AMP and IMP Dissociate Actomyosin into Actin and Myosin

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Received February 29, 2008; Accepted May 16, 2008; Online Publication, August 7, 2008

We investigated to determine why heating of squid muscle at 60℃ induced the liberation of actin from myofibrils. When a mixture of a myofibrillar fraction and a low-molecular sarcoplasmic fraction prepared from squid muscle was heated at 60℃, actin liberation occurred. When a myofibrillar fraction was heated with ATP, AMP, or IMP, actin liberation occurred. Hence, AMP is perhaps one of the factors causing actin liberation in postmortem squid muscle. It was found that AMP and IMP reversibly dissociated actomyosin of chicken, bovine, and porcine skeletal muscles into actin and myosin on incubation at 0℃ at pH 7.2 in 0.2M KCl. These results led us to conclude that AMP and IMP were the most responsible factors causing actin liberation from myofibrils in the heated muscle and causing reversible dissociation of actomyosin on storage of skeletal muscle at a low temperature. Hence, AMP and IMP are possible factors causing the resolution of rigor mortis in muscles.

Key words: actomyosin dissociation; actin liberation; rigor mortis resolution; meat cooking; IMP induced actomyosin dissociation

When animal and fish meats are processed by vacuum cooking, i.e., heating at a low temperature, around 60℃, for a long time, the texture of meats is known to be softer than that of meats cooked traditionally at higher temperatures. Many studies have been conducted to examine a softening phenomenon in vacuum-cooked meats, using chicken,1–5) pork,2) and squid muscle.6–8) Our previous study demonstrated that heating of squid mantle muscle,9) beef, pork, and chicken at 60℃ or 65℃ caused liberation of a marked amount of actin from actomyosin in myofibrils (unpublished data), and suggested that such a reaction was responsible for softening the vacuum-cooked meat.

Changes in the microstructure of myofibrils10) and in SDS-polyacrylamide gel electrophoretic patterns of whole muscle proteins11) were examined previously in heated squid muscle, but there have been no reports concerning with actin liberation and the responsible factor.

One of authors (A. Okitani) found that incubation of actomyosin from rabbit skeletal muscle at 4℃ at pH 5.7 in 0.6M KCl caused irreversible dissociation into actin and myosin,12,13) and that such dissociation did not occur on incubation at 25℃.14,15) Thus the irreversible liberation of actin caused by heating at around 60℃ appeared to be unexplainable by the heat denaturation of actomyosin in an acidic region.

The present study was conducted to identify the factor responsible for heat-induced actin liberation from actomyosin in myofibrils.

Materials and Methods

Materials. The Japanese common squid Todarodes pacificus harvested the day before was procured from a local market and used immediately. The average weight was approximately 200 g. The squid were gutted and skinned. The mantles were minced with a meat chopper. Breast meats (M. pectoralis superficials) of chicken and loins (M. longissimus thoracis) of beef and pork were purchased from retail shops and minced with a meat chopper. The chicken samples were Japanese domestic broiler meats. The beef samples were from Japanese domestic Holstein cattle. The pork samples were from Japanese domestic Berkshire pigs. ATP, ADP, AMP, IMP, adenosine, and inosine were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade. Centriflo CF25 ultrafiltration membranes were obtained from Millipore, Billerica, MA, USA.

Preparation and incubation of squid muscle homogenates. Forty grams of minced squid muscle (to be regarded as 40 ml of 0.2M KCl solution) were mixed with 4.0 ml of 2.0M KCl, 1.6 ml of 1.0M Tris–HCl buffer (pH 7.2)/100 mM NaN3, 4.0 ml of 100 mM EDTA, and 30.4 ml of distilled water, and homogenized twice at 17,000 rpm for 1 min with a homog-

