Heat-induced Gelation of Myosin Filaments[†]

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Received February 12, 1988

Various lengths of myosin filaments were prepared by changing the speed of lowering the ionic strength. Short filaments were formed by rapid dilution and the filament length became longer with decreasing the speed of lowering the ionic strength. The average length of myosin filaments ranged from 0.5 to 3.0 μm with varying the speed of dilution. Myosin filament suspensions were heated to form gels, and those gel strengths were evaluated as rigidity. The rigidity depended on the length of the filaments before heating. The longer the filaments, the higher the rigidities. The gels with higher rigidity had a fine strand-like network structure. In contrast, that showing lower rigidity had a coarse aggregated structure. When low protein concentrations of myosin filament suspensions were heated at 40°C, myosin filaments associated with increasing the incubation time. When heating was done at 60°C, aggregation of myosin filaments occurred in a short time. Electron microscopic observation suggests that the association and the following aggregation of myosin filaments *per se* cause formation of a whole network structure. Short myosin filaments began to aggregate randomly in an early stage of heating and they made clusters. But long filaments did not form a cluster; instead, some of them associated side-by-side to form bundles. These differences in the mode of aggregation among various lengths of myosin filaments seemed to be one of possible reasons for the different network structures observed in the gels.

Water-holding capacity and binding properties are important meat processing characteristics. These characteristics are substantially derived from myosin, the major protein component of striated muscle myofibrils.¹⁾ Myosin molecules are packed in the thick filaments of myofibrils under physiological condition. But myosin molecules are dissociated from the filaments and disperse as monomers upon increasing the ionic strength above 0.3. The transformation between filaments and monomers can occur reversibly *in vitro* by changing the ionic strength.²⁾

Myosin forms gels upon heating under appropriate conditions regardless of its monomeric or filamentous state.^{3~7)} There are discernible differences in the rheological parameters of heat-induced gels between mono-

meric and filamentous myosins.^{3~5)} Ishioroshi *et al.*³⁾ showed that the rigidity of heat-induced gels of myosin at pH 6.0 remained at a constant level from 0.4 to 1.0 M KCl, but the rigidity increased below 0.4 M KCl, reached a maximal value at 0.2 M KCl, and it again decreased below 0.2 M. This finding suggests that the gel strength depends on the state of the myosin molecules; in other words, filamentous myosin forms more rigid gels than monomeric myosins. Furthermore, the rigidity of filamentous myosin shows ionic strength dependency, in contrast with that of the monomeric one.^{3,4)}

One can imagine that the ionic strength dependency of the rigidity of myosin filaments is due to the architecture of the filaments, because the architecture of myosin filaments is varied by factors such as ionic strength and

[†] This work was supported by a Grant-in-Aid for Scientific Research (Project No. 61430025, 1986) from the Ministry of Education, Science and Culture of Japan.

pH,⁸⁾ the presence of Mg^{2+} and ATP,^{9,10)} and the speed of lowering the ionic strength.^{9,11,12)} The last factor especially affects the length of the filaments.

In our previous paper, we reported that the long myosin filaments prepared by dialysis formed more rigid gels than the short ones prepared by rapid dilution.¹³⁾ We have now prepared various lengths of myosin filaments and studied the characteristics of their heat-induced gels. In addition, we have done morphological observations on the filaments along the course of aggregation upon heating. We demonstrate that the strength and the structure of heat-induced gel of myosin filaments depend on the filament length before heating.

MATERIALS AND METHODS

Protein preparations. DEAE-Sephadex column-purified myosin was prepared from rabbit back and hind leg muscles by the method of Offer *et al.*¹⁴⁾ The myosin preparation was free from C-protein and H-protein, which are minor protein components in the thick filaments of myofibrils. Myosin was stored as 55% ammonium sulfate precipitates at 4°C until use. It was dialyzed against 0.3 M KCl and clarified just before use.

Myosin filaments were prepared by lowering the ionic strength by dilution or dialysis. The speed of dilution was controlled by a peristaltic pump. The final condition of the solvent was 0.1 M KCl and 20 mM K phosphate (pH 6.0).

