Cardiac troponin-C: a rapid and effective method of purification

Jolanta Szynkiewicz, Dariusz Stępkowski, Hanna Brzeska and Witold Drabikowski

Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warsaw, Poland

Received 4 December 1984

A new purification procedure for cardiac troponin-C is described which has several advantages over previous methods. High purity of the final product was assessed by electrophoretic, enzymatic and spectroscopic methods.

Cardiac muscle *Troponin-C*

1. INTRODUCTION

Cardiac troponin-C (cTN-C) calcium-binding protein has been less studied than troponin-C from skeletal muscle. The main factor limiting studies on cTN-C is the low yield obtained from its preparation. All isolation procedures described so far are based on the extraction of troponin complex and subsequent separation of cTN-C [1,2]. Although these procedures yield 1600 mg troponin complex per kg cardiac muscle [1] the yield of cTN-C is very low (10–15 mg/kg muscle) [1,2]. Similar purification procedures applied to skeletal muscle [3,4] yield about 200 mg troponin-C per kg tissue.

Here, we describe a procedure which yields 160 mg cTN-C per kg muscle. The purification procedure is based on the extraction of cTN-C from muscle with a solution containing 6 M urea, followed by two rapid chromatographic steps on DEAE-Sephadex and Phenyl-Sepharose columns.

2. MATERIALS AND METHODS

2.1. *Purification of cTN-C*

The whole purification procedure was carried out at 4°C. Cardiac troponin-C was isolated from bovine hearts cleaned of fat and connective tissues. Fresh (or stored frozen at −30°C) bovine heart muscle (250 g) was minced in a meat grinder and then homogenized for 1 min in an Atomix blendor at high speed in 500 ml of solution I (0.1 M KCl, 10 mM EDTA, 10 mM β-mercaptoethanol, 50 mM Tris–HCl; pH 7.5). The suspension was centrifuged for 1 h at 24000 × *g*, the sediment was homogenized again in 500 ml of solution I and centrifugation was repeated. The sediment obtained was homogenized for 1 min in 500 ml of solution II (0.2 M KCl, 6 M urea, 10 mM EDTA, 10 mM β-mercaptoethanol, 50 mM Tris–HCl; pH 7.5), diluted twice with the same solution and stirred for 30 min. Insoluble components were spun down at 24000 × *g* for 1 h and the viscous, brown supernatant was mixed with 20 g (dry wt) of DEAE-Sephadex A-25 equilibrated with solution II. The suspension was gently stirred with a glass rod for 1 h, allowed to sediment and the Sephadex residue was washed several times with solution II to obtain a colourless solution above the ion exchanger layer. Proteins bound to Sephadex were eluted with 400 ml of solution III (0.8 M KCl, 6 M urea, 10 mM β-mercaptoethanol, 50 mM Tris–HCl; pH 7.5). The eluate was dialysed first against 0.1 M KCl, then against 2 mM NH_4HCO_3 and concentrated to 50 ml on a PM 10 Diaflo ultrafiltration membrane (Amicon Corp.). $CaCl_2$, Tris–

Abbreviations: CD, circular dichroism; UV, ultraviolet

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

HCl (pH 7.5) and solid NaCl were added to the concentrated protein solution to obtain final concentrations 15 mM, 50 mM and 0.5 M, respectively. The above mixture was applied to a Phenyl-Sepharose column (2 × 22 cm) equilibrated with solution IV (15 mM $CaCl_2$, 50 mM Tris–HCl, pH 7.5). The unbound proteins were washed out with 280 ml solution IV and cTN-C was eluted subsequently with 350 ml solution V (15 mM EDTA, 50 mM Tris–HCl, pH 7.5). The presence of cTN-C in collected fractions was determined by SDS electrophoresis. Fractions containing pure cTN-C were pooled, concentrated as described above, and desalted on a Sephadex G-25 column equilibrated with 2 mM NH_4HCO_3. About 40 mg cTN-C was routinely obtained from 250 g muscle.

