# Physicochemical Changes and Mechanism of Heat-Induced Gelation of Arrowtooth Flounder Myosin

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Physicochemical changes of myosin during heating were investigated to elucidate the mechanism of heat-induced gelation of arrowtooth flounder (ATF) myosin at high ionic strength. Changes in dynamic properties indicated ATF myosin formed a gel in three different stages as shown by the first increase in $G'$ (storage modulus) at 28 °C, followed by the decrease at 35 °C and the second increase at 42 °C. DSC thermogram showed the onset of myosin denaturation at 25 °C with two maximum transition temperatures at 30 and 36 °C. The decrease in α-helical content indicated ATF myosin began to unfold at 15 °C and the unfolding continued until it reached 65 °C. Turbidity measurement showed myosin began to aggregate at 23 °C and the aggregation was complete at 40 °C. Surface hydrophobicity increased consistently in the temperature range studied, 20−65 °C. Sulfhydryl contents decreased significantly at 20−30 °C due to the formation of disulfide linkages but remained constant at temperatures >30 °C. ATF myosin was shown to be extremely sensitive to heat, resulting in denaturation at lower temperature than other fish myosin. Denaturation was initiated by unfolding of the α-helical region in myosin followed by exposure of hydrophobic and sulfhydryl residues, which are subsequently involved in aggregation and gelation processes.

**Keywords:** *Arrowtooth flounder; denaturation; gelation; myosin; surimi*

## INTRODUCTION

Heat-induced gelation of surimi, an intermediate product produced by repeated washing of minced fish flesh and mixing with cryoprotectants to extend its frozen shelf life, is a fundamentally important step in the manufacture of a variety of surimi seafoods such as kamaboko, fish meat gel, and crab and other shellfish analogues. Particularly, the formation of a protein network in these final products contributes not only to the elastic texture but also to other functional properties that are important to simulate the appearance, flavor, and texture of the natural meat counterparts (Park et al., 1997).

Heat-induced gelation of surimi is a complex physicochemical process involving structural and functional changes of myofibrillar proteins. The formation of myofribrillar networks can be represented by three stages, that is, dissociation, thermal denaturation, and aggregation (Roussel and Cheftel, 1990). In the presence of salts, muscle fibers and proteins undergo major structural changes, leading to the solubilization of myosin, actin, and a number of other myofibrillar constituents (Parsons and Knight, 1990). Partial unfolding of the protein structure is accelerated by an increase in temperature, resulting in the aggregation of unfolded regions between protein molecules to form a three-dimensional network.

Formation of fish meat gel has been characterized, with respect to changes in three-dimensional structure, by three stages as heating proceeds: "suwari", "modori", and "kamaboko" (Suzuki, 1981). Suwari, the gel-setting stage, describes the formation of a loose protein network when fish mince paste containing 2−3% salt is heated to 40−50 °C. It is mediated by transglutaminase and considered as the reaction wherein a three-dimensional network is formed and subsequently acts as the backbone of the final gel (Niwa et al., 1995). Modori describes the partial disruption of a loose protein network as the temperature is further increased to 50−60 °C. Modori is associated with the action of endogenous heat-activated proteinases and/or the thermal behavior of myofibrillar proteins alone (Niwa, 1992). Kamaboko or gel enhancement refers to the formation of an ordered, strong, and elastic gel at temperatures >65−70 °C.

The ability of surimi to form an elastic gel is largely derived from myosin (Sano et al., 1988). Myosin comprises 55−60% of the myofibrillar proteins. It is a multidomain protein with two large heavy chains and four light chains arranged into an asymmetrical molecule with two globular heads attached to a long α-helical rodlike tail (Privalov, 1982; Lopez-Lacomba et al., 1989). Myosin has been shown exclusively to possess gelling ability, in contrast to other protein components. In surimi, myosin is present in a complex form with actin and other proteins, collectively called actomyosin, which could form an elastic gel (Niwa, 1992). Because actin itself could not form a strong gel on heating, it is thought that the gelling characteristics of actomyosin are derived from the myosin portions. However, binding with actin has been shown to modify the gelling characteristic of myosin (Yasui et al., 1980). The changes

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in rheological properties of myosin and actomyosin, when heated under similar conditions, indicated that myosin produces more elastic gels than actomyosin and the gelation reflected three steps of texture formation previously described using fish mince (Sano et al., 1988).