Protein concentration of myosin solution was measured spectrophotometrically using an extinction coefficient at 280 nm of $0.52 \text{ (mg/ml)}^{-1} \text{ cm}^{-1}$.¹⁵⁾

Measurement of rigidity. The protein concentration of myosin filament suspension was adjusted to 2.5 mg/ml. A suspension was heated at 65°C for 20 min in a glass cuvette, which was placed in the thermostatically controlled cell holder. Rigidity of the gel was measured with a band type viscometer by the method of Yasui *et al.*¹⁶⁾

Scanning and transmission electron microscopies. The structure of heat-induced gels of myosin filaments was observed in a scanning electron microscope (JEOL, JSM-T200) by the method of Yasui *et al.*¹⁶⁾ The negatively stained myosin filaments were observed by a transmission electron microscope and the filament lengths were measured as previously described.¹³⁾

RESULTS

Turbidity is widely used as one of the pa-

TABLE I. TURBIDITY OF MYOSIN FILAMENT SUSPENSIONS AND FILAMENT LENGTH AT VARIOUS SPEEDS OF LOWERING THE IONIC STRENGTH

Speed (ml/min)	Turbidity ^a (A_{350})	Length ^b (μm)
> 70	0.964	0.4 ~ 0.7
2.6	0.950	0.6 ~ 0.9
0.95	1.010	0.8 ~ 1.1
0.33	1.105	1.0 ~ 1.6
Dialyzed	1.245	2.0 ~ 3.5

^a All samples contained 0.1 M KCl, 20 mM K phosphate (pH 6.0), and 2.5 mg/ml of myosin. Light path was 1 cm.

^b Lengths were expressed as a range of average lengths obtained from several independent experiments.

FIG. 1. Rigidity of Heat-induced Gel of Myosin Filaments at Various Filament Lengths.

Myosin filament suspensions (0.1 M KCl, 20 mM phosphate, pH 6.0, and 2.5 mg/ml) were heated at 65°C for 20 min to allow gelation. Unheated myosin filaments were observed in a transmission electron microscope and average length was estimated.

rameters which indicate dissociation or association of protein molecules. As shown in Table I, the speed of dilution affected turbidities of the myosin filament suspensions. Turbidities increased with slowing the speed. Since these changes in turbidity indicate that there are some morphological differences among myosin filaments, the filaments were observed in a transmission electron microscope and their lengths were measured. When the speed of dilution was very rapid, such as over 70 ml/min, the average filament length obtained from several independent experiments was from 0.4 to 0.7 μm . Upon slowing the speed of lowering the ionic strength, fila-

FIG. 2. Scanning Electron Micrographs of Heat-induced Gel of Myosin Filaments.

a, b, and c represent gels which were formed from average lengths of 0.6, 1.2, and 2.5 μm of filaments, respectively. Scale bar indicates 5 μm .

ments became longer. These results were consistent with the results reported previously.^{9,11,12} There were rather large variances in the average filament lengths of several preparations by dialysis. This may be due to the difference in the rate of exchanging the solvents across the dialysis membrane which may be changed by the stirring speed of the dialysate in each experiment.

Suspensions of various lengths of myosin filaments were heated to form gels. The rigidity was measured and the morphology of the gels was observed. All of the filament suspensions were gelled by heating at 65°C for 20 min. Figure 1 shows the relationship between rigidity and filament length. The rigidities of short myosin filaments such as 0.5 μm were about 6,000 dyn/cm². The rigidities increased with lengthening of the filaments. The gels formed by long (over 2.5 μm) filaments had 12,000 ~ 14,000 dyn/cm² of rigidity, which was almost twice that formed by short filaments. These results indicate that the rigidities of heat-induced gels of myosin filaments are intimately related to the length of filament before heating.

The structures of heat-induced gels of myo-

FIG. 3. Changes in Temperature of Myosin Filament Suspensions.

One ml of myosin filament suspension was incubated at 40°C (●) or 60°C (○). Arrowheads denote the position where a sample of 50 μl of the suspension was withdrawn for transmission electron microscopy.

sin filaments were observed in a scanning electron microscope to know how the rigidity is related to the structure of the gels (Fig. 2). When short filaments were heated, the gel showed a coarse, aggregated and rather porous structure as shown in Fig. 2a. In addition, the strands which organize a whole network structure of the gel looked knotty. With increasing lengths of myosin filaments, the network of the gels became finer; furthermore, the strands became thinner and smoother (Fig. 2, b and c). The gel made from long myosin

FIG. 4A.

FIG. 4B.

FIG. 4. Course of Changes in Myosin Filaments upon Heating at 40°C.

