Polymerization Behavior of Various Actomyosin through Disulfide Bonding upon Heating

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To make clear the polymerization behavior of myosin heavy chain (HC) through SS bonding upon heating, SDS-PAGE and the measurement of total SH groups (TSH), reactive SH groups (RSH) and surface hydrophobicity were performed on those actomyosin (AM, 0.5% protein) from carp, flying fish and rabbit which were heated for 1 h at temperatures ranging from 10 to 80°C.

Results were similar in common among three kinds of AM.

In the presence of EDTA (1 mM), surface hydrophobicity and RSH content enormously increased during the heating at the temperature between 30 and 40°C followed by the gradual increase with the rise in temperature up to 80°C without the decrease in TSH.

In the presence of Cu²⁺ (6 μM), TSH content decreased obviously with the rise in temperature above 30°C and RSH decreased above 40-50°C, although surface hydrophobicity behaved as in the presence of EDTA. HC dimer through SS bonding was observed up to 30°C, though its amount was unchanged without decrease in TSH upon heating. Above 30°C, the higher molecules than HC dimer such as trimer, tetramer and so on were found and the polymerization of HC was promoted with the rise in temperature. Actin was also polymerized above 30°C.

These results suggest that up to 30°C the dimer of HC was formed through the oxidation of SH groups without the unfolding of the protein and above 30°C HC polymer was formed along with the oxidation of SH groups which were emerged with the unfolding. In addition, it was suggested that actin is oxidized to high polymer above 30°C as well as HC.

Key words: actomyosin, myosin heavy chain, polymerization, disulfide bond, sulfhydryl group, surface hydrophobicity, heat denaturation

It is well-known that heat-gelation properties of myofibrillar proteins especially of actomyosin, the major constituent protein of muscle, play an important role in meat protein processes involving heat treatment. Furthermore, studying in detail showed that myosin is an essential protein for the gel formation. Hence, the understanding of the role of its behavior upon heating would give a better knowledge of gel formation in meat jelly products during such processes.

Heat-induced gelation of the protein involves in unfolding of proteins followed by orientation of unfolded molecules during aggregation, influencing the development of a gel network. Disulfide bond is one type of bond between molecules, which occurs at some stage in the heat gelation of protein and is often related to the hardness of the product.

Previously, Itoh et al. suggested that disulfide bonding can contribute to the gel formation of actomyosin upon heating and is also responsible for the polymerization of the protein. As this polymerization behavior was examined by a gel filtration method, it is not clear which constituent of actomyosin is responsible for the polymerization. In the recent researches using SDS-PAGE in our laboratory, Kishi et al. elucidated that the polymerization of myosin heavy chain through disulfide bonding occurs during the heating of carp myosin at 80°C. While Somponge et al. found that the dimer of myosin heavy chain is mainly formed during the ice storage of carp actomyosin and myosin owing to the oxidation of sulfhydryl groups on myosin. However, the polymerization behavior with the rising in temperature has not been studied yet.

The present study aimed to make clear the polymerization behavior of myosin heavy chain through disulfide bonding upon heating of actomyosin by analyzing SDS-PAGE patterns and by measuring sulfhydryl content and surface hydrophobicity in the presence of EDTA to inhibit oxidation and in the presence of copper ion to promote it. Since the thermal stability of muscle proteins varies between species, this study was conducted on actomyosin from 3 species (carp, flying fish, and rabbit). Carp was used since we have been using living fish in our series of experiments. Flying fish was selected from our experience as a fish in which the degradation of myosin heavy chain does not occur upon heating. Rabbit was used as a tradi-
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Materials and Methods

Materials

Dorsal muscles of live carp Cyprinus carpio and prerigor chilled flying fish Cypselurus hiraii, and frozen rabbit hind leg muscle were used for preparing natural actomyosin.

