Heat-induced Gelation of Myosin Filaments at a Low Salt Concentration†

Makoto ISHIOROSHI, Kunihiro SAMEJIMA* and Tsutomu YASUI

Department of Animal Science, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan
*Department of Dairy Science, The College of Dairying, Ebetsu 069-01, Japan

Received May 23, 1983

The heat-induced gelation properties of myosin in low salt concentration were studied. Freshly prepared myosin formed gels with an extremely high rigidity in 0.1 to 0.3 M KCl at pH 6.0 on heating. This high heat-induced gel formability of myosin filaments diminished during storage, concomitant with the loss of the filament formability inherent in the native myosin. Presumably intermolecular aggregation was the cause of this loss during storage. The difference in the heat-induced gelation of myosin filaments at a low salt concentration (0.2 M KCl) and that of myosin monomers at a high salt concentration (0.6 M KCl) was clearly distinguishable from their gelling behavior. The high gelation ability of freshly prepared myosin filaments upon heating seems to develop through the interfilamental head-head aggregation on the surface of the filaments without involving the tail portion of the molecule.

Skeletal muscle myosin in salt solution (i.e., 0.6 M NaCl or KCl) forms a gel upon heating. It is well known that the heat-induced gelation of myosin plays a decisive role in the development of binding properties of meat products such as sausages.† The salt added in the commercial production of meat products is commonly at a concentration range between 2.0 and 3.0%. Molar concentrations calculated from these percentages gives 0.47 ~ 0.68 molarities, assuming that skeletal muscle contains 75% water. At this salt concentration range, myosin molecules are released from thick filaments of the myofibrils and disperse in the solution largely as monomers. Most earlier studies2~7) on the heat-induced gel formability of myosin have also been carried out in salt solutions of 0.5 ~ 0.6 M in which myosin molecules exist as monomeric species.8~10) We have studied the heat-gelling properties of myosin11,12) as well as the effects of actin and regulatory proteins13~16) on gelation of myosin in 0.6 M KCl.

In some our recent work,12) we have already reported that freshly prepared myosin formed a gel with an extremely high rigidity on heating at 0.1 ~0.3 M KCl, a salt concentration range in which it is in the form of filaments,8~10) and that this gel-forming ability disappeared gradually with storage time. It was suggested that the loss of the gel formability might be correlated with the changes in the filament formability of myosin during storage.

In this paper, the heat-gelling properties of myosin at a low salt concentration (0.2 M KCl) and the changes in physicochemical properties of myosin during storage are reported. Several new features have been found in the heat-induced gelation of myosin (filament or self-associated type myosin) in low salt media.

† This work was supported by a Grant-in-Aid for Scientific Research (Project No. 5843002, 1983) from the Ministry of Education, Science and Culture of Japan.

Abbreviations: ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; Pi, inorganic orthophosphate; DTT, dithiothreitol; PMSF, phenylmethyl sulphonyl fluoride; EDTA, ethylenediamine tetraacetate; SDS, sodium dodecyl sulfate.
These features are distinctly different from those developed by myosin (monomer or dispersed type myosin) in high salt solutions.

MATERIALS AND METHODS

Reagents. ATP, DTT, and PMSF were purchased from Sigma Chemical Co., Ltd. All other chemicals used were of the highest purity.

Proteins. Myosin was prepared essentially as described by Perry\(^{17}\) from rabbit muscles (hind leg and back). The prepared myosin was either used soon after preparation for gelation studies at different concentrations ranging from 0.1 to 0.6 M KCl or kept in 0.6 M KCl for storage study. The pH of the KCl solution and storage temperature were always 6.5 and 0°C unless otherwise mentioned. The concentration of protein in the gelation study was always maintained at 4.5 or 5.0 mg/ml.

Actin was extracted from acetone-treated dried muscle powder with a cold solution containing 0.2 M ATP and 5 mM Tris-HCl (pH 8.0) and purified according to the method of Spudich and Watt.\(^{18}\)

Solubility. To examine the alterations in filament formability of myosin during storage, changes in solubility were investigated by centrifuging myosin in the presence of various salt concentrations at 10,000 × g for 30 min. The solubility of myosin has been expressed as: [(Supernatant protein concentration)/(Initial protein concentration)] × 100. It may be emphasized that the solubility here refers to myosin monomers and/or oligomers.

