BBA 45953

EXHAUSTIVE EXTRACTION OF THE TROPONIN–TROPOMYOSIN FACTOR FROM THE I–Z–I BRUSH OF STRIATED MUSCLE*

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(Received February 13th, 1970)

SUMMARY

1. I–Z–I brushes were prepared completely free of myosin A, and exhaustively disassembled with mild solvents at 0°. Changes in the fine structure of the I–Z–I brush were observed after each of eleven mild short-term low-temperature extractions, until only single loose I-filaments remained.

2. High yields of the Ca^{2+}-sensitizing protein (troponin–tropomyosin factor) were obtained in Extractions I–IV, during the major changes in the loosening of the I-bands. Initially, almost all of the extracted protein was troponin–tropomyosin factor in nature and the yields of the troponin–tropomyosin factor, as a percent, were nil after Extraction VII.

3. Extracts I–III were higher in troponin than Extracts IV–VII.

4. α-Actinin-like protein[23] was very low in yield until Extraction V, after which it held to a high level through Extraction IX. This means that α-actinin-like protein, discussed elsewhere[23], was increasing in yield as Z-band integrity was disappearing.

INTRODUCTION

The I–Z–I brushes[1,2] consist of Z-bands with attached I-filaments on both sides. As a result of two gentle extractions of I–Z–I brushes, we[1,2] have obtained a preparation which appears closer, than does native tropomyosin[3], to the natural form of the protein complex which conveys Ca^{2+} sensitivity to a reconstituted actomyosin system. This protein shows a high degree of purity through ultracentrifugation and is outstanding in its Ca^{2+}-sensitizing ability. Through the employment of several techniques we concluded that this preparation, which we termed troponin–tropomyosin factor, represents a tighter and more effective complex of troponin and tropomyosin than occurs in the native tropomyosin[1,2]. The fact that this complex is completely extractable by short-term low-temperature (0°) extraction steps but still contains tropomyosin as a constituent, which had previously been thought to be extractable

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

* Published with the approval of the Director of Research Programs, College of Agricultural and Life Sciences, University of Wisconsin.

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only at room temperature[4], led us to consider the gentle disassembly of the I–Z–I brush. We have also previously indicated that the retention of myosin influences the extraction of protein from the I-filament[2]. Further, the use of acetone and/or high-temperature extraction represents rigorous handling and may materially alter proteins from their native forms. The isolation procedure, however, for actin[5], tropomyosin[6], native tropomyosin[3], troponin[7], and α-actinin[8] all involve one or more of these potential hazards. Our approach, therefore, was to prepare I–Z–I brushes which were completely free of myosin A and to exhaustively disassemble these I–Z–I brushes with mild solvents at 0°. We have followed the changes in the fine structure of the I–Z–I brush after each of eleven extractions, until only single loose I-filaments remained and simultaneously applied molecular criteria to the characterization of the Ca^{2+}-sensitizing portion of the extracted proteins.

MATERIALS AND METHODS

Rabbits were given sodium pentobarbital (90 mg) and (+)-tubocurarine chloride (1.5 mg) prior to exsanguination. All skeletal muscles were excised immediately, immersed in ice and coarsely (2 mm) ground. All subsequent preparation, extraction and handling procedures were carried out at 0–1°.

Preparation of I–Z–I brushes

The separate preparation of I–Z–I brushes in bulk was performed by the procedure of FUKAZAWA *et al.*[2], which essentially consisted of the preparation of myofibrils, exhaustive removal of myosin A, and extensive and special washing and handling procedures.

Extraction of I–Z–I brushes

Each extraction of the I–Z–I brushes consisted of the following: (a) addition of 0.5 vol. of a solution which was 0.2 mM ATP and 0.2 mM ascorbate (pH 7.5); (b) stirring slowly for 30 min; (c) centrifugation at 37000 × *g* for 60 min.

The supernatant after each centrifugation was given its respective extract number and the residue was subjected to a repetition of the extraction procedure.

Electron microscopic observations

I–Z–I brushes were centrifuged at 3000 × *g* for 15 min and washed once with 0.1 M phosphate buffer (pH 7.0). Particles of the I–Z–I brushes were then immersed in cold 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After the glutaraldehyde was drawn off the particles, they were washed twice with 0.1 M phosphate buffer (pH 7.0) and fixed for 2 h with cold 1 % OsO_4, buffered with veronal acetate (pH 7.3). The fixed particles were then dehydrated in graded ethanol and propyleneoxide and embedded in an Epon-812 mixture. Sections were cut with a glass knife in a Porter-Blum MT-2 ultramicrotome and stained with 1 % solution of uranyl acetate and undiluted lead citrate[9]. All electron micrographs were taken with a Hitachi Hu-11 B instrument.

Fractionation of the extracts

Each extract was fractionated into α-actinin-like and troponin–tropomyosin-containing proteins by the procedure of EBASHI AND EBASHI[3]. The troponin–tropo-

myosin complex was again subjected to additional $(NH_4)_2SO_4$ to give the equivalent of the combined Fractions I and II of WATANABE AND STAPRANS[10], EBASHI *et al.*[11] and FUKAZAWA *et al.*[2]. These combined fractions were isoelectrically separated into tropomyosin and troponin according to EBASHI *et al.*[11].

