コイ筋原線維中および単離したα-アクチニンに対するカルパインの作用

誌名	日本水産學會誌
ISSN	00215392
巻/号	576
掲載ページ	p. 1133-1139
発行年月	1991年6月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

Nippon Suisan Gakkaishi 57(6), 1133-1139 (1991)

Action of Calpain on α-Actinin within and Isolated from Carp Myofibrils

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(Received November 24, 1990)

When purified α-actinin from carp ordinary muscle was treated with calpain type II from the same muscle, SDS-PAGE revealed that α-actinin subunits were cloven into fragments of slightly lower molecular mass. On Sephacryl S-300 gel filtration, however, calpain-treated α-actinin was eluted at the same position as that of the native one, suggesting that the dimeric subunit composition still remained after calpain digestion under the physiological condition. The binding ability of α-actinin to F-actin at low temperature was slightly increased by calpain treatment.

It was also found that calpain clove not only isolated α-actinin but also α-actinin within carp myofibrils. In addition calpain showed an another function to solubilize and release both native and digested α-actinins from the myofibrils.

These results suggested that α-actinin removing action of calpain rather than α-actinin cleavage would be expected to contribute to the weakening of Z-line.

It has been established that α-actinin in vertebrate skeletal muscle including fish muscle is a major constituent of Z-line of the myofibril and has a molecular weight of 200,000 and two identical subunits.[1-5] The physiological role of α-actinin is involved in the anchorage of F-actin filaments at Z-line.[2-4]

Postmortem fragmentation of the myofibrils is directly related to the degradation and alteration of Z-line structure. Therefore, α-actinin probably plays a key role in postmortem changes of the Z-line structure.

Calpain, a calcium dependent neutral proteinase, that exists intracellularly in the muscle tissue, degraded many myofibrillar proteins and removed Z-line from intact myofibrils.[6-10] It has been, however, reported that calpain released α-actinin from Z-line without proteolysis.[6,7,10] Whereas in this paper we found that α-actinin within and isolated from carp myofibrils was a substrate of calpain in addition to the action of calpain to release α-actinin from Z-line of myofibrils.

Materials and Methods

Fish

The ordinary muscle was excised from live specimens of carp *Cyprinus carpio* and used to prepare myofibrils, α-actinin, actin, and calpain.

Myofibril Preparation

Myofibrils were prepared according to the method of Perry and Grey[11] as mentioned in the previous paper.[12] Briefly, minced muscle was washed three times with 3 volumes of cold buffer (0.1 M KCl, 5 mM EDTA, and 39 mM borate buffer at pH 7.1). The muscle tissue was homogenized for 30 s in a blender and centrifuged at 1,600×g for 10 min. The pellet was suspended in 5 volumes of buffer containing 0.1 M NaCl-20 mM Tris-acetate (pH 7.0)–5 mM 2-mercapto ethanol, and then centrifuged. These procedures repeated three more times and the last centrifugation was done at 180×g for 10 min. At this phase, the rate of fragmentation of the myofibrils prepared was 10% or less as judged by phase contrast microscopy.

Actin and α-Actinin Preparations

Actin was extracted from the acetone powder[13] and purified by the method of Spudich and Watt.[14] α-Actinin was extracted and purified by DEAE-cellulose chromatography as described previously.[5]

Calpain Preparation and Assay

Calpain type II (EC 3.4.22.17) was prepared by the method of Sakamoto *et al.*[15] Calpain activity was measured with casein as a substrate at 25°C.[15] Acid soluble products formed were determined as

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the absorbance at 278 nm. One unit of the calpain activity was defined as the activity that increased the absorbance at 278 nm by 1.0 in 1 h.

Calpain Treatment of Myofibrils and α-Actinin

The myofibrils or α-actinin was incubated at 25°C with calpain under the following conditions: 5 mg/m*l* myofibrils (or 1 mg/m*l* α-actinin), 0.1 M NaCl, 5 mM $CaCl_2$, 5 mM 2-mercaptoethanol, and 20 mM Tris-acetate (pH 7.0). The incubation was terminated by the addition of 10 mM EDTA (final concentration).

