A NEW METHOD OF PREPARATION OF TROPONIN I (INHIBITORY PROTEIN) USING AFFINITY CHROMATOGRAPHY. EVIDENCE FOR THREE DIFFERENT FORMS OF TROPONIN I IN STRIATED MUSCLE

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1. Introduction

The methods currently in use for the preparation of the components of the troponin complex involve the preliminary isolation of the troponin complex and its subsequent fractionation into individual components. The procedure is satisfactory for rabbit skeletal muscle and often can be modified for other muscles. In some cases, however, the troponin preparations particularly from smooth muscle usually contain proteins in addition to the troponin I, troponin C and troponin T which leads to difficulties in the isolation of pure samples of the individual components. The relatively long period of isolation required by these procedures often leads to some degradation of the components, especially of troponin I which is particularly susceptible to breakdown by endogenous cathepsins.

The report that troponin C and troponin I form, in the presence of Ca^{2+}, a specific complex that is stable in high urea concentrations [1–3] and which can be dissociated by ethanedeoxybis- (ethylamine) tetra-acetic acid (EGTA), presents an ideal system for the application of affinity chromatography for the isolation of these two components. A method using troponin C linked to Sepharose is described for the rapid isolation of pure troponin I by a one-stage procedure from a few grams of whole muscle. Application of the procedure to the striated muscle of rabbit has indicated that there are at least three forms of troponin I present in this tissue, each form being specific for the type of muscle from which it is isolated.

2. Methods

2.1. *Preparation of muscle proteins*

Troponin was prepared by the method of Ebashi et al. [4], and fractionated into its components by chromatography on DEAE-cellulose equilibrated with 8 M urea, 50 mM Tris –HCl buffer, (pH 8.0), containing 15 mM 2-mercaptoethanol [5]. Under these conditions troponin I passed unretarded through the column followed by the successive elution of troponin T and troponin C on application of a linear gradient to 0.5 M NaCl, 8 M urea, 50 mM Tris –HCl buffer (pH 8.0).

Desensitized actomyosin and tropomyosin were prepared by the procedures described by Cummins and Perry [6].

Extracts for affinity chromatography were prepared by homogenising, immediately after removal from the animal, 4–6 g of muscle in 10 vol of 8 M urea, 75 mM Tris–HCl buffer (pH 8.0), containing 15 mM 2-mercaptoethanol and 1 mM $CaCl_2$, using a small Waring Blender type of homogeniser. The homogenate was clarified by centrifugation, followed by filtration through glass wool. To ensure equilibration and dissociation the clear extract was dialysed against the urea buffer in a rocking dialyser for several hours prior to chromatography.

2.2. *Affinity column*

The procedure of Porath, Axén and Ernbäck [7] was used to couple troponin C to Sepharose 4B (Pharmacia). 20 g wet weight (see below) of Sepharose were washed with distilled water and suspended in

distilled water to a total volume of 50 ml. The suspension was stirred gently at 20°C and the pH adjusted to 11.0 by the addition of 6 M NaOH. After the addition of 4 g of powdered cyanogen bromide the pH of the suspension was maintained between 11.0 and 11.5 by the addition of 6 M NaOH. During cyanogen bromide activation the temperature was prevented from rising above 25°C by the addition of ice. The activated Sepharose was washed successively with 20 vol of cold distilled water and cold 0.1 M $NaHCO_3$ and finally resuspended in 10 ml of cold 0.1 M $NaHCO_3$. The coupling was started by the addition of 5 ml of troponin C (20 mg/ml) dissolved in 0.1 M $NaHCO_3$ containing 5 mM $CaCl_2$, and allowed to proceed for 20 hr with gentle stirring at 4°C. The troponin C–Sepharose conjugate was washed with cold distilled water followed by 0.1 M $NaHCO_3$ and finally equilibrated with the urea buffer used to prepare the muscle extracts. The amount of troponin C coupled to the Sepharose was estimated routinely as the difference between the total amount added and that eluted from the conjugated Sepharose by washing with distilled water and 0.1 M $NaHCO_3$, determined from the extinction at 280 nm. Direct estimation of the amount of protein coupled was carried out by amino acid analysis under the conditions described by Wilkinson et al. [8]. Under the conditions described 1.5 mg of troponin C were coupled to 1 g wet weight of Sepharose. In all cases wet weight refers to the weight of Sepharose which had been sucked to dryness on a glass sinter. If stored in the urea buffer troponin C–Sepharose could be used with no apparent loss in effectiveness one month after preparation and after twenty cycles of operation.