ATPase assay. The inorganic phosphate (Pi) liberated during the hydrolysis of ATP by myosin was measured by the method of Fiske and Subbarow.\(^{19}\) The reaction was terminated by adding an equal volume of 15% trichloroacetic acid.

Fluorescence and light scattering intensities. Fluorescence and light scattering intensity measurements were made on a Hitachi fluorescence spectrophotometer type 650-60. Excitation was at 278 nm. Changes in the fluorescence intensity of myosin solutions were estimated at 335 nm, the temperature being 25°C. The light scattering intensity (90°) of myosin solution was determined at 535 nm at 25°C.

Viscosity. Viscosity measurements were made with an Ostwald type viscometer at 25°C. The flow rate for water was 45 sec.

Gelation. The gelation of myosin was measured using the band type viscometer reported earlier.\(^{11}\)

RESULTS

Effect of storage

The data presented in Fig. 1(A) show that freshly prepared myosin, though it formed quite a firm gel on heating in low salt concentration (0.1 ~ 0.3 M KCl, pH 6.0), reached its maximum rigidity (~ 8,000 dyn/cm\(^2\)) at 0.2 M KCl. This substantiates our previous findings.\(^{12}\) However, with the increase of salt concentration beyond 0.2 M, rigidity of freshly prepared myosin declined sharply. It reached a minimum value of about 1,800 dyn/cm\(^2\) at 0.4 M and thereafter it remained constant with further increases in the salt concentration up to 0.6 M.

On the other hand, the gel firmness of stored myosin was significantly lower than that of the freshly prepared one under similar conditions.

![Fig. 1. Effects of Salt Concentration and Storage Time on Changes in the Rigidity (A) and Percentage of Myosin Monomers and/or Oligomers (B).](attachment:image.png)
The gel strength was the weakest at 0.1 ~ 0.2 M KCl of 10 ~ 15 days stored myosin samples. However, at high molarity (> 0.3 M), gelation strength of stored myosin was still appreciable (Fig. 1(A)).

Figure 1(B) demonstrates the changes in solubility of stored myosin on dilution to different salt concentrations as compared to the solubility of freshly prepared myosin diluted to the same molarity of salt. The freshly prepared myosin remained 100% soluble at about 0.25 M or above KCl concentrations. The stored myosin exhibited significantly reduced solubility at low salt concentrations (0.1 ~ 0.3 M), although its solubility at high salt concentrations (> 0.3 M) remained equivalent to that of freshly prepared myosin. However, the solubility characteristics of myosin do not seem to correlate with its gel formability, especially at high salt concentration.

**Effects of temperature, pH and DTT**

Figure 2 depicts the gelation profiles of freshly prepared myosin on heating in 0.2 M KCl (in the presence and absence of the disulfide linkage cleaving agent DTT), as compared to those in 0.6 M KCl. The gelation profile of myosin in 0.2 M KCl at pH 6.0 was quite distinct from that in 0.6 M KCl. Myosin in 0.2 M KCl at pH 5.0 developed a substantial rigidity even at 20°C and attained its maximum value at about 60°C, whereas myosin in 0.6 M KCl attained far less values at corresponding temperatures. Besides, the presence of DTT in the system did not produce any effect on the gelation rate of myosin in 0.2 M KCl, but it greatly depressed the gelation profile of myosin in 0.6 M KCl, especially at high temperatures (> 30°C). Its effect seems to be independent of the pH value of the system. However, the influence of pH on rigidity appears to be affected by the ionic strength of system.

Figure 3 shows pH dependent, heat-induced gelation of myosin at 65°C in the presence of low and high salt concentrations. The highest gelation of myosin occurred at pH 5.5 in 0.2 M KCl and at pH 6.0 in 0.6 M KCl, respectively.