Estimation of Molecular Weight

The molecular weight (MW) of sample proteins was determined by gel filtration on a Sephacryl S-300 column (2.6×45 cm) equilibrated with 0.1 M NaCl-20 mM Tris-acetate (pH 7.5). Proteins used as standards were aldolase (MW 158,000), bovine serum albumin (MW 67,000), and chymotrypsinogen (MW 25,000).

Binding Assay of Calpain-Treated α-Actinin and F-Actin

Interaction of calpain-treated α-actinin and native F-actin was measured by the methods of turbidity and sedimentation experiments at low temperatures.[5] Calpain-treated α-actinin (0.2 mg/m*l*) was mixed with F-actin solution (1 mg/m*l*) containing 0.1 M KCl, 1 mM $MgCl_2$, 0.15 mM ATP, 0.06 mM $CaCl_2$, and 20 mM Tris-acetate (pH 7.5). The turbidity of the mixture was measured at 330 nm decreasing its temperature from 30°C to 5°C at a rate of 0.3°C/min. The same mixtures were separately incubated at 25°C and 2°C for 20 min; then F-actin and associated proteins were pelleted by centrifugation at 280,000 ×g for 1 h at 25°C and 2°C, respectively. The amount of free calpain-treated α-actinin in the solution was measured by SDS-PAGE of the solutions taken before and after the centrifugation.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sample proteins were treated in 8 M urea, 2% SDS, 2% 2-mercaptoethanol, and 20 mM Tris-HCl (pH 8.0) at 100°C for 1 min. SDS-PAGE was done according to the Weber and Osborn,[16] and Laemmli[17] procedures. In most cases, a 7.5% acrylamide slub gel was used. Gels were stained with Coomassie Brilliant Blue. Densitometric tracings were carried out and values were obtained as a difference between absorbances at 640 nm and 700 nm with Simadzu CS-710 densitometer. Proteins were determined either by biuret method[18] or by microbiuret method[19] with bovine serum albumin as a standard.

Results

Calpain Treatment of Isolated α-Actinin

α-Actinin isolated from carp muscle was treated with calpain at 25°C and then examined by SDS-PAGE. Figure 1 shows a very fast disappearance of the α-actinin subunit (100,000 Da) with the concomitant increase of a fragment with a molecular mass of about 96,000 Da, but no detectable small fragments. The subunit fragment produced was resistant to calpain and seemed to accumulate during the incubation.

Since it is known that α-actinin is composed of two identical subunits, attempts have been made to relate the breakdown of the subunits due to calpain treatment as shown in Fig. 1 to the changes in α-actinin molecule. Changes in molecular

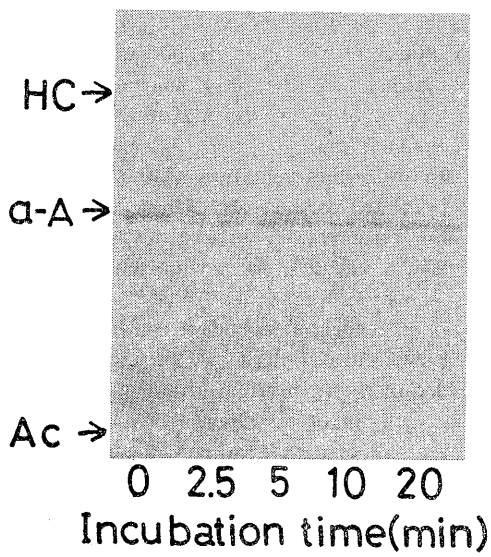

Fig. 1. Cleavage of carp α-actinin by calpain treatment.

Carp α-actinin (1 mg/m*l*) was incubated at 25°C with carp calpain (0.3 unit/m*l*) in the medium containing 0.1 M NaCl, 5 mM $CaCl_2$, 5 mM 2-mercaptoethanol, and 20 mM Tris-acetate (pH 7.0). The reaction was terminated by the addition of 10 mM EDTA. Loads of 0.35 μg/lane were run on a 7.5% polyacrylamide slub gel. Arrows show the positions of α-actinin (α-A), myosin heavy chain (HC), and actin (Ac).

weight of α-actinin during calpain treatment were analyzed by Sephacryl S-300 gel filtration under the physiological condition, *i.e.* in 0.1 M NaCl-20 mM Tris-acetate at pH 7.5 (Fig. 2). The protein samples loaded separately on the gel column were native and calpain-treated α-actinins whose subunit or fragment compositions were shown on SDS-PAGE inserted in Fig. 2.

α-Actinin was eluted from the gel column at the elution volume of 127 m*l*; that is at the position giving a molecular weight of 200,000. Calpain-treated α-actinin in 50% digestion (▲) was eluted as a single peak at the same position as that of native α-actinin. The same result was also obtained in the case of calpain-treated α-actinin which had been converted completely into the fragments as shown on SDS-PAGE (Fig. 2, ●).

These results indicate that calpain clove α-actinin subunits into unique fragments of slightly lower molecular mass, however, the dimeric composition of the molecule was still remained under physiological condition. Therefore, calpain is involved in the production of a species of α-actinin with "nicked" subunits.

Effect of Calpain Treatment on the Interaction of α-Actinin and F-Actin

It is well known that α-actinin causes the gelation of F-actin solution with increasing turbidity at low temperatures.[2-5] Thus, the turbidity of the mixture of F-actin and calpain-treated α-actinin at the molecular ratio of 5: 1 was measured at 330 nm with decreasing temperature from 30°C to 5°C at a rate of 0.3°C/min, then keeping constant at 5°C (Fig. 3).

Although no detectable change in turbidities of F-actin, α-actinin, and calpain-treated α-actinin alone was found at all temperature ranges employed, the turbidity of the mixture of F-actin and α-actinin solution began to increase below 13°C and reached to a half maximum value at 8.3°C. The similar increase in the turbidity of the mixture of F-actin and calpain-treated α-actinin was found, but with a half maximum value at 10°C.

The same mixture mentioned above was centrifuged at 280,000×g for 1 h at 25°C and 2°C, separately, to investigate binding ability of calpain-treated α-actinin to F-actin. The results were shown in Fig. 4. The centrifugation did not sediment 15% at 2°C and 6.5% at 25°C of the protein in tube containing only F-actin, whereas all protein remained in supernatant in tube con-

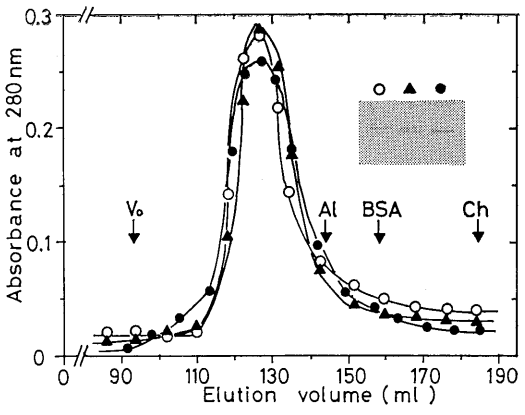

Fig. 2. Comparison of molecular weights of α-actinin and calpain-treated α-actinin on Sephacryl S-300.

Carp α-actinin was digested by calpain as shown in Fig. 1. After termination, the protein (9 mg) was loaded on Sephacryl S-300 column (2.6×45 cm) equilibrated with 0.1 M NaCl-20 mM Tris-acetate (pH 7.5), and then eluted with the same buffer at a flow rate of 27 m*l*/h. ○, native α-actinin; ▲, α-actinin digested for 2.5 min; ●, α-actinin digested for 30 min. The inserted figures show the protein composition of these samples on SDS-PAGE. The positions of void volume, aldolase (MW 158,000), bovine serum albumin (MW 67,000), and chymotrypsinogen (MW 25,000) are also shown by the arrows with V_0, Al, BSA, and Ch, respectively.

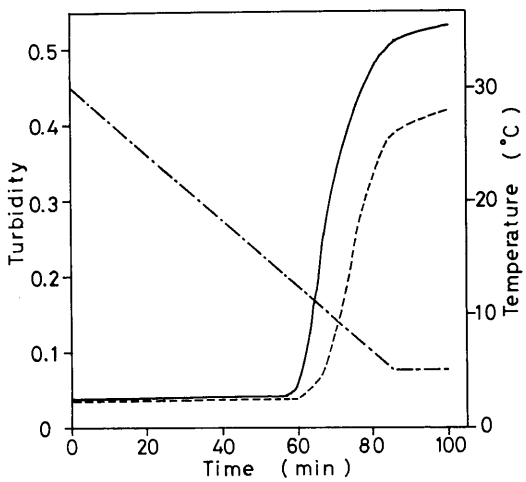

Fig. 3. Temperature dependent changes in turbidity of mixtures of F-actin and either α-actinin or calpain-treated α-actinin.

Turbidity change of the mixtures containing 1 mg/m*l* F-actin and either 200 μg/m*l* α-actinin (---) or calpain-treated α-actinin (—) in the presence of 0.1 M NaCl, 1 mM $MgCl_2$, 0.06 mM $CaCl_2$, and 20 mM Tris-acetate (pH 7.5) was measured at 330 nm decreasing the temperature (-·-) from 30°C to 5°C at a rate of 0.3°C/min. The calpain-treated α-actinin used was the same preparation as that (●) in Fig. 2.

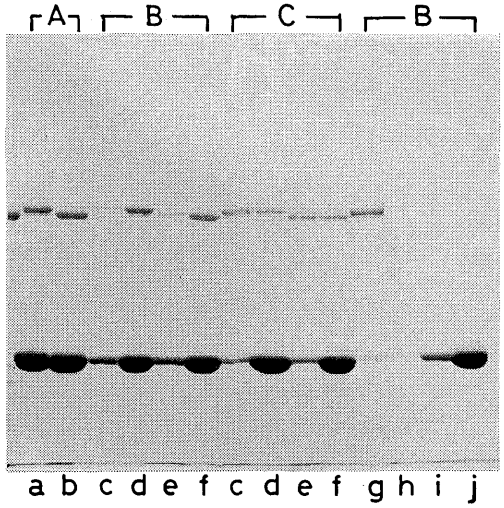

Fig. 4. Binding of F-actin and either α-actinin or calpain-treated α-actinin.

The same mixture (1 m*l*) as used in Fig. 3 was centrifuged at 280,000×g for 1 h at 2°C and 25°C, and then the supernatant and sediment obtained were applied on SDS-PAGE using a 7.5% polyacrylamide slub gel with the same volumes equivalent to those before centrifugation. (A) Before centrifugation; (B) after centrifugation at 2°C; (C) after centrifugation at 25 °C; a, mixture of F-actin (1 mg/m*l*) and α-actinin (0.2 mg/m*l*); b, mixture of F-actin (1 mg/m*l*) and calpain-treated α-actinin (0.2 mg/m*l*); c, supernatant of a; d, sediment of a; e, supernatant of b; f, sediment of b; g, supernatant of α-actinin; h, sediment of α-actinin; i, supernatant of F-actin; j, sediment of F-actin.

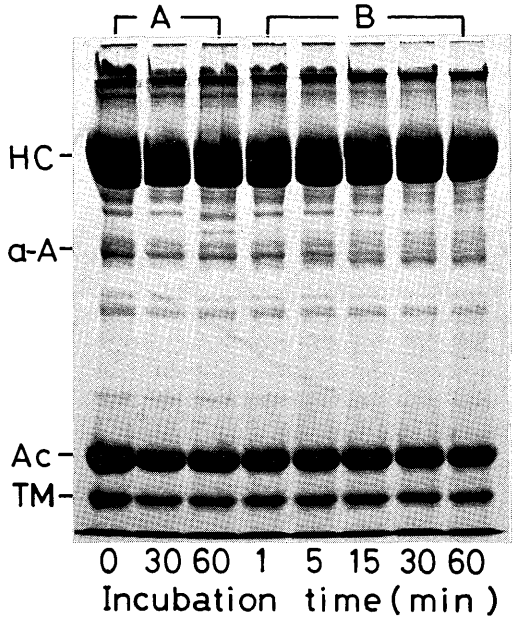

Fig. 5. Changes in SDS-PAGE patterns of myofibrils during incubation with calpain.

Carp myofibrils (5 mg/m*l*) were incubated with calpain (0.25 unit/m*l*) at 25°C in the medium containing 0.1 M NaCl, 5 mM $CaCl_2$, 5 mM 2-mercaptoethanol, and 20 mM Tris-acetate (pH 7.0). The reaction was terminated by the addition of 10 mM EDTA. Protein (50 μg/lane) was applied on a 7.5% polyacrylamide slub gel. (A) myofibrils incubated without calpain; (B) myofibrils incubated with calpain; HC myosin heavy chain; α-A, α-actinin; Ac, actin; TM, tropomyosin.

taining only α-actinin or calpain-treated α-actinin alone. In the case of experimental tubes containing the mixture, 88% α-actinin and 92% calpain-treated α-actinin were cosedimented with F-actin at 2°C (Fig. 4 B, c-f). However, only 40% α-actinin and 46% calpain-treated α-actinin were cosedimented with F-actin at 25°C (Fig. 4 C, c-f).

The results of turbidity and sedimentation experiments indicate that binding ability of α-actinin to F-actin was slightly increased by calpain treatment.

Calpain Treatment of α-Actinin within the Myofibril

Myofibrils were incubated with calpain in the medium containing 0.1 M NaCl, 5 mM $CaCl_2$, 5 mM 2-mercaptoethanol, and 20 mM Tris-acetate (pH 7.0) at 25°C for period up to 60 min as shown in Fig. 5. At given times, fixed quantities of the assay medium were removed to analyze by SDS-PAGE. Figure 5 shows the SDS-PAGE patterns; α-actinin subunits disappeared giving the fragments, similar to the calpain treatment of the isolated α-actinin, whereas the other major proteins of the myofibril such as actin, myosin heavy chain, and tropomyosin were apparently unaffected by calpain at an early stage of the incubation. Myofibrils treated with calpain showed a release of soluble proteins depending on incubation time (Fig. 6). A small amount of soluble protein was evident even in the controls and was represented in the 0-time values. SDS-PAGE analysis of the soluble fractions shows that a number of proteins were solubilized and released from the myofibrils by calpain treatment (Fig. 7(1)).

The major protein released was α-actinin fragments and increased with the incubation

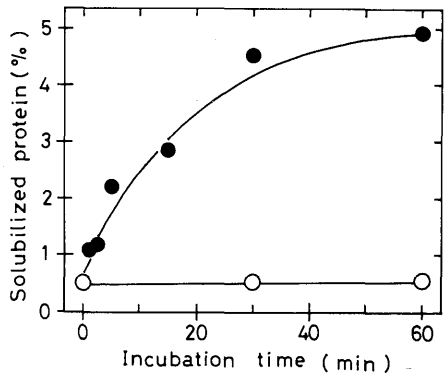

Fig. 6. Increase in the amount of proteins solubilized and released from myofibrils during incubation with calpain.

The assay condition was the same as in Fig. 5. After termination, the reaction medium was centrifuged at 2,200×g for 20 min; the protein concentration in the supernatant thus obtained was determined. ●, Myofibrils incubated with calpain; ○, myofibrils incubated without calpain.

time to 1.3% on the total staining intensity of myofibrillar proteins basis (Fig. 7(2)). The amount of intact α-actinin released was rapidly increased only at first stage of the incubation and then decreased to the initial level. These results suggest that the release of the fragments along with intact α-actinin from the myofibrils was not directly resulted from the digestion of α-actinin within the myofibrils, but may be dependent on calpain's ability to cause the release of the soluble proteins from Z-line.

Figure 8 shows changes in the amount of α-actinin and its fragments remaining in the pelletted myofibrils after calpain treatment. With incubation time, α-actinin rapidly disappeared from the myofibrils, whereas the fragments were increased up to 35% of the initial amount of α-actinin present in the myofibrils. Since the fragments produced were stable to calpain digestion as shown in Fig. 1, the sum of the amount of α-actinin and the fragments were expected to be approximately equivalent to the initial amount of α-actinin at any time during the incubation. Therefore, the difference between the initial amount of α-actinin and the sum of the remaining α-actinin and the fragments in the myofibrils expressed the amount of α-actinin released from the myofibrils during incubation with calpain. On the basis of this calculation, about 40% α-actinin was solubilized and released from the myofibrils for first 30 min incubation with calpain.

Discussion

It is well established in higher vertebrates that calpain removes Z-line from the intact myofibrils with concomitant release of α-actinin

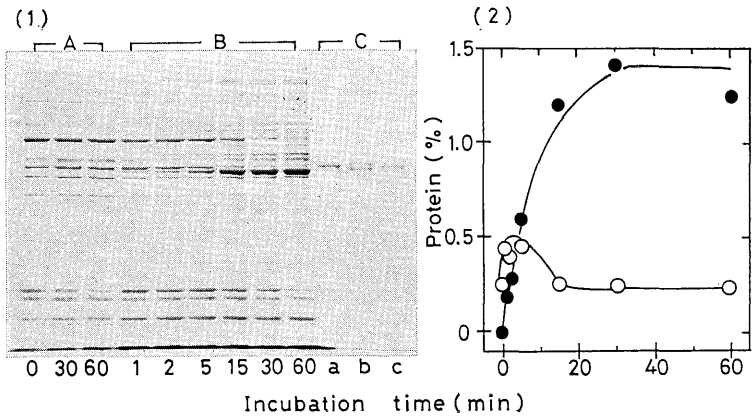

Fig. 7. Changes in SDS-PAGE patterns of solubilized proteins during incubation of myofibrils with calpain.

The solubilized and released proteins into the supernatant as shown in Fig. 6 were analyzed by SDS-PAGE on a 7.5% polyacrylamide slub gel. (1) A, proteins released from myofibrils during incubation without calpain. B, proteins released from myofibrils during incubation with calpain. C, standards for comparison: a, α-Actinin; b, α-actinin in 50% digestion; c, fragments produced from α-actinin by calpain digestion. (2) Amounts of solubilized and released α-actinin (○) and its fragments (●) were expressed as a percent to the total myofibrillar proteins before incubation.

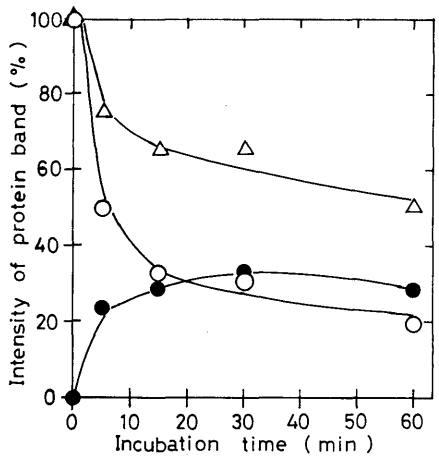

Fig. 8. Changes of amount of α-actinin remaining in the myofibrils during calpain digestion.

Calpain-digested myofibrils were centrifuged down to remove the solubilized proteins and then analyzed by SDS-PAGE on a 7.5% polyacrylamide slub gel. The relative intensities of protein bands to the initial intensity of α-actinin in the myofibrils (%) were plotted against incubation time. ○, α-Actinin; ●, fragments produced from α-actinin by calpain digestion; △, ○ (α-actinin)+● (fragments).

without proteolysis.[7-10] The similar result has been reported on carp calpain-treated myofibrils by us.[6] However, the results of the present study show that calpain degraded not only α-actinin isolated from carp myofibrils but also α-actinin within the myofibrils. The apparent discrepancy in calpain action on carp α-actinin is probably caused by the different analytical methods of SDS-PAGE. In the present study, we selected and used a 7.5% polyacrylamide slub gel according to the method of Laemmli[17] which gave an improved resolution of closed protein bands in the region of 100,000 Da than 5% polyacrylamide disk gel system of the method of Weber and Osborn[16] used previously.[6]

In the calpain treatment, two identical α-actinin subunits were cloven into calpain resistant fragments and no further degradation was detected, suggesting a limited hydrolysis. We confirmed another action of calpain in the present study (Fig. 7), which had been already reported,[6] on removing α-actinin without proteolysis from the myofibrils, but the released α-actinin was digested instantly by calpain. These results demonstrate that the digestion of α-actinin is not essential for removing it from the Z-line of myofibrils.

Sephacryl S-300 gel filtration (Fig. 2) showed that molecular mass of α-actinin was not altered by calpain treatment. This result suggests that a dimeric composition of α-actinin may be still remained with noncovalent interactions after calpain digestion. Furthermore, it should be noted that the formation of the fragments did not impair the binding ability of α-actinin to F-actin. In other words, cleavage of α-actinin by calpain did not disrupt the quaternary structure of the α-actinin molecule, nor F-actin binding sites under physiological condition. Hence, we presumed that α-actinin removing action of calpain rather than cleavage of α-actinin would be expected to contribute to the weakening of the Z-line.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture.

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