Preparation of well-washed myosin B

Myosin B was prepared essentially according to SZENT-GYORGYI[12] with slight modification[13] and converted to desensitized well-washed actomyosin by the procedures of PERRY *et al.*[14].

Preparation of trypsin-treated myosin B

Myosin B was prepared essentially according to SZENT-GYORGYI[12] with slight modification[13] and subjected to trypsin treatment, within 2 days of preparation, by the procedure of WATANABE AND STAPRANS[10]. Myosin B suspensions (1 mg protein per ml in 0.1 M KCl and 20 mM Tris–maleate buffer (pH 7.0)) were incubated with trypsin (10 μg/ml) for 10 min at 25°. The reaction was terminated by the addition of trypsin inhibitor (20 μg/ml). Subsequently the suspensions were washed twice with 0.1 M KCl and adjusted to 0.6 M KCl with a 3 M KCl solution. All trypsin-treated myosin B samples were used within 3 days of preparation.

Protein concentration

All protein concentrations were determined by the biuret method[15] after calibration against a nitrogen analysis (micro-Kjeldahl). Bovine serum albumin preparations were also used as standards.

Turbidimetric test for superprecipitation

Superprecipitation was assayed by the turbidimetric test of EBASHI[16] and as modified by YASUI AND WATANABE[17] and SERAYDARIAN *et al.*[18]. The composition of the reaction mixture will be specified in the description of individual experiments.

Ultracentrifugal analysis

All samples were given ultracentrifugal inspection in a Spinco Model E ultracentrifuge. The conditions of these observations will likewise be described in the appropriate sections.

RESULTS AND DISCUSSION

Ultrastructural changes

A typical I–Z–I brush is shown in Fig. 1. It corresponds in appearance to those of HUXLEY[19] except for a retention of the myofibrillar alignment. Both the Z-bands and I-filaments are about typical in their appearance. In our earlier work[1,2], we found that two extractions, of the I–Z–I brush, with 0.5 vol. of 0.2 mM ATP and 0.2 mM ascorbate yielded large quantities of the troponin–tropomyosin factor. This preparation presents some unusual features in its crystal structure[1,20]. Large globules appear near the center of certain ribs of the crystal lattice and may represent the basis of the troponin periodicity in the natural I-filament[21]. The subjection of the brushes to two extractions, however, does not lead to any major disruption of the ultra-

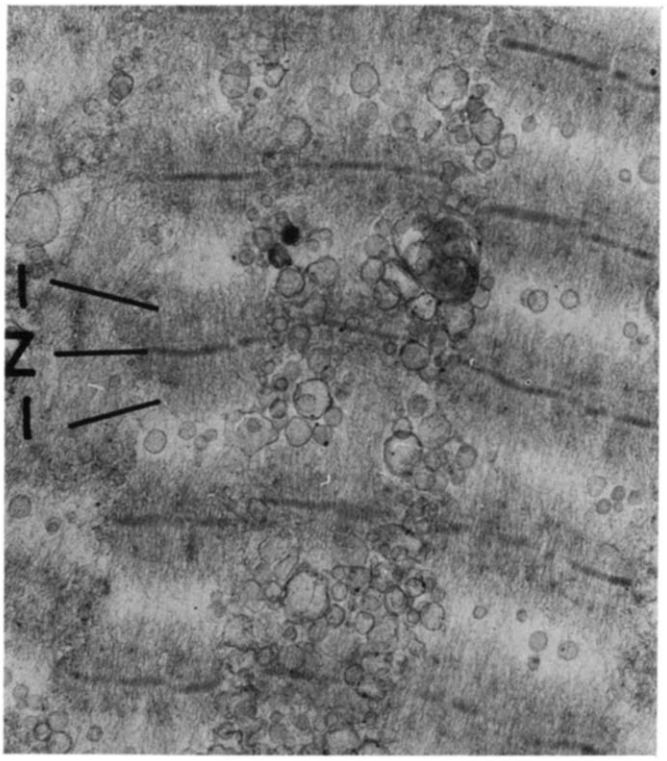

Fig. 1. I–Z–I brushes after myosin A extraction. Z-lines and I-bands are still intact (× 17000).

structure of the brushes (Fig. 2), at most there may be some general loosening of the I-bands. After three extractions there is some loosening of the Z-band structure, although the intensity of its zigzag configuration remains extremely high. After five extractions (Fig. 3) both the I- and Z-bands show further loosening; however, the zigzag configuration and the integrity of the Z-band are still distinct. The next electron micrograph (Fig. 4) was taken after seven extractions and one can see that a substantial part of the Z-line is being removed. After the eighth extraction (Fig. 5), the Z-line has been almost completely removed, with only an occasional trace of Z-line location (not shown), although some of the I-filaments are still in their original alignment. However, after eleven extractions the I-filaments are all individually separated and in partial to complete disarray (Fig. 6). All of these ultrastructural changes will be discussed in terms of the nature of the protein that is found in each extract.