According to the studies on physicochemical changes using myosin and its subfragments from several species of fish, that is, flying fish (Taguchi et al., 1987), carp (Sano et al., 1990a), croaker (Hamada, 1992), cod, and herring (Chan et al., 1992; Gill et al., 1992; Chan and Gill, 1994), it is agreed that heat-induced gelation of myosin under high-salt conditions involves two processes including denaturation and aggregation (Stones and Stanley, 1992). In denaturation, myosin undergoes conformational changes, exposing functional groups, such as hydrogen bonds and hydrophobic groups. In aggregation, denatured myosin molecules align themselves and interact with each other to form a three-dimensional network. The interactions involved in gelation have been shown to be dependent upon the species of fish employed (Taguchi et al., 1987; Sano et al., 1990b; Chan et al., 1993). Because myosin heavy chain is the main subunit involved in gelation (Samejima et al., 1984), differences in the composition and structure of myosin heavy chain among various fish species may be responsible for species-to-species differences in the cross-linking ability and gelation properties of fish muscle under the same conditions.

Arrowtooth flounder (ATF) is an abundant fish species caught off the North America/Pacific region. However, its utilization has been hampered by softening of muscle by heat stable proteases during heating (Greene and Babbitt, 1990; Wasson et al., 1992). To provide basic information for future utilization of this fish species, we investigated physicochemical changes of ATF myosin during heating and its gelation mechanism under the high ionic strength condition normally used for surimi gel products.

## MATERIALS AND METHODS

**Reagents.** Potassium chloride (KCl), sodium azide ($NaN_3$), Tris base, 1-(L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), 2-mercaptoethanol ($\beta$ME), ethylene glycol bis($\beta$-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), magnesium acetate, Tris maleate, adenosine triphosphate (ATP), sodium bicarbonate, magnesium chloride, sodium phosphate, potasium phosphate, glycerol, 8-anilino-1-naphthalenesulfonic acid (ANS), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO). Phenylmethanesulfonyl fluoride (PMSF) was purchased from Calbiochem Co. (La Jolla, CA). Sodium dodecyl sulfate (SDS) and urea were purchased from Bio-Rad Laboratories (Hercules, CA).

**Sample.** Arrowtooth flounder were obtained from the National Marine Fisheries Service, Utilization Research Laboratory, Kodiak, AK. Trawl-caught arrowtooth flounder were obtained and manually filleted. The fillets were vacuum-packed in polyethelene bags, frozen at −20 °C, and transported in dry ice to the OSU Seafood Laboratory. Frozen fillets were kept at −50 °C until used.

**Myosin Preparation.** Myosin was extracted according to the method described by Martone et al. (1986) with slight modifications. All steps were performed at 0−4 °C to minimize proteolysis and protein denaturation. ATF fillets were finely chopped and added with 10 volumes of buffer A (0.10 M KCl, 1 mM PMSF, 10 $\mu$M E-64, 0.02% $NaN_3$, and 20 mM Tris-HCl, pH 7.5). After incubation on ice for 10 min with occasional stirring, washed muscle was recovered by centrifugation at 1000*g* for 10 min. The pellet was suspended in 5 volumes of buffer B [0.45 M KCl, 5 mM $\beta$ME, 0.2 M $Mg(CH_3COO)_2$, 1 mM EGTA, and 20 mM Tris-maleate, pH 6.8], and ATP was added to a final concentration of 10 mM. The mixture was kept on ice for 1 h with occasional stirring and centrifuged at 10000*g* for 15 min. Supernatant was recovered, and 25 volumes of 1 mM $NaHCO_3$ was slowly added; the mixture was incubated for 15 min on ice. Precipitated myosin was collected by centrifugation at 12000*g* and resuspended gently with 5 volumes of buffer C (0.50 M KCl, 5 mM $\beta$ME, and 20 mM Tris-HCl, pH 7.5), followed by the addition of 3 volumes of 1 mM $NaHCO_3$and $MgCl_2$ to the final concentration of 10 mM. The mixture was incubated overnight and centrifuged at 22000*g* for 15 min. Myosin was recovered in the pellet and used immediately or stored in 50% glycerol at −20 °C. The purity of extracted myosin was estimated by SDS−PAGE and densitometric analysis of the gels.

**Dynamic Rheological Analysis.** Rheological changes of myosin during thermal gelation were analyzed using a Bohlin CS-50 rheometer (Bohlin Instruments, Inc., Cranbury, NJ), which operated in the small-amplitude oscillatory mode according to the method described by Xiong (1993) with a slight modification. The rheometer was equipped with 20-mm parallel plate geometry with a gap of 1 mm. Samples were prepared as myosin suspension at 45 mg/mL in 0.6 M KCl/20 mM phosphate buffer, pH 7.0. Sample was sheared at a fixed frequency of 0.1 Hz to minimize stress on the sample during network with a maximum strain amplitude of 0.015. These parameters have been previously determined to give a linear response in the viscoelastic region (data not shown). Samples were heated from 25 to 80 °C at 1 °C/min using a Bohlin temperature control unit. To avoid sample evaporation during heating, a plastic cover was used. Data were collected every 60 s during shearing measurements. Network development was studied in real time by measuring changes in storage modulus ($G'$), loss modulus ($G''$), and loss tangent (tan $\delta$) as a function of temperature.

