# SEPARATION AND CHARACTERIZATION OF THE 37 000 DALTON COMPONENT OF THE TROPONIN SYSTEM

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## 1. Introduction

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 [1–4]. One component, troponin A, has no influence on desensitized actomyosin alone, but in combination with troponin B causes the ATPase activity of desensitized actomyosin to become dependent on calcium concentration. The other, troponin B is composed of two major components of approximate mol. wt 37 000 and 23 000 daltons [5, 6]. The 23 000 component has been implicated to be the major active fraction in inhibiting the $Mg^{2+}$ activated ATPase activity of synthetic actomyosin and tropomyosin enhances this inhibition. The protein of mol. wt 37 000 daltons, also referred to as TN–T*, is present in all troponin complex preparations [3, 4, 6, 7]. As yet there is no general agreement on its biological role, although it may have a special affinity for tropomyosin. The main problem in its detailed physicochemical and biological characterization appears to be the difficulties experienced by different workers in cleanly separating this component from other members of the troponin complex.

The present investigation represents the first physico–chemical characterization of a homogeneous preparation of TN–T prepared by CM cellulose chromatography. The measurements include SDS acrylamide gel electrophoresis, low speed sedimentation equilibrium, circular dichroism, amino acid analysis and parallel biological activity studies in the presence and absence of tropomyosin.

* *Abbreviations:* DAM, desensitized actomyosin; SDS, sodium dodecyl sulphate; CM cellulose, carboxymethyl cellulose; ATPase, adenosine triphosphatase; DTT, dithiothreitol; TN–T, tropomyosin binding component of troponin.

## 2. Materials and methods

Crude Troponin B prepared from rabbit skeletal muscle using the method of Hartshorne and Mueller [1] was further purified by CM cellulose chromatography as described earlier [8]. SDS polyacrylamide gel electrophoresis and molecular weight determinations were carried out according to Weber and Osborn [9].

Assays of ATPase activity were carried out by electrometric titration following proton liberation accompanying ATP hydrolysis in the pH-stat (Radiometer TTT1, equipped with a titrator and titrigraph). The reaction mixture consisted of 0.2 mg DAM at 25°C, containing 2.5 mM ATP, 2.5 mM $MgCl_2$, 1 mM Tris–HCl buffer, pH 7.6. The total volume of the reaction mixture was 10 ml. Actomyosin was desensitized by repeated washings with 2 mM Tris–HCl according to the procedure of Schaub and Perry [2].

Circular dichroism measurements were made on a Cary model 6001 CD attachment to a Cary 60 recording spectropolarimeter in accordance with the methodology of Mani et al. [8]. Protein concentrations were determined in the ultracentrifuge employing the Rayleigh interference optical system assuming 41 fringes equivalent to 10 mg/ml [10]. For CD and sedimentation equilibrium studies the protein was initially dissolved in $10^{-4}$ N HCl and then dialysed against the buffer. CD measurements were made in 0.5 M KCl, 0.05 M potassium phosphate, pH 7.0.

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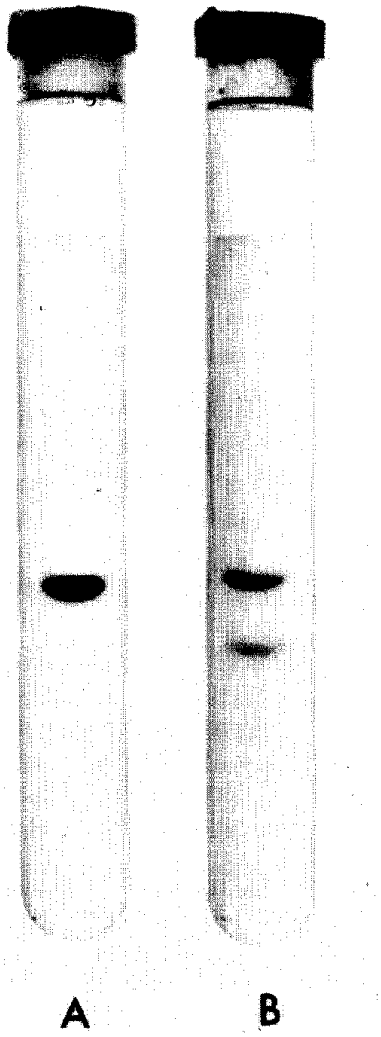


Fig. 1. 10% sodium dodecyl sulfate polyacrylamide gels showing (a) 20 μg of 37 000 component of troponin prepared by CM cellulose chromatography and (b) 10 μg of the same sample after standing at room temperature for a week.