Protein yield and relative composition

Table I shows the yield and fractionation of the protein that was removed with each extract. While total extractable protein increased through Extraction IV and then declined slowly to Extraction XI, the yields in Extracts II–IX were all of high magnitude. The supernatant after $(NH_4)_2SO_4$ fractionation represents troponin–tropomyosin factor protein which was in high yield in Extracts II–IV and sharply declining yields after Extraction V, with hardly any in Extracts VIII–XI.

Fig. 2. I–Z–I brushes after two extractions. Z-line loosening becomes apparent (× 36000).

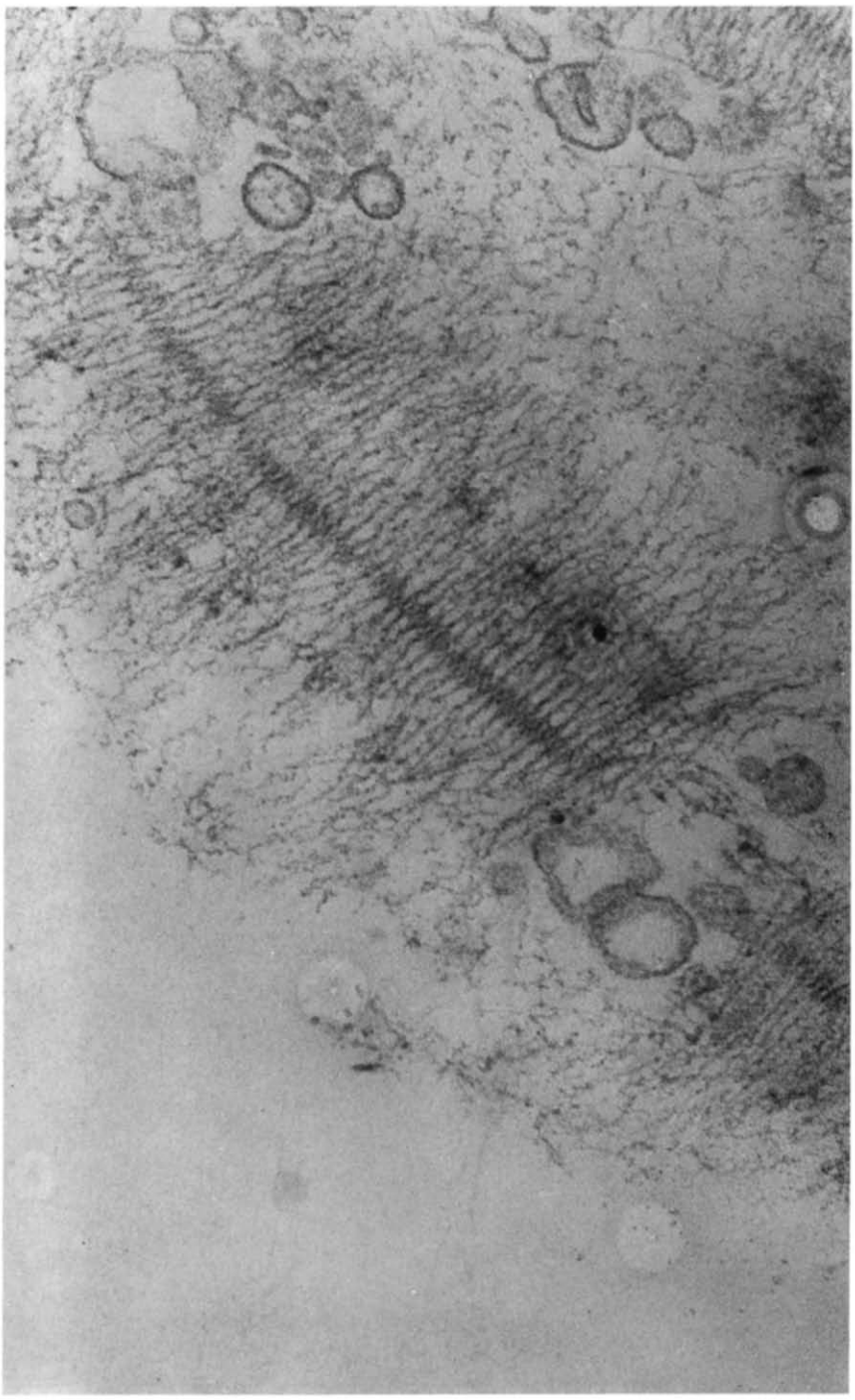

Fig. 3. I–Z–I brushes after five extractions. Z-lines and I-filaments have a greater degree of looseness (× 67500).