A, Myosin filaments were prepared by either dialysis (a~d) or dilution (e~h). a and e, unheated; b and f, c and g, and d and h are myosin filaments heated for 2, 10, and 20 min, respectively. Scale bar indicates 0.5 μ m: B, Myosin filaments prepared by dilution at high magnification. a, unheated; b, heated for 10 min at 40°C. Scale bar indicates 0.2 μ m.

filaments prepared by dialysis showed a fine strand-like network structure (Fig. 2c).

Myosin filaments aggregated, instead of forming a gel, upon heating if the protein concentration was low, such as 0.2 mg/ml in this study. Morphological changes in myosin filaments in a process of aggregation during heating was followed in a transmission electron microscope. One ml of myosin filaments suspensions, which were prepared by rapid dilution or dialysis, were heated at 40°C or 60°C. Figure 3 shows the changes in tempera-

ture of the sample held in a water bath. Fifty μ l of the suspensions were withdrawn from the stock at appropriate time during heating. They were immediately cooled in an ice bath, then prepared for electron microscopy.

In the case of heating at 40°C, no marked changes in the filaments were observed after 2 min (Fig. 4A, b and f). However, the filaments became to associate with increasing heating time (Fig. 4A, c and g for 10 min; d and h for 20 min). As shown in Fig. 4B, the projections were frayed out from the surface of

FIG. 5. Course of Changes in Myosin Filaments upon Heating at 60°C.

Myosin filaments were prepared by either dialysis (a ~d) or dilution (e ~h). a and e, unheated; b and f, c and g, and d and h are myosin filaments heated for 30 sec, 1 min, and 2 min, respectively. Scale bar indicates 0.5 μ m.

each filament in the unheated filaments. However, the filaments became knobby after 10 min of heating, suggesting that partial denaturation of subfragments 1 and 2, which are responsible for the projections of the myosin filament took place. Although these morphological changes were observed in the filaments heated at 40°C, in general, individual filaments were distinguishable even after 20 min of heating.

When myosin filament suspensions were heated at 60°C, aggregation seemed to be completed within 1 min (Fig. 5). The surface of the filaments looked knobby 30 sec after heating; simultaneously, association of filaments took place (Fig. 5, b and f). Myosin filaments themselves were disordered after 1 min of heating at 60°C regardless of their lengths (Fig. 5, c and g). Besides irregular aggregation of long filaments, many filaments seemed to associate side-by-side. A typical example is shown in Fig. 5c. In contrast, short filaments associated randomly and made clusters (Fig. 5, e~h) in the earlier stage of heating. A detailed observation on the structure of negatively stained aggregates of myosin filaments in a transmission electron microscope was difficult because of the high electron density of the aggregates; only the peripheral parts of the aggregate was observable. Neither marked shortening nor thinning was observed in any lengths of myosin filaments during heating. Instead, the filaments looked thickened upon heating. These observation suggest that no dissociation of myosin molecules from the filaments occurred.

DISCUSSION

The water holding and binding capacities of processed meat are based on the capability of myosin to form thermal gels.^{17,18)} Rheological properties of heat-induced gels of myosin are known to be affected by various factors, such as ionic strength, pH, protein concentration, and heating temperature.^{3~6,19)} Among these factors, ionic strength and pH are also critical ones which determine whether the myosin

molecules are monomers or filaments. When the ionic strength is above 0.3 and pH is neutral, myosin molecules are dispersed as monomers, but they are assembled into filaments upon lowering the ionic strength. The equilibrium between monomers and filaments shifts to higher ionic strength with lower pH.⁸⁾ It should be noted that the pH of meat, namely post-mortem muscle, falls usually to about 5.4 to 5.7, because of accumulation of lactic acid produced by glycolysis. From these points, studies on heat-induced gelation of myosin in filamentous state would be important.

In our previous paper, we demonstrated that short myosin filaments formed less rigid gels than long ones.¹³⁾ The present results confirm our previous result and also reconcile the discrepancy between the structural difference of heat-induced gels of myosin at low ionic strength reported by Ishioroshi *et al.*³⁾ and Hermansson *et al.*⁵⁾ The former authors have shown an aggregated structure in a gel, but a fine strand-like network structure has been shown by the latter. These authors did not observe their myosin filament preparations by a transmission electron microscope; however, our results suggest that myosin filaments prepared by Ishioroshi *et al.* are short and those of Hermansson *et al.* are long.

