SEPARATION AND CHARACTERIZATION OF THE CONSTITUENTS OF TROPONIN

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1. Introduction

Hartshorne and coworkers [1] have found recently that troponin, a protein which in the form of a complex with tropomyosin sensitizes actomyosin to changes in concentration of calcium ions (for review see [2]) can be separated at pH 1.0 into two fractions. One of them, troponin B, inhibited the Mg^{2+}-stimulated ATPase activity of DAM* [3] independently of the concentration of Ca^{2+} and of the presence of tropomyosin whereas a second fraction, troponin A, had no influence on DAM *per se*, but sensitized troponin B to Ca^{2+} ions in the presence of tropomyosin. Two similar fractions were later obtained by Schaub and Perry [4] with the use of SE-Sephadex chromatography.

Our previous experiments also showed that preparations of troponin are heterogenous; moreover, disc electrophoresis and chromatography on DEAE-Sephadex-A-50 column indicated the presence of more than two fractions [5]. By using the latter method the protein fractions were obtained and some of their properties are described in this paper.

2. Experimental

Preparations of troponin were usually obtained from "native tropomyosin" extracted at low ionic strength [6]. To remove contaminating nucleic acid $(NH_4)_2SO_4$ was added to the supernatant obtained at pH 4.6 to 70% saturation and the precipitate was collected. Troponin obtained in this way revealed in disc electrophoresis the presence of 5 bands, whether or not urea was present and this pattern did not change in the presence of 1 mM dithiothreital (fig. 1a). Most of the first band, A, remains in the spacer gel, sometimes moving only slightly into the separating gel. The fasted band, E, moves with the mobility of the marker dye.

Chromatography of troponin on a DEAE-Sephadex-A-50 column in the presence of 4 M urea with continuous KCl gradient usually gave 4 protein peaks. The first one was non-retarded, the other three were eluted at 0.06–0.10, 0.20–0.25, 0.30–0.34 M KCl, respectively. Sometimes a fifth small peak appeared at 0.65–0.70 M KCl, especially when troponin was prepared from crude tropomyosin extracted with 1 M KCl from alcohol–ether muscle powder [6]. Fig. 1b shows the disc electrophoresis pattern of the peaks. When chromatography on DEAE-Sephadex was performed in the presence of 0.1 ml EDTA a better separation of the protein fractions was achieved and peak IV contained no more band C (fig. 1c). Consequently, EDTA was added together with urea to the eluent (fig. 2).

Material present in peak I is soluble, whereas those of peak II and III are insoluble in water. Therefore in the case of peaks II and III after chromatography urea and EDTA were removed by dialysis against 0.3 M KCl. Peak IV is soluble both in water and in 0.3 M KCl. Fig. 1 shows that protein present in peak I corresponds to band A of the whole troponin preparations. Peak II contains a protein fraction moving slowly as a broad band, corresponding to band B. Sometimes its pattern in electrophoresis is similar to that of peak III. Peak III contains a single protein fraction moving with a mobility

* *Abbreviations:*
DAM : desensitized actomyosin;
EGTA: ethylene glycol bis-(β-aminoethylester)-*N,N'*-tetra-acetic acid.