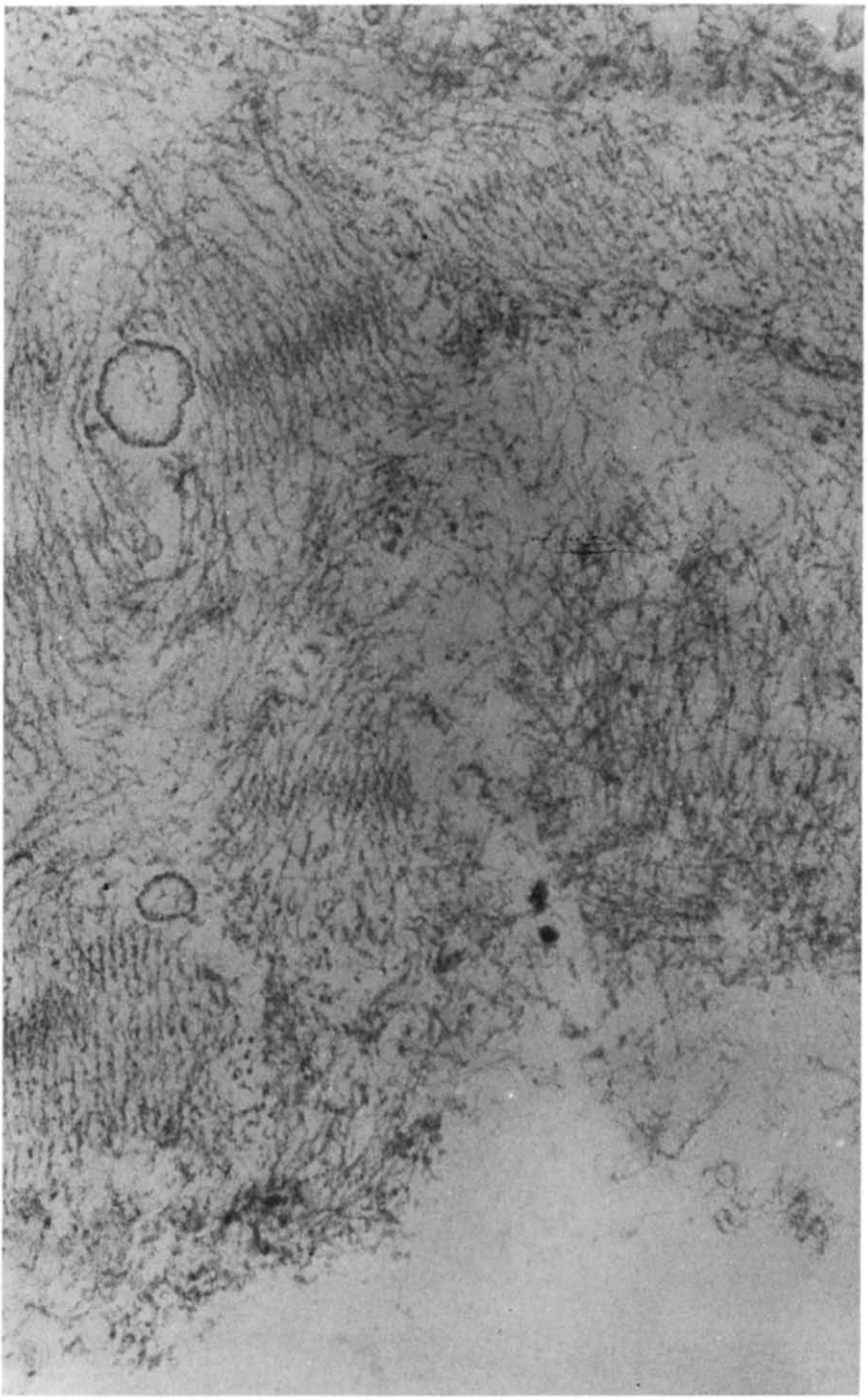

Fig. 4. Appearance of I–Z–I brushes after seven extractions. Intact zigzag configurations have disappeared from the Z-lines. I-bands have a greater degree of looseness than can be seen in Fig. 3 (× 67 500).