**Differential Scanning Calorimetry (DSC).** DSC measures energy changes associated with phase transition that occur in protein molecules when subjected to heating (Davies et al., 1994). DSC studies were performed in a DuPont 910 differential scanning calorimeter (DuPont Co., Wilmington, DE). To eliminate water condensation in and under the DSC cell, 40 mL/min of helium gas was purged through the purging port. The system was calibrated using DuPont calibration software with an indium thermogram. Myosin solutions, prepared at 45 mg/mL in 0.6 M KCl/20 mM potassium phosphate, pH 7.0, were sealed in hermatic DSC pans and accurately weighed to 14−20 mg wet weight on a semimicro balance (Mettler Instrument Corp., Highstown, NJ). All samples were scanned at 10 °C/min over the range of 2−100 °C using a DuPont mechanical cooling accessory. A hermatic empty pan was used as a reference. Five determinations were made, and the most representative thermograms with average onset and transition temperatures were reported.

**Circular Dichroism (CD).** Secondary structures of proteins were analyzed using a JASCO A-500 spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan) equipped with a temperature control unit and operated under constant nitrogen flush. Myosin pellet was dissolved in cold 0.6 M KCl/20 mM potassium phosphate, pH 7.0, and centrifuged at 7800*g* for 10 min. Supernatant was used for CD analysis after dilution to 0.4 mg/mL in the same buffer. A circular quartz cell (path length of 0.1 cm) with a jacket for temperature control was used. The temperature inside the cell was monitored using a thermocouple. Thermal scanning experiments were performed from 10 to 65 °C at an average heating rate of 1.2 °C/min. Scan speeds and time constant were chosen to allow sufficient response time and achieve a favorable signal-to-noise ratio. Molar ellipticities at 222 nm ($[\theta]_{222}$) were calculated using eq 1 and the mean residue molecular weight

$$[\theta]_{222} = (\theta_{obs} \times 115)/(10 \times C'') \quad (1)$$

$$\alpha\text{-helical content} = 100([\theta]_{222}/-40000) \quad (2)$$

of myosin of 115 (McCubbin and Kay, 1982). α-Helix content

was estimated using eq 2 on the basis of $[\theta]_{222}$ of poly(L-glutamic acid), which possesses a completely helical structure (Holzwarth and Doty, 1965).

**Turbidity Measurement.** Myosin solution was prepared at 1 mg/mL in chilled 0.6 M KCl/20 mM potassium phosphate, pH 7.0, and centrifuged at 7800*g* for 10 min to remove insoluble debris. Myosin solution were placed in a cuvette (light path length of 1 cm), covered with Parafilm, and heated linearly at an average rate of 1.3 °C/min from 18 to 65 °C. Turbidity was measured as absorbance at 350 nm as heating proceeded using a spectrophotometer (Beckman Instrument, Inc., Redmond, WA) equipped with a temperature-controlled unit. Results were reported as absorbance per protein concentration ($A_{350}$/mg/mL).

**Surface Hydrophobicity.** Surface hydrophobicity ($S_0$ ANS) was determined according to the method of Li-Chan et al. (1985) with slight modifications. Myosin solution was diluted to 0.125, 0.25, 0.5 and 1 mg/mL in 0.6 M KCl/20 mM phosphate, pH 7.0, and aliquoted into 2 mL in borosilicate tubes. Parafilm was placed on the tubes to prevent evaporation during heating. The tubes were placed in a circulating water bath at 20 °C and allowed to equilibrate for 5 min prior to heating. A thermometer was used to monitor the temperature of protein solution in culture tubes placed in the water bath. Temperatures were recorded with 30 s intervals. The temperature rise in each run, from 20 to 80 °C, was shown to be linear, and the heating rate was estimated as 1.2−1.5 °C/min. To 2.0 mL of diluted myosin solutions was added 10 $\mu$L of 10 mM ANS dissolved in 50 mM phosphate, pH 7.0. Fluorescence intensity was measured using a luminescence spectrophotometer (LS 50B, Perkin-Elmer Ltd., Beaconsfield, U.K.) at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. Fluorescence intensity was plotted against protein concentration, and the slope of regression line was reported as $S_0$ ANS.