This study also indicates that the rigidity of heat-induced gels of myosin filaments depends on the filament length (Fig. 1). One possible reason for the differences in the rigidities of heat-induced gels of various lengths of filaments is at least the structure of the gels. Upon heating, long myosin filaments formed a gel which has a fine strand-like network structure with high rigidity. In contrast, short filaments form less rigid gel, which has a knotty aggregated structure (Fig. 2). The number of strands in a unit volume of gel is larger in a fine strand-like network structure than that in a coarse aggregated one. Therefore, physical stress applied to a gel may be dispersed by a number of strands in a fine network structure; and so the strength of a gel having such structure would be higher than that having an aggregated one.

The difference in the structures of heat-induced gels among various lengths of myosin filaments can be explained as follows: short filaments may be able to move much more easily in a solvent than the longer ones during heating. Assuming the protein concentrations of the filament suspensions are the same, the number of short filaments in a unit volume is larger than that of longer ones. As a result, some of the short filaments would associate to form clusters, in which the filaments are randomly oriented, in an early stage of heating. This seems to occur above 40°C (Fig. 4). Then, a whole structure is organized by connecting such clusters during subsequent heating. Consequently, a knotty aggregated structure is formed upon heating of short filaments. In contrast, random movement of long filaments in a solvent is probably more restricted than that of shorter ones. This means that long filaments aggregate throughout in a system rather than by the local formation of clusters. Some of the long filaments seemed to form bundles with lateral cross-linking (Fig. 5, c and d); suggesting that these bundles caused formation of strands which make up a whole network structure. It was not clear whether a strand could be made by just one myosin filament. However, it is likely that some of strands in a fine network structure are made by more than two myosin filaments at least.

Yasui and his associate have proposed a mechanism of heat-induced gelation of myosin as follows;^{3,4,20,21} when myosin molecules are dispersed as monomers at high ionic strength, aggregation of head portions of the myosin molecules, to which sulfhydryl groups contribute, occurs first; then, cross-linking of rods of myosin molecules follows. The latter reaction involves helix-coil transformation. On the other hand, in the case of gelation of myosin filaments at low ionic strength, they have suggested that gelation occurs chiefly by interfilamentous cross-linking through myosin heads; the role of the rod portion, which constitutes a shaft of the filament, is less.⁴⁾

Our results are not contradictory to this hypothesis. Neither thinning nor shortening of

myosin filaments was observed during heating, indicating that the filaments *per se* are subject to heat-denaturation, even if partial dissociation of myosin molecule from the filament occurred. Since myosin heads project from the surface of the filaments, it is probable that head portions of myosin molecules play some role in interfilamentous cross-linking, which involves side-by-side, end-to-end or random orientation of the filaments, on heat-induced gelation of myosin filaments. As shown in the electron micrographs in Figs. 4 and 5, the projections which frayed out from the surface of the filament became to show knobby appearance before disordering of the shaft of the filament upon heating. These results suggest that myosin heads are more sensitive to heat than the tail portion of the molecule. This is consistent with the results of thermal denaturation studies of myosin and its subfragments.^{22,23)} The disordering of the shaft of the filament upon heating may be due to shortening of the tail of the myosin molecule.²⁴⁾ The beginning of the structural changes in the projections and the association of the filaments in an early stage of heating seemed correlative.

Recently, Morita *et al.*²⁵⁾ have reported on the heat-induced gelation of chicken myosin at 0.6 M KCl and acidic pH. When a myosin solution in 0.6 M KCl is heated, only a weak gel is formed in the pH range from 6.0 to 6.5. The rigidity of the gel below pH 6.0 is much greater than that at higher pH range. An interesting point is that myosin molecules are assembled into filaments even at 0.6 M KCl when the pH is below 6.0. It should be noted that myosin molecules exist as monomers at this KCl concentration and neutral pH. The filaments formed at pH 5.4 are longer than those at pH 5.7; coincidentally, the rigidity of the filaments at pH 5.4 is higher than that at pH 5.7. The above observation indicates that, even at high ionic strength, the characteristics of thermal gels of myosin depends on a state of myosin assembly before heating, as well as those at low ionic strength.

In conclusion, the thermal gel strength of myosin closely correlates with the structure of

the gel which is determined by a state of assembly of myosin molecules before heating.

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