]	0.071[a]	4.8[a]	5.47[a]
	gastrocnemius	1.136[a]	0.054[a]	5.2[a]	4.99[a]
55	pectoralis	0.776[a]	0.126[a]	11.3[a]	1.96[b]
	iliotibialis	1.007[a]	0.068[a]	5.6[b]	2.63[b]
	gastrocnemius	1.135[a]	0.096[a]	3.4[b]	5.82[a]

[a−c] Means within a column, for each temperature, with different superscripts are significantly different ($P < 0.05$). [d] Nonlinear regression parameters represent the following: β_1 is the optical density at end of time period, β_2 is the optical density at zero time, β_3 is the location of the inflection point, and β_4 is the slope or rate.

Several other studies have evaluated changes in turbidity under dynamic temperature conditions. Chicken breast salt-soluble proteins were found to have lower onset temperatures and have a higher rate of aggregation at 40−45 °C than salt-soluble proteins from the leg (Xiong and Brekke, 1990a). In a later study, Xiong and Brekke (1990b) used derivative curves of dA_{320}/dT to determine transition points. At pH 6.0 there were three thermally induced transitions for the breast salt soluble proteins and two for leg. The first two transition temperatures of breast salt-soluble proteins were similar to those of the leg, but the third transition temperature occurred at higher temperature. At pH 6.5, there was a shift in transition temperatures. Breast salt-soluble proteins first transition occurred at approximately 45 °C, whereas the first transition for the leg salt-soluble protein occurred at approximately 50 °C. Our myosin data are different than the SSP at pH 6.0 but similar to the SSP at pH 6.5. The reasons for these differences cannot be deduced at present.

For all myosins, the maximum aggregation rate was 5.5−5.8 min^{-1} (Table 2). The highest aggregation rates were observed at lower temperatures for pectoralis and iliotibialis myosin and decreased as the temperature was increased. The gastrocnemius muscle myosin aggregation rates were high and somewhat similar for the two temperatures, 50 and 55 °C, where aggregation occurred.

CONCLUSIONS

Distinct differences in denaturation and aggregation are found among white muscle (pectoralis) and two red muscle (iliotibialis and gastrocnemius) myosins from chickens. The denaturation and aggregation processes for pectoralis and iliotibialis myosins are similar in rates and transitions; however, pectoralis myosin is shifted to a lower temperature range in the aggregation process. Gastrocnemius myosin had the most unique denaturation and aggregation properties of the three myosins

investigated. The significance of these isoform-associated differences in unfolding and aggregation relative to gelation will be addressed in a subsequent manuscript.

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Received for review June 6, 1995. Revised manuscript received February 6, 1996. Accepted March 18, 1996.[⊗] This work was supported in part by a USDA National Needs Graduate Fellowship. Paper FSR-95-13 of the Journal Series of the Department of Food Science, North Carolina State University, Raleigh, NC. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of products named or criticism of similar ones not mentioned.

JF9503422

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