**Determination of Sulfhydryl (SH) Content of Myosin.** Total and surface reactive SH groups were determined using DTNB according to the method of Ellman (1959). Aliquots (2 mL) of myosin solution (1 mg/mL) were linearly heated from 20 to 80 °C in a temperature-controlled water bath as described previously under Surface Hydrophobicity. Samples were cooled immediately, mixed, and aliquoted into two portions to determine total and surface reactive SH. For total SH determination, 1 mL of heated sample was added to 3 mL of 0.2 M Tris-HCl, pH 6.8, containing 8 M urea, 2% SDS, and 10 mM EDTA, followed by 0.4 mL of 0.1% DTNB solution and incubation at 40 °C for 25 min. Absorbance was measured at 412 nm and used to calculate total SH content, using the molar extinction of 13600 $M^{-1}$ $cm^{-1}$. Surface reactive SH was determined by incubating 1 mL of the heated sample in 3 mL of 0.2 M Tris-HCl, pH 6.8, containing 2% SDS and 10 mM EDTA at 5 °C for 25 min. Surface reactive SH content was calculated from absorbance using the molar extinction of 14150 $M^{-1}$ $cm^{-1}$ according to Riddles et al. (1983). A blank was prepared by replacing sample with 0.6 M KCl/20 mM phosphate buffer, pH 7.0. Both total and surface reactive SH contents were expressed as moles per $10^5$ g of protein.

**Protein Determination.** Protein concentration was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. Myosin was first solubilized in 5% (w/v) SDS solution at 95 °C for 5 min and further incubated at 85 °C for 30 min. Insoluble debris was removed by centrifugation at 7800*g* for 10 min, and supernatant was used for protein analysis.

**Statistical Analyses.** Obtained data were analyzed statistically by analysis of variance (ANOVA). Mean differences were determined using the least significant difference (LSD) multiple-range test (Statgraphics version 6.0, Manugistics Inc., Rockville, MD). Significance of difference was established at $p \leq 0.05$.

## RESULTS

**Myosin Extract.** The purity of extracted myosin was 85% as estimated by the densitometric analysis of SDS−PAGE gel. The electrophoretic pattern showed that myosin of arrowtooth flounder was composed of heavy chains (MHC) and at least two different types of light chains (Figure 1). Their molecular weights were estimated as approximately 205, 23, and 21 kDa, respectively. Because no notable changes in the electrophoretic patterns of myosin were observed by incubation for up to 30 min at 55 °C, extracted myosin was postulated to be free of endogenous protease. The appearance of smear bands in the control group indicated the degradation products accumulated prior to myosin extraction. MHC was the main target of proteolytic activity in which its band disappeared completely by heat treatment for 20 min (Wasson et al., 1992). The results indicated the myosin preparation was free of endogenous enzymes, such as proteases and transglutaminase, and suitable for further analyses.

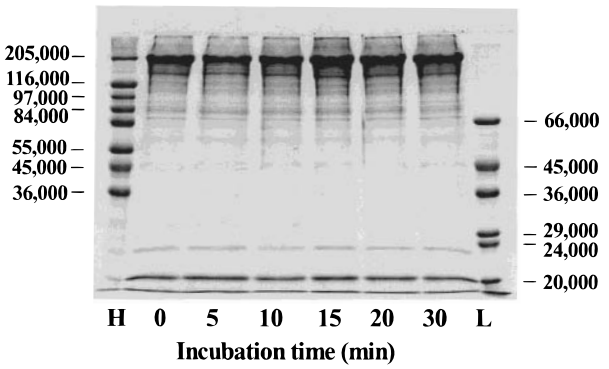


**Figure 1.** SDS−PAGE pattern of ATF myosin before and after incubation at 55 °C for up to 30 min. H and L designate high and low molecular weight protein standards, respectively.

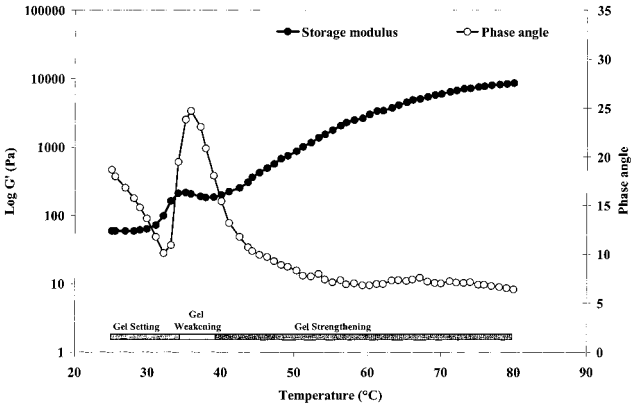


**Figure 2.** Changes in rheological properties of ATF myosin suspension (45 mg/mL) heated from 25 to 80 °C at a rate of 1 °C/min.