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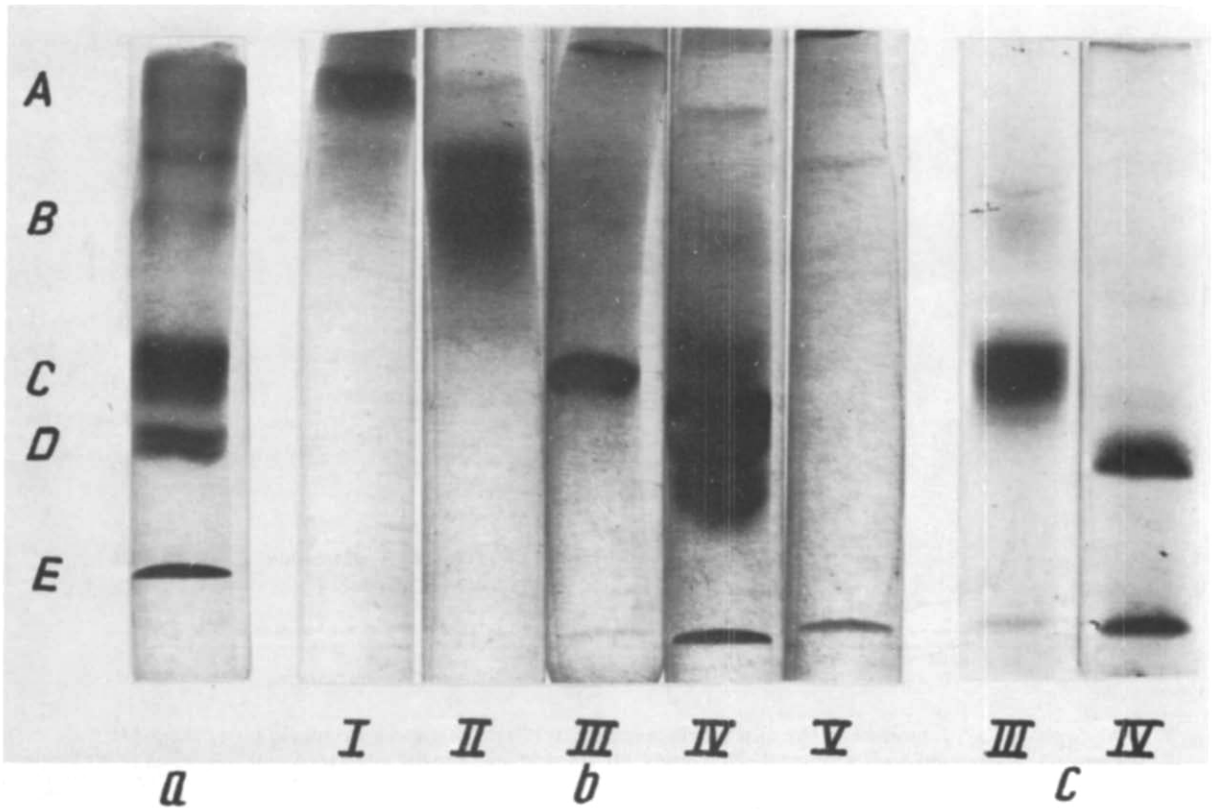

Fig. 1. Acrylamide gel electrophoresis of troponin (a), peaks obtained on DEAE-Sephadex-A-50 column in the absence (b), and in the presence of 0.1 mM EDTA (c), respectively. Disc electrophoresis was performed on 7.5% polyacrylamide gels essentially according to Davis [17] with tris-glycine buffer, pH 8.5 in the presence of 4 M urea. 100 μg of protein samples were applied. Stained with Coomasie blue. Figures refer to the numbers of peaks.

corresponding to band C. The material of peak IV, when obtained from the columns eluted with EDTA, shows two bands, D and E.

Recently we have shown that when disc electrophoresis of troponin is performed in the presence of 1 mM EGTA only the mobility of band D, and possibly that of E is changed [7]. Electrophoresis of the peaks supports that observation. In the presence of EGTA only material of peak IV changes its electrophoretic pattern so that band D moves faster and disc E usually disappears. Peak V, if present, contains material most of which passes through the dialysis bags. Preliminary analysis indicates the presence of only disc E in this peak.

The absorption spectra of peaks I, II and III have maxima at 278 nm, although the protein fractions present in the peaks differ somewhat in the E_{278}/E_{260} ratio. These are 1.7–1.9, 1.5–1.6 and 1.4–1.5 for peaks I, II and III, respectively. Peak IV has a E_{278}/E_{260} ratio of about 1.0 and has four maxima at 253, 259, 265 and 269 nm, indicating a high content of phenylalanine [c.f. 8, 9].

In agreement with previous observations [1, 6, 10] our preparations of troponin inhibit the Mg^{2+}-stimulated ATPase activity of DAM in the presence of tropomyosin and EGTA. In the absence of these factors the effect differs somewhat from preparation to preparation, but in general at low concentrations of troponin some stimulation of the ATPase activity is usually observed [c.f. 1]. At higher concentrations of troponin this stimulation disappears and finally, at concentrations higher than 300 μg/ml, there is some inhibition.