3. Results and discussion

When purified components of the troponin complex from rabbit skeletal muscle were applied individually to the troponin C–Sepharose column in 8 M urea, 50 mM Tris–HCl buffer (pH 8.0), 15 mM 2-mercaptoethanol, 1 mM $CaCl_2$, only troponin I was bound and subsequently eluted by addition of 10 mM EGTA to the buffer. The capacity of the troponin C–Sepharose conjugate was approximately 0.25 mg troponin I per gram of Sepharose containing approximately 1.5 mg of bound troponin C per g wet weight. On application of the troponin complex from white skeletal muscle of the rabbit to the column, troponin I was selectively bound as was the case when troponin I alone was passed down the column. This indicates that the Sepharose-bound troponin C possessed a sufficiently strong affinity for troponin I to remove this protein preferentially from the troponin I–troponin C complex [1–3] that exists in the aqueous phase in the presence of excess Ca^{2+}. In the presence of $CaCl_2$ the troponin I was strongly bound to the conjugated Sepharose for it could not be removed by increasing the ionic strength of the eluting solution by adding 0.5 M KCl to the buffer. Nevertheless addition of 10 mM EGTA effectively eluted troponin I when 0–0.5 M KCl was added to the buffer. If the urea concentration of the buffer in which the applied troponin complex was dissolved was less than 8 M some troponin C was also eluted with the troponin I when EGTA was added to the buffer. As troponin C is not bound to the column independently this suggests that in addition to the specific Ca^{2+}-requiring interaction between troponin I and troponin C, there is a non-specific interaction that can be broken down by the higher urea concentration. This observation also suggests that this interaction occurs independently of the Ca^{2+}-requiring troponin I–troponin C interaction, by means of which troponin I is held in the column (see [3, 5]).

Troponin I bound to the troponin C–Sepharose column and subsequently eluted by addition of 10 mM EGTA to the buffer had the same specific inhibitory activity [3] on the Mg^{2+}-stimulated ATPase of desensitized actomyosin as the protein before application. It was also indistinguishable in electrophoretic mobility on electrophoresis in 0.1% sodium dodecyl sulphate, 100 mM sodium phosphate buffer (pH 7.0) on 10% polyacrylamide gels [9] from troponin I that had not been subjected to affinity chromatography. Troponin I isolated from troponin by affinity chromatography also formed the complex with troponin C in 8 M urea, 25 mM Tris 80 mM glycine buffer (pH 8.6), 5 mM $CaCl_2$ [2] which migrated as a single band on polyacrylamide gel electrophoresis under these conditions with mobility identical with that of the complex obtained with unchromatographed troponin I.

When whole muscle, removed from a rabbit

immediately after death, was homogenized in 8 M urea, 75 mM Tris–HCl buffer (pH 8.0), 15 mM 2-mercaptoethanol, 1 mM $CaCl_2$, as described in the Methods, and applied to a troponin C–Sepharose column most of the protein passed through without binding. After washing with buffer until the extinction of the eluate was identical with that of the buffer applied, addition of 10 mM EGTA to the buffer caused the elution of a small peak which in the case of rabbit psoas muscle amounted to about 0.25–0.5 mg of protein per gram wet weight of muscle applied. (fig. 1) The protein migrated as a single band on

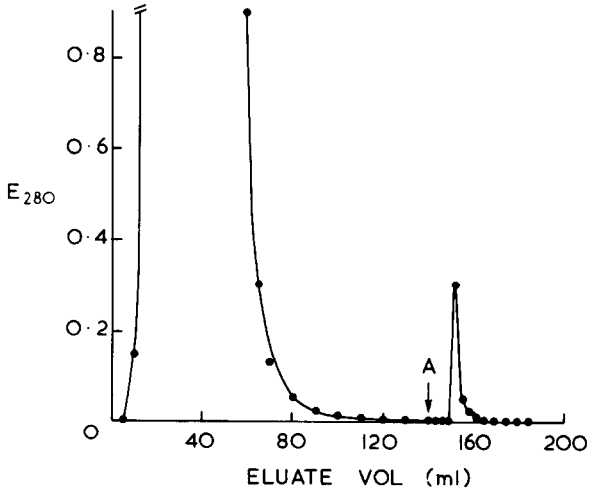

Fig. 1. Chromatography of an extract of rabbit soleus muscle on a troponin C–Sepharose column, 4 g of rabbit soleus muscle dispersed in 40 ml of 8 M urea, 75 mM Tris–HCl buffer (pH 8.0), 1 mM $CaCl_2$, 15 mM 2-mercaptoethanol as described in the Methods and applied to a column of troponin C–Sepharose (8 cm × 2 cm) At point A elution of bound protein is carried out with the same buffer containing 10 mM EGTA.

polyacrylamide gel electrophoresis in sodium dodecyl sulphate, and in 6 M urea, 5% acetic acid, pH 3.5 [10]. It formed the characteristic complex with troponin C from rabbit skeletal muscle and 8–10 μg of the protein inhibited the Mg^{2+}-stimulated ATPase of 300 μg desensitized actomyosin by 50% in the presence of excess tropomyosin. In these properties and amino acid analysis it was identical with samples of inhibitory protein isolated from the troponin complex by the standard procedure.

Comparison of troponin I from rabbit psoas, with that isolated from soleus and cardiac muscles of the same species, indicated that marked differences existed between the protein isolated from each of these striated muscles. On electrophoresis in sodium dodecyl sulphate, troponin I from psoas and soleus muscle migrated with similar mobilities corresponding to an apparent molecular weight of 23 000 whereas the cardiac troponin I was clearly different as has been reported elsewhere [2, 11–13] with an apparent molecular weight of 29 000. Likewise on electrophoresis in 15% polyacrylamide gels containing 6 M urea, 5% acetic acid (pH 3.2), the preparations of troponin I from psoas and soleus muscles had similar mobilities whereas cardiac troponin I migrated about 15% slower (fig 2a, b).

Although apparently similar when compared by electrophoresis alone, the troponin I of psoas and soleus were not identical, for the complexes formed in each case with troponin C from rabbit white skeletal muscle [2, 3] migrated with different mobilities (fig. 2a). It was noted that the troponin I from soleus muscle, judging by the mobilities of the bands formed on complexing with troponin C from white skeletal muscle consisted of two types, a minor component apparently identical with that obtained with troponin I from white rabbit muscle, and a major component which formed a complex of mobility similar to that obtained with cardiac troponin I and white skeletal muscle troponin C. Cardiac troponin I appeared to be relatively homogeneous as judged by these criteria.

These results strongly suggested that the troponin I of white skeletal and cardiac muscles and the major component of troponin I of soleus muscle were not identical.

This conclusion was confirmed by gel electrophoresis of the cyanogen bromide digest of troponin I isolated from each of the three muscles by the method of Gross and Witkop [14]. The patterns of such digests were very different (fig. 2b) suggesting that the primary structural differences between the three proteins are considerably greater than one might expect in simple polymorphic forms of a given protein. This implies that the synthesis of this protein is under the control of at least three structural genes.

The structural differences were reflected in the biological activity of the preparations. When tested