**Dynamic Rheological Properties.** Typical changes in rheological properties of ATF myosin during thermal scanning are shown in Figure 2. Of the rheological parameters assessed, storage modulus ($G'$) and phase angle were used to evaluate gel formation. An increase in storage modulus ($G'$), a measure of energy recovered per cycle of sinusoidal shear deformation, indicated the increase in rigidity of the sample associated with the formation of elastic gel structure (Egelandsdal et al., 1995). The first-derivative plot showed that the apparent onset of heat-induced elastic myosin gel formation occurred at 28 °C, where $G'$ began to rise, and reached a maximum at 34 °C. An increase in $G'$ (~3% of the maximun $G'$) found in these temperature ranges was referred to as "gel setting", in which the loose gel

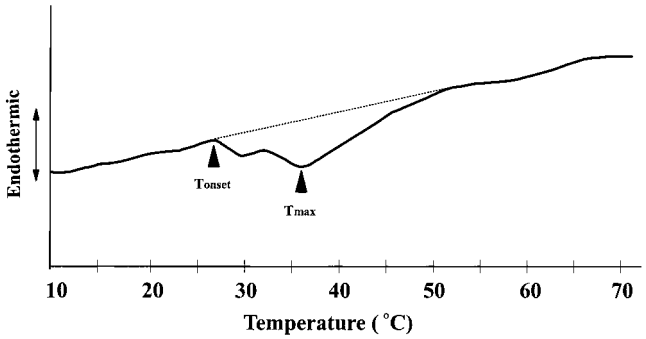


**Figure 3.** DSC endotherm of ATF myosin in 0.6 M KCl/20 mM sodium phosphate buffer, pH 7.0, heated from 2 to 80 °C at a rate of 10 °C/min.

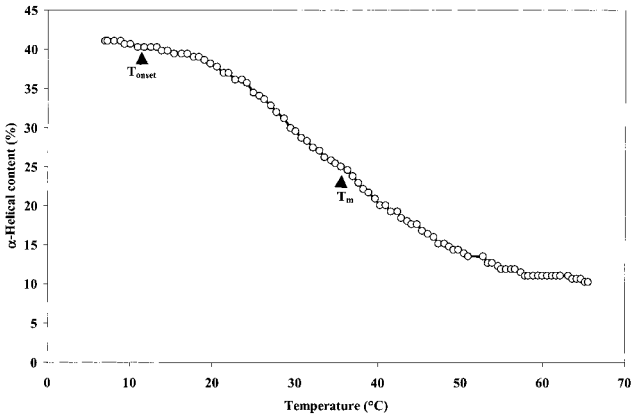


**Figure 4.** α-Helical content as a function of temperature of ATF myosin in 0.6 M KCl/20 mM sodium phosphate buffer, pH 7.0, heated from 2 to 80 °C at a rate of 1.3 °C/min. Each point is the mean of two determinations.

structure was formed. However, the term should be distinguished from "suwari", observed with surimi mediated by endogenous fish transglutaminase. The slight decrease in $G'$ at 34−40 °C was referred to as "gel weakening". Because ATF myosin used in this study was confirmed to be free of proteolytic activity, disruption of the initial gel structure was not due to the action of heat-activated proteases responsible for modori in surimi. Similar to the $G'$ drop observed in chicken breast myosin (Xiong, 1997), it was postulated to be due to helix-to-coil transformation of myosin, which leads to a large increase in fluidity of the semigel and may disrupt some of the protein network already formed (Sano et al., 1988). The onset of the second increase in $G'$ was noticed at 42 °C, at which $G'$ continuously increased thereafter. This final step was referred to as "gel strengthening", and it was ascribed to both an increase in the number of cross-links between protein aggregates and a deposition of additional denatured proteins in the existing protein networks to strengthen the gel metrix (Xiong, 1997).

The phase angle decreased from 18° to 10° by heating from 25 to 32 °C and increased rapidly to a maximum of 25° at 35 °C. The phase angle continuously decreased thereafter to a minimum of 7° at 50 °C and remained constant until the final stage of heating. The use of phase angle to evaluate network characteristics has the advantage of incorporating the contributions of both $G'$ and $G''$ into a single parameter to evaluate the final network (Egelandsdal et al., 1995). Changes in the phases angle reflected a transition of the viscous myosin sol to the elastic myosin gel, which correlated to the changes of $G'$ observed during heating.

**DSC Studies.** ATF myosin was found to undergo a multistage denaturation process as characterized by an endothermic trough and peaks (Figure 3). The onset of denaturation was observed at 25 °C, whereas the maximum transition temperature ($T_{max}$) was observed at 36 °C with one transition peak detected at 30 °C. The observed $T_{max}$ was within the temperature range (25−46 °C) observed among various fish species (Ogawa et al., 1993) and closely related to those of other cold-water fishes, that is, cod and herring (Davies et al., 1994). However, it was lower than those of myosins extracted from chicken, turkey, beef, and pork, of which transition normally occurred at 45−55 °C. The results suggested that ATF myosin is highly unstable to heat and its thermal susceptibility might be related to the habitat temperature of the living animal (Johnston et al., 1973).

