KOREAN J. FOOD SCI. TECHNOL
Vol. 6, *No.* 2 (1974)

근원섬유 단백질에 관한 연구

(제 1 보) 근원섬유에 관한 형태학적 연구

연세대학교 이공대학 식품공학과
양 융·김 철 재·문 윤 희·유 주 현

(1974년 3월 23일 수리)

Studies on the Myofibrillar Proteins

Part I. Phase Microscopy of Myofibrils from Rabbit Muscle**

by

Ryung Yang, Chuljai Kim, Yoonhee Moon* and Juhyun Yu

Department of Food Engineering, Colleage of Science and Engineering, Yon Sei University, Seoul Korea

(Received March 23, 1974)

Abstract

To obtain further information concerning the nature myofibrillar proteins in a food system, an investigation has been conducted to compare the change in the biochemical property of the myofibril with the changes in the morphological structure of the myofibril.

When myofibrils were prepared with 0.16 M KCl-0.04 M Tris-HCl, the band pattern was clear and distinct. There was a uniform thickening of A-band, a sharp appearence of Z-lines and a wide I-band. The band pattern of myofibrils was changed as the composition of extraction solution was changed. Also the ATPase activity of myofibril changed as the length of sarcomere changed.

When myofibrils were treated with a low concentration of trypsin, myofibrils turned in the contracted state. With the progress of prolonged trypsin treatment, most of myofibrils exhibited a pattern of alternating light and dark bands, supercontracted pattern.

Although myofibrils exhibited a supercontracted band pattern, the ATPase activity of myofibril continued to increase with the progress of trypsin treatment.

An assumption was made that tropomyosin may be located in Z-line and that troponin-tropomyosin complex can inhibit the ATPase activity of myofibrils through the structural alternation of myofibril.

Introduction

The physical and chemical changes accompanying muscle contraction and rigor-mortis have received a great deal of attention, but the molecular events occuring during contraction-relaxation cycle remain an enigma.

As results of studies on the myofibrillar in the past two decades, however, it has been generally accepted today that muscle is highly integrated chemical machine [1], and that the contraction of all muscle is brought about by the interaction of actin, myosin

**Present address: Colleage of Animal Husbandry, Kon Kuk University.*

***Presented at Meeting of Korean Society of Food Science and Technology, Kwangju, October, 13, 1973.*

and ATP [2], and these interaction of actin, myosin and ATP result in an efficient transduction chemical into mechanical energy.

It has been also reported recently that the interactions of actin, myosin and ATP are controlled or influenced by some of minor proteins of the myofibril[3,4,5].

The proteins of the myofibrils that have been identified to date include myosin[2] actin, tropomyosin [6], α-actinin [3,7], β-actinin[8], troponin[4,5,9], and M-protein[10]. For a detailed discussion of the myofibrillar proteins, the reader is referred to our recent review[1].

Much of the present knowledge of the fundamental morphological structure of the myofibril takes its foundation from the early work of Huxley[12] and his electron microscope study of the organization of the myofibril has contributed substantially to the basic information on the morphological structure of the myofibril as we know it today[13,14] (Fig.1). A-bands

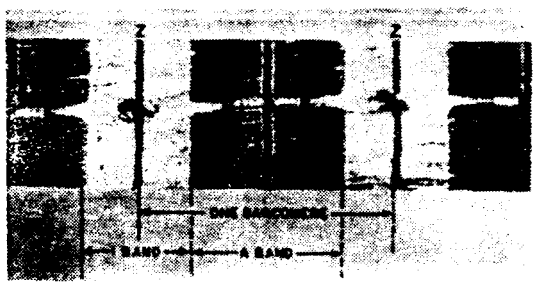

Fig. 1. The Fundamental Cotractile Unit Enclosed by two Membranes, the Z-lines[14]

consist of thick filaments, approximately 100 Å in diameter and 1.5μ in length, and the thick filaments primarily contain myosin[13,14]. Conversely, I-bands consis of thin filaments, approximately 80 Å sn diameter and 1~2 in length, and thin filaments contain actin, tropomyosin and troponin [15].