2.2. *Spectral measurements*

Fluorescence measurements were recorded in a 1 cm thick cell with a Perkin-Elmer MPF-3 spectrofluorometer and the UV absorption spectra were obtained with a Cary 219 (Varian) spectrophotometer in 0.5-cm thick cells at 20°C. The UV absorption spectra were corrected for solution turbidity according to [5] by plotting the dependence of log absorbance of solution vs log wavelength and extrapolating the linear dependence between these values from the range 320–400 nm to the absorption range 255–310 nm. The CD spectra were measured with a Jasco J-41A instrument calibrated according to Cassim and Yang [6]. The measurements were performed in a 0.176 mm thick cell at 10°C.

2.3. *Determination of cTN-C concentration*

The concentration of purified cTN-C was determined using an extinction coefficient of $E^{1\%}_{276\,nm}$ = 3.0 in the absence and $E^{1\%}_{276\,nm}$ = 3.1 in the presence of Ca^{2+}. The concentration of cTN-C used to calculate the above values was obtained by amino acid analysis using a Beckman 119 CL instrument.

2.4. *Isolation of other proteins and enzymatic assays*

Rabbit skeletal troponin-1 and bovine brain calmodulin were prepared as in [4,7]. Phosphodiesterase preparation and assays were performed as described by Cheung [8] and inorganic phosphate was determined by the method of Fiske and SubbaRow [9].

2.5. *Electrophoresis*

SDS- and urea-polyacrylamide gel electrophoresis was performed according to Weber and Osborn [10] and Perrie and Perry [11] with the modification described by Drabikowski et al. [12], respectively.

3. RESULTS AND DISCUSSION

There are 3 major steps in our purification procedure for cTN-C here. Firstly, a muscle homogenate was washed with a solution containing EDTA which removes most of the sarcoplasmic proteins. This treatment is especially important in washing out calmodulin [13]. Fig.1A shows that the discarded fraction contains a protein which migrates in urea gel with a mobility identical with that of calmodulin. Secondly, the insoluble fraction containing the myofibrillar proteins was solubilized in 6 M urea and 0.2 M KCl. Under these conditions cTN-C was predominantly bound to the DEAE-Sephadex whereas the majority of proteins were not (fig.1B, sample I, II). The third step is based on the observation that cTN-C, like calmodulin [14] and skeletal troponin-C [7], is able to bind hydrophobically to phenyl-Sepharose in the presence of Ca^{2+} while the other acidic proteins cannot. The high A_{260}/A_{280} ratio observed in the fractions eluted from the phenyl-Sepharose column with a calcium-containing solution indicates that under these conditions nucleotide-containing material is also removed. Cardiac TN-C is released from phenyl-Sepharose by EDTA treatment because of its lower hydrophobicity in the absence of Ca^{2+} (fig.1B, sample III).

The purity of this preparation was checked electrophoretically, by phosphodiesterase assay and spectral measurements. The cTN-C preparation migrates as one band in SDS- as well as in urea-polyacrylamide gel electrophoresis (fig.1B, sample III, fig.1C, sample I, II). As shown previously the mobility of cTN-C in urea electrophoresis depends on the presence of Ca^{2+} (fig.1C, sample I, II) [13]. The ability of cTN-C to form a complex with troponin I was examined electrophoretically in the presence of 5 M urea and Ca^{2+}. The appearance of the cTN-C–troponin-I complex is shown in fig.1C, sample III.

The absence of traces of calmodulin, a protein with physicochemical properties very similar to