**CD Measurements.** An asymmetric configuration of myosin molecule with a relatively high content of α-helix allows application of CD to monitor changes in secondary structure induced by heat. By heating linearly from 9 to 65 °C at 1.5 °C/min, ATF myosin showed a decrease in helicity as temperatures increased (Figure 4). The melting temperature ($T_m$), at which 50% of the initial helical structure is lost, was determined to be 34 °C. The first-derivative plot shows the α-helical content started to decrease at 10 °C and proceeded to at least three major stages before completely unfolding at 65 °C. According to Ogawa et al. (1993), the pattern of α-helical decrease in fish myosins by heating is classified by three types depending on the initial helicity and the number of transitions involved in the decrease. The pattern determined with ATF myosin was similar to that of walleye pollack. It exhibited slightly lower initial helicity prior to heating and decreased gradually during heating.

Thermal denaturation shown by the endothermic peaks of myosin (Figure 3) was indicated due to the disruption of α-helical structure. However, the onset temperature of the unfolding process detected by CD was found to be at 10 °C, 15 °C lower than that detected by DSC. CD may be more sensitive to the detection of small changes than DSC, but differences in conditions used in the measurements, that is, protein concentration and heating rate, may have contributed to the discrepancy. Increasing heating rate does affect the unfolding rate of the protein. An upward shift in transition temperature ($T_{max}$) was commonly observed by the increase in heating rate (Park and Lanier, 1990). Compared to heating rate, the change in myosin concentration has little effect on transition temperatures (Goodno and Swenson, 1975; Chan et al., 1992). On the basis of the same heating rate used for CD and the dynamic test, 1 °C/min, it was concluded that denaturation of ATF myosin occurred before development of $G'$ was noticeable.

**Turbidity Measurements.** Turbidity development of ATF myosin was monitored from 20 to 65 °C (Figure 5). An increase in absorbance of heated fish myosin solution resulted from the formation of myosin aggregates (Gill et al., 1992), which were large enough to cause light scattering. The formation of myosin aggregates was enhanced sharply by an increase in temperature. The first-derivative plot of the extent of the turbidity−temperature curve showed the onset temperature of thermal aggregation was 23 °C. A maximum was found at 40 °C with two transition peaks at 25 and 30 °C. Thermal aggregation of fish myosin is

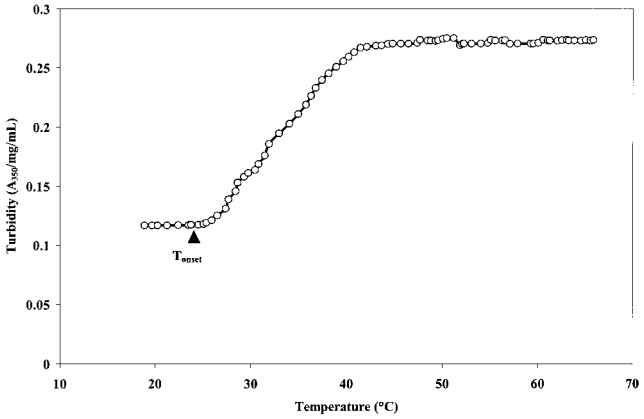


**Figure 5.** Effect of temperature on turbidity of ATF myosin solution (1 mg/mL). Protein solutions were continuously heated from 20 to 65 °C at a rate of 1.3 °C/min.

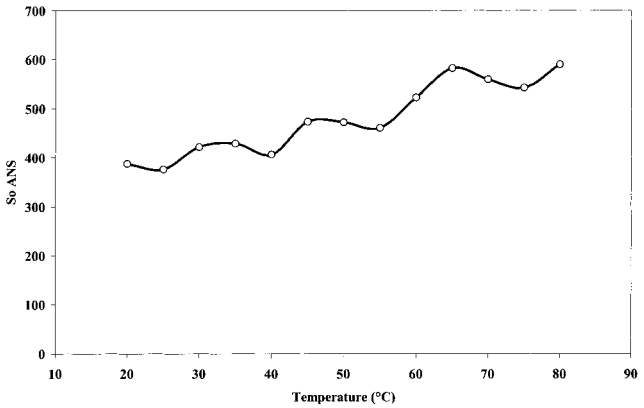


**Figure 6.** Effect of temperature on surface hydrophobicity of ATF myosin solution (1 mg/mL). Protein solutions were continuously heated from 20 to 65 °C at a rate of 1.3 °C/min.

an ordered process in which the rate and extent of aggregation are specific to species and dependent on concentration used (Chan and Gill, 1994). Species difference is attributed to the ability of MHC to form cross-linking (Chan et al., 1992). Compared with the results of a thermal-unfolding profile of heat-treated myosin molecule (Figure 4), it is suggested that some conformational changes in myosin molecules seem to be the driving force for the acceleration of myosin−myosin interactions shown at the transition temperature (Ishioroshi et al., 1979). The temperature at which the maximum turbidity was noticed would be higher if a higher concentration of myosin was used.