Figs. 3 and 4 show the effect of the DEAE-Sepha-

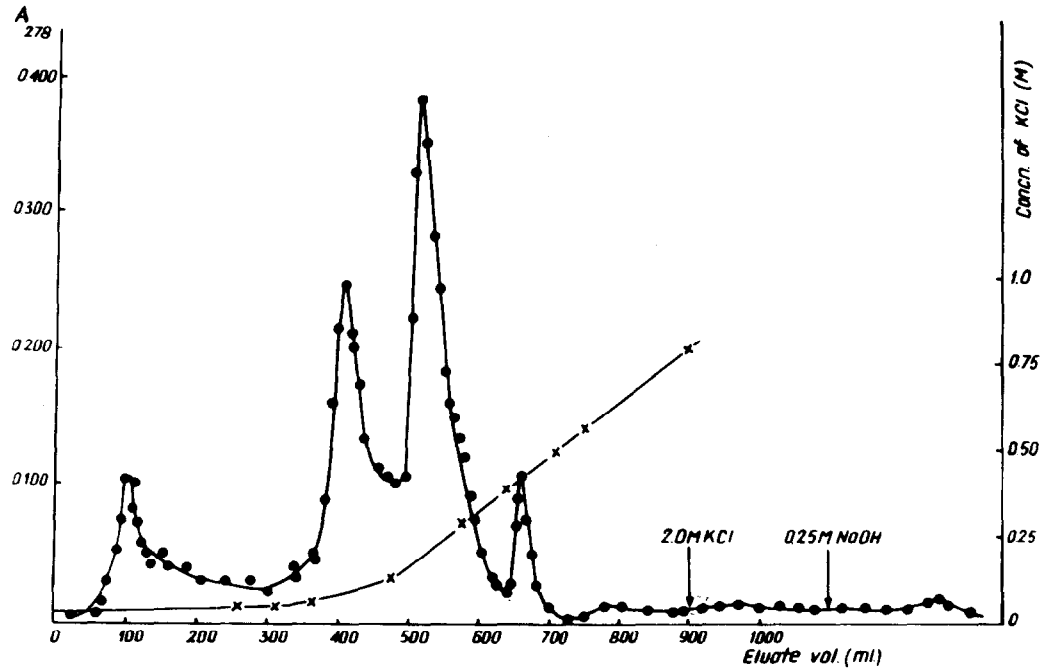

Fig. 2. DEAE-Sephadex A-50 chromatography of troponin preparation. 120 mg of protein was loaded on a column (40 × 2.5 cm) of DEAE-Sephadex-A-50 equilibrated against 50 mM tris-HCl, pH 7.5, 4 M urea and 0.1 mM EDTA and was eluted by KCl gradient.

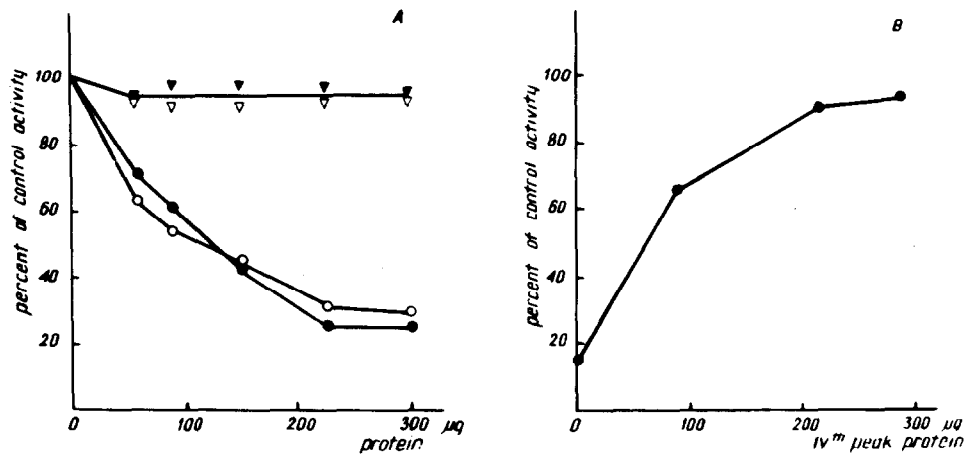

Fig. 3. A) Effect of peak I and peak IV on the Mg^{2+}-stimulated ATPase activity of DAM. Reaction mixtures contained 0.50 mg/ml DAM, 0.03 M KCl, 0.01 M tris-HCl, pH 7.5, 1 mM ATP, 1 mM $MgCl_2$ and the protein from peak I (○, ●) or peak IV (▽, ▼), as indicated in the figure. Full symbols denote samples in which 0.1 mM EGTA and tropomyosin in the amounts corresponding to 0.66 of the protein from the particular peak were present.

B) Effect of peak IV on the inhibition by peak I of the Mg^{2+}-stimulated ATPase of DAM in the absence of EGTA. The assay was carried out under the same conditions as in fig. 4A.

dex peaks on the Mg^{2+}-stimulated ATPase activity of DAM. The protein of peak I strongly inhibits ATPase activity whether or not EGTA and tropomyosin are present and in this respect it corresponds to troponin B of Hartshorne et al. [1] or to the inhibitory factor of Schaub and Perry [4] (fig. 3A). Peak IV, on the other hand, has no effect on the ATPase activity under these conditions, but it abolishes the inhibitory effect of peak I in the absence of EGTA and tropomyosin (fig. 3B). Thus, the material present in this peak behaves similarly to troponin A [1] or to calcium sensitizing factor [4]. The proteins present in peak II and III also have an effect on the ATPase activity of DAM (fig. 4), a stimulatory one at low concentrations and an inhibitory at higher ones. On the other hand, in the presence of tropomyosin and EGTA both peaks inhibit the ATPase activity of DAM although somewhat weaker than peak I.