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EDTA was used to inhibit myosinases I and II in a squid muscle as myosin hydrolyzing enzymes. The homogenate obtained contain 0.5 g/ml of meat in 0.2 M KCl/20 mM Tris–HCl buffer (pH 7.2)/5 mM EDTA/2 mM NaN₃. The pH of the homogenates was 6.1 to 6.3 at 0°C. Each 2 g of the homogenate was put in a test tube and incubated in a water bath at 60°C for a defined period. After incubation, the homogenate was cooled in ice water, mixed with 18 ml of 20 mM Tris–HCl buffer (pH 7.2)/5 mM EDTA/2 mM NaN₃ (solution A), and homogenized 2 times at 17,000 rpm for 1 min. A small portion of the homogenate was separated out for electrophoretic analysis, and the remainder was centrifuged at 12,000 × g for 20 min. The supernatant obtained and the separated homogenate were diluted with 3.5 volumes of 10 mM Tris–HCl buffer (pH 7.2) solution containing 0.1 M NaCl/1 mM NaN₃ (solution NT), and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The sample (13.3 μl) loaded on each well contained the proteins derived from 148 μg of minced muscle.

Fractionation of squid muscle. Minced squid muscle was fractionated into myofibrillar, high-molecular sarcoplasmic, and low-molecular sarcoplasmic fractions by the following method: The squid muscle homogenate (80 ml) was prepared from 40 g of minced muscle by the method described above and centrifuged at 39,000 × g for 20 min. The proteins of the supernatant obtained (30 ml) was concentrated 2-fold using a Centriflo CF 25 ultrafiltration membrane, and dialyzed overnight against solution A. The dialysate (15 ml) was a high-molecular sarcoplasmic fraction occurring in solution A at the same concentration as in the minced muscle.

The sediment (20 g) obtained after centrifugation of the squid muscle homogenate was mixed with 80 ml of solution A, homogenized at 17,000 rpm for 20 s, and then centrifuged at 39,000 × g for 20 min. The sediment obtained was mixed with 80 ml of solution A, homogenized at 17,000 rpm for 10 s, and then centrifuged at 39,000 × g for 20 min. The sediment obtained was a myofibrillar fraction occurring in solution A at the same concentration as in the minced muscle.

Minced squid muscle (20 g) was mixed with 60 ml of distilled water and homogenized twice at 17,000 rpm for 1 min. After centrifugation of the homogenate at 12,000 × g for 20 min, the supernatant obtained (39 ml) was filtrated with a Centriflo CF 25 ultrafiltration membrane. The filtrate (20 ml) was concentrated in the solid state with a rotary evaporator. The dried material was solubilized with 5 ml of Tris–HCl buffer (pH 7.2) solution containing 5 mM EDTA/2 mM NaN₃. The solution obtained was a low-molecular sarcoplasmic fraction occurring in solution A at the same concentration as in the minced muscle.

Incubation of squid muscle fractions. The squid muscle fractions prepared as described above were incubated separately or in combination, as follows: One gram of a myofibrillar fraction was mixed with 1 ml of solution A, a low-molecular sarcoplasmic fraction, or a high-molecular sarcoplasmic fraction, and incubated at 60°C for 0 and 30 min, and then cooled in ice water. Each milliliter of a low-molecular sarcoplasmic fraction and a high-molecular sarcoplasmic fraction was mixed with 1 ml of solution A and incubated at 60°C for 0 and 30 min, and then cooled in ice water. Thus each fraction was incubated in solution A at a concentration half that in the minced muscle. The incubated sample was mixed with 18 ml of solution A and homogenized 2 times at 17,000 rpm for 1 min. After centrifugation of the homogenate at 12,000 × g for 20 min, the supernatant obtained was diluted with 4 volumes of solution NT and then subjected to SDS–PAGE. The sample (13.3 μl) loaded on each well contained the proteins derived from 148 μg of minced muscle.