**Hydrophobicity.** Surface hydrophobicity, determined by the ANS probe, increased nonlinearly with an increase in temperature (Figure 6). The increased hydrophobicity indicated the structural and conformational changes of ATF myosin during heating, causing the hydrophobic groups to become more exposed and bind with ANS. As heating proceeded, the slight decreases in surface hydrophobicity observed at 25, 45, 55, and 75 °C were probably due to the aggregation of myosin, which competed for the same hydrophobic binding sites for ANS. The increased hydrophobicity during heating indicated the involvement of hydrophobic interactions in myosin gel formation at both gel-setting and -strengthening stages.

**Total and Surface Reactive Sulfhydryl (SH) Contents.** The total SH content of ATF myosin was estimated as 6 mol/$10^5$ g of protein, comparable to those

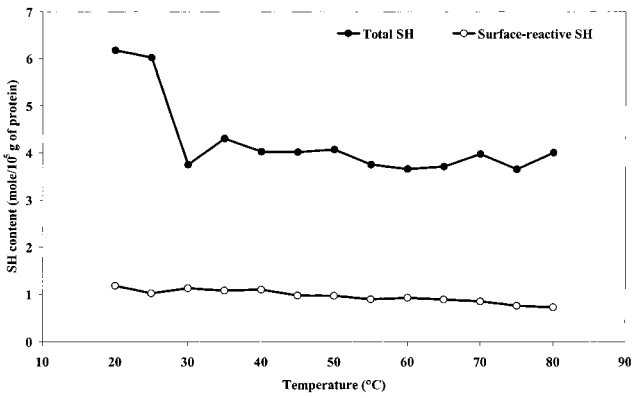


**Figure 7.** Changes in total and surface SH groups of ATF myosin, heated from 20 to 65°C at a rate of 1.3 °C/min.

of salmon (Lin and Park, 1998) and carp (Tsuchiya and Matsumoto, 1975) myosin. It was measured in the absence of urea; thus, ∼15% of the total SH contents was supposed to be the surface reactive SH group. The total sulfhydryl content of ATF myosin decreased significantly with heating from 20 to 30 °C but remained relatively constant thereafter (Figure 7). A decrease in SH content was reported to be due to the formation of disulfide bonds through oxidation of SH groups or disulfide interchanges (Hayakawa and Nakai, 1985). The implication is, therefore, that disulfide bonds are involved in the thermal aggregation of fish myosin. No significant changes were observed for surface reactive SH content during heating.

## DISCUSSION

Heat-induced gelation of ATF myosin is a complex process composed of several physicochemical changes. The observed denaturation and aggregation processes were similar to the mechanism proposed by Stone and Stanley (1992). ATF myosin was highly sensitive to heat, resulting in denaturation at lower temperature compared with other fish myosin reported. Denaturation was initiated at 15 °C by the unfolding of the α-helical region in myosin followed by exposure of hydrophobic and sulfhydryl groups, which are subsequently involved in the formation of intermolecular bonding in the aggregation process. Continuation of aggregation of myosin at higher temperature resulted in the development of three-dimensional structure as shown by a significant increase in $G'$ and a decrease in phase angle.

The ATF myosin molecule is thought to consist of a number of discrete, cooperative domains that could independently unfold during heating, similar to myosin from mammals (Privalov, 1982; Lopez-Lacomba et al. 1989), poultry (Wang and Smith, 1994), and fishes (Ogawa et al., 1993; Davies et al., 1994). The one large endothermic peak of ATF myosin observed in this study was thought to be a composite of several transitions deriving from each domain of myosin molecule, which has been found to unfold in different temperature ranges. Discrepancies in species and experimental conditions, such as pH, ionic strength, and heating rate (Park and Lanier, 1990), used in different studies make it difficult to determine the melting order of the various specific regions of ATF myosin by comparing its DSC thermogram with those of myosin from other fish. However, it was postulated that a hinge region near the center of the myosin rod may be the first domain to