The second characteristic feature of troponin is the binding of calcium [10–13]. Our preparations of troponin usually contain 2–3 moles of exchangeable calcium per 10^5 g protein, not removable by Dowex 50 [12]. When troponin is fractionated according to Hartshorne et al. [1] most of this Ca is found in troponin A. Examination of the calcium content in the peaks obtained from DEAE-Sephadex chromatography showed that peak IV contains 3–4 moles of exchangeable Ca per 10^5 g protein, whereas the other peaks contain only traces.

The other specific property of troponin is the interaction with tropomyosin which is shown by the increase in viscosity of tropomyosin on adding troponin [13–15] or by gelation at higher concentrations [15]. Preliminary experiments seem to indicate that the material of peaks I, II and III interact with tropomyosin. The addition of peak I to tropomyosin at low ionic strength produces precipitation, whereas peaks II and III cause gelation of tropomyosin solutions. The protein of these two peaks becomes soluble in the absence of salt when tropomyosin is present. Peak IV seems not to interact with tropomyosin.

The present results clearly show that troponin preparations contain more than two protein fractions as reported by other workers [1, 4]. Troponin A and B were found heterogenous in disc electrophoresis [5, 16], and our present experiments show that troponin B contains besides peak I, the material of peaks II and III, which is probably responsible for the solubility

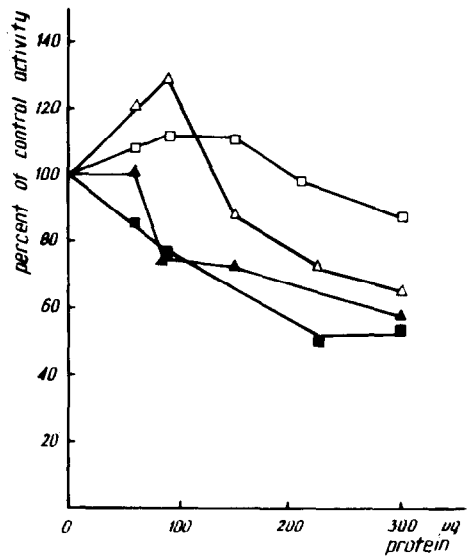

Fig. 4. Effect of peak II and III on the Mg^{2+}-stimulated ATPase activity of DAM. Peak II (□, ■), peak III (△, ▲). Full symbols denote the samples containing EGTA and tropomyosin. Conditions of the assay as the legend to fig. 3A.

of troponin B which differ from those of peak I. Also troponin A is not purely peak IV but contains only a predominance of the material of that peak. Thus the present procedure leads to much better separation of individual components of troponin than those previously used [1, 4].

Protein present in peak I is a powerful inhibitor of the Mg^{2+}-stimulated ATPase activity of DAM, whereas the material present in peak IV seems to be the only one responsible for those properties of troponin which are directly related to calcium ions. These are the binding of calcium, changes in the electrophoretic mobility in the absence of Ca^{2+} and modification of the inhibitory effect of peak I on the ATPase activity of DAM in the presence of Ca^{2+}. It remains to be elucidated which protein, that of band D, or band E, or both, are responsible for these properties.

For the property of troponin-sensitization of DAM to calcium ions in the presence of tropomyosin, the combination of the proteins from peak I and IV seems to be sufficient. What then is the significance of the protein(s) present in peaks II and III? These fractions also inhibit ATPase activity of DAM in the presence of EGTA and tropomyosin and, in addition, seem to be responsible for the activating effect of troponin in

the absence of EGTA. It is quite possible that both peaks II and III contain the same protein and the separation on DEAE-Sephadex column into two peaks is due to some equilibrium between aggregated and dissaggregated forms, but the definite role of this protein factor in troponin – tropomyosin system remains to be elucidated.

In spite of heterogeneity of troponin preparations one should emphasize the fact that all constituents interact mutually and form a kind of complex, since on a calibrated Sephadex G-100 column troponin gives only one fairly symmetrical peak, found by us at an elution volume corresponding to a molecular weight about 70,000.

Acknowledgement

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