Tropomyosin may also be located in the Z-line, as originally proposed by Huxley [13].

Many investigations on the myofibrillar proteins in a food system have been widely studied for many years, but the actual physicochemical changes which lead to tenderization remains unclear. In order to obtain information concerning the nature of myofibrillar proteins in a Food system, an investigation has been conducted to compare the changes in the biochemical property of the myofibril with the changes in the morphological structure of the myofibril. This paper describes the obvious changes occourred during the preparation of myofibrils and the structural alterations by trypsin treatment.

Materiais and Methods

Muscle The longissimus dorsi muscle was removed from a newly killed rabbit, trimming off fat and connective tissue, and chopped.

Preparation of Myofibril: Myofibrils were prepared by th method of Yang[16]

The flow sheet showing the preparation of myofibril are as follow;

Step I. Chopped meat
↓ Add 80 volumes of 0.16M KCl (w/v)-0.04M Tris-HCl (pH 7.5)
↓ Homogenize for 1 min
↓ Centrifuge at 2000 x g for 10min
↓ Sediment / Supernatant (discard)

Step II. Sediment
↓ Add 80 volumes of 0.16M KCl(w/v)
↓ Centrifuge at 2000 x g for 10 min
↓ Sediment / Supernatant (discard)

Step III. Sediment
↓ Add 80 volumes of 0.16M KCl(w/v)
↓ Centrifuge at 2000 x g for 10 min

Step IV. Sediment
↓ Repeat Step III twice

Step V. Sediment
↓ Add 80 volumes of 0.16M KCl(w/v)
↓ Filter by 6 mesh nylon net
The filtrate
(Myofibrils)

For the preparation of the contraction state-Myofibrils, 0.16M KCl-5mM $CaCl_2$-0.04M Tris-HCl(pH7.5) was used in spite of 0.16M KCl-0.04M Tris-HCl (pH 7.5) in Step I. Also for the preparatoin of the relaxation state-myofibrils, 0.16M KCl-1mM EDTA-0.04 M Tris-HCl (pH 7.5) was used in Step I.

Trypsin treatment: Myofibrils (5mg/ml) in 150mM KCl-15mM Tris-HCl (pH 7.5) were incubated with trypsin (Sigma & Co., recrystallized) at trypsin to myofibril ratio of 1 to 2000 at 25°C for specified periods of time. The tryptic action was stopped by the

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

addition of 2-fold amount of trypsin inhibitor (Sigma & Co., twice recrystallized). The resultant suspension was provided for experiments.

ATPase activity measurement: The reaction mixture composed of myofibrils (0.25mg/ml), 1mM $MgCl_2$ or 1mM EDTA, 1mM ATP and 15mM Tris-HCl (pH 8.0) was incubated at 25°C for 5 min. The reaction was stopped by the addition of trichloroacetic acid (final concentration of 4%).

Composition of the incubation mixture is shown in Tables. ATPase activity was expressed as μ moles of inorganic phosphorus liberated per 1 min by 1 mg of protein.

Protein concentration estimation: Protein concentration was estimated by biuret method which was standardized by micro-kjeldahl method.

Phase observation: All phase observation were made with Olympus trinocular microscope Moder ECEtr equipped with photomicrographic apparatus, model P M-6, and phase contrast equipment A.

For examination and photography with phase micro scope, myofibrils suspended in 0.16M KCl were stirred thoroughly with a glass rod and a drop of the myofibrillar suspension adhering to the rod was placed on a pre-cleaned slide (special slide for phase observations), and a coverslide was lowered onto the suspension.

Results and Discussion

Phase-contrast micrographs of Myofibrils: When myofibrils were prepared with 0.16M KCl-0.04M Tris-HCl (pH 7.5), the band pattern was clear and distinct, as indicated by a wide I-bandand the presence of H-zones (Fig.2). There was a uniform thickening of A-band and also a sharp appearence of Z-line (Fig.2).