**Effects of actin, ATP and inorganic pyrophosphate**

The addition of a small amount of actin was reported to enhance the heat-induced gel formability of myosin in 0.6 M KCl by forming actomyosin complexes in the system. However, the data in Fig. 4, show that the addition of actin to the system in 0.2 M KCl progressively inhibited the gelation of myosin.
This may imply that the mechanism for the network formation by the filament myosin system is different from that of the monomer myosin system, and that the association of F-actin with myosin heads plays a dominant role in this inhibitory effect.

The addition of ATP in the system also instantly caused a drastic decrease in the rigidity (Fig. 5). However, the suppressing effect of ATP on the gelation of fresh myosin in 0.2 M KCl diminished with time of incubation at 25°C before heating. The restoration of the rigidity to the initial value clearly paralleled the liberation of Pi from ATP during incubation. After complete hydrolysis of the added ATP, the suppressing effect of ATP on gel-strength completely leveled off (Fig. 5).

It may also be emphasized that the addition of pyrophosphate whose effect on the heat-induced gelation of myosin in 0.6 M KCl is known to be similar to that of ATP,\textsuperscript{14} showed no adverse effect on the gelation of myosin in 0.2 M KCl, nor did the presence of ADP in the system have any influence. These results imply that this inhibitory effect is ATP-specific and may be closely related to complex formation at the enzymic active site(s) of the myosin head with ATP.

The rigidity values attained by the addition of either F-actin (weight ratio of myosin/actin = 1.5) or ATP (1 mm) were ~1,000 and ~1,800 dyn/cm\(^2\), respectively. These data (Figs. 4 and 5) suggest that heat-induced gelation of myosin in 0.2 M KCl possibly involves actin binding site(s) as well as ATP binding site(s). For ascertaining the validity of this proposition another experiment was designed to examine the effect of added F-actin and ATP on gelation of myosin in 0.2 M KCl. The inhibitory effect of actin on gelation of myosin was again quite obvious (Fig. 6). Surprisingly, the effect of ATP was additive in preventing the gelation of myosin in the presence of actin. It was expected that in the presence of ATP, at least the inhibitory effect of actin on myosin gelation would be eliminated, although the actin-induced inhibition could reappear again as ATP is hydrolyzed to ADP and Pi with incubation at 25°C before heating. These expectations were based on the
Heat-induced Gelation of Myosin

Vertical arrows, (actin and ATP), show the addition of F-actin (weight ratio of myosin to actin = 5.5) and subsequent addition of 2 mM ATP with 2 mM MgCl₂ to myosin before thermal treatment. For other conditions see Figs. 4 and 5. (○) The rigidity of thermally treated myosin at 65°C for 20 min. (●) Pi liberation during incubation with ATP.

fact that ATP prevents actomyosin complex formation and brings about dissociation of this complex. Figure 6 shows that as ATP was hydrolyzed to the level as low as about 1/3~1/4 of the initial concentration, the rigidity value once suppressed by ATP (~1,000 dyn/cm²) was rapidly restored to the initial value (~5,000 dyn/cm²).

Changes in physicochemical properties

Figure 1 showed that the heat-induced gel formability of stored myosin (at 0°C) gradually diminished with time and lost completely in about 2 weeks. On storage at higher temperatures such as 25°C, the gel formability of myosin was lost even within 10 hr. This suggests that the rate of loss in the gel formability of myosin in 0.2 M KCl is temperature-dependent. Figure 7 presents the result of parallel experiments on the solubility, actin-activated Mg²⁺-ATPase and EDTA-ATPase activities of myosin, stored in 0.6 M KCl (pH 6.5) and at 25°C. Changes in solubility of myosin always preceded the decrease in the gelation strength in low salt concentrations. The rate of decrease in actin-activated Mg²⁺-ATPase activity (△) was determined at 25°C in a medium containing 0.5 mg/ml myosin, 0.2 mg/ml actin, 30 mM KCl, 20 mM Tris-HCl buffer (pH 7.5), 1 mM ATP and 1 mM MgCl₂. EDTA-ATPase (▲) was estimated at 25°C in a medium containing 0.5 mg/ml of myosin, 0.5 mM KCl, 20 mM Tris-HCl buffer (pH 7.5), 1 mM ATP and 1 mM EDTA.