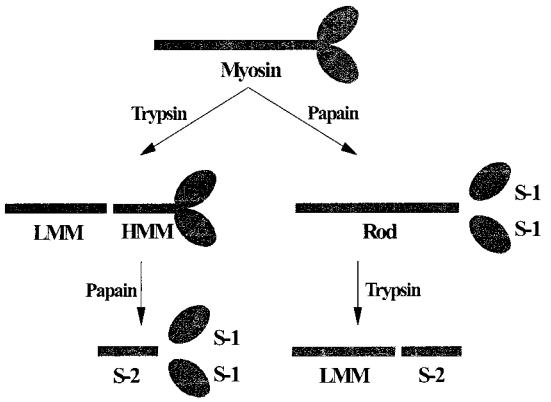


**Figure 8.** Schematic diagram of a myosin and its subfragments [modified from Pearson and Young (1989)].

unfold because of its lowest thermal stability and susceptibilty to proteolysis (Wang and Smith, 1994).

Nonheated myosin molecules are known to be present in a monomeric form at high salt concentration (ionic strength >0.5) and at pH 6.0 or above (Morita et al., 1987). Therefore, an increase in absorbance with heated fish myosin solution resulted from the formation of myosin aggregates (Gill et al., 1992). ATF myosin began to aggregate at 23 °C prior to the completion of the denaturation process. Because myosin is a relatively large molecule, various proteolytic subfragments of myosin have been prepared and used to study their structure and function (Figure 8). The heavy chain can be cleaved by papain near the globular end of the rod to produce a myosin rod and subfragment 1 (S-1) or by trypsin at the hinge region to produce light meromyosin (LMM) and heavy meromyosin, which can be further cleaved to yield S-1 and subfragment 2 (S-2) (Rattrie and Regenstein, 1977). However, the role of each discrete region of myosin in aggregation has not been clearly determined. Gill and Conway (1989) concluded that the tail rather than the head portion of myosin was involved in the thermal aggregation of cod myosin in accordance with Sano et al. (1990a,b), who proposed that the initial stage of gel formation at 30−44 °C of carp myosin was mainly attributed to the interaction of LMM. In contrast to those studies, Taguchi et al. (1987) proposed that aggregation of fish myosin was initiated by the inteaction between S-1 and the oxidation of SH groups followed by unfolding and interaction of LMM to form a gel network, similar to the mechanism proposed by Samejima et al. (1981) for rabbit myosin.

Because most of the sulfhydryl groups (~68%) in myosin are located in the head portion of myosin (Lowey et al., 1969), the significant decrease in SH groups observed at 20−30 °C not only indicated the formation of disulfide bonds during gel formation but also implied the role of myosin heads at the initial aggregation, as reported by Taguchi et al. (1987) and Chan et al. (1993). Irreversible aggregation of S-1 from rabbit skeletal myosin (Samejema et al., 1981) and chicken breast muscle myosin (Smyth et al.,1998) at 45 °C was associated with the oxidation of SH groups. In addition to disulfide linkage, hydrophobic interaction may also be involved in the initial aggregation of myosin. Compared to the rod portion, which contains a large portion of charged amino acids, the head portion is relatively rich in hydrophobic amino acid residues (Maita et al., 1991). Dissociation of light chains from the head of myosin may lead to irregular refolding (Hamai and Konno, 1989) and create hydrophobic patches for intramolecular and intermolecular head association (Sharp and Offer, 1992) during the initial stage of heating.

It is evident that hydrophobic interaction is a major force involved in myosin aggregation at high temperature. The exposure of hydrophobic domains has been suggested as a prerequisite for the formation of large myosin aggregates (Wicker et al., 1986; Chan et al., 1992). The concomitant increase in surface hydrophobicity of myosin aggregate suggests the presence of hydrophobic interactions taking place in the thermal aggregation of fish myosins. Several studies have suggested that hydrophobic interactions are involved in the heat-induced gelation of fish myosin. Gill and Conway (1989) showed that the thermal aggregation of the cod MHC was the result of noncovalent forces and that the forces were weakened in the presence of hydrophobic, apolar substances. Hamada (1992) reported that hydrophobic bonds were found to play a principal role in the gelation of myosin. An increase in protein hydrophobicity was shown to be responsible for the subsequent increased rigidity of myosin gels from fish, rabbit, and chicken (Wicker et al., 1986) and other functional properties of muscle foods including emulsifying capacity (Li-Chan et al., 1985).

## CONCLUSION

ATF myosin prepared free of endogenous proteinases showed that thermal gelation of arrowtooth flounder involved denaturation and aggregation of myosin molecules. Denaturation was initiated by the unfolding of the α-helical region in myosin followed by exposure of hydrophobic and sulfhydryl groups, which are subsequently involved in the formation of intermolecular bonding in the aggregation process to form three-dimensional structure. In the absence of endogenous proteinases, the thermal susceptibilty of ATF myosin provided the base to form high-quality protein gels.

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