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Studies on the Myofibrillar Proteins*

Part 2. New Procedure for the Extraction of Regulatory Proteins from Myofibrils

by
Ryung Yang, Chuljai Kim, Juhyun Yu, Hyuksin Lee**
and Youngdong Cho***
Department of Food Engineering, College of Science and Engineering, Yon Sei University, Seoul 120, Korea.

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근원섬유단백질에 관한 연구

(제 2 보) 근수축 조절단백질의 새로운 정제방법

양 융 · 김철재 · 유주현 · 이혁신** · 조영동***
연세대학교 이공대학 식품공학과 · ***연세대학교 이공대학 생화학과

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ABSTRACT

An attempt was made to study on new method for the extraction of the regulatory proteins from myofibrils, and the procedures for the preparation of desensitized actomyosin and for complete extraction of troponin-tropomyosin complex were developed.

When myofibrils were treated through the procedures developed in this study, actomyosin obtained had no Ca-sensitivity, indicating that Ca-sensitizing protein factor had been removed completely from myofibril. Consequently, it was concluded that the procedures developed in this study were convenient to test whether Ca-sensitizing proteins has been removed or not.

When Mg-activated ATPase activity of myofibril were measured, the myofibrillar ATPase turned into the actomyosin type ATPase with the progress of the treatment. This result was interpreted to show that the regulatory proteins of the myofibril seems to play a cementing role on the structure of myofibril.

When supernatant containing the regulatory proteins were fractionated with $(NH_4)_2SO_4$ saturation solution, regulatory proteins, α-actinin and troponin-tropomyosin complex, could be obtained and they showed their typical phyoislogical activity which modify the actin-myosin interaction.

The amount of troponin-tropomyosin complex in myofibril was 72 mg per g myofibril. This result was in good agreement with the results reported by many investigators, and therefore it was concluded that our procedures for the extraction of troponin-tropomyosin complex were desirable to study on the quantitative analysis of troponin-tropomyosin complex.

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** Present address: College of Animal Husbandry, Kon Kuk University.
*** Department of Biochemistry, College of Science and Engineering, Yon Sei University, Seoul 120, Korea.

INTRODUCTION

It has been well proved that the interaction of myosin and actin in the presence of MgATP plays an essential role on the molecular basis of muscular contraction. Moreover, there have been discovered recently several new structural proteins, which exert regulatory functions on the ATP-myosin-actin system, directly or indirectly. Although their functions are so varied, they are called experimentally regulatory proteins.

It has been said that the regulatory proteins of the myofibrils, which influence the interaction of myosin and actin, are α-actinin, tropomyosin and troponin. α-actinin was originally discovered by Ebashi group[1]. In the early stages of work on α-actinin, it was considered that α-actinin was essential for pure actomyosin to superprecipitate. Later it has been reported that α-actinin accelerates the gelation of F-actin and therefore influences the superprecipitation[1,2]. Also, it has been demonstrated that α-actinin can be separated into two components, 6S and 10S, and that 6S-α-actinin is important for actomyosin to superprecipitate[3]. Briskey et al.[4] have demonstrated the evidence of α-actionin causing the extensive cross-linkage of F-actin, and suggested that it probably is logicalized at the Z-band. Therefore, α-actinin may be considered to play a role as a cementing substance in holding thin filaments at the Z-band. The function of 10S α-actinin has not yet been found.

From the physiological point of view, troponin is the most important component of all regulatory proteins because it is able to modify the interaction of myosin and actin[5,6]. Troponin has been shown to possess a unique calcium-binding capacity by Ebashi et al.[7] and Fushs et al.[8] Among the various structural proteins, troponin is the only protein to bind calcium ion, a capacity that is not modified in the presence of tropomyosin, F-actin, myosin or ATP.

It has been demonstrated clearly that two molecules of troponin and two molecules of tropomyosin locate along the entire length of the F-actin filament with 400Å periodicity in a side-by-side way[6]. The association of troponin with the thin filaments is made through its binding to tropomyosin which have a length of over 400Å and are in turn bound to F-actin directly[6]. Troponin can modify the structure of actin only indirectly through intermediation of tropomyosin[6]. The triggering action of Ca ion on muscular contraction is attributed to the troponin-tropomyosin complex, which is distributed along the entire length of the thin filament[9].

Ebashi et al.[10] have originally discovered the true Ca-sensitizing complex, and they termed this complex native tropomyosin. Essentially it represented a room-temperature extraction of a myosin-free mince, after which the native tropomyosin in the extract was separated from α-actinin by ammonium sulfate. Afterwards, Ebashi et al.[5] have shown that native tropomyosin is a complex of tropomyosin and troponin.

The isolation and purification of these regulatory proteins have been described by many workers. Robson[11] has shown the procedure of extracting α-actinin and troponin-tropomyosin complex from intact rabbit muscle in his studies on purification of α-actinin. Briefly, this procedure consists of preparation of myofibrils, followed by washing the myofibrils with water to lower the ionic strength, and then extraction of the swollen myfiobrils by 1-2 mM Tris at pH 8.5 and 2°C for 64-72 hours. These crude extracts were then fractionated between 0% and 30% and between 30% and 75% ammonium sulfate saturation to prepare a crude α-actinin extract (0~30%) and a crude troponin-tropomyotin complex (30~75%). Also, Arakawa et al.[12,13] have reported recently that α-actinin and troponin-tropomyosin complex can be extracted by the low ionic strength extraction of myofibrils. Furthermore, Perry et al.[14] have proclaimed that troponin-tropomyosin complex could be removed from myofibrils with treating weak alkaline solution extraction. Ebashi group[6] has demonstrated that troponin-tropomyosin complex can be extracted from myosin B or myofibril which are treated with either weak alkaline sloution extraction or mild trypsin treatment.

The purpose of this work is to study the new procedure for the preparation regulatory proteins, especially the procedure for complete extraction of troponin-tropomyosin complex.

MATERIALS AND METHODS

All solutions used were prepared with the deionized, distilled water.

Muscle

The longissimus dorsi muscle was removed from a newly killed rabbit, immersed in ice, and then trimmed free of fat and connective tissue, and chopped. To prevent putrefaction, 10 mM sodium azide solution was sprayed on the surface of meat and the container.

Preparation of Myofibril

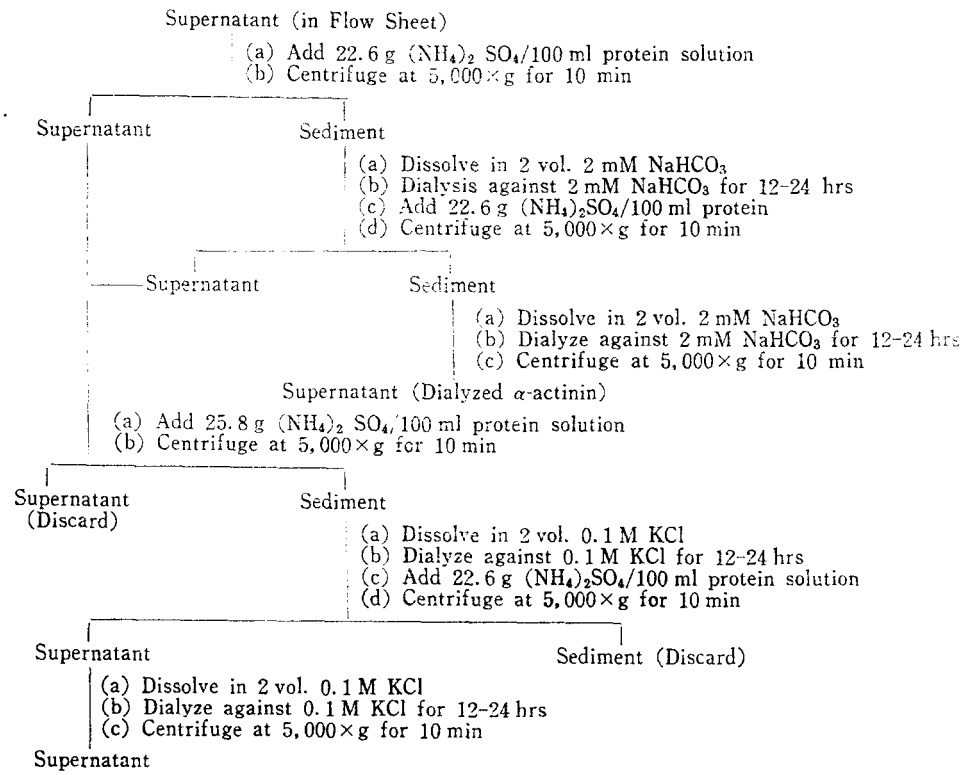
Myofibrils were prepared as described previously[15].

Preparation of Regulatory Proteins of Myofibril

Regulatory proteins were extracted according to the new procedure developed in this study (see Results and Discussion).

The supernatant collected in Flow Sheet was fractionated into 10-40% and 40-80% ammonium sulfate saturation fraction.

The flow sheet showing ammonium sulfate fractionation are as follow;



ATPase Activity Measurement

The reaction mixture composed of 0.25 mg/ml myofibril (or 0.125 mg/ml myofibril, 0.125 mg/ml actomyosin), 1 mM $MgCl_2$ or 1 mM EDTA (or 1 mM EGTA), 1 mM ATP and 10 mM (or 25 mM) Tris-HCl (pH 8.0) was incubated at 25°C for 5 min. The reaction was stopped by the addition of trichloro-acetic acid (final concentration of 4%). The composition of the incubation mixture is shown in Figures. The ATPase activity was expressed as μmoles of phosphorus liberated by 1 mg of protein for 1 min.

Protein Concentration Estimation

The protein concentration was determined by the biuret method which was standardized by micro-Kjeldahl method. The protein concentration of supernatant collected in Flow Sheet, if necessary, was determined by the method of Lowry et al.[13]

Ultracentrfiugal Analysis

Ultracentrifugal analysis was performed with a Hitachi UCI analytical ultracentrifuge equipped with diagonal schieren optics and facilities continuous temperature measurement.

Sedimentation coefficient was calculated by the following equation,

$$S=\frac{1}{\omega^2}\times\frac{2(x_2-x_1)}{(x_2+x_2)(t_2-t_1)}$$

where ω is the angular velocity in radians per second, and x_2 and x_1 are distances in centimeter of sedimentation peak from the center of rotation, when times after reaching full speed are t_2 and t_1.

RESULTS AND DISCUSSION

Flow Sheet for the Preparation of Regulatory Proteins of Myofibrils

Robson[11] reported that α-actinin and troponin-tropomyosin complex could be obtained by treating the myofibrils of rabbit muscle with the low ionic strength extraction, and Arakawa et al.[12] described the same results as Robson. The procedure outlined in this study is essentially the same procedure of Arakawa et al.[12], but some modification were made. The procedure developed for the preparation of regulatory proteins involves the preparation of myofibrils, removing the divalent metal ions which have any undesirable influence upon the protein-protein interactions by the addition of 1 mM EDTA as shown in Step I, washing the myofibrils with water to lower the ionic strength until the myofibrils are swollen, and then diluting the swollen mass with the weak alkaline solution, sodium bicarbonate with which Ebashi group had made use of extraction of troponin-tropomyosin complex from the myofibrils, as shown in Step III, Step VI, Step VIII, Step X, Step XI, Step XII, and Step XIV of Flow Sheet. The aim of addition of 50 mM KCl in some steps to the swollen mass were to cause the shrinkage of the swollen myofibril, thereby facilitating centrifugal precipitation of the unextracted residue.

The main aim of the procedure described in this study was the exhaustive extraction of troponin-

Myofibrils
↓ Centrifuge at 2,000×g for 10 min

Supernatant (save) | Sediment I
↓ Resuspend in 100 vol. 1 mM EDTA(V/W myofibril). Centrifuge at 3,000×g for 10 min

Supernatant (save) | Sediment II
↓ Resuspend in 100 vol. H_2O(V/W myobril). Centrifuge at 5,000×g for 30 min

Supernatant (save) | Sediment III
↓ Resuspend in 100 vol. 0.05 M KCl(V/W myofibril). Centrifuge at 5,000×g for 20 min

Supernatant (save) | Sediment IV
↓ Resuspend in 100 vol. H_2O (V/W myofibril). Centrifuge at 5,000×g for 20 min

Supernatant (save) | Sediment V
↓ Resuspend in 100 vol. H_2O (V/Wmyofibril). Centrifuge at 10,000×g for 30 min

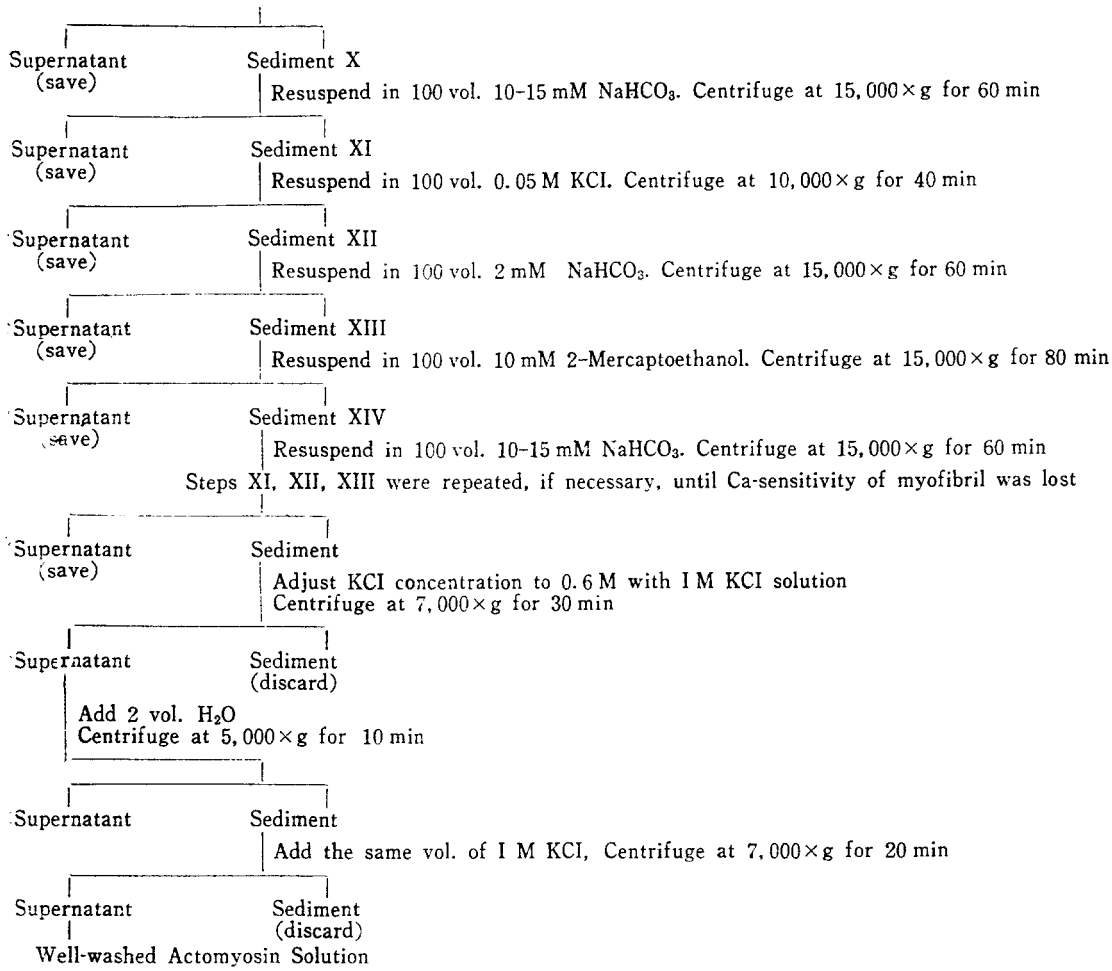
Supernatant (save) | Sediment VI
↓ Resuspend in 100 vol. 0.05 M KCl (V/W). Centrifuge at 5,000×g for 20 min

Supernatant (save) | Sediment VII
↓ Resuspend in 100 vol. H_2O(V/W). Centrifuge at 10,000×g for 30 min

Supernatant (save) | Sediment VIII
↓ Resuspend in 100 vol. 2 mM $NaHCO_3$(V/W). Centrifuge at 15,000×g for 60 min

Supernatant (save) | Sediment IX
↓ Resuspend in 100 vol. 10 mM 2-Mercaptoethanol. Centrifuge at 15,000×g for 80 min

* Abbreviations used in this paper are:
ATP, adenosine triphosphate; Tris, tris-(hydroxymethyl)-aminomethane; EGTA, 1,2-bis-(2-dicarboxymethyl-aminoethoxy)-ethane; Pi, inorganic phosphorus; MF, myofibrils; AM, actomyosin; EDTA, ethylenediamine-tetraacetic acid.



Flow Sheet Showing Preparation of Well-washed Actomyosin and Supernatant Containing Regulatory Proteins.

tropomyosin complex from the myofibrils.

To test whether Ca-sensitizing protein factor has been completeley removed or not, the Mg-activated ATPase activities of the myofibrils were measured and also the effect of EGTA on the Mg-activated ATPase activities were checked in each step of Flow Sheet.

Changes in Mg-activated ATPase Activity of Myofibrils

A great number of studies reported on the effects of Ca and Mg ions on the ATPase activity of myosin, actomyosin and myofibril in relation to the physiological role of the divalent metal ion in muscle function [17,18,19]. Since Mg ion enhances the ATPase activity of actomyosin at very low KCl concentration whereas they inhibit it at higher KCl concentration, and actin is necessary for this dual effect of Mg ion [20]. It may be considered that the Mg-activated ATPase activity of myofibrillar protein reflect the interaction of myosin and its allosteric effector [21], actin, and that a low rate of ATP hydrolysis is associated with the dissociation of actomyosin into its components.

When myofibrils were treated through the procedure outlined in above section, the Mg-activated ATPase activity of the myofibril at low ionic strength increased, as shown in Fig. 1. Fig. 1 shows that the Mg-activated ATPase activity of the myofibril at low ionic strength increases to 240% of the initial value. On the other hand, the Mg-activated ATPase activity of the myofibril at higher ionic strength increased slightly at early stages of the treatment and thereafter decreased to 20% of the initial value at 20 steps of

the procedure (Fig. 1).

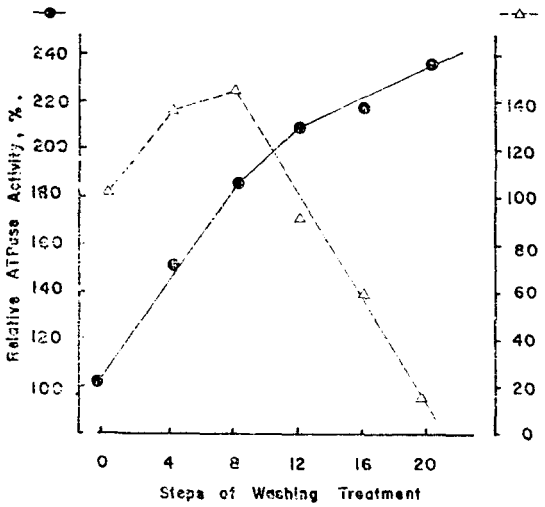


Fig. 1. Effect of Washing Treatment of Mg-modified ATPase Activity of Myofibril.
ATPase assay : 0.25 mg/ml myofibril, 1 mM $MgCl_2$, 1 mM ATP, 10 mM Tris-HCl (pH 8.0) and 0.01 M KCl (filled circles) or 0.1M KCl (dotted lines) at 25°C for 5 min.

Since the Mg-activated ATPase activity of actomyosin exhibits a biphasic response, that is, high at low ionic strength and low at high ionic strength[22]. The result in Fig. 1 may be interpreted to show that the myofibrillar ATPase turns into the actomyosin type ATPase with the progress of the treatment.

Since the Mg-activated activity of actomyosin reflects the interaction of actin and myosin, and the low rate of ATP hydrolysis is associated with the dissociation of actomyosin into its components[23]. The result that the dependence of myofibrillar ATPase activity on KCl concentration becomes greater may be considered to show that some modification in actin-myosin interaction occurs, and therefore myofibrils become more susceptible to ATP-induced transformation with the progress of washing treatment.

Our previous report[15] showed that myofibrils were composed of two kinds of filaments which bind laterally by such a substance as Z-line. Therefore, it occurred to us that both myosin and actin in the myofibril might be considered not to be so transformable as in the isolated actomyosin system. Also, Yang et al.[24] suggested that troponin-tropomyosin complex of the myofibril is the protein factor which support the fine structure of myofibril rigidly and consequently, repressed the Mg-activated ATPase activity of myofibril.

Since the regulatory proteins of the myofibril *perse* are water-soluble proteins[1,6,10], the washing treatment through the procedure developed in this study may result in the elimination of the regulatory proteins from the myofibril. Accordingly, the change in Mg-activated ATPase activity by the washing treatment may assume to be mainly due to the elimination of the regulatory proteins from the myofibril because the regulatory proteins are built into the structure of the myofibril and also able to modify the actin-myosin interaction[25,26].

Changes in EDTA-enhanced ATPase Activity of Myofibrils

Friess[27] reported that, in the absence of Ca ion, EDTA above concentration of 10^{-4} M accelerated the dephosphorylation rate of myosin and about 10^{-2} M achieved a maximum acceleration of approximately 400%. Moreover, Bowen and Kerwin[28] showed that the addition of actin to myosin ATPase activity. Therefore, it is clear that EDTA-enhanced ATPase activity of myofibrillar proteins is ascribed to myosin components of myofibrillar proteins. Since ATPase

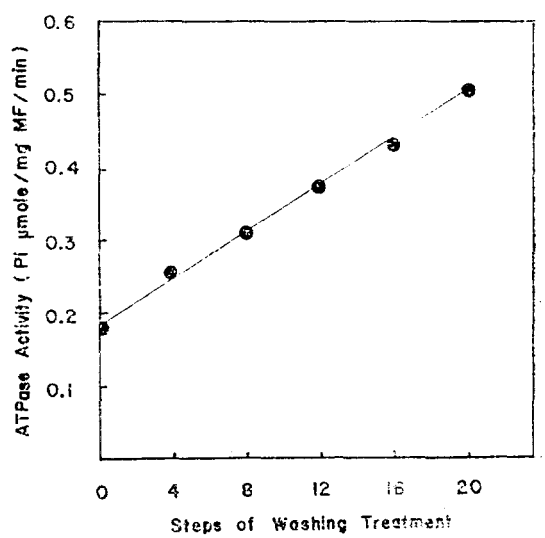


Fig. 2. Effect of Washing Treatment on EDTA-enhanced ATPase Activity of Myofibril.
ATPase assay : 0.25 mg/ml myofibril, 1 mM EDTA, 1 mM ATP, 25 mM Tris-HCl(pH8.0) and 0.6 M KCl, 25°C. 5 min.

activity of myosin reflected a remnant of the more fundamental interaction between myosin and ATP in the living muscle[29], it might be considered to be of significance to investigate on the myosin ATPase. Thus, we checked the EDTA-enhanced ATPase activity of myofibrils.

As shown in Fig. 2, EDTA-enhance ATPase activity of the myofibril increased to 250% of the initial value with the progress of washing treatment. It is noteworthy that the increment of EDTA-ATPase activity is in good agreement with the result of Mg-ATPase activity (cf. Fig. 1 and Fig. 2). The result that the myosin type ATPase activity of myofibril continues to increase with the progress of washing treatment may be due partly to the change in the content of myosin per gram of protein and partly to lowering the inhibitory action of actin on myosin ATPase. The latter suggestion may be assumed to be in accordance with the alteration in actin-myosin interaction. The results presented in this section are consistent with the result obtained from Mg-ATPase activity in above section.

Effectof EGTA on Mg-ATPase Activity of Myofibrils

Booler and Watanabe[29,30] showed early that EDTA or EGTA had brought about the relaxation of glycerated muscle fiber. Moreover, it was found that the contractile state of muscle was governed by changes in Ca concentration in the myofibrillar space[7,8]. Since the control of muscle contraction is mediated through troponin, a protein component of the thin filaments, which has a unique affinity for Ca ion, the effect of EGTA on the Mg-activated ATPase activity of myofibril has been interpreted to reflect the association of troponin to contractile process.

As shown in Fig. 3, when Mg-activated ATPase activity of myofibril was tested, the Mg-activated ATPase activity of myofibril was inhibited in the presence of EGTA. It is generally accepted that actomyosin is activated by Mg ion and may or may not be regulated by Ca ion, depending upon whether or not the Ca-sensitizing protein factor is present in the complex. Furthermore, the regulation of this ATPase activity and superprecipitation of actomyosin depends upon the free Ca ion concentration. In muscle, regulation of the free Ca ion concentration is the

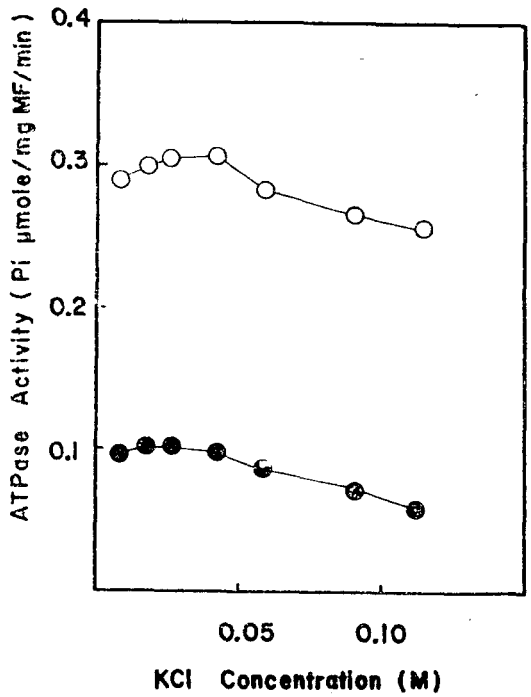


Fig. 3. Effect of EGTA on Mg-modified ATPase Activity from Myofibril.

ATPase assay : 0.25 mg/ml myofibril, 1 mM $MgCl_2$, 1 mM ATP, 10 mM Tris-HCl(pH 8.0) and KCl at the concentration cited on abscissa in the absence(open circles) or presence (filled circles) of 1 mM EGTA.

function of the sarcoplasmic reticulum, while *in vitro* the free Ca ion concentration can be be regulated by isolated vesicles of the sarcoplasmic reticulum, or by metalchelators such as EGTA or EDTA. Fig. 3, shows that the Ca-sensitizing protein factor, ie., troponin-tropomyosin complex is pressent in the myofibrils because the Mg-activated ATPase activity of myofibrils is depressed in the presence of EGTA.

Ebashi et al.[31] reported that in the absence of native tropomyosin, actin and myosin superprecipitate at low ionic strength and clearing could be observed at high ionic strength.

When myofibrils were treated through the procedures developed in this study, the inhibitory effect of EGTA on the Mg-activated ATPase activity was completely eliminated (Fig. 4). This result indicates that there is no troponin-tropomyosin complex in the well-washed myofibrils.

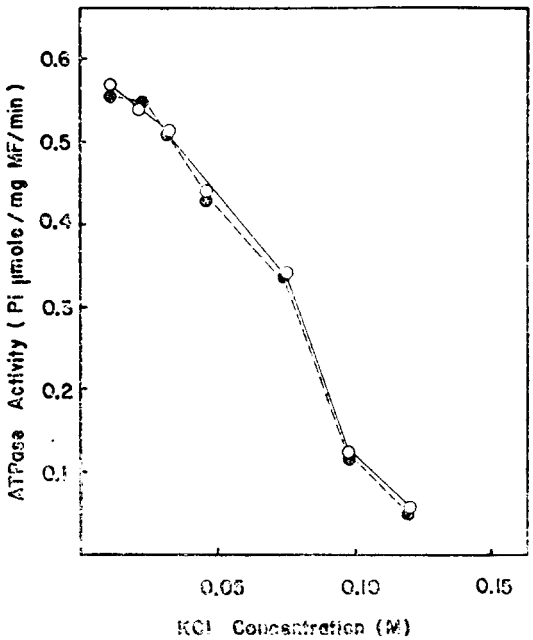


Fig. 4. Mg-modified ATPase Activity of Well-washed Myofibril.

ATPase assay : 0.125 mg/ml myofibril, 1 mM $MgCl_2$, 1 mM ATP, 25 mM Tris-HCl (pH 8.0) and KCl at the concentration cited on abscissa in the presnce (filled circles) or absence (open circles) of 1 mM EGTA.

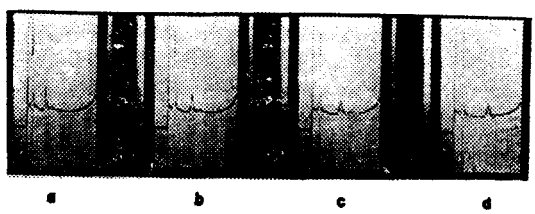


Fig. 5. Ultracentrifugal Sedimention of Residue Actomyosin from Myofibril.

Preparation : Actomyosin, 4.3 mg/ml in 0.6M KCl-20 mM Tris-Maleate (pH7.0).
Times after reaching 55,430 rpm.
a ; 15 min, b ; 20 min, c ; 25 min, d ; 30 min.

Fig. 5 shows the sedimentation diagrams of the well-washed myofibrils by the procedures developed in this study. The well-washed myofibril consits of three components of 21.2 S, 17.5 S and 5.6 S (Fig.5). This ultracentrifugal sedimentation pattern is in good agreement with that of natural actomyosin extracted from musccle directly[32]. Therefore, it was concluded that the procedures developed in present study were desirable to obtain the actomyosin from which troponin tropomyosin complex is eliminated completely.

Fractionation of Regulatory Proteins from Myofibrils

Regulatory proteins were extracted according to the procedures described in above section. The supernatant of each step in Flow Sheet was collected and fractionated between 10% and 40% and between 40% and 80% ammonium sulfate saturation to prepare a α-actinin (10-40%) and a troponin-tropomyosin extract (40-80%). Hereafter in this presentation, we will refer to these fractions as the P_{10-40} and P_{40-80} fractions. The effect of P_{10-40} on the Mg-activated ATPase activity of actomyosin at various levels of KCl concentration are shown in Fig. 6. P_{10-40} caused an increase in the Mg-activated ATPase activity of actomyosin suspensions. This result is in agreement with the results of Maruyama and Ebashi[34], Briskey et al[35], Maruyama[36].

Since α-actinin accelerates the Mg-activated ATPase activity of actomyosin and the effect of α-actinin on

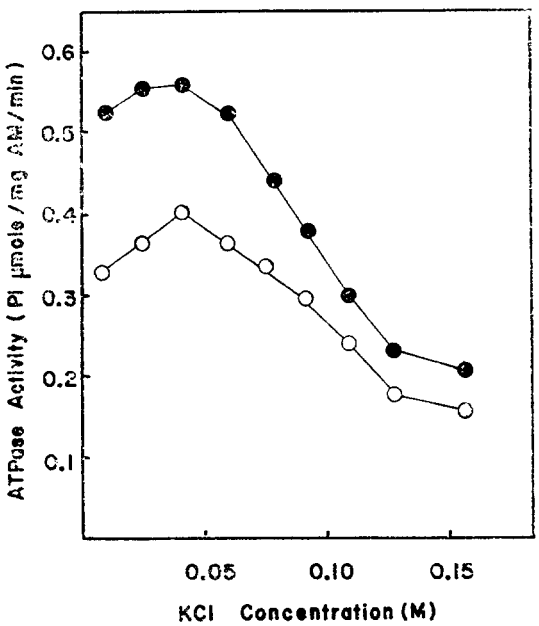


Fig. 6. Effect of α-actinin on Mg-ATPase Activity of Residue Actomyosin.