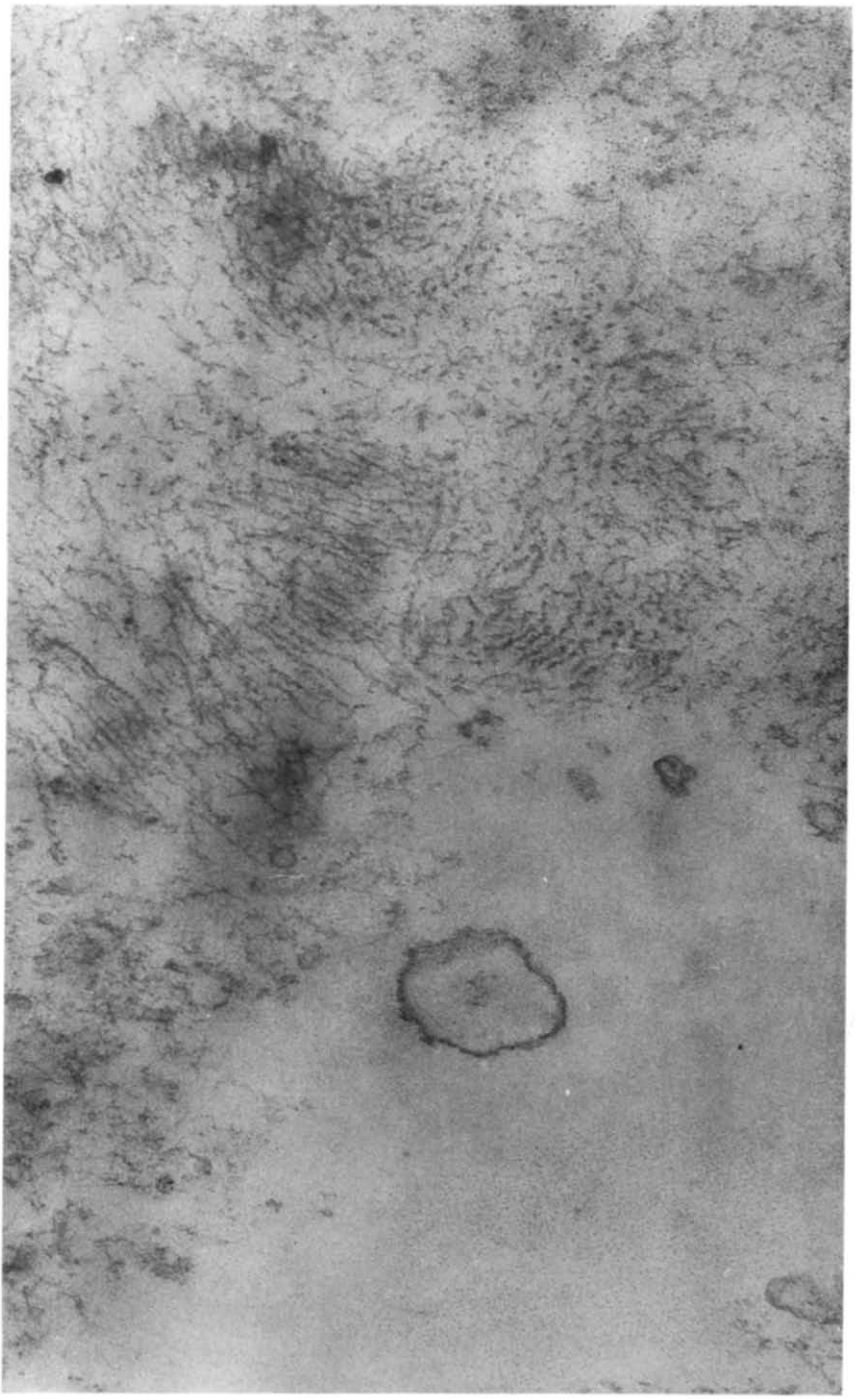

Fig. 5. Appearance of I–Z–I brushes after eight extractions. Note that the constituents of Z-lines have been removed (× 67500).

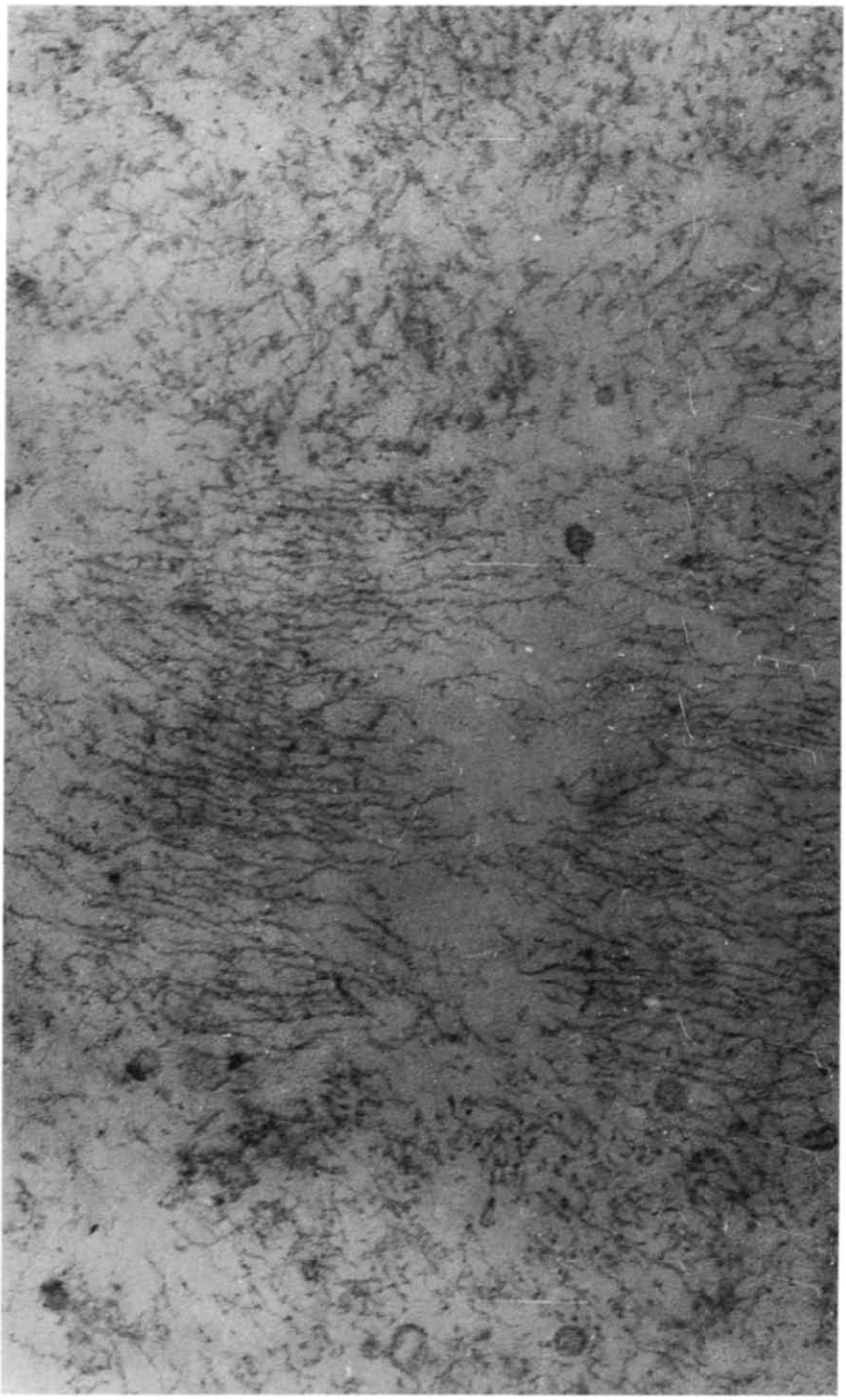

Fig. 6. Appearance of I–Z–I brushes after eleven extractions (× 67500).

TABLE I

QUANTITY AND GENERAL NATURE OF EXTRACTED PROTEIN

400 g muscle mince.

Extraction No.	*Total protein extracted (mg)*	*Fractionation (22.5 g $(NH_4)_2SO_4$ per 100 ml)*		*Combined troponin–tropomyosin factor and α-actinin-like protein (mg)*
		*troponin–tropomyosin factor protein (mg)**	*α-actinin-like protein (mg)***	
I	313.88	241.4	45.9	287.3
II	667.50	575.4	55.4	630.8
III	636.00	378.0	99.8	477.8
IV	984.00	409.0	189.3	598.3
V	923.00	284.4	352.3	636.7
VI	624.00	196.6	408.3	604.9
VII	764.00	137.8	410.6	548.4
VIII	602.00	59.9	436.0	495.9
IX	609.80	20.9	456.8	477.7
X	407.50	6.3	313.2	319.5
XI	342.00	4.8	259.0	261.8

* That fraction of the extracted protein that precipitates between 22.5–34.5 g $(NH_4)_2SO_4$ per 100 ml extract.

** That portion of the extracted protein that precipitates with 22.5 g $(NH_4)_2SO_4$ per 100 ml extract and remains soluble during dialysis.

TABLE II

QUANTITY OF TROPONIN AND TROPOMYOSIN IN TROPONIN–TROPOMYOSIN FACTOR PREPARATIONS FROM TWO GROUPS OF EXTRACTS OF I–Z–I BRUSHES

Extract group	*Initial quantity of crude troponin–tropomyosin factor*	*$(NH_4)_2SO_4$ fraction****			
		I		*II*	
		Troponin (mg)	*Tropomyosin§ (mg)*	*Troponin (mg)*	*Tropomyosin (mg)*
A*	330	49.7	37.8	14.8	63.9
B**	2366	123	116	61	324

* Extracts I–III.

** Extracts IV–VIII.

*** See fractionation procedure of FUKAZAWA *et al.*[2].

§ According to EBASHI *et al.*[11].

It should be noted that the high yields of the troponin–tropomyosin factor were obtained during the major changes in the loosening of the I-bands. The precipitate, which represented α-actinin-like protein was very low in yield until Extraction V, after which it held to a high level through Extraction IX. This means that α-actinin-like protein was increasing in yield as Z-band integrity was disappearing (Figs. 7 and 8). When the α-actinin-like protein was dialyzed to removed the $(NH_4)_2SO_4$, a certain amount of protein precipitated and has not been included in the figures for yield of α-actinin-like protein. As the quantity of α-actinin-like protein increased,

so this precipitable protein did also, which accounts for the increasing difference between the total extractable protein and the combined total of the troponin–tropomyosin factor and α-actinin-like protein. Some of this protein may represent either (a) protein still unidentified to date, (b) partially denatured actin (although thought unlikely because of the mild extraction conditions) or (c) actin which has been precipitated by either α-actinin or the troponin–tropomyosin factor[22]. If the latter factor was the major cause of the precipitation of the protein during dialysis,

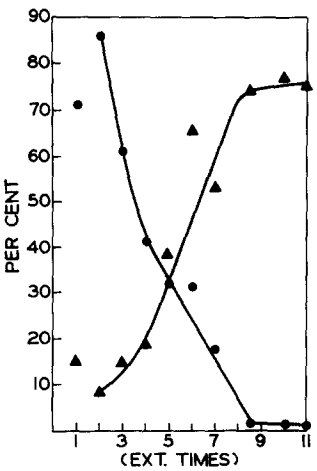

Fig. 7. Yield (as a percentage of combined proteins) of troponin–tropomyosin factor and α-actinin-like protein during each of eleven extractions of the I–Z–I brush. ●—●, troponin–tropomyosin factor protein represents that fraction of extracted protein that precipitates between 22.5–34.5 g $(NH_4)_2SO_4$ per 100 ml extract; ▲—▲, α-actinin-like protein, representing that portion of the extracted protein which precipitates with 22.5 g $(NH_4)_2SO_4$ per 100 ml extract and remains soluble during dialysis against a 1 mM $KHCO_3$ solution.

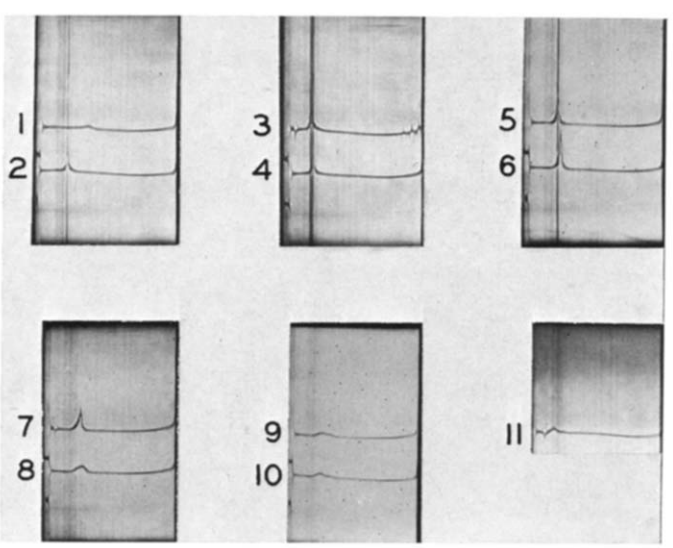

Fig. 8. Ultracentrifugal sedimentation of crude Extracts I–XI. Extract number indicated. Preparation: I, 0.65 mg/ml; II, 2.98 mg/ml; XI, 4.38 mg/ml; all the rest 5.0 mg/ml in 0.1 M KCl and 20 mM Tris–maleate buffer (pH 7.0); 33 min after reaching full speed, 60000 rev/min.

it would seem that α-actinin-like protein would be more involved in this phenomena than the troponin–tropomyosin factor because the discrepancy becomes greater as α-actinin-like protein is removed from the Z-band.

When the yields of α-actinin-like protein and the troponin–tropomyosin factor are added together and expressed as a percent of this total, we can get a clear picture of the preferential removal of each of these proteins or complexes with each succeeding extraction (Fig. 7). One can see that, initially almost all of the extractable protein is troponin–tropomyosin factor in nature, and that the majority of the troponin–tropomyosin factor comes out in Extractions I–V and the yields of the troponin–tropomyosin factor, as a percent, are nil after Extraction VII. Conversely, most of the α-actinin-like protein is removed after Extraction V, with the protein in extracts beyond Extract VII being almost entirely α-actinin in nature. In general terms, as the troponin–tropomyosin factor is extracted the I-bands loosen, and as α-actinin-like protein is removed the Z-bands disappear. The question still remains as to whether these proteins or complexes each change in relative composition, *i.e.* tropomyosin and troponin, *etc.*, with succeeding extractions. For purposes of clarity in this manuscript, a further assessment of the composition and nature of the α-actinin extract will be reported elsewhere[23] and the remainder of this paper will be devoted to the characterization of the troponin–tropomyosin factor preparations from Extracts I–XI.

Ultracentrifugal analysis

Crude extracts (I–XI). Fig. 8 shows the crude extracts as they appear during ultracentrifugation under the same conditions. The protein concentrations of Extracts I and II were lower but Extracts III–X were all 5.0 mg/ml, with Extract XI being 4.38 mg/ml. It can be seen that the pictures of Extracts I–VI appear quite typical for the troponin–tropomyosin factor[2], with only a small amount of the troponin–tropomyosin factor existing in Extract VII. Conversely, extracts after Extract VII show a predominance of α-actinin-like protein[18]. These techniques offer opportunities for the complete quantitative assessment of these proteins in their near native states, and this work is in progress.

Purified troponin–tropomyosin factor ($(NH_4)_2SO_4$ fraction, 22.5–34.5 g/100 ml) Fig. 9 shows the purified troponin–tropomyosin factor of Extracts I–VIII. Beyond Extract VIII, the yield of the purified troponin–tropomyosin factor was so low it could not be ultracentrifugally assessed. This fraction in Extract I appears to be high in troponin, sedimenting like Fraction I of regular troponin–tropomyosin factor, which is made from two combined extracts of the I–Z–I brushes[2]. Extract II shows an increase in tropomyosin, whereas Extracts III, IV and V are typical of the troponin–tropomyosin factor and consist of troponin to tropomyosin in about a 1:2 ratio on a weight/weight basis.