Fig. 6. Changes in the Inhibitory Effect of F-Actin in the Presence of ATP on the Heat-induced Gelation of Myosin in 0.2 M KCl (pH 6.0).

Fig. 7. Changes in the Gel Formability, Solubility and ATPase Activities of Myosin during Incubation.

Freshly prepared myosin (15 mg/ml) in 0.6 M KCl and 5 mM Tris-maleate buffer (pH 6.5) was incubated at 25°C in the presence (○, △) and absence (●, □, ▲) of 1 mM PMSF before each measurement. The rigidity (○, ●) was measured in 0.2 M KCl under the same conditions as in Fig. 1. Solubility (△) of myosin was recorded in 0.2 M KCl. Actin-activated Mg²⁺-ATPase activity (□) was determined at 25°C in a medium containing 0.5 mg/ml myosin, 0.2 mg/ml actin, 30 mM KCl, 20 mM Tris-HCl buffer (pH 7.5), 1 mM ATP and 1 mM MgCl₂. EDTA-ATPase (▲) was estimated at 25°C in a medium containing 0.5 mg/ml of myosin, 0.5 mM KCl, 20 mM Tris-HCl buffer (pH 7.5), 1 mM ATP and 1 mM EDTA.

Fig. 8. Changes in Fluorescence, Light Scattering Intensity and Viscosity of Myosin during Incubation.

Freshly prepared myosin (5 mg/ml) in 0.6 M KCl and 5 mM Tris-maleate buffer (pH 6.5) was incubated at 25°C before each measurement. Fluorescence (○) and light scattering intensity (△) were determined at 25°C in a medium containing 0.5 mg/ml of myosin, 0.6 M KCl, and 5 mM Tris-maleate buffer (pH 6.5). Viscosity (□) was measured at 25°C in a medium containing 2 mg/ml myosin, 0.6 M KCl and 20 mM phosphate buffer (pH 7.0).
Mg$^{2+}$-ATPase activity of myosin was relatively slower than that of solubility and rigidity. But EDTA-ATPase activity showed little change during storage.

Changes in fluorescence, light scattering intensity, and viscosity of myosin incubated under the same conditions are shown in Fig. 8. Although fluorescence intensity did not change, light scattering intensity and viscosity showed remarkable increases corresponding to the decline in solubility and gel formability of myosin (Fig. 7).

**DISCUSSION**

It is accepted that isolated myosin forms uniform thick filaments in low salt concentrations (<0.3 M KCl)$^{8,9}$ of the same size as those of myofibrils at physiological ionic strength, but myosin disperses as monomers in solutions of ionic strength greater than 0.4. The results in Fig. 1 suggest that the interactions which occur in the heat-induced gelation of the myosin filaments at low salt concentrations are different from those which are responsible for the gelation of myosin monomers in high salt concentrations. The heat-induced gel formability of myosin monomers was not affected by storage of myosin samples in a high salt solution (0.6 M KCl, pH 6.5). On the other hand, the gel-forming ability of myosin filaments in low salt concentrations was extremely high within 24 hr after preparation of the myosin, but decreased rapidly with storage.

Figures 2～5 also indicate characteristic differences in the heat-gelling properties of the myosin filaments (in 0.2 M KCl) and those of myosin monomers (in 0.6 M KCl), and suggest that the gelation of myosin filaments and monomers on heating develops through a different process. At high salt concentrations the tail portion of the myosin monomer is free in the solution to play an essential role in the heat-induced gelation of myosin through a partially irreversible helix-coil transition process followed by formation of a three-dimensional network.$^{20,21}$ Since the myosin tail remains packed in the axis of the myosin filament assembly in low salt concentration, it may not be contributing to the gelation process in the same manner as it does in the case of myosin monomers (0.6 M KCl), unless dissociation of the filament into monomers occurs prior to the gelation upon heating. Earlier studies$^{20,21}$ on the heat-induced gelation properties of the subfragments from the tail portion of myosin molecule showed that they could not form heat-set gels but precipitated at lower salt concentrations than 0.3 M KCl.