One gram of a myofibrillar fraction was mixed with 1 ml of solution A containing 4 or 16 mM ATP or ATP-related compound and incubated at 60°C for 30 min, and then cooled in ice water. Thus a myofibrillar fraction was incubated with 2 or 8 mM ATP or ATP-related compound in solution A at a concentration half that in the minced muscle. The incubated sample was treated and subjected to SDS–PAGE by the method described above.

Preparation of actomyosin. Following the conventional method, minced chicken, beef, and pork was extracted for 24 h with 6 volumes of Weber-Edsall solution (0.6 M KCl/0.04 M NaHCO₃/0.01 M Na₂CO₃). The brei was centrifuged and diluted to a concentration of 0.2 M of KCl with distilled water. After dilution, the precipitate was collected in 0.6 M KCl. The protein was refined by this dilution method 3 times successively.

Incubation of actomyosin with ATP and ATP-related compounds. Actomyosin solution (0.4 ml of 5 mg protein/ml in 0.6 M KCl) was mixed with various regents to prepare 1.2 ml of an incubation mixture containing 1.67 mg/ml of actomyosin/0.2 M KCl/20 mM Tris–HCl buffer (pH 7.2)/2 mM NaN₃/2, 8 mM ATP, or an ATP-related compound. The mixture was incubated at 0°C for up to 16 h and then centrifuged at 12,000 × g for 20 min. The supernatant obtained was diluted with 0.7 volume of solution NT and then subjected to SDS–PAGE. The sample (13 μl) loaded on each well contained the proteins derived from 13.4 μg of actomyosin.

In order to remove ATP-related compounds, the 16 h-incubated mixture was dialyzed against 1 liter of 20 mM Tris–HCl buffer (pH 7.2) solution containing 0.2 M KCl/2 mM NaN₃ for 24 h at 4°C. The dialysate was analyzed by the method described above.

SDS–PAGE. SDS–PAGE was carried out by the method of Laemmli using 10% separating and 5%
stem polyacrylamide gels. The gels were stained with Coomassie brilliant blue R250. As molecular weight (Mr) markers, cytochrome c (Mr = 12,500), trypsin inhibitor (20,100), α-chymotrypsinogen (25,000), carbonic anhydrase (29,000), ovalbumin (45,000), bovine serum albumin (67,000), and phosphorylase b (97,000) were used.

**Protein determination.** The protein concentration was determined by the Biuret method using bovine serum albumin as a standard.

**Results**

**Actin liberation from myofibrils induced by heat-treatment of squid muscle homogenates**

Our previous study indicated that a noticeable amount of actin was liberated irreversibly from myofibrils by heating of minced muscle and muscle pieces of squid mantle at 60 °C. It was examined whether such a reaction would happen under 60 °C incubation of squid muscle homogenate and similar conditions in postmortem muscle in situ, i.e., at pH 6 in 0.2 M KCl.

The incubated homogenate was diluted 10-fold with solution A and centrifuged. The supernatant obtained was subjected to SDS–PAGE to detect liberated actin. This method, used in a previous study, is essentially based on the well-known fact that under centrifugation of myofibrillar proteins in 0.2 M KCl at neutral pH, native actomyosin is obtained in the precipitate while native myosin or actin is obtained in the supernatant. The results obtained are shown in Fig. 1. The protein bands corresponding to actin and myosin heavy chain were not observed in the supernatant from an unheated sample (a in Fig. 1). Hence it appears that actin and myosin formed actomyosin precipitating at 0.2 M KCl, and that the bands observed in the gel were to be ascribed to sarcoplasmic proteins. On the other hand, in the supernatants from the samples heated for 2 to 60 min (b, c, d, e, and f), a dense band of actin corresponding to Mr of 42,000 was observed, while the band of myosin heavy chain was not. Since in the heated whole homogenate before centrifugation (g, h, and i in Fig. 1) myosin heavy chain was observed, the proteolytic action of myosinas I and II was found to be somewhat inhibited with EDTA. The density of the actin band in the unheated whole homogenate (g) was lower than that in the heated whole homogenates (h and i). This appears to have been caused by the fact that the unheated sample was not finely homogenized enough to react with SDS as much as the heated samples in the dilution process of SDS–PAGE.