Actomyosin Preparation

Actomyosin was prepared according to the method of Takashi et al.18) with minor modifications as follows. Actomyosin was extracted with 0.6 M NaCl-phosphate buffer (3.38 mM NaH$_2$PO$_4$·2H$_2$O, 15.5 mM Na$_2$HPO$_4$, pH 7.5) for 20 h at 4°C. The actomyosin precipitate was obtained by diluting the extract and then adjusting the pH to 6.8. The precipitate was adjusted to 0.6 M in NaCl concentration and to approximately 0.5% in protein concentration. The obtained solution was clarified by centrifugation at 10,000 x g for 30 min and the supernatant was used as a natural actomyosin.

Treatments

In the case of carp actomyosin, prior to the next step, it was mixed with leupeptin 100 μg/ml of actomyosin solution to inhibit proteolysis which occur around 50°C (data were not shown). The carp actomyosin was exposed to 3 different conditions, i.e., with no additive, mixing with 1 mM EDTA, and mixing with 6 μM CuCl$_2$·2H$_2$O. Flying fish and rabbit actomyosins in which proteolysis was not observed, were treated under the same conditions as carp actomyosin except that leupeptin and EDTA were not added. Then each 9 ml of the samples (5 mg protein/ml) was heated in controlled water baths for 1 h at the temperatures ranging from 10 to 80°C at 10° intervals.

Protein Content

Total nitrogen content (N) was determined by the micro-Kjeldahl method.20) Protein content was calculated from N x 6.0.

SDS-PAGE

One part of the treated sample was solubilized in 9 parts of 8 M urea-2% SDS-50 mM phosphate buffer (pH 6.8) containing 0.3 mM NEM. To prepare the reduced samples, ME was added to the above solution to obtain a final concentration of 10%. Samples were heated in the boiling water for 2 min. Then both reduced and unreduced samples were dialyzed separately against 10 volumes of 2% SDS-50 mM phosphate buffer (pH 6.8) overnight at room temperature.

Inner dialysates of the unreduced sample and the reduced sample (4 ml) were mixed with 1 ml of 50% glycerol-5% SDS-0.25% BPB-50 mM phosphate buffer (pH 6.8) without and with 5% ME, respectively. The sample (50 μl) was subjected to SDS-PAGE using 3% polyacrylamide gel according to the method of Weber and Osborn.20) The protein subunit contents were estimated from densitograms scanned at 640 nm on a Shimadzu CS-9000 chromatoscanner. Staining density of the polymer on the top of gels (top polymer) was calculated by the equation (1) and the relative density of HC and actin was calculated by the equation (2):

\[
\text{Top polymer} = (A_{UR} - A_{UR - top band})_{UR}
\]

(1)

\[
\text{Relative density of HC or actin} = \frac{(D_{UR})_H}{(D_{UR})_H} \times 100
\]

(2)

where A is total staining density, D is staining density of HC or actin band in SDS-gel, UH is unheated sample, H is heated sample at any temperatures, R is reduced sample, UR is unreduced sample.

Sulfhydryl (SH) Content

Total SH content was determined according to the method of Ellman21) with minor modifications. One part of treated sample was mixed with 9 parts of 8 M urea-2% SDS-10 mM EDTA in 0.1 M phosphate buffer (pH 6.8). To 4 ml of the mixture, 0.4 ml of 0.1% DTNB solution was added and incubated at 40°C for 15 min. The absorbance at 412 nm was measured to calculate SH content using the molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ for 2-

![Fig. 1. SDS-PAGE patterns of carp actomyosin heated at various temperatures.](image)

a, No additive; b, With copper ion; c, With EDTA; HC, myosin heavy chain; Ac, actin; (HC)$_2$, heavy chain dimer.
nitro-5-thiobenzoic acid at this wavelength. Reactive SH content was determined in the same manner as the total SH content except that the solvent used was a urea-SDS free solution containing 0.6 M NaCl-50 mM phosphate buffer-10 mM EDTA (pH 6.8). Besides, the reaction with DTNB was carried out at 4°C for 1 h.

