SEPARATION AND CHARACTERIZATION OF THE INHIBITORY FACTOR OF THE TROPONIN SYSTEM

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Received May 24, 1971

SUMMARY. Several fractions possessing troponin-like activity have been isolated by Sephadex G-200 and QAE*-Sephadex A-25 column chromatography in non-denaturing solvents. SDS acrylamide gel electrophoresis indicated the presence of three major components in crude troponin and the chromatographically isolated biologically active fractions contained different amounts of these three constituents; they possessed molecular weights of 40,000, 25,000, and 22,000, and were arbitrarily referenced as components I, II, and III, respectively. The percentage compositions of these components in the various troponin fractions isolated were correlated with their biological activity as measured by their ability to inhibit superprecipitation of desensitized actomyosin. Extent of inhibition of superprecipitation was found to increase with increasing amounts of component II in the preparations, whereas components I and III had no apparent role in this regard. Component II of molecular weight 25,000 is thus implicated to be the inhibitory factor of previously reported troponin preparations.

The muscle protein, troponin, which in combination with tropomyosin renders actomyosin ATPase activity sensitive to calcium concentration, has been shown to contain several protein components. Hartshorne and Mueller (1) separated troponin into two components by pH 1.0 treatment. One component, troponin B, inhibited the Mg^{+2} activated ATPase activity of desensitized actomyosin independently of calcium concentration and of the presence of tropomyosin (2). The other, troponin A, had no influence on desensitized actomyosin alone, but in combination with troponin B caused the ATPase activity of desensitized actomyosin to become dependent on calcium concentration. Similar components were found by Schaub and Perry (3) using SE-Sephadex chromatography in 6 M urea.

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* Abbreviations: QAE, quaternary aminoethyl; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

More recently Drabikowski et al. (4) have separated troponin into at least four components by chromatography on DEAE-Sephadex in the presence of 4 M urea. Ebashi et al. (5) using a combination of Sephadex G-150 and SE-Sephadex column chromatographies in 6 M urea have shown the existence of three protein components constituting troponin. The first two (troponins I and II) are found in Mueller's troponin B. Troponin I, of molecular weight 40,000, showed an affinity for tropomyosin. Troponin II, of molecular weight 22,000, bound calcium. Another fraction of molecular weight 17,000, corresponding to Mueller's troponin A was also obtained. Ebashi concluded that troponins I and II were required for biological activity.

Most of the aforementioned preparative methods for dissociation and separation of the components of troponin have employed denaturing solvents such as 6 M urea. The present study describes the use of a more gentle preparative approach, that of Sephadex G-200 and QAE-Sephadex chromatographies under non-denaturing conditions which enable the isolation of several fractions possessing troponin-like activity. By correlating profiles from SDS acrylamide gels with biological activities as measured by inhibition of superprecipitation of desensitized actomyosin, an attempt has been made to pinpoint the component required for this inhibitory activity.

EXPERIMENTAL. Troponin was prepared as follows. Muscle powder, prepared using the Bailey sequence of organic solvents (6) was extracted 18 hrs at room temperature with 7 volumes of a solution containing 1 M KCl, 50 mM Tris buffer at pH 7.6 and 1 mM DTT. The supernatant after centrifugation was cooled to 4°C and the pH was lowered to 4.6 to remove tropomyosin. After returning the pH to 7, and dialysis against 2 x 10 volumes distilled water, the precipitate between 40 and 60% saturated ammonium sulfate was collected. This was dissolved in 50 mM Tris buffer at pH 7.6 containing .5 mM DTT, made 1 M with respect to KCl and the pH was lowered to 4.6. Any precipitate was discarded. The supernatant was neutralized, dialyzed extensively against distilled water, and lyophilized. This resulted in a yield of 2-3 gm of

"crude troponin" per 150 gm muscle powder.

Troponins A and B were prepared from crude troponin by the method of Hartshorne and Mueller (1). Actomyosin was prepared by the method of Weber and Edsall as documented by Szent-Györgyi (7).

All chromatographies were carried out at 4°C in the usual manner. Constant flow rates were maintained by L.K.B. peristaltic pumps.

For SDS acrylamide gels methodology similar to that of Shapiro et al. (8) was used. Protein samples were heated at 80°C for 10 minutes in 1% SDS,1 mM DTT, electrophoresed on 5% gels for 2-1/4 hr. at 5 mA/tube, stained with Coomassie Blue for 3 hrs., and destained with a Canalco horizontal destainer. Gels were scanned in a Gilford 240 spectrophotometer at 550 mμ. Relative quantities of each band present were determined planimetrically by measuring the area under the peak after scanning.