The above results indicate that actin was liberated from myofibrils whereas myosin was not when squid muscle homogenate was heated to 60 °C at pH 6 in 0.2 M KCl, similarly to minced muscle and muscle pieces. Hence it appears that squid muscle homogenates can be employed to identify the factor responsible for actin liberation in the subsequent work.

![Fig. 1. SDS–PAGE of Squid Muscle Homogenate Heated to 60 °C.](image1)

Squid muscle homogenate was heated at pH 6.1–6.3 to 60 °C, and then centrifuged in 0.2 M KCl. The whole homogenate and the supernatant obtained by centrifugation were subjected to SDS–PAGE. The details are described in “Materials and Methods.” a–f, supernatant from muscle homogenate heated for a, 0 min; b, 2 min; c, 5 min; d, 10 min; e, 30 min; f, 60 min; g–i, whole homogenate from muscle homogenate heated for g, 0 min; h, 10 min; i, 30 min. j, molecular weight marker; MHC, myosin heavy chain.

![Fig. 2. SDS–PAGE of Low- and High-Molecular Sarcoplasmic Fractions of Squid Muscle after Heating to 60 °C.](image2)

Each sarcoplasmic fraction was heated at pH 7.2 to 60 °C, and then centrifuged in 0.2 M KCl. The supernatant obtained was subjected to SDS–PAGE. The details are described in "Materials and Methods." a, molecular weight marker; b and c, low-molecular sarcoplasmic fraction heated for b, 0 min; c, 30 min; d and e, high-molecular sarcoplasmic fraction heated for d, 0 min; e, 30 min.

**Squid muscle fraction responsible for heat-induced liberation of actin from myofibrils**

The three squid muscle fractions, myofibrillar, high-molecular sarcoplasmic, and low-molecular sarcoplasmic fractions, were incubated at 60 °C separately or in combination and subjected to SDS–PAGE to identify the fraction containing the factor responsible for the heat-induced liberation of actin from myofibrils.

As shown in Fig. 2, no protein bands were observed in the low-molecular sarcoplasmic fraction before (b in Fig. 2) or after incubation (c). Many protein bands, except for the actin band, were observed in the high-molecular sarcoplasmic fraction before incubation (d), but no bands were observed after incubation (e), indicating insolubilization caused by heat denaturation.
The myofibrillar fraction (MF) was heated at pH 7.2 to 60 °C with and without another fraction, and then centrifuged in 0.2 M KCl. The supernatant obtained was subjected to SDS–PAGE. The details are described in “Materials and Methods.” a, molecular weight marker; b and c, MF without another fraction heated for b, 0 min; c, 30 min; d and e, MF with the low-molecular sarcoplasmic fraction heated for d, 0 min; e, 30 min; f and g, MF with the high-molecular sarcoplasmic fraction heated for f, 0 min; g, 30 min; MHC, myosin heavy chain.

As shown in Fig. 3, four thin bands were observed in the myofibrillar fraction before incubation (b in Fig. 3), and one of the four bands corresponded to actin. After incubation (c), the other three bands became dense, but the actin band did not. When the myofibrillar fraction was incubated with the low-molecular sarcoplasmic fraction (e), the actin band became markedly dense. On the other hand, the density of the actin band did not change when the myofibrillar fraction was incubated with the high-molecular sarcoplasmic fraction (g).

These results indicated that the factor responsible for the heat-induced liberation of actin from the myofibrils of squid mantle muscle belonged to the low-molecular compounds in the sarcoplasm.