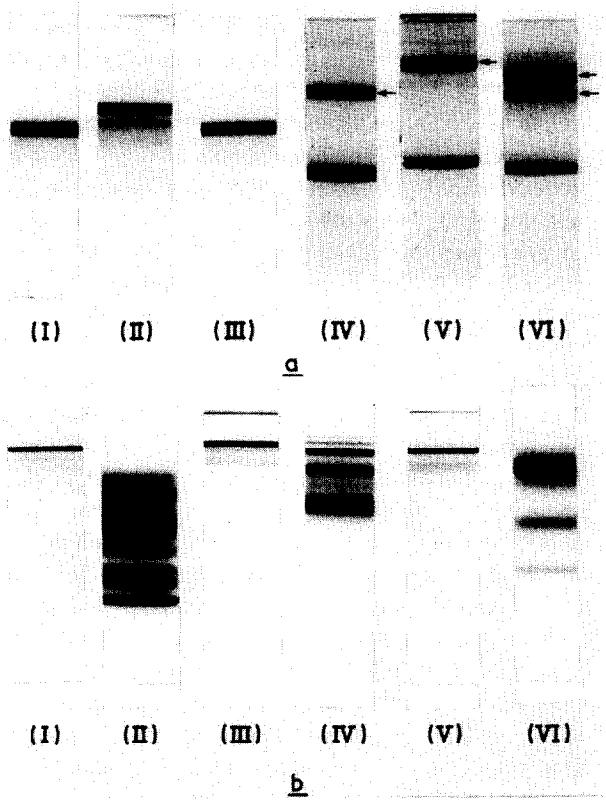

Fig. 2. Polacrylamide gel electrophoresis of troponin I(TN-I) preparations isolated by affinity chromatography from different striated muscles of the rabbit. Electrophoresis conditions: a. (i)–(iii) in sodium dodecyl sulphate (pH 7.0); (iv)–(vi) 6 M urea, 25 mM Tris–80 mM glycine (pH 8.6). b. (i)–(vi) 6 M urea, 5% acetic acid (pH 3.2). In a (iv) to (vi) the fastest band is excess troponin C(TN-C) and the arrows indicate the complex of troponin C and troponin I. a. (i) 40 μg L. dorsi TN-I; (ii) 60 μg heart TN-I; (iii) 40 μg soleus TN-I; (iv) 40 μg L. dorsi TN-I + 80 μg muscle skeletal TN-C; (v) 25 μg cardiac TN-I + 40 μg TN-C; (vi) 60 μg soleus TN-I + 80 μg TN-C. b. (i) 40 μg L. dorsi TN-I; (ii) *(i)* after cyanogen bromide digestion; (iii) 50 μg cardiac TN-I; (iv) *(iii)* after cyanogen bromide digestion; (v) 40 μg soleus TN-I; (vi) *(v)* after cyanogen bromide digestion.

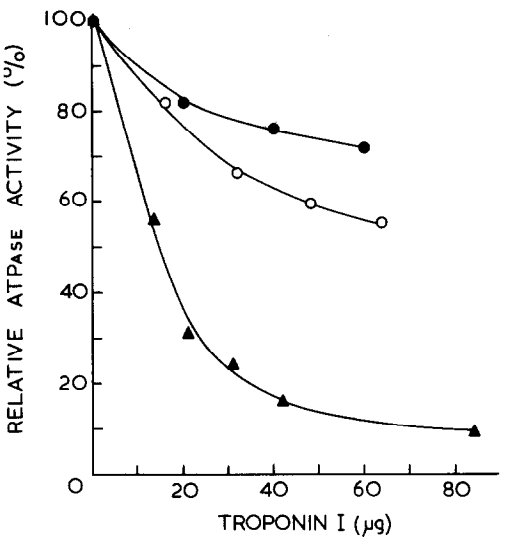

Fig. 3. Inhibitory activity of troponin I preparations from different striated muscles on the Mg^{2+}-stimulated ATPase of desensitised actomyosin. ATPase assays carried out in 25 mM Tris–HCl (pH 7.6), 2.5 mM ATP, 2.5 mM $MgCl_2$, 320 μg desensitised actomyosin. Tropomyosin: troponin I = 2:1 by weight. Total vol 2 ml incubated 5 min at 25°C. Activities expressed as percentage of that obtained in absence of troponin I. (▲) L. dorsi; (O) Soleus; (●) Cardiac.

against desensitized actomyosin of rabbit white skeletal muscle using tropomyosin from the same muscle (consisting of 80% α subunit, and of 20% β subunit [6, 15]) the inhibitory activities of the troponin I preparations were in the order white skeletal > red skeletal > cardiac muscle (fig. 3). The significance of these differences cannot yet be determined for as yet we have no information whether troponin I shows any specificity for full biological activity for the myosin and tropomyosin polymorphs characteristic of the muscle cell type from which it is derived.

These examples give some indication of the value of the method we have described for the direct isolation of troponin I from relatively small samples of muscle. Apart from simplicity and speed the method also has the additional advantage that possible degradative changes by tissue enzymes are reduced to a minimum by rapid homogenization of the whole muscle immediately after removal from the animal into strong urea solution. With more sensitive methods of detection of troponin I the procedure could be scaled down for use for biopsy samples of 30 to 40 mg of muscle. The problem of scaling up are simply those of preparing enough troponin C to prepare large amounts of troponin C–Sepharose. As the Ca^{2+}-dependent interaction of troponin C with troponin I is not species or muscle type specific the well-characterized and readily prepared troponin C

from rabbit white skeletal muscle can be used for preparing columns for general application.

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