Superprecipitation of desensitized actomyosin in the presence of purified troponin–tropomyosin factor from Extracts I–VIII

Figs. 10A–10C show the effect of the troponin–tropomyosin factor fraction, which has been purified in the range of 22.5–34.5 g/100 ml, upon superprecipitation of desensitized actomyosin. All of these fractions from Extracts I–VII have excellent Ca^{2+}-sensitizing activity and cause a complete clearing in and delay of superprecipitation. The control studies with and without ethyleneglycol-bis-(β-aminoethyl ether)-

N,N'-tetraacetic acid (EGTA) show that the actomyosin has been completely desensitized. Extract VIII, however, does not convey Ca^{2+} sensitivity to the actomyosin system. On the basis of this finding and its appearance during ultracentrifugal inspection, we imagine that it is primarily tropomyosin, without sufficient troponin to convey Ca^{2+} sensitivity to the system. Studies directed, however, to the precise characterization of this fraction are presently in progress.

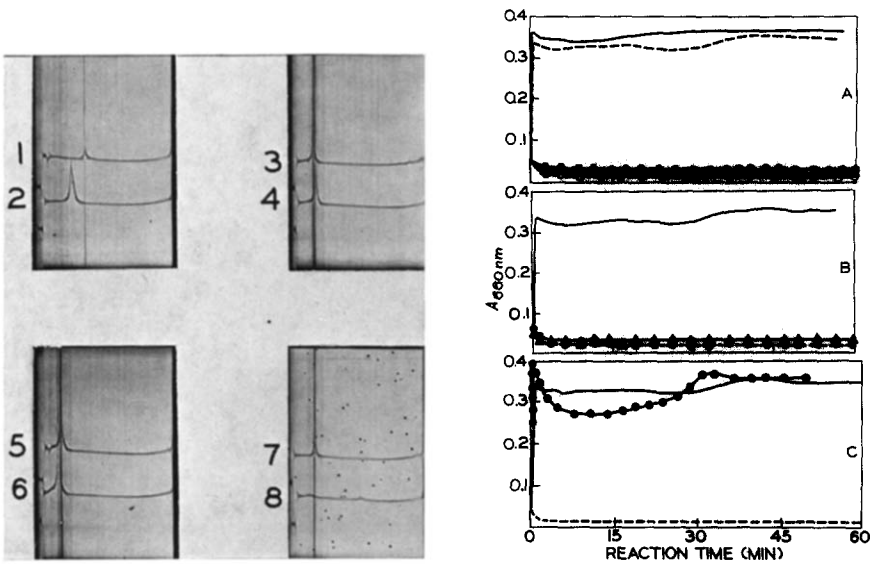

Fig. 9. Ultracentrifugal sedimentation of purified troponin–tropomyosin factor from Extracts I–VIII. Extract number indicated. Preparation: I, 1.92 mg/ml; II, 3.40 mg/ml; all the rest 0.1 mM $KHCO_3$ and Tris–maleate buffer (pH 7.0); 25 min after reaching full speed, 60 000 rev/min. Temperature 20°.

Fig. 10. Effect of purified troponin–tropomyosin factor from Extracts I–VIII on the superprecipitation of desensitized actomyosin. Final concns.: desensitized actomyosin, 0.33 mg/ml; KCl, 0.035 M; Tris–maleate buffer (pH 7.0), 20 mM; $MgCl_2$, 1 mM; ATP, 1 mM; EGTA, 25 μM (except for control); troponin–tropomyosin factor protein, 25 μg/ml. A. Extracts I–III. ———, control, without EGTA; – – – – –, control with EGTA; ●—●, Extract I; ▲—▲, Extract II; ○—○, Extract III. B. Extracts IV–VI. ———, control with EGTA; ▲—▲, Extract IV; ●—●, Extract V; – – – – –, Extract VI. C. Extracts VII and VIII. ———, control with EGTA; – – – – –, Extract VII; ●—●, Extract VIII.

Quantitation of troponin and tropomyosin in various groups of extracts of I–Z–I brushes (Table II)

Extracts I–III were pooled as were Extracts IV–IX. Troponin–tropomyosin factor Fractions I and II were prepared from each of the pooled extracts according to the procedures of FUKAZAWA *et al.*[2]. Each fraction was then isoelectrically separated into troponin and tropomyosin according to the procedure of EBASHI *et al.*[11]. It can be seen that Fraction I is rich in troponin, which supports our previous suggestion on the general composition of Extract I, based on its appearance during ultracentrifugal inspection. Ultimately, in the work in progress, we will obtain an improved quantitation of troponin and tropomyosin in each of the extracts; however, we can generalize from the pooled data that the Extracts I–III have a higher percentage

(38%) of troponin in relation to tropomyosin than do Extracts IV–VIII (29%). The properties of the α-actinin-like proteins in the extracts have been discussed elsewhere[23].

ACKNOWLEDGMENTS

This study was supported in part by Public Health Service Research Grant FD-00107-11 and a research grant from AMIF, Chicago, Ill.

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