Superprecipitation of desensitized actomyosin was measured by the turbidometric assay of Ebashi (9). The reaction was monitored by means of a Beckman DBG spectrophotometer with recorder attachment. Actomyosin was desensitized by trypsin treatment (10).

RESULTS AND DISCUSSION. Sephadex G-200 column chromatography of crude troponin resulted in two major peaks, 1 and 2 (Figure 1). These peaks were subsequently rechromatographed on Sephadex G-200, and essentially Gaussian distributions resulted. Peak 1 was farther fractionated into at least four components on QAE-Sephadex (Fig. 2).

The composition of crude troponin as judged by SDS-acrylamide gels can be seen in Figure 3. There are three major bands corresponding to fractions with molecular weights of 40,000 (I), 25,000 (II), and 22,000 (III). The relative amounts of these components were determined by densitometric scanning. Crude troponin was found to consist of approximately 43% component I, 27% component II, and 25% component III (Table 1).

The SDS acrylamide gels of peaks 1 and 2 from Sephadex G-200 are shown in Figure 3. Peak 1 is similar in composition to crude troponin (Table 1).

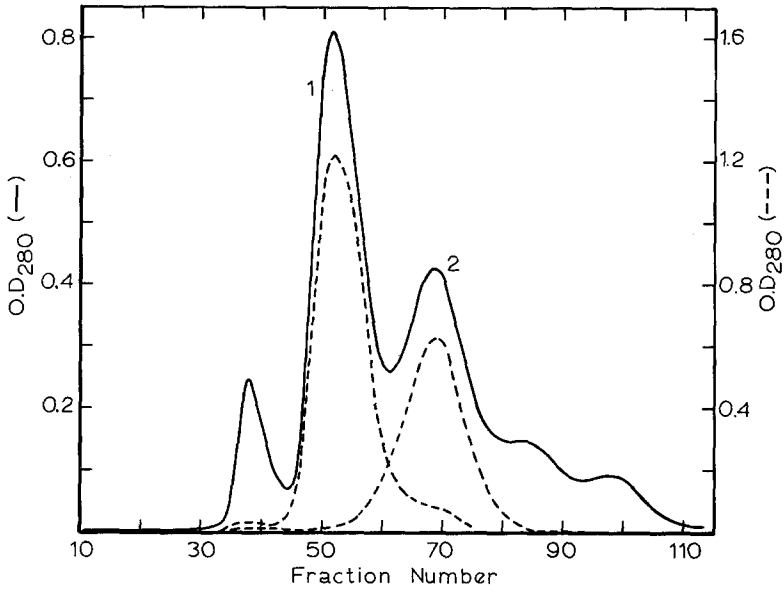

Figure 1: Sephadex G-200 treatment of crude troponin. About 100 mg protein in 4 ml buffer (0.5 M KCl, 0.05 M PO_4, pH 7, .5 mM DTT) was applied to a column of dimensions 2.5 cm x 100 cm equilibrated against the same buffer and eluted at 13.7 ml/hr. Fraction size was 4.57 ml. Rechromatography is indicated by the broken line.

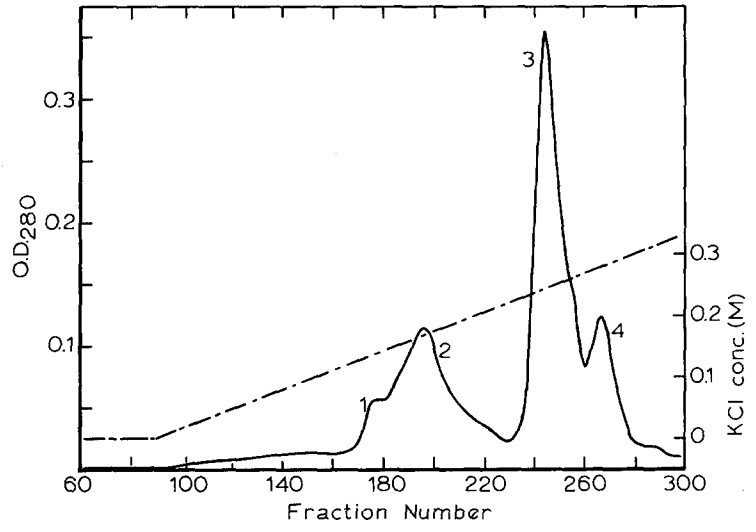

Figure 2: QAE-Sephadex A-50 column chromatography of peak 1 from Sephadex G-200. 150 mg protein was applied to a 2.5 cm x 40 cm gel bed equilibrated with 50 mM Tris, pH 7.6, .5 mM DTT and eluted with a gradient of KCl (broken line).