**Surface Hydrophobicity**

Surface hydrophobicity was assessed by measuring the fluorescence intensity using ANS as a fluorescent probe according to the method of Niwa.²²) The relative fluorescence intensity of each sample of protein series was plotted as a function of the protein concentration and the slope of the plots was expressed as the surface hydrophobicity.

**Results and Discussion**

**SDS-PAGE Patterns and Densitometric Analysis**

Figure 1 illustrates SDS-PAGE patterns of the unreduced and the reduced samples of carp actomyosin heated at various temperatures, and Fig. 2 does those of actomyosin from flying fish and rabbit. From their densitometric analysis, the behavior of HC and the formation of polymer were shown in Fig. 3.

In the unreduced samples of actomyosin from carp with no additive (Fig. 1a), the dimer of myosin heavy chain (HC)₂ was observed as a polymer of HC below about 30°C, though its amount seems to be unchanged upon heating up to 30°C. In these reduced samples, the dimer could not be seen. This means that the dimer is formed by SS bonds between HC molecules, since ME disrupts the disulfide bond to two SH groups²³) owing to its reducing ability.

At the higher temperatures, polymerization of HC clearly occurred through SS bonding. Above 30 to 60°C, the formation of myosin heavy chain polymer (HC)ₙ, such as (HC)₂ and (HC)₄, as well as a polymer at the top of the gels were observed along with the decrease in HC, as shown in the unreduced samples. At 70 and 80°C, these polymers could not be seen except one at the top of the gels. In addition, actin decreased slightly upon heating. This means that polymer through SS bonding is formed mainly with HC. In their reduced samples, almost the same patterns were obtained at any temperatures except at 60–80°C where some protein bands which were higher in molecular weight than HC were observed. On these polymers, detail experiments should be carried out to identify the bonding, though it might be a bond other than SS bonding, since these polymers were observed in the reduced samples containing 2% SDS and 1–2% ME. Furthermore a band which was observed just above HC monomer at 40°C or above in the unreduced samples was not confirmed in its protein composition this time. This band seems to be composed of HC and actin because its molecular weight is estimated at about 260 K from the mobility and also seems to be bound mainly by SS bond.

Figure 1b shows the results corresponding to a stronger oxidation owing to the addition of copper ion. Up to 30°C, the formation of (HC)₂ was promoted only a little in spite of the presence of copper ion. Above 30°C, the polymerization was promoted as resulting in the disappearance of HC at 60°C.

![Fig. 2. SDS-PAGE patterns of actomyosin from flying fish and rabbit heated with and without copper ion at various temperatures.](image)

Fig. 2. SDS-PAGE patterns of actomyosin from flying fish and rabbit heated with and without copper ion at various temperatures. a, Flying fish; b, Rabbit. Abbreviations are the same as shown in Fig. 1.
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Fig. 3. Staining density of HC, (HC)_2, top polymer of the unreduced samples of three kinds of actomyosin heated at various temperatures.

In the presence of EDTA (Fig. 1c), on the contrary, HC was hardly polymerized upon heating. From this result it was confirmed that the polymerization of HC in the absence of EDTA is due to the oxidation of SH groups between molecules, since EDTA has inhibitory effect on the oxidation by chelating some metal catalysts.

The results of SDS-PAGE patterns of flying fish and rabbit actomyosin were almost in the same manners as carp actomyosin (Fig. 2).

The formation of top polymer at the temperature above 30°C discussed above was also clearly confirmed from a densitometric analysis of the unreduced samples, while the formation of (HC)_2 seems to be promoted only a little even in the presence of copper ion upon heating, though the amount of (HC)_2 increased a little more than that before adding copper ion (Fig. 3).

The changes in the remaining actin in the unreduced samples upon heating were shown compared with those in the remaining HC in Fig. 4. Actin as well as HC was almost unchanged up to 30°C and remarkably decreased above 50°C, though the decreasing ratio of actin was less than that of HC. This means that HC is mainly oxidized but actin is also oxidized to polymer upon heating (Fig. 4).