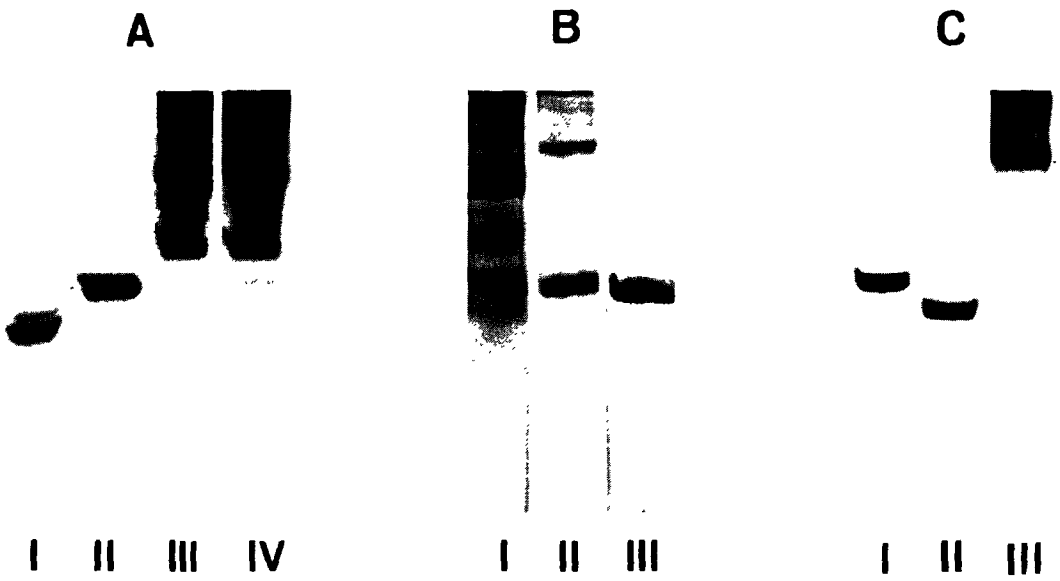

Fig.1. Polyacrylamide gel electrophoresis. (A) In the presence of urea; calmodulin + 2 mM $CaCl_2$ (I), calmodulin + 2 mM EDTA (II), supernatant discarded after first step of preparation + 2 mM $CaCl_2$ (III) or + 2 mM EDTA (IV). (B) In the presence of SDS; soluble fraction of urea suspension before mixing with Sephadex (I), fractions eluted from DEAE A-25 column with solution containing 6 M urea and 0.8 M KCl (II), and fractions eluted from phenyl-Sepharose column with solution containing EDTA (III). (C) In the presence of urea; cTN-C + 2 mM $CaCl_2$ (I), cTN-C + 2 mM EDTA (II), cTN-C + skeletal troponin-I + 2 mM $CaCl_2$ (III).

those of cTN-C, in the cTN-C preparation was checked by phosphodiesterase activity test. Our cTN-C, at a concentration 1000-times higher than that of calmodulin which saturated the enzyme, did not result in detectable activation of phosphodiesterase.

Cardiac troponin-C contains 3 tyrosine and 8 phenylalanine residues but no tryptophan [15]. The high phenylalanine to tyrosine ratio is reflected in the UV absorption spectra (fig.2). The UV absorption as well as fluorescence (not shown) spectra of the cTN-C preparation do not reveal the presence of tryptophan-containing proteins.

The CD spectra of cTN-C (fig.3) show that its secondary structure is highly ordered, which excludes the possibility that significant structural changes occurred during purification. The $[\theta]_{222}$ values (table 1) are lower than those given in [2,16], which is mainly attributed to the lower temperature of measurements [17].

As reported by others, the binding of Ca^{2+} to cTN-C causes changes in its UV absorption, fluorescence and CD spectra [2,16,18]. We have observed all these changes for cTN-C prepared by our method (table 1).

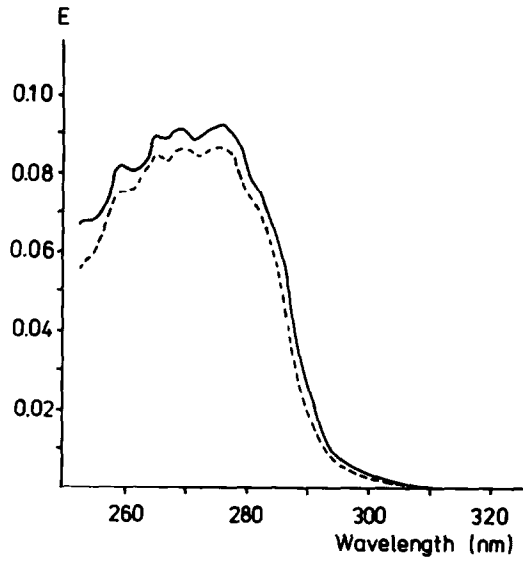

Fig.2. UV absorption spectra of cardiac troponin-C. Protein concentration was 0.58 mg/ml. Measurements were performed in a 0.5 cm thick cell in 20 mM Hepes-NaOH (pH 7.0) in the presence of 2 mM $CaCl_2$ (——) or 2 mM EDTA (– – –) at 20°C.