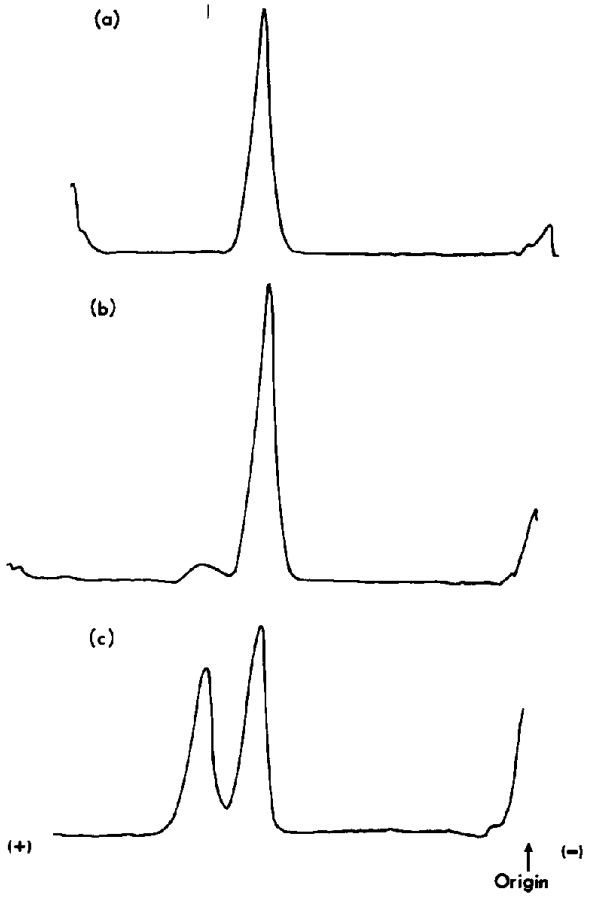


Fig. 2. (a) Densitometer scan of the 37 000 component of troponin after electrophoresis on 10% polyacrylamide gel in the presence of SDS. Direction of migration is from right (−) to left (+). (b) Incubation at 80°C for 1 hr in 1% SDS, 1 mM DTT. (c) Incubation for two weeks in 1% SDS, 1 mM DTT at room temperature.

Low speed sedimentation equilibrium studies were carried out in a Beckman Model E analytical ultracentrifuge according to methodology described earlier [8].

Amino acid analysis of the protein was effected in a Beckman Model 121 analyzer on samples hydrolyzed for 24, 48, and 72 hr. The values reported are averaged values for the three hydrolysis times. Threonine and serine values were obtained by extrapolating to zero time. For valine and isoleucine the 72 hr value was used.

## 3. Results and discussion

CM cellulose column chromatography of crude troponin B resulted in 3 peaks [8] with the protein eluting in peak 3 corresponding to TN–T. In this investigation, it was observed that with freshly prepared eluents all the protein fractions were eluted faster and poor resolution was obtained. In order to get good resolution it was found necessary to prepare the eluents two or three days in advance since the elution profile was affected by the degree of ester formation in the eluent. SDS acrylamide gel electrophoresis revealed the protein to be homogeneous (fig. 1) and as

Table 1
Amino acid content of TN–T (mol. wt 37 000 g).

| Amino acid | This study | Wilkinson et al. [5] |
|---|---|---|
| Lys | 47.9 | 42.7 |
| His | 7.4 | 6.4 |
| Arg | 30.2 | 27.4 |
| Asp | 24.8 | 26.6 |
| Thr | 4.8 | 7.1 |
| Ser | 8.6 | 11.0 |
| Glu | 73.2 | 76.2 |
| Pro | 12.1 | 11.1 |
| Gly | 10.1 | 10.5 |
| Ala | 31.4 | 33.7 |
| Val | 12.4 | 13.9 |
| Met | 6.05 | 5.6 |
| Ile | 10.2 | 9.4 |
| Leu | 24.7 | 23.8 |
| Tyr | 4.8 | 4.8 |
| Phe | 5.5 | 5.6 |
| Tryp | 2.2 | – |
| Cys | – | 0.6 |

can be seen from the densitometer scan of the gel (fig. 2a) there is no indication of any minor contaminants (in particular, the inhibitory protein) in our preparation. The molecular weight estimated from the SDS acrylamide gel was 37 000 ± 1 000 daltons. If the protein in 1% SDS, 1 mM DTT was left standing at room temperature for a few days or at 80°C for 1 hr, upon electrophoresis, another band of mol. wt around 20 000 daltons was seen. The proportion of this low molecular weight species was found to increase with time of incubation in SDS. Fig. 2b and c shows this effect. This time dependent fragmentation of the protein molecule might be due to either one of several factors such as: (a) proteolysis; (b) acid hydrolysis and (c) presence of a very labile bond. In regard to these possibilities, Drabikowski et al. [11] have already established that TN–T is very susceptible to proteolysis by neutral cathepsins. Acid hydrolysis of the peptide bond linking aspartic acid with proline in proteins is known to take place at acid pH [12], and since TN–T has a high content of proline (table 1), this mechanism is certainly possible. Consistent with our gel patterns, low speed sedimentation equilibrium studies on solutions of TN–T, showed the presence of heterogeneous material. Fig. 3 shows a typical ln absorbance versus $r^2$ plot that was obtained. Weight average molecular weights ranged from 15 000–50 000 daltons, from

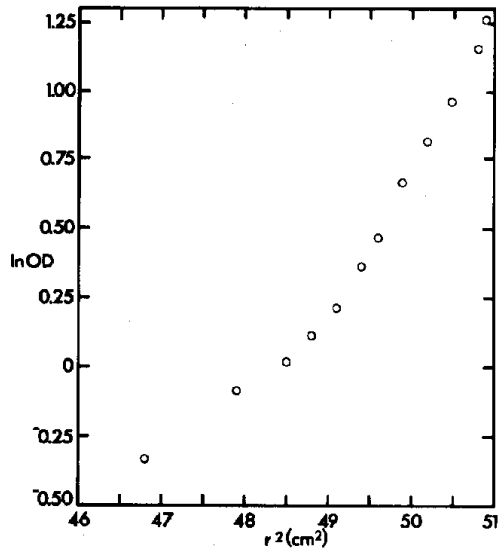


Fig. 3. Plot of the natural log of the concentration (absorbance) as a function of the square of the distance from the axis of rotation for TN–T dialyzed against 0.5 M KCl, 0.05 M potassium phosphate, 1 mM DTT (pH 7.0) for 48 hr. The rotor speed was 16 000 rev/min.

which it is evident that the TN–T component, in addition to containing low molecular weight material, also exhibits aggregation. Susceptibility of TN–T to aggregation is in agreement with the observations of Greaser and Gergely [13].

The ultraviolet spectrum of the preparations was typical of that of a protein with a maximum at 278 nm and an inflexion around 290 nm due to tryptophan, of which there appears to be approximately two residues per mole as determined by the method of Bencze and Schmid [14].

Bioassays were carried out on TN–T samples dissolved in $10^{-4}$ N HCl. The protein was found to inhibit the $Mg^{2+}$ stimulated ATPase activity of DAM in the presence and absence of EGTA (fig. 4). Tropomyosin had no significant influence on the inhibitory effect of TN–T. For maximum inhibition it was noted that nearly 60 μg of TN–T was required per 0.2 mg of DAM, i.e. 300 μg per 1 mg, whereas with the inhibitory protein only 25 μg was required per 1 mg of DAM for a comparable extent of inhibition [8]. Since the TN–T preparations employed in this study were found to be homogeneous by SDS gel electrophoresis the observed biological activity cannot be

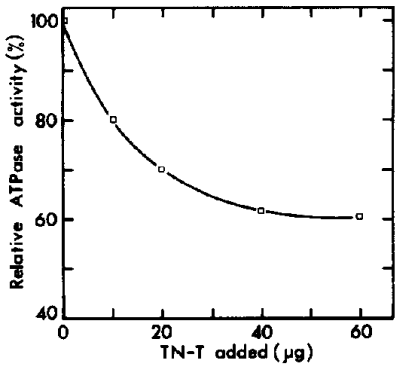


Fig. 4. Effect of the TN–T component of troponin on the $Mg^{2+}$-stimulated ATPase activity of DAM.

due to contamination with the inhibitory protein. Control experiments conducted with DAM and tropomyosin alone showed no alteration in ATPase activity. This would suggest that the DAM preparations employed were essentially devoid of inhibitory protein. The inhibitory effect thus would appear to be a real property of the TN–T molecule; however it should be noted that this activity is only one eighth that of the inhibitory protein when compared on a molar basis.

In the far ultraviolet region the CD spectrum of the protein possesses two negative dichroic peaks, located at 221 and 206.5 nm, band positions typical of a protein containing α-helix. The $[\theta]$ values at these two wavelengths were $-7800 \pm 200$ and $-10\,000 \pm 200$ $(deg \cdot cm^2)/dmole$ respectively. The low ellipticity values obtained are indicative of a low helix content in the protein consistent with its large content of proline (table 1). In the near ultraviolet region the protein did not have any significant ellipticity. Addition of $Mg^{2+}$ had no significant effect on the CD spectrum of the protein in contrast to troponin A and the inhibitory protein which are known to undergo conformational changes with divalent metal addition [4, 8, 15].

Amino acid composition of the protein, presented in table 1, is very similar to that reported by Wilkinson et al. [5]. However, small discrepancies do arise, the most notable of which is the lower content of threonine and serine in our preparation.

From this study it is clear that even though the protein undergoes harsh treatments during its isolation, it seems to be stable as long as it is in the form of a complex. However, once it is separated from the complex, it is quite labile. In order to understand the possible role of TN–T in muscle contraction, it is essential to study the interaction of this protein with the other components of the troponin complex as well as tropomyosin. Such studies are presently under investigation in our laboratory.

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