Changes in SH Content

To confirm the formation of disulfide bonds during heating, the changes in SH content were examined and shown in Fig. 5.

As TSH was determined in the presence of urea-SDS, thus the decrease in TSH shows the oxidation of SH groups. Whereas RSH was done without the denaturants, changes in RSH content show the behavior of the surface SH groups of the molecules upon heating.

In the presence of EDTA (Fig. 5. carp), the sudden increase in RSH was observed above 30°C followed by gradual increase up to 80°C, while TSH was kept stable upon heating. This clearly indicates that without oxidative conditions, SH groups emerged from the inside of molecule along with the unfolding of the protein structure upon heating above 30°C.

In no additive samples, TSH was almost unchanged (8.51 to 8.43 mol/10^5 g in the case of carp) up to 30°C and significantly decreased above 30°C. RSH was stable at low temperatures and started increasing at the range of 30 to 40°C (carp) or 30 to 50°C (flying fish and rabbit). At the higher temperatures, RSH decreased with the rise in temperature accompanying the parallel decrease in TSH. Copper ion adding showed the strong oxidation in carp and rabbit actomyosin above 30°C, though the oxidation was very weak at 30°C or under. These results suggest that the
exposed SH groups were oxidized to form SS bonds upon heating above 30°C, and also support well the polymerization behavior of actomyosin through SS bonding.

In addition, the present results of the behavior of TSH and RSH without EDTA agreed well with those which were reported by Itoh et al.,24) Lanier et al.25) and Sano et al.26)

Behavior of Surface Hydrophobicity

In order to make sure the unfolding of protein molecules upon heating, surface hydrophobicity was measured by using ANS. The results in Fig. 6 showed that the intensity highly increased at the temperature ranging of 30 to 40°C in the case of carp and of 30 to 50°C in the case of flying fish and rabbit, following by gradual increase with the rise of temperature, as already reported on flatfish actomyosin and myosin by Niwa et al.22,27) and on carp actomyosin by Sano et al.26) These behavior corresponded well to the behavior of RSH upon heating in the presence of EDTA. Therefore, it was concluded that the increase in RSH is due to the unfolding of protein structure. Regardless of the presence of copper ion, almost the same behavior of surface hydrophobicity was observed as in the absence of copper ion. Niwa et al.27) reported that the hydrophobic interactions increased more intensively upon heating at 40°C, and this increase is presumably related to the formation of intermolecular interactions. Thus, it would appear likely that the increase in those hydrophobic interactions enhance the polymerization through SS bonding.

Considering all results, at low temperatures below about 30°C, (HC)2 through SS bonding existed without accompanying the structure changes and the increase in its amount. At high temperatures above about 30°C, unfolding of HC structure emerged the inner SH groups that normally existed inside the molecule and resulted in the formation of polymer through oxidation of these exposed SH groups. Furthermore, the polymerization through SS bonding was occurred much more strongly above 30°C than below 30°C. These results suggest that the mechanisms in SS bonding at high temperatures above 30°C are different from below 30°C.

Recently, Somponge et al.14,11) found that (HC)2 is formed owing to the oxidation of myosin tail portion during the ice storage of carp actomyosin. Furthermore, Kishi et al.29) reported that rod portion is responsible for the dimer formation of HC and S-1 is responsible for the polymer formation of HC during heating at 80°C. Thus the responsibility of myosin subfragments, S-1 and rod, for the polymerization of HC has been clarified only during ice storage and at 80°C. Therefore, the further study should be conducted to clarify the behaviors of head and tail portions of HC accompanied by the rising of heating temperature.
Fig. 6. Changes in surface hydrophobicity of three kinds of actomyosin upon heating at various temperatures.

- UH, unheated sample; - no additive; - plus Cu++; - plus EDTA.

Temperature (°C)

**References**