As clearly shown in Fig. 4, F-actin exerted a strong inhibitory effect on the heat-induced gelation of the myosin filaments, whereas the gel formability of myosin monomers in 0.6 M KCl was enhanced by the addition of F-actin or actomyosin.$^{13～15}$ Previous studies on superprecipitation (an in vitro model of muscle contraction) have indicated that, under physiological conditions, in the absence of ATP, F-actin (thin filaments) binds to myosin heads which are projected from the surface of the bipolar myosin (thick) filaments so as to form a rigor complex.$^{22}$ Upon the addition of ATP, actin filaments dissociate from myosin filaments and subsequently with hydrolysis of ATP reassociate to superprecipitate (contract). Since the actin binding sites as well as the enzymic active sites are located in the “head” region of myosin,$^{10,23}$ it may well be presumed from the specific inhibitory effect of actin (Fig. 4) and ATP (Fig. 5) on the heat-induced gelation of myosin filaments that both these sites may be involved in the interfilamental head-head interactions for network formation in the system upon heating. The specific binding of actin and ATP to the “head” makes the condition unfavorable for head-head interactions between myosin filaments. The results in Fig. 6 strongly support this contention.

The decline in heat-induced gel formability of myosin filaments during storage of myosin in solution either at 0°C or at 25°C (Figs. 1 and 7) seems to correlate with the changes in filament formability of myosin before heating. In fact, Yasui et al.$^{24}$ have already demon-
Heat-induced Gelation of Myosin

...strated the loss in the filament formability of myosin during the initial stage of denaturation prior to losing its Ca$^{2+}$-ATPase activity. Kimura et al.\textsuperscript{25} reported that when heat denaturation of myosin was judged from actin-activated Mg$^{2+}$-ATPase activity, inactivation of myosin occurred in two steps, \textit{i.e.}, an initial fast phase followed by a slow phase. They indicated a possible relationship between the former inactivation and the loss of filament formability of myosin.

Very recently, Pinset-Häström and Whalen\textsuperscript{26} investigated the decay of the thick filament formability due to aging of myosin. They have reported that the loss of filament formability during storage resulted from degradation of the DTNB-light chain by a protease which had contaminated their myosin preparation. We checked our myosin samples before and after storage for the likelihood of such a contamination. However, no sign of proteolytic degradation was apparent on SDS gel electrophoretograms of the myosin samples, although they had lost both filament and gel formability (data are not shown here for brevity). The presence of PMSF, an inhibitor for serine proteases, in myosin preparation during incubation did not prevent the loss in gel formability of myosin filament (Fig. 7).

In the case of smooth muscle, phosphorylation and dephosphorylation of the P-light chain regulate the filament formability of myosin.\textsuperscript{27} Since the DTNB-light chain of skeletal muscle can also be phosphorylated\textsuperscript{28} and the DTNB-light chain in our myosin preparation was in the unphosphorylated form, we also examined the effects of phosphorylation of DTNB-light chain on the gelation of myosin filament. The results obtained, however, have indicated that phosphorylation of the DTNB-light chain has no influence on the gelation of myosin filaments (data are not shown here). Thus, what causes a rapid fall in the gel formability of myosin filaments during storage remains to be elucidated.

The changes in light scattering intensity and viscosity of myosin preparations on incubation implicate the formation of aggregates without the exposure of hydrophobic residues of the myosin molecules (Fig. 8). Denaturation studies\textsuperscript{24,29} on myosin reported previously have demonstrated that the aggregation of the head portion of myosin molecule in solution at high salt concentration took place at such relatively low temperature as 35°C. The loss of the characteristic filament formability (\textasciitilde 1.5 μm long) of the stored myosin during incubation followed by the depression in heat-induced gel formability of myosin filaments may be ascribed to the progressive polymerization of myosin monomers. It is to be emphasized that the heat-induced gelling properties of the myosin filaments observed in this study may provide a clue to elucidate quality characteristics of unsalted cooked meat, because the state of the thick filaments and interactions between thick and thin filaments in the myofibrils of meat is considered to play a key role in determining the functional properties of meat.

REFERENCES