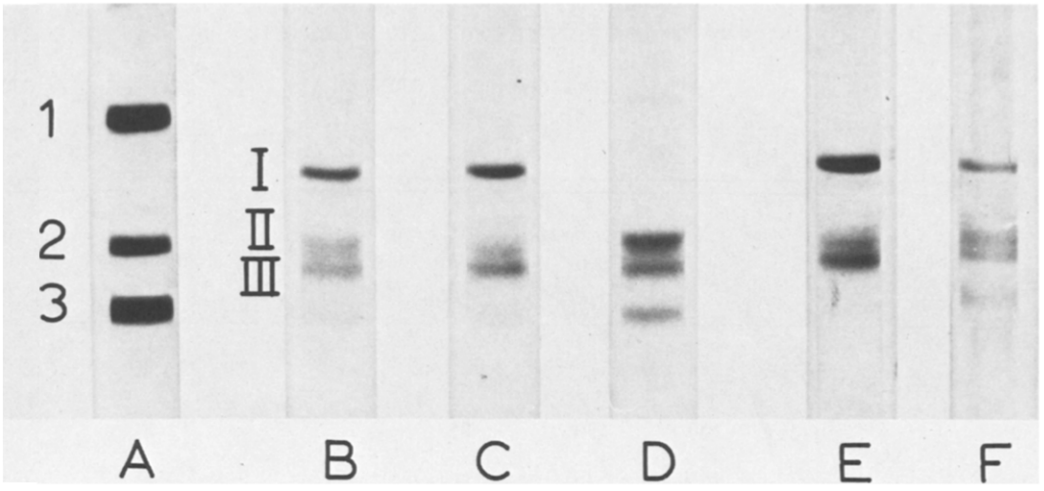

Figure 3: SDS acrylamide gels of crude troponin (gel B), peaks 1 and 2 from Sephadex G-200 (gels C and D respectively), and peaks 3 and 4 from QAE-Sephadex (gels E and F respectively). About 10 µg of protein was applied. Gel A contains a standard mixture of: 1) bovine serum albumin, 2) chymotrypsinogen A and 3) sperm whale myoglobin.

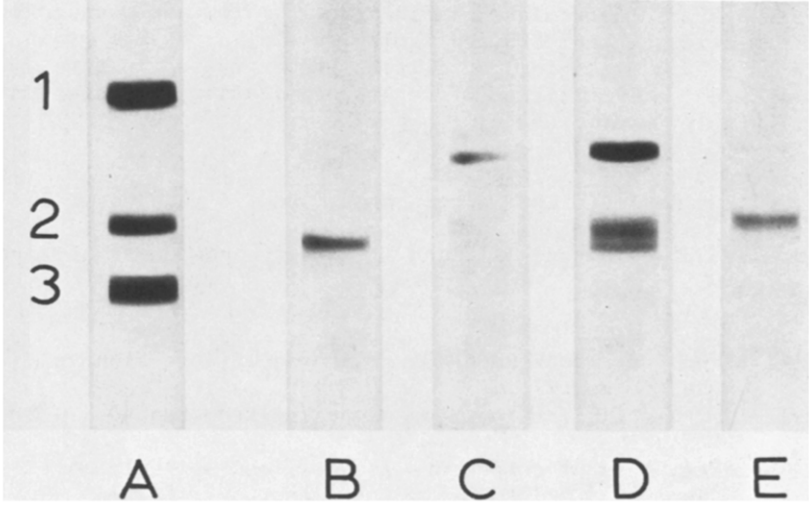

Figure 4: SDS acrylamide gels of troponin A (gel B), troponin B (gels C and D) and troponin B supernatant (gel E). Gels B, C, and E contained 5-10 µg protein; gel D about 20 µg. Gel A contained a standard mixture of: 1) bovine serum albumin, 2) chymotrypsinogen A and 3) sperm whale myoglobin.

TABLE 1

CORRELATION OF COMPOSITION OF THE VARIOUS TROPONIN FRACTIONS WITH THEIR BIOLOGICAL ACTIVITY*

	% composition of components of molecular weight			Biological Activity
	40,000	25,000	22,000	
Crude troponin	43	27	25	+++
Peak 1 from Sephadex G-200	50	25	24	++
Peak 2 from Sephadex G-200	<2	50	25	++++
Peak 3 from QAE-Sephadex	48	26	26	+++
Peak 4 from QAE-Sephadex	39	20	31	++
Troponin A	<2	<2	90	0
Troponin B	51	35	10	
Troponin B supernatant	5	85	<2	+++++

* % compositions were determined planimetrically from areas under the peaks of densitometric scans of SDS acrylamide gels. Values are probably accurate to ± 5%. Biological activities are expressed in time (+ represents about 4 minutes) of inhibition of superprecipitation of desensitized actomyosin by 16 µg/ml protein.