**Effect of ATP-related compounds on actin liberation from squid myofibril**

Subsequent work was conducted to determine the factor responsible for actin liberation in the low-molecular sarcoplasmic fraction of squid muscle. The low-molecular compounds that increase markedly in postmortem muscle are known to be lactic acid and breakdown products from ATP among many compounds in muscles.20) The latter compounds, ATP-related compounds are thought to have higher affinity to actomyosin than lactic acid, because it has been found that the myosin-ADP-inorganic phosphate complex is formed at the initial stage of ATP hydrolysis by actomyosin.21) Hence the effects of ATP and ATP-related compounds were investigated by incubation at 60 °C for 30 min with the myofibrillar fraction free from the low-molecular sarcoplasmic fraction. The result obtained is shown in Fig. 4.

As shown in Fig. 4, actin was liberated slightly by incubation with 2 mM ATP (c in Fig. 4), and markedly by incubation with 8 mM ATP (d). A concentration of 8 mM for ATP and ATP-related compounds is the highest value observed in normal muscle.20,22) Actomyosin is known to be dissociated into actin and myosin with ATP in 0.2 M KCl and a neutral pH region in the absence of Ca ions.20 In the present incubation in the presence of a Ca chelator EDTA, actomyosin was dissociated into actin and myosin by ATP. The liberated actin was solubilized in 0.2 M KCl, while the liberated myosin appeared to be insolubilized in 0.2 M KCl by heat denaturation.

Incubation with AMP and IMP exhibited actin liberation in a dose-dependent manner (e, f, g, and h in Fig. 4). On the other hand, incubation with ADP, adenosine, and inosine had no effects on actin liberation (not shown in figure).

These results indicate that AMP and IMP among ATP-related compounds had an ability to liberate actin from myofibril. AMP has been found to be the dominant ATP-related compound in postmortem squid muscle.23) Hence AMP is perhaps one of the factors causing the liberation of actin from myofibrils of squid muscle vacuum-cooked at 60 °C. However, in order to determine whether AMP is the major responsible factor, further work should be conducted to investigate the contribution rate of AMP and the involvement of other substances contained in the low-molecular sarcoplasmic fraction.

**Effect of ATP-related compounds on actomyosin from skeletal muscles of vertebrates**

It was found that actomyosin from the muscle of squid, an invertebrate, was dissociated into actin and myosin by two kinds of mononucleotide, AMP and IMP, as described above. The effects of AMP and IMP on actomyosin from the skeletal muscles of vertebrates were hence examined. After such actomyosins were
incubated with 8 mM ATP or ATP-related compounds at 0°C for 16 h, the supernatants obtained by centrifugation of the incubated mixture were subjected to SDS–PAGE. The results for chicken actomyosin are shown in Fig. 5. When actomyosin was incubated alone, SDS–PAGE of whole actomyosin exhibited a typical pattern, in which actin and myosin heavy chain were observed (a in Fig. 5), while that of the supernatant from actomyosin exhibited some amount of actin and no myosin heavy chain (b). When actomyosin was incubated with ATP, ADP, adenosine, and inosine (c, d, g, and h, respectively), some amount of actin was observed in the supernatant, but the myosin heavy chain was not. Since EDTA was not added in the incubation medium used here, it was assumed that dissociation of actomyosin with ATP did not occur. On the other hand, when actomyosin was incubated with AMP and IMP (e and f in Fig. 5), large amounts of actin and myosin heavy chain were observed. This result indicates that incubation with AMP and with IMP at 0°C caused dissociation of actomyosin into actin and myosin in a form soluble to the medium at pH 7.2 at 0.2 M KCl.

The duration of incubation necessary for the dissociation of actomyosin was examined, and the result is shown in Fig. 6. When chicken actomyosin was incubated with 8 mM IMP, the dissociation of actomyosin occurred partially immediately after incubation (c in Fig. 6), and occurred almost completely after 2 (d) and 16 h of incubation (e). Thus, it was found that incubation for a period of 2 h was necessary for actomyosin to be dissociated fully.

The effects of ATP and ATP-related compounds on bovine and porcine actomyosin were examined by the same method as used for chicken actomyosin. The results, shown in Fig. 7, indicate that AMP and IMP (e and f in Fig. 7) caused dissociation of both actomyosins, as in the case of chicken actomyosin (data for bovine actomyosin not shown in figure).

The effects of removal of AMP and IMP by dialysis from the dissociated actomyosin were examined using porcine actomyosin (Fig. 7). As shown in lanes b and e in Fig. 8, actin and myosin heavy chain were not observed in the supernatant obtained by centrifugation of actomyosin in 0.2 M KCl after removal of AMP and IMP, indicating that association of actin and myosin occurred. The experiment using bovine actomyosin exhibited the same result as that for porcine actomyosin (not shown in figure). Thus it was demonstrated that the dissociation of skeletal muscle actomyosin induced by AMP and IMP was reversible under such conditions, preventing denaturation of actin and myosin at pH 7.2 at 0°C.
weakening of Z-line structure in myofibrils. Many studies have been dedicated to determining the factor responsible for these reactions. Possible factors are several muscle proteinases, including cathepsins B, D, L, and H, calpain, and Ca ions increasing post-mortem in a sarcoplasm.

The present study indicates for the first time that AMP and IMP accumulated postmortem in the Mollusca and in vertebra muscles respectively should be added to the list of possible factors in the resolution of rigor mortis induced by the dissociation of actomyosin. Study is now being conducted to gain more direct evidence for AMP and IMP as the agents resolving rigor mortis.

References

10) Kugino, M., and Kugino, K., Microstructural and

Fig. 8. SDS–PAGE of Dissociated Actomyosin after Removal of AMP and IMP.

Porcine actomyosin (AM) was incubated at pH 7.2 at 0°C with 8 mM AMP or IMP for 16 h, and then dialyzed against the buffer solution without AMP or IMP. The dialysate was centrifuged in 0.2 mM KCl. The supernatant obtained was subjected to SDS–PAGE. The details are described in “Materials and Methods.” a, whole AM after dialysis of AM incubated with AMP; b, supernatant after dialysis of AM incubated with AMP; c, supernatant before dialysis of AMP incubated with AMP; d, whole AM after dialysis of AM incubated with AMP; e, supernatant after dialysis of AM incubated with IMP; f, supernatant before dialysis of AM incubated with IMP; g, molecular weight marker; MHC, myosin heavy chain.

Discussion

The present study indicates for the first time that mononucleotides such as AMP and IMP are capable of dissociating actomyosin into actin and myosin in a reversible manner in 0.2 mM KCl at pH 7.2 at 0°C. Since dissociation was observed for an invertebrate muscle, squid mantle, and vertebrate skeletal muscles such as chicken, beef, and pork, it is possible that such a reaction is common to actomyosins of various animals. Work to determine the mechanism of the reaction in detail is being conducted. The results so far indicate that the reaction in postmortem squid muscle is accelerated by raising temperature from 0°C to 60°C, accompanied with insolubilization of liberated myosin (unpublished data).

It is well known that table meats such as chicken, beef, and pork contain significant amounts of IMP as ATP breakdown compound, instead of AMP. Therefore, it is suggested that a softness of such meats produced by vacuum cooking should be ascribed to an irreversible dissociation of actomyosin into actin and myosin induced by heating at low temperatures, around 60°C, in the presence of IMP. This suggestion has been demonstrated to be correct by further study (unpublished data).

It is well known that skeletal muscle enters rigor with depletion of ATP postmortem, resulting in a loss of meat tenderness. This rigor mortis is thought to be caused by tight binding of actin and myosin, composing actomyosin, induced by ATP depletion. When muscle in rigor mortis is stored further, it becomes as tender as pre-rigor muscle. Such improvement in tenderness is supposed to be caused by a resolution of rigor induced by weakening the interaction between actin and myosin and by a