ATPase assay : 0.125 mg/ml actomyosin, 1 mM $MgCl_2$, 1 mM ATP, 25 mM Tris-HCl (pH 8.0) and KCl at the concentration cited on abscissa in the absence (open circles) or presence (filled circles) of α-actinin (0.05 mg/ml).

the Mg-activated ATPase activity is specific for the actomyosin system[1,25], P_{10-40} fraction was interpreted to be α-actinin component. To confirm that P_{10-40} fraction is to be α-actinin, the ultracentrifugal analysis was conducted.

Fig. 7 shows the ultracentrifugal sedimentation diagrams of P_{10-40} fraction and P_{40-80} fraction. Upper traces were the sedimentation profile of P_{40-80} fraction and lower traces were the sedimentation profile of P_{10-40} fraction.

Lower component had an $S_{20,w}$ of 6.3. Since $S_{20,w}$ of α-actinin is 6.2[33], it was concluded that P_{10-40} fraction contains 6S-α-actinin. Accordingly, it was confirmed that the procedures developed in present study were desirable to obtain α-actinin from myofibrils.

Upper component had an $S_{20,w}$ of 3.6, when calculated by the equation presented in materials and methods.

Since native tropomyosin, troponin-tropomyosin complex, had an $S_{20,w}$ of 3.7[10] or more, depending upon that the share of troponin in the complex is poorer or richer, it was considered that P_{40-80} fraction consisted of troponin-tropomyosin complex. It is clear that P_{40-80} fraction has little protein other than troponin-tropomyosin complex because the sedimentation profile of the protein other than troponin-tropomyosin complex is not detected (Fig. 7). Thus, it was concluded that the procedures developed in present study were desirable to prepare troponin-tropomyosin complex from myofibrils. Our procedure for the extraction of regulatory proteins aimed to place greater emphasis upon the complete extraction of troponin-tropomyosin complex.

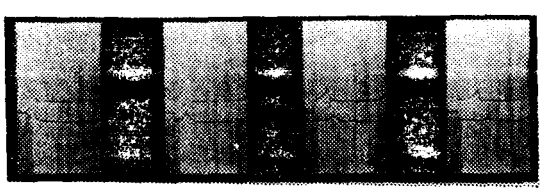


Fig. 7. Ultracentrifugal Sedimentation of Fraction $_{10-40}$% and Fraction$_{40-80}$% at Various Times after Reaching 55,430 rpm.

Upper traces : Fraction $_{40-80}$%, 2.4 mg/ml in 0.1 M KCl-10 mM Tris-Maleate (pH 7.0).

Lower traces : Fraction $_{10-40}$%, 2.5 mg/ml in 0.1 M KCl-10 mM Tris-Maleate (pH 7.0).

a ; 15 min, b ; 35 min, c ; 59 min, d ; 90 min.

When the amount of troponin-tropomyosin complex extracted was estimated, troponin-tropomyosin complex of 72 mg per gram of myofibril was obtained, as presented in Table I. This result is in good agreement with the result of Maruyama et al.[37], considering that some loss during the preparation of the myofibril may occur. Therefore, we concluded that our procedure for the preparation of the regulatory proteins was sufficient enough to study on the quantitative analysis of troponin-tropomyosin complex.

Table I. Troponin-tropomyosin Complex Contents in Myofibrils

Naruyama et al. [37]	80 mg/g Myofibril
Yang et al.	72 mg/g Myofibril

〈요 약〉

근육의 수축 및 사후강직은 myosin과 actin 그리고 ATP와의 상호작용에 의한 것임은 널리 알려진 定說이다. 최근 myosin과 actin 및 ATP의 상호작용이 근조절단백질의 지배를 받고 Ca ion이 관여하고 있다는 것이알려졌다. 그런데 이들 조절단백질은 수용성단백질로써의 성질을 가지고 있음에도 불구하고 염용성단백질 구분에 들어 있다. 본 연구의 목적은 염용성단백질 구분에 들어 있는 이들 조절단백질의 새로운 분리정제방법을 연구하는 데 있었다.

이를 위하여 본 연구에서는 새로운 정제방법의 flow sheet를 작성하였다.

이 제안된 새로운 정제방법은 근원섬유중의 조절단백질의 함량을 정량적으로 추적할 수 있는 장점을 가지고 있다는데 그 특색이 있다.

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