However, peak 2 is deficient in component I and is made up of 50% component II, 25% component III, and 15-20% of a component of molecular weight 16,000.

Peaks 3 and 4 from QAE-Sephadex treatment (Figure 2) have profiles on SDS acrylamide gels similar to those of peak 1 from Sephadex G-200. Peak 4 contains slightly less of components I and II and slightly more component III

For comparison sake, troponin A and troponin B were prepared from crude troponin (1). The material remaining after removal of troponin B was saved and will be referred to as troponin B supernatant. SDS acrylamide gels on these Hartshorne-Mueller preparations are shown in Figure 4. Troponin A is

essentially a single band of molecular weight 22,000 (component III). Troponin B is heterogeneous, containing two major species of molecular weight 40,000 (component I) and 25,000 (component II), the former in excess. Troponin B supernatant contains a major band of molecular weight 25,000 (component II) and traces of components with molecular weights 40,000 (component I) and 16,000.

Biological activities of the various fractions isolated were measured as the extent of inhibition of superprecipitation of desensitized actomyosin in the absence of calcium. This inhibition did not require the presence of tropomyosin, as previously noted (2-4). For illustration purposes, the results of a typical assay of material taken from peak 2, obtained by Sephadex G-200 chromatography of crude troponin, are portrayed in Figure 5. Curves 2 and 3, which represent the respective addition of 16 and 32 µg/ml of material illustrate the linearity of the relationship between concentration

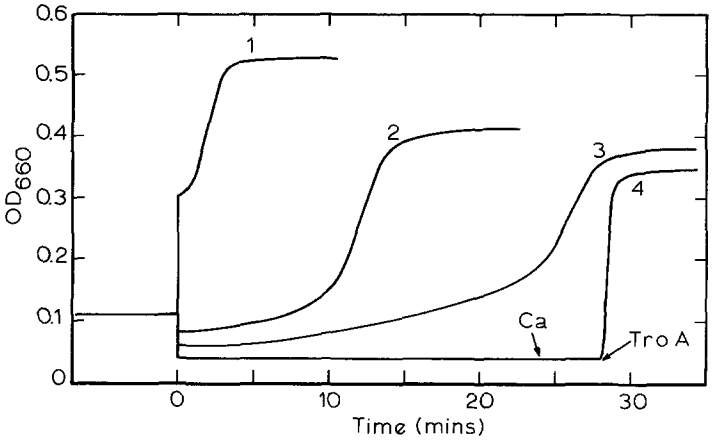

Figure 5: Effects of troponin fractions on superprecipitation of trypsin-desensitized actomyosin.
The reaction mixture contained: 20 mM imidazole, pH 7.0, 1.3 mM $MgCl_2$, 6.5 x 10^{-5} M EGTA, 33 mM KCl and 0.8 to 1.0 mg/ml actomyosin. At time 0 the mixture was made 0.33 mM in ATP.
Curve 1 represents the control with or without .1 mM $CaCl_2$. Curves 2 and 3 result when 16 and 33 µg/ml respectively of peak 2 material from Sephadex G-200 are added to the system. Curve 4 shows the effect of addition of 50 µg/ml troponin B. $CaCl_2$ (at a final concentration of .1 mM) and troponin A (50 µg/ml) were added at the times indicated.

of active fraction and time of inhibition of superprecipitation, at least over the range of concentrations used. It is noteworthy that troponin B (curve 4) caused calcium insensitive inhibition of superprecipitation which was overcome by the addition of troponin A.

Superprecipitation was semiquantitated in terms of the time required to reach maximum $OD_{660\ m\mu}$. The biological activities of the materials under study are summarized in the final column of Table 1 along with approximate percentages of each of the components of molecular weights 40,000 (I), 25,000 (II), and 22,000 (III), as determined from densitometric scans of the SDS acrylamide gels. The data suggest that there is a direct relationship between the amount of component II present and the extent of inhibition of superprecipitation. At the same time components I and III appear to play no apparent role in this inhibition. Component II of molecular weight 25,000 is thus implicated to be the inhibitory factor of previously reported troponin preparations (1,3,4). Furthermore, the present chromatographic separation procedure has the virtue over all others (3-5) in that it is effected in the absence of denaturants such as 6-8 M urea, and hence should result in components that are more typically native.

ACKNOWLEDGEMENTS. The authors are grateful to Mr. A. Keri and Mr. V. Ledsham for excellent technical support and to Dr. W. D. McCubbin for stimulating discussions. This investigation was generously supported by the Muscular Dystrophy Association of Canada, the Medical Research Council of Canada (MRC-MT 1223), the Life Insurance Medical Research Fund and the Alberta Heart Foundation. A. C. Murray is indebted to the Canadian Heart Foundation for receipt of a predoctoral fellowship.

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