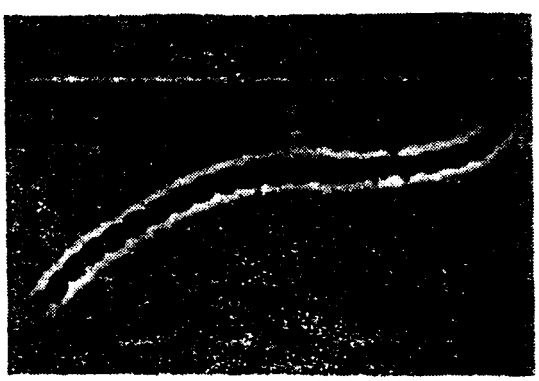

Fig. 2. Myofibrils prepared with 0.16M KCl-0.04M Tris-HCl(pH 7.5) photo: x 1500

This band pattern is typical of myofibril and consistent with Huxley's interdigitating filament model[14].

However, the myofibrils prepared with 0.16M KCl-5mM $CaCl_2$-0.04M Tris-HCl (pH 7.5) were in a contracted state (Fig. 3). There was a uniform thickening

Fig. 3. Myofibrils prepared with 0.16M KCl-5 mM $CaCl_2$-0.04M Tris-HCl (pH 7.5) photo:x 1500

of A-band, a concomitant shortening of the I-band and a disappearance of Z-line (Fig. 3). This result shows that Ca ion has the contraction-triggering function, as described early by Ebashi [5]. On the other hand, the myofibrils prepared with 0.16M KCl-1mM EDTA-0.04M Tris-HCl (pH 7.5) were relaxed, as indicated by a wide I-band and a fuzzy, broaden appearance of Z-line (Fig. 4).

Fig. 4. Myofibrils prepared with 0.16M KCl-1mM EDTA-0.04M Tris-HCl (pH7.5 photo: x 1500

Since Ca ion has the muscle contraction-triggering function,[5] the removal of Ca ion by EDTA treatment may result in the relaxation of myofirbril and therefore the myofibril prepared with 0.15M KCl-1mM EDTA-0.04M Tris-HCl solution may be relaxed. Bozler

and Watanabe also showed early that EDTA or EGTA had brought about relaxtion of glycerated muscle fibers [17]. Consequently, it is apparent that $CaCl_2$ can be used to bring about the rapid and extensive contraction of muscle and that EDTA can be useful to prevent the contraction during the preparation of myofibril.

Effect of the contraction of muscle on the Mg-activated ATPase activity of Myofibirls: Since the myofibrils prepared with 0.16M KCl-5mM $CaCl_2$-0.04M Tris-HCl solution were in the contractved state (Fig.3), the effect of contraction during the preparation of myofibril on Mg-activated ATPase activity was investigated.

As presented in Table 1, the Mg-activated ATPase activity of myofibril treated with 5mM $CaCl_2$ was the lowest at low ionic strength and that of the myofibril treated with 1mM EDTA was the highest. At the high ionic strength of 0.15, however, the difference in the Mg-activated ATPase activity among myofibrils became negligible (Table 1).

Table 1. Mg-activated ATPase activiy of the myofibrils in ihe presence of Ca ion or not
(Pi μmole/mg MF/min)

Treatment	voat ionic strength of 0.05	at ionic strengh of 0.15
None	0.195	0.095
5mM $CaCl_2$	0.105	0.095
1mM EDTA	0.275	0.120

ATPase assay: 0.25 mg/ml myofibril, 1mM $MgCl_2$, 1mM ATP 15mM Tris-HCl (pH 8.0) and 0.05M or 0.15M KCl, 25°C

It is clear that the dependence of myofibrillar AT Pase activity on KCl concentration becomes greater with progress of relaxation of myofibril. Hayashi et al showed that the maximum value of ATPase activity of glycerol-treated muscle fiber was observed in a range of sarcomere lengths of 2.0 to 2.5μ and that in the region where the sarcomere length was less than 2.0μ, the ATPase activity decreased as the sarcomere length decreased[18]. Our result presented in Table 1 are in good agreement with the result of Hayashi et al., even though we do not measure the exact length of sarcomere of the Myofibrils.