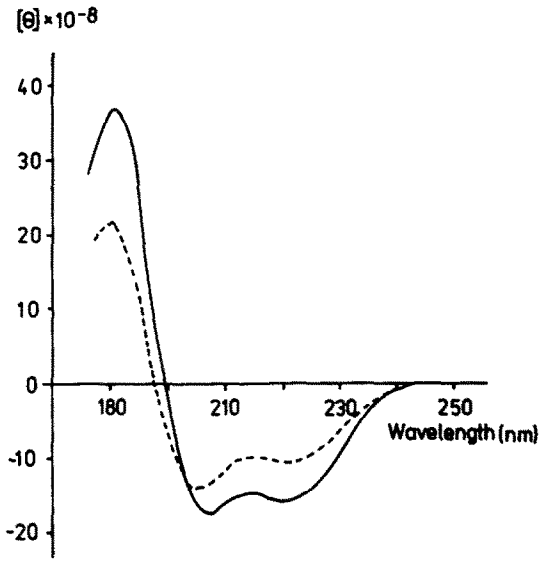

Fig.3. CD spectra of cardiac troponin-C. Measurements were performed in 20 mM Hepes-NaOH (pH 7.0) in the presence of 2 mM $CaCl_2$ (——) or 2 mM EDTA (– – –) at 11°C.

Table 1

Physicochemical properties of cardiac troponin-C

	In the presence of	
	EDTA	Ca^{2+}
$[\theta]_{222}$	−10500	−15750
$E^{1\%}_{276\,nm}$	3.0	3.1
Mobility in urea electrophoresis, R_f	0.44	0.51
Relative tyrosine fluorescence intensity at 306 nm	1.0	1.6

The fluorescence measurements were performed in 50 mM Hepes-NaOH (pH 7.0) in the presence of 2 mM EDTA or 2 mM $CaCl_2$ at room temperature. The excitation wavelength was 286 nm. The fluorescence intensity for sample containing EDTA was taken as unity. For conditions of other measurements see legends to figs 1–3

The calcium-dependent change in the $[\theta]_{222}$ value (53%) is higher than those reported by Leavis and Kraft [2] (33%) and Burtnick et al. [16] (28%) and is close to those observed for skeletal troponin-C [19] (50%). In the case of skeletal troponin-C the changes in the CD spectra reflect the conformational changes occurring in its calcium-binding domains III and IV [19]. The high homology in amino acid sequence [15] and similar affinity for Ca^{2+} [2,19] of these domains in skeletal and cardiac troponin-C lead one to expect similar changes in the CD spectra of both proteins upon binding of Ca^{2+}.

The method of preparation of cTN-C described above has some advantages over previous methods. The yield is more than 10-times higher than reported so far and is comparable to that of skeletal muscle troponin-C. The procedure is short and the final product practically free of calmodulin.

ACKNOWLEDGEMENTS

We thank Dr S.Yu. Venyaminov for performing some of the spectral experiments and Dr I. Kąkol, J. Kuźnicki and M.G. Sarzała for helpful discussion and critical reading of the manuscript.

REFERENCES

[1] Brekke, C.J. and Greaser, M.L. (1976) J. Biol. Chem. 251, 866–871.
[2] Leavis, P.C. and Kraft, E.L. (1978) Arch. Biochem. Biophys. 186, 411–415.
[3] Greaser, M.L. and Gergely, J. (1971) J. Biol. Chem. 246, 4226–4233.
[4] Drabikowski, W., Dąbrowska, R. and Barylko, B. (1973) Acta Biochim. Polon. 20, 181–199.
[5] Winder, A.F. and Gent, W.L.G. (1971) Biopolymers 10, 1243–1251.
[6] Cassim, J.Y. and Yang, J.T. (1974) Biochemistry 8, 1947–1951.
[7] Brzeska, H., Szynkiewicz, J. and Drabikowski, W. (1983) Biochem. Biophys. Res. Commun. 115, 87–93.
[8] Cheung, W.Y. (1971) J. Biol. Chem. 246, 2859–2869.
[9] Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–380.
[10] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[11] Perrie, W. and Perry, S.V. (1970) Biophys. J. 119, 31–38.
[12] Drabikowski, W., Kuźnicki, J. and Grabarek, Z. (1977) Biochim. Biophys. Acta 485, 124–133.
[13] Drabikowski, W., Kuźnicki, J. and Grabarