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Calmodulin-free skeletal-muscle troponin C prepared in the absence of urea

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A method is described for the rapid preparation of electrophoretically pure troponin C from rabbit skeletal-muscle myofibrils that avoids the use of urea. The three-step procedure includes extraction of the myofibrils with EDTA-containing buffers, one-step elution from DEAE-Sephadex and Sephadex G-100 chromatography in the presence of EDTA. The procedure gives yields comparable with those of currently used methods that involve dissociation of the troponin complex with urea. Except for the thiol-group reactivity, troponin C produced by our method is physicochemically and functionally indistinguishable from that obtained by the classical procedure. Purified troponin C always contains traces of calmodulin. However, this contamination can be decreased to <0.02% by means of a second Sephadex G-100 chromatography step in the presence of EDTA.

Two different purification procedures are currently used for the preparation of troponin C from rabbit skeletal muscle. The classical procedure involves the extraction of troponin from native tropomyosin (Schaub & Perry, 1971) or from myofibrils (Ebashi *et al.*, 1971). The troponin complex is then dissociated with 6M-urea and the subunits further fractionated by DEAE-Sephadex gradient chromatography and gel filtration (Greaser & Gergely, 1971; Eisenberg & Kielley, 1974). A more recent method involves direct solubilization of whole muscle in 6M-urea followed by affinity chromatography on immobilized troponin I (Head *et al.*, 1977). Both procedures yield 200–300mg of troponin C/kg of muscle. The disadvantages of these methods are that the first is time-consuming and the second only applicable on a small scale. They both make use of urea, which may alter the physicochemical properties of troponin C. Moreover, it is open to question whether such preparations are not contaminated by calmodulin. Indeed, the presence of calmodulin has been demonstrated in skeletal muscle (Yagi *et al.*, 1978; Nairn & Perry, 1979), and the separation of the two proteins is hampered by their close structural similarity (Stevens *et al.*, 1976) and biological cross-reactivity (Amphlett *et al.*, 1976).

The primary goal of the present study was to develop a simple, large-scale and rapid procedure to produce high-purity troponin C, i.e. in which the calmodulin contamination can be controlled and kept very low; yields should be comparable with those of the existing purification procedures. The study also aimed at comparing the properties of the final product with those of troponin C prepared in the presence of urea, and to answer the question raised by Murray & Kay (1971) and Greaser *et al.* (1973), whether troponin C, purified under denaturing conditions, is still representative of the native protein.

Abbreviation used: Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

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Materials and methods

Proteins

Rabbit skeletal-muscle myofibrils were obtained by eight successive washings of the homogenized muscle with 5vol. of 40mM-Tris/HCl buffer, containing 25mM-KCl, 1mM-mercaptoethanol, 20μM-phenylmethanesulphonyl fluoride, 0.1μg of pepstatin/ml and 0.6mM-NaN_3, pH 7.0. During a ninth wash, the myofibrils were transferred in 5vol. of 20mM-Tris/HCl buffer, pH 7.8, containing 15mM-mercaptoethanol, 20μM-phenylmethanesulphonyl fluoride, 100μg of pepstatin/litre and 0.6mM-NaN_3 (buffer A). Rabbit skeletal-muscle troponin C and troponin I, prepared from troponin complex with the use of urea by the method of Ebashi *et al.* (1971), were provided by Dr. E. H. Fischer, University of Washington, Seattle, WA, U.S.A. Troponin C was also purified by the method of Schaub & Perry (1971), except that urea was omitted from the purification step involving ion-exchange chromatography. Bovine brain calmodulin and calmodulin-

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free phosphodiesterase were prepared by the method of Watterson *et al.* (1976). The concentration of calmodulin and of different preparations of troponin C were measured spectrophotometrically, assuming $A_{276}^{1\%}$ values of 1.8 and 2.0 respectively (Watterson *et al.*, 1976), or by the method of Lowry *et al.* (1951) or by that of Bradford (1976) with troponin C as standard.

Phosphodiesterase activity

This was assayed by the method of Boudreau & Drummond (1975). In assays with various concentrations of troponin C, the total protein content was kept constant by adding bovine serum albumin. Since up to 3mg of proteins in the assay (300μl) do not interfere with the enzymic test, and half-maximal activation of phosphodiesterase occurs at 15ng of calmodulin per assay, the limit of detection of contamination by calmodulin in a protein sample is very low, approx. 5p.p.m. (w/w).

Disc-gel electrophoresis

This was performed on 15% gel columns in the presence of 0.1% sodium dodecyl sulphate, 1mM-EDTA or 1mM-$CaCl_2$, as described by Laemmli (1970). The complex-formation between troponin C and troponin I was monitored by electrophoresis as described by Head & Perry (1974).

Apparent molecular weight determinations

These were performed on a column (2cm × 140cm) of Sephadex G-100 with bovine serum albumin (mol.wt. 67000), ovalbumin (43000), chymotrypsinogen (25000), myoglobin (17800) and cytochrome *c* (12400) as markers. The total and void volumes were determined with free Ca^{2+} and Blue Dextran respectively. The partition coefficients between the liquid and gel phases, $K_{av.}$, were calculated as described by Fischer (1969).

Thiol-group titration

This was carried out by the method of Habeeb (1972) with Nbs_2 from Calbiochem (San Diego, CA, U.S.A.).

Metal-binding

This was determined by equilibrium dialysis as described previously (Wnuk *et al.*, 1979). Portions (1ml) of troponin C (0.5mg/ml) were dialysed against 25mM-Tricine {*N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine}, pH7.4, containing 80mM-KCl, 0.1mM-dithiothreitol, 50μM-EGTA and the desired amounts of $CaCl_2$ and/or $MgCl_2$. After 48h of dialysis, the amount of Ca^{2+} and Mg^{2+} bound to the protein was calculated from the difference between inside and outside solutions, as measured with a Perkin–Elmer 303 atomic-absorption spectrophotometer. The free Ca^{2+} concentration was calculated by using the association constants of Martell & Smith (1974).

Results

Effect of troponin and troponin C on phosphodiesterase

A typical preparation of troponin, obtained by the method of Schaub & Perry (1971), activates bovine brain phosphodiesterase, but at concentrations 5×10^5-fold higher than those of bovine brain calmodulin (3mg of troponin per test versus 6ng of calmodulin for the same activation). When troponin was heated at 100°C for 2min before assay, activation occurred at concentrations (protein present before the heat step) 10-fold lower than those of native troponin, suggesting that the activating factor is released on heating. Troponin C obtained by the modified method of Schaub & Perry (1971) as described in the Materials and methods section activates the enzyme at concentrations 300-fold higher than those of calmodulin, whereas troponin C prepared by the method of Ebashi *et al.* (1971) activates at concentrations 1000–2000-fold higher than those of brain calmodulin. The high variance of these results suggests that troponin and troponin C lack the intrinsic ability to stimulate phosphodiesterase, but are contaminated by variable calmodulin. Indeed, when samples of troponin C, prepared by the modified method of Schaub & Perry (1971), or by that of Ebashi *et al.* (1971), were chromatographed on a Sephadex G-100 column equilibrated in a buffer containing EDTA, the endogenous factor, which activates phosphodiesterase, eluted as a symmetrical peak distinctly beyond the peak of troponin C (Fig. 1). The activity of the sample charged on the column is entirely recovered in the second peak without a shoulder at the elution position of troponin C. Furthermore, the $K_{av.}$ value of the activating factor corresponds to that of bovine brain calmodulin (Fig. 1*a*). These results show that the activation of phosphodiesterase attributed to troponin C is due to contaminating calmodulin.

Apparent molecular weights of troponin C and calmodulin

The separation by gel filtration of two proteins of nearly identical molecular weights, namely troponin C and calmodulin, can be explained in the light of their abnormal behaviour during Sephadex chromatography, especially in the presence of EDTA. Fig. 2 shows the elution profile of a mixture of equal amounts of troponin C and calmodulin on a Sephadex G-100 column equilibrated with buffer A containing Ca^{2+} or EDTA. In the presence of Ca^{2+} (Fig. 2*b*), the two proteins elute with $K_{av.}$ values of

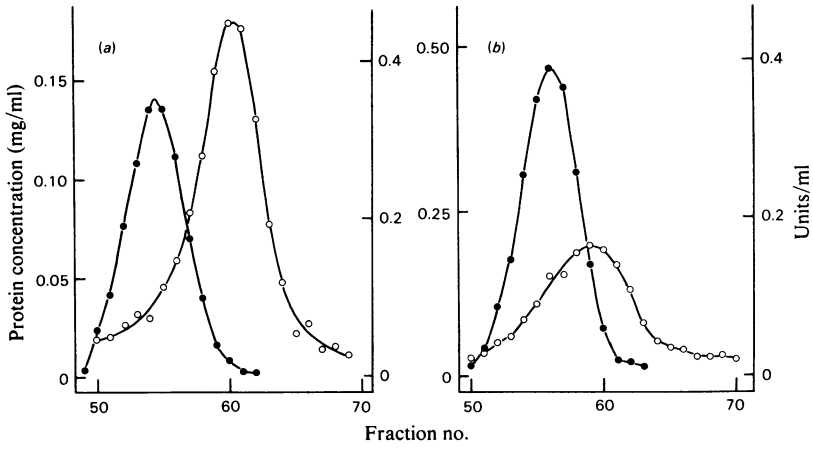

Fig. 1. *Sephadex G-100 chromatography of troponin C prepared by the modified method of Schaub & Perry (1971) (a) and that of Ebashi et al (1971) (b)*

A column (2cm × 140cm) was equilibrated in 20mM-Tris/HCl buffer, pH 7.8, containing 15mM-mercaptoethanol, 20μM-phenylmethanesulphonyl fluoride, 200μg of pepstatin/litre, 0.6mM-NaN_3 and 5mM-EDTA. Total protein concentration (●) was measured by the method of Bradford (1976). The activating factor (○) was monitored by the phosphodiesterase assay. One unit corresponds to 1μg of calmodulin.

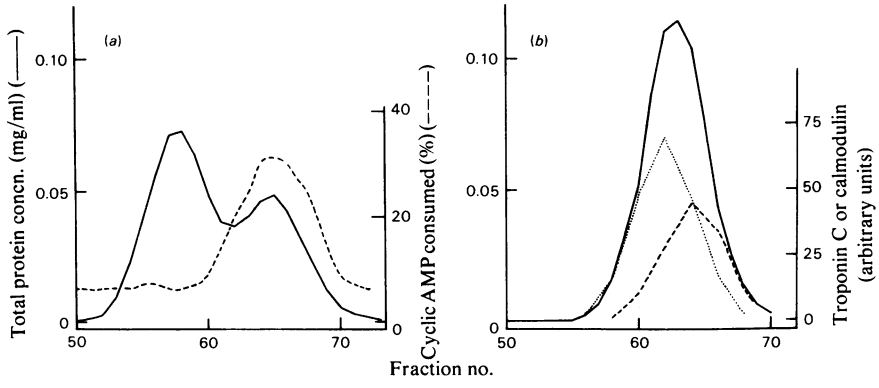

Fig. 2. *Elution behaviour of troponin C and calmodulin on a Sephadex G-100 column (2cm × 140cm) in the presence of 5mM-EDTA (a) or 150μM-$CaCl_2$ (b)*

Total protein concentration (——) was measured by the method of Bradford (1976). (*a*) In the presence of EDTA, the elution profile of calmodulin was monitored by the phosphodiesterase assay. (*b*) In the presence of $CaCl_2$, the elution profile of troponin C (· · · ·) and calmodulin (– – – –) were monitored by gel scanning and expressed in arbitrary units.

0.39 for troponin C and 0.41 for calmodulin; this corresponds to apparent mol.wts. of 37000 and 33000 respectively. In the presence of EDTA (Fig. 2*a*), however, the proteins have $K_{av.}$ values of 0.34 for troponin C and 0.44 for calmodulin, corresponding to apparent mol.wts. of 43500 and 31000, respectively. A similar difference in the elution of troponin C in the presence or absence of Ca^{2+} was observed by gel chromatography on Sephadex G-200 (Head & Perry, 1974).

Purification of troponin C from myofibrils without the use of urea

Preparation of electrophoretically pure troponin C. In preliminary attempts to purify actomyosin-contaminated troponin on DEAE ion-exchange columns without urea, some troponin C dissociated from the complex, provided that EDTA was included in the column buffer. Furthermore, when myofibrils from 200g of muscle were repeatedly extracted with 400ml of buffer A containing

5 mM-EDTA, each extraction yielded an amount of troponin C proportional to the quantity of troponin C present in the myofibrils (Table 1) and obeyed the equation for partition of one component between two immiscible phases (Vogel, 1956):

$$Q_n = \frac{KQ_T}{(1+K)^n} \quad (1)$$

where Q_n is the quantity of troponin C obtained at the nth extraction, Q_T the quantity of total extractable troponin C and K the partition coefficient. Plotted according to eqn. (1), the data of Table 1 show that the myofibrils from 200 g of muscle contain 120 mg of extractable troponin C; a very similar value was obtained by gel scanning after disc-gel electrophoresis by the method of Head & Perry (1974) on non-extracted myofibrils in the presence of urea and EDTA, with troponin C as a standard. Total muscle contains about twice as much troponin C (Head & Perry, 1974; Geigy, 1963), suggesting that during the preparation of myofibrils part of troponin C dissociates. Table 1 shows that seven extractions are sufficient to extract 90% and three extractions nearly two-thirds of the troponin C present.

On the basis of this observation, an efficient and simple purification procedure was devised. The pooled supernatants of three myofibrillar extractions were dialysed against buffer A containing 5 mM-EDTA and 0.2 M-NaCl, and loaded on a 25 ml column of DEAE-Sephadex A25 equilibrated in the same buffer. The gel was washed until no more proteins were eluted, as monitored by the method of Bradford (1976). The troponin C-containing fractions obtained by one-step elution with the column buffer containing 0.7 M-NaCl were brought to 6 mM-$CaCl_2$, dialysed against water to remove EDTA and freeze-dried. The protein, resolubilized in water, was passed through a Sephadex G-100 column (2 cm × 140 cm) equilibrated in buffer A containing 5 mM-EDTA. Troponin C was eluted after a peak with a nucleotide-like spectrum and was followed by a shoulder contaminated with calmodulin (Fig. 3). All fractions of troponin C without visible contamination by calmodulin (as monitored

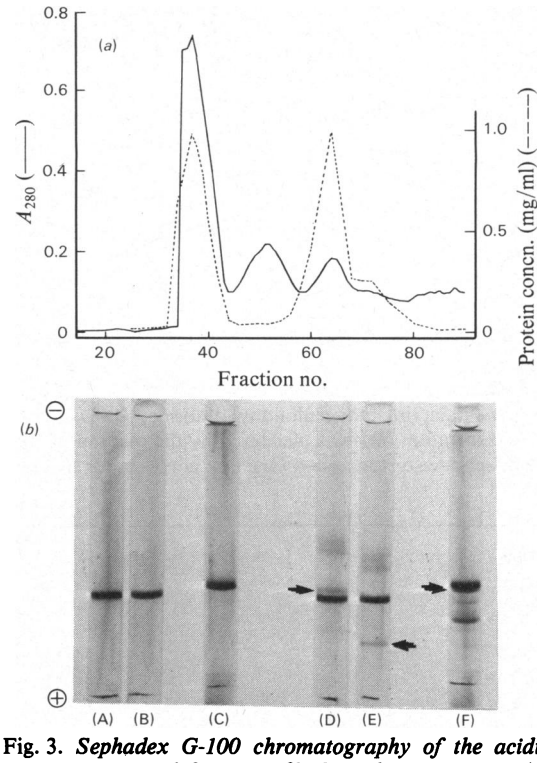

Fig. 3. *Sephadex G-100 chromatography of the acidic proteins extracted from myofibrils with 5 mM-EDTA (a) and disc-gel electrophoresis of selected fractions (b)* Protein concentration was measured by the method of Bradford (1976) with troponin C as a standard. A photograph of disc-gel electrophoresis on 15% gel columns of fraction nos. 64 (A, B and C) and 68 (D, E and F) in the presence of 1 mM-$CaCl_2$ (A and D), 1 mM-EDTA) (B and E) or 0.1% sodium dodecyl sulphate (C and F) is shown in (*b*). The arrows indicate the position of bovine brain calmodulin in the three different systems.

Table 1. *Yield of troponin C after successive extractions of myofibrils from 200 g of rabbit skeletal muscle with 400 ml of EDTA-containing buffer*

Extraction	Volume (ml)	Total protein extracted (mg)*	Troponin C extracted (mg)†
1st	540	166	33.4
2nd	350	109	22.1
3rd	375	95.5	17.7
4th	360	102	14.0
5th	385	91	9.8
6th	375	63	5.7
7th	390	57	4.7

* Determined by the method of Bradford (1976) with bovine serum albumin as standard.

† Determined by disc-gel electrophoresis in the presence of 1 mM-EDTA and subsequent scanning with troponin C as a standard. Results are mean values for duplicate experiments.

by disc-gel electrophoresis) were dialysed against water and freeze-dried.

Preparation of calmodulin-free troponin C. Electrophoretically pure troponin C, obtained as described above, still contains appreciable amounts of calmodulin as shown by the phosphodiesterase test. To decrease this contamination, the freeze-dried protein was dissolved in 2ml of water and passed again through the same Sephadex G-100 column (2cm x 140cm) equilibrated in buffer A containing 5mM-EDTA. Fig. 4 shows that the contamination by calmodulin in the first half of the troponin C peak is <0.02%, which represents the lower limit of detection by the phosphodiesterase test at the troponin C concentration used in this experiment. Contamination by calmodulin increases markedly in the second half of the peak. Hence, the purity of the troponin C samples depends on which fractions are included in the pool. For instance, when fractions 52 to 61 were pooled, the contamination amounted to 0.014% as determined by the phosphodiesterase test.

The balance-sheet of the purification of troponin C from myofibrils (Table 2) indicates that troponin C virtually free of calmodulin can be obtained in reasonably good yields as compared with the currently used techniques, even though no more than three extractions were performed.

Comparison of troponin C prepared with and without the use of urea

Troponin C isolated with the use of 6M-urea might have different properties from the native C subunit of troponin, especially if renaturation is not fully reversible. As the procedure described here yields a troponin C that was never in contact with urea, a comparison of the two samples was of interest. Both proteins migrate on polyacrylamide-disc-gel electrophoresis in the presence of EDTA or Ca^{2+}, with indistinguishable mobilities. Circular dichroism from 200 to 250nm showed no significant difference in the spectra of the two proteins, indicating a very similar secondary structure. Equilibrium dialysis experiments as described in the Materials and methods section indicated that both troponin C samples contain two Ca^{2+}/Mg^{2+} sites, two Ca^{2+}-specific sites and one to two Mg^{2+}-specific sites (Table 3), in good agreement with the results of Potter & Gergely (1975). The same complexes with rabbit skeletal-muscle troponin I (Fig. 5) were obtained by both troponin C preparations when monitored by electrophoresis by the method of Head & Perry (1974).

However, there is one property that may be altered by urea treatment, namely the reactivity of thiol groups. On titration with Nbs_2, troponin C prepared by the procedure described here contains

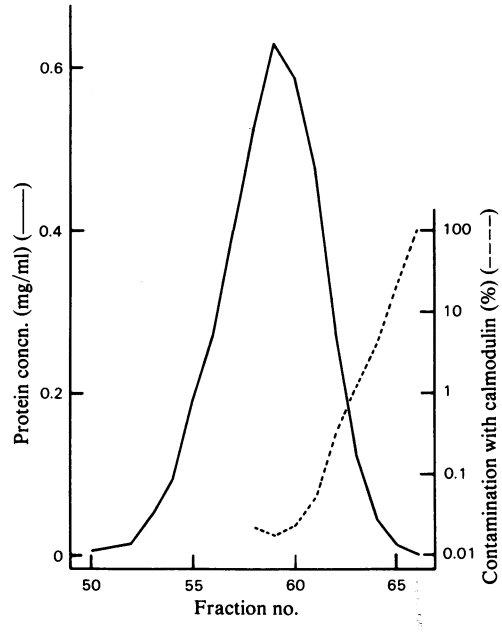

Fig. 4. *Second Sephadex G-100 chromatography of the fractions from Fig. 3 containing troponin C*

Protein concentration was monitored by the method of Bradford (1976) with troponin C as a standard; the calmodulin contamination was measured by the phosphodiesterase test with bovine brain calmodulin as a standard. Fractions 53–57 do not activate significantly phosphodiesterase, even when as much as 150μl per test was added. Therefore the calmodulin contamination of these fractions is not depicted.

Table 2. *Summary of a typical purification of troponin C from myofibrils present in 1 kg of rabbit skeletal muscle*

	Total proteins (mg)*	Troponin C (mg)	Yield (%)	Purity (%)
Three EDTA extractions	1556	336†	100	23
DEAE-Sephadex	563	361†	98.5	64
First Sephadex G-100	227	223†	62.0	98.5‡
Second Sephadex G-100	147	147*	40.0	99.98‡

* Determined by the method of Bradford (1976).
† Determined by gel scanning after disc-gel electrophoresis in the presence of 1mM-EDTA, with troponin C as standard.
‡ With respect to calmodulin contamination determined by the phosphodiesterase test.

Table 3. *Ca^{2+} and Mg^{2+} binding to troponin C prepared with or without the use of urea*
Equilibrium dialysis in 25 mM-Tricine buffer, pH 7.4, containing 80 mM-KCl and 0.1 mM-dithiothreitol.

	Bound Ca^{2+} (mol/mol of protein)		Bound Mg^{2+} (mol/mol of protein)	
	+Urea	−Urea	+Urea	−Urea
$CaCl_2$ (0.1 mM)	3.98	3.90	—	—
$CaCl_2$ (0.1 mM) + 1 mM-$MgCl_2$	3.96	4.04	1.05	1.29
$MgCl_2$ (1 mM) + 1 mM-EGTA	—	—	3.62	4.44
$CaCl_2$ (33 nM)*	0.72	0.76	—	—
$CaCl_2$ (0.28 μM)*	1.36	1.31	—	—

* Free Ca^{2+} concentration in the presence of a 50 μM-EGTA buffer.

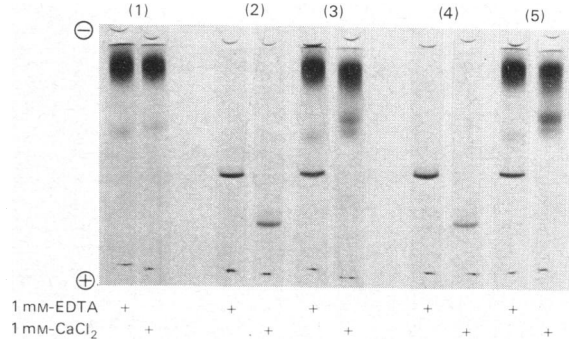

Fig. 5. *Complex-formation between troponin C (3 μg/assay) and troponin I (13 μg/assay) as monitored by disc-gel electrophoresis (15% gels) in the presence of 6 M-urea and EDTA or $CaCl_2$*
(1) Troponin I; (2) troponin C prepared without use of urea; (3) troponin I + troponin C prepared without use of urea; (4) troponin C prepared with use of urea; (5) troponin I + troponin C prepared with use of urea.

0.98 thiol group/molecule. In contrast, troponin C prepared in the presence of urea no longer possesses a reactive thiol group; at a protein concentration of 30 μM, and in the presence of 2 mM-EDTA and 6 M-urea, the increase in A_{412} is negligible. The effect of urea on thiol-group reactivity is not always consistent, as Potter *et al.* (1976) claimed to have titrated a thiol group of urea-prepared troponin C. In the presence of Ca^{2+}, the thiol group of troponin C prepared without urea can be fully titrated with a second-order rate constant of $106\ M^{-1} \cdot s^{-1}$, a value that compares well with that of Potter *et al.* (1976).

Discussion

The purification of troponin C described here is based on the finding that, in the presence of EDTA, much more than in the presence of Ca^{2+}, myofibrils gradually release their total troponin C content, which can be removed by centrifugation. No other components of the troponin complex are released since no significant complexation of troponin C occurs in the presence of Ca^{2+} when the supernatants are monitored by electrophoresis (results not shown). The amount of troponin C in the supernatants follows the partition law indicating an equilibrium between bound and free troponin C. Our results indicate that, in the presence of EDTA, the association of troponin C to the myofibrils is weak. Potter & Gergely (1974), as well as van Eerd & Kawasaki (1973), showed by polyacrylamide-disc-gel electrophoresis in the absence of urea but in the presence of EGTA the existence of complexes between troponin C and troponin I or T. However, these complexes are in equilibrium with considerable amounts of non-associated proteins (van Eerd & Kawasaki, 1973). The arrangement of troponin in the thin filaments is such that in the presence of Ca^{2+} troponin C and troponin I are in close contact, which is not the case in the presence of EGTA (Sutoh, 1980).

From Table 1, it can be deduced that about seven successive extractions would be sufficient to remove nearly all the troponin C present in the myofibrils. Calmodulin is also extracted, but, in contrast with troponin C, it is predominantly released during the first extraction with EDTA-containing buffer. Hence, when the desired end product is calmodulin-free troponin C, the first extract can be discarded and the subsequent ones used for further purification. These extracts still contain important amounts of proteins other than troponin C and calmodulin. To decrease the concentration of bulk proteins and to perform the Sephadex G-100 chromatography under optimal conditions, these contaminating proteins were removed by a fast DEAE ion-exchange chromatography with one-step elution. The remaining contaminants, i.e. calmodulin and a protein that co-migrates with the light-chain 3 of myosin, were finally removed by Sephadex G-100 chromatography. Dedman *et al.* (1977) reported that rabbit skeletal-muscle troponin C fully stimulated bovine

brain phosphodiesterase, although at a 600-fold higher concentration than calmodulin; this is apparently due to the presence of contaminating calmodulin.

Another goal of this study, besides developing a rapid and large-scale procedure for the purification of calmodulin-free troponin C, was to evaluate the extent of renaturation of troponin C after treatment with 6 M-urea, which results in extensive unfolding of the peptide backbone (Nagy & Gergely, 1979). The secondary structure, metal-binding properties and complex-formation with troponin I of troponin C samples prepared with or without the use of urea appear identical. Tensiometric measurements performed on skinned muscle fibres soaked with troponin I showed that both troponin C preparations are equally efficient in relieving the inhibition of tension development (experiments kindly performed for us by Dr. G. Kerrick at the University of Washington, Seattle, WA, U.S.A.). However, the thiol-group reactivity shows significant differences between the two troponin C samples, which might be explained by carbamoylation of the thiol group (Stark, 1964) on prolonged exposure of the protein to high concentrations of urea. Apparently the extent of this reaction, which results in the disappearance of the titrable thiol group, varies in different laboratories. The procedure described here offers the advantage of being independent of urea and hence eliminates the risk of protein modification or incomplete refolding.

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