The structural alterations of Myofibrils by trypsin treatment: Several reports showed that Z-lines in myofibrils could be removed easily by a mild trypsin treatment[19,20] and that trypsin might be a useful tool for eliminating the physiological activity of troponin-tropomyosin complex [5,21].

Since troponin-tropomyosin complex is distributed along the entire length of thin filament at approximately 400Å periodicity[5], the removal of troponin-tropomyosin complex by trypsin treatment may result in the obvious structural alteration of myofibrils. Thus, we studied the structural alterations by trypsin treatment.

When the myofibril prepared with 0.16M KCl-0.04M Tris-HCl solution (Fig. 5a) were incubated with a low

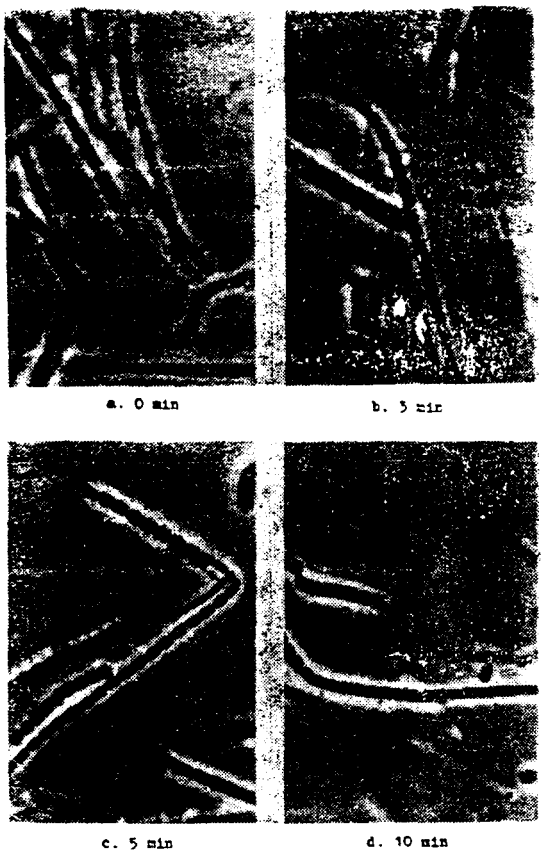

Fig. 5. Myofibrils were treated with trypsin for specified periods of time in the presence of 0.15M KCl-10mM Tris-HCl (pH 7.2) at 25°C photo: x 1500

concentration of trypsin at trypsin to myofibril ratio of 1 to 2000 for 3 min, the myofibrils were in the contracted state (Fig. 5b). Although intact myofibrils had a typical band pattern of the relaxed myofibril, as indicated by a wide I-band and a clear appearence of Z-line (Fig. 5a), the myofibril treated by trypsin turned contracted, as indicated by a uniform thickening of A-band, a concomitant shortening of I-band and

a disappearance of Z-line(Fig. 5b). Similar situations were also observed with the myofibrils treated by trypsin for 5 min or 10 min (Fig. 5c or Fig. 5d).

When trypsin treatment was prolonged under the same conditions, the treated myofibrils were fragmented (Fig. 6a and Fig. 6b). This result is in good agreement with the results that Z-lines in myofibrils can be removed easily by a mild trypsin treatment[19,20].

Since α-actinin assumed to be one of the components of Z-lines is fairly resistant to tryptic action [3], this result that Z-lines is easily removed by a mild trypsin treatment may be considered to support the hypothesis that a protein other than α-actinin, fairly labile to tryptic action, is built into the structure of Z-lines. Tropomyosin may be considered to be the component responsible for the tryptic degradation of Z-lines because the location of tropomyosin in Z-lines has been already proposed by Huxley and also tropomyosin is fairly labile to tryptic action [6,22]. However, for the detailed discussion, further investigation in detail may be needed.

When myofibrils were incubated with a concentration of trypsin for more prolonged, period most of myofibrils were supercontracted, exhibiting a pattern of alternating light and dark bands (Fig. 6c and 6d). It is noteworthy that trypsin treatment can induce the myofibrils supercontracted. Those obvious structural alterations of myofibrils were reinvestigated by measuring the changes in ATPase activity of myofibril by trypsin treatment.

When myofibrils were treated by trypsin, the Mg-activated ATPase activity of myofibrils at low ionic strength continued to increase during 80 min of the treatment, as presented in Table 2. The ATPase activity of myofibrils increased to approximately 500% of the initial value (Table 2). We showed in the above section that the contracetd myofibrils had a lower ATPase activity than the relaxed myofibril did (Table 1). Nevertheless, the Mg-activated ATPase activity of the myofibrils contracted by trypsin treatment increased with the progress of contraction induced by trypsin treatment (Fig. 5 and 6, Table 2). Since troponin could be digested selectively among myofibrillar proteins [4,5], the myofibrils treated by trypsin might be considered to differ from the myofibrils contracted by Ca ion because although the myofibrils were in the contracted state, troponin in the myofibrils treated by trypsin was eliminated by trypsin treatment. This assumption was supported by the results that when the activation of ATPase activity of the myofibril by trypsin was at maximal level, effect of EGTA on the Mg-activated ATPase activity of myofibrils was completely eliminated (Table 3), and that the Mg-activated ATPase

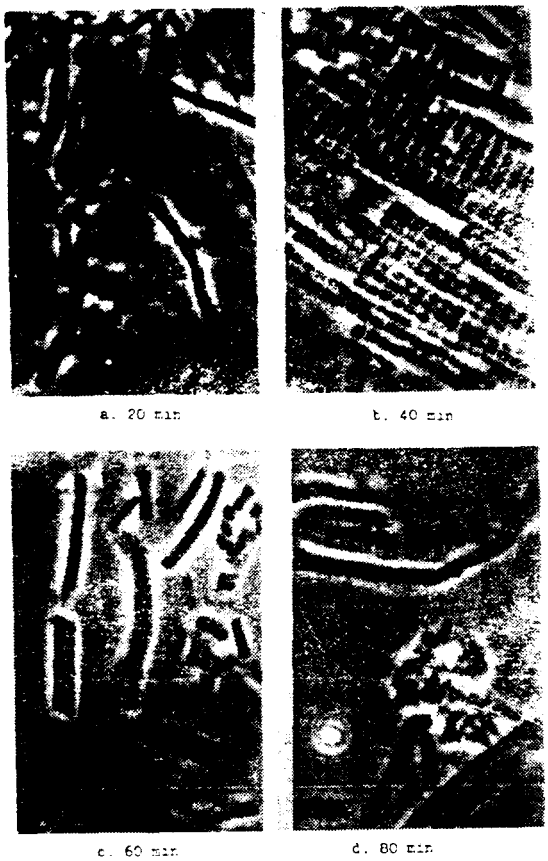

Fig. 6. Myofibrils were treated with trypsin for specified periods of time in the presence of 0.15M KCl-10mM Tris-HCl (pH 7.2) at 25°C photo: x 1000

Table 2. Effect of trypsin treatment on the Mg-activated ATPase activity of Myofibrils

Time of treatment (min)	0	3	5	10	20	30	40	60	80
ATPase activity (Pi μmoles/mgMF/min)	0.105	0.180	0.225	0.265	0.305	0.350	0.395	0.450	0.465

ATPase assay: 0.25mg/ml Myofibril, 1mM $MgCl_2$, 1mM ATP, 15mM Tris-HCl (pH 8.0) and 0.01MKCl, 25°C

Table 3. ATPase activity of the trypsin-treated myofibrils in the presence or absence of EGTA

(Pi μmoles/mgMF·min)

Time (min) of trypsin treatment	0	5	10	20	30	40	60	80
None	0.105	0.230	0.295	0.395	0.450	0.447	0.455	0.450
EGTA	0.060	0.195	0.280	0.395	0.448	0.450	0.453	0.452

ATPase assay: 0.25 mg/ml myofibrils, 1mM $MgCl_2$, 1 mM ATP, 0.01M KCl and 15mM Tris-HCl (pH 8.0) in the presence or absence of 1mM EGTA

activity of the myofibril treated by trypsin for 20 min was decreased by the addition of troponin tropomyosin complex (Fig. 7).

Since troponin has no ability to inhibit the Mg-ATPtrase activity of myofibril [6], the increase in Mg-activated ATPase activity of myofibrils by trypsin may be

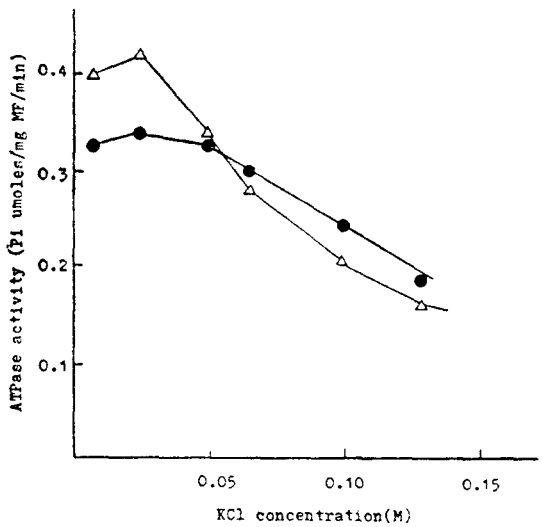

Fig. 7. Effect of troponin-tropomyotin complex on the Mg-activated ATPase activity of trypsin-treated myofibrils

△—△: the myofibrils treated with trypsin for 20 min

●—●: the myofibrils treated with trypsin for 20 min aplus troponin-tropmyofisin complex (0.25mg)

considered to be due to the structural alteration of myofibrils induced by tryptic proteolysis. Drabikowski et al reported that it was difficult to seperate tropomyosin from actin in the presence of troponin [23]. Therefore, troponin seems to play a cementing role in preventing the dissociation of tropomyosin from actin. This assumption has been made already in our previous study [16].

요 약

근원섬유단백질의 생화학적 성질에 대하여서는 아직도 불명한 점이 많고, 특히 식품으로써의 근원섬유단백질의 저장중의 변화에 대하여서는 규명되어야 할 점이 너무나 많다.

본 연구는 근원섬유단백질의 저장중의 변화를 추구하기 위한 기초작업으로서 subcelular structure인 근원섬유를 재료로 하여 그 형태학적 측면과 생화학적 측면의 상관성을 비교 검토하였다.

근원섬유의 조제방법에 따라서 근원섬유의 筋節(sarcomere)의 길이는 변화하고 있었고 근절의 길이의 변화는 생화학적 성질, 즉 ATPase활성에 현저한 변화를 일으켰다. 근원섬유를 저농도의 trypsin으로 처리하면 근원섬유의 ATPase활성은 현저히 증가하나, 근원섬유의 位相差현미경 상은 수축상태의 상을 나타내었다가 처리시간의 연장에 따라 sarcomere가 fragmentation을 나타냄을 보여주었다. 얻어진 결과로 근원섬유중에는 ATPase활성을 저해하는 factor가 존재하며, 이 fator는 단백질의 inhibitory action은 아니고 steric effect임을 추정하였다. 또한 Z-line의 구성물질중에, troponin의 관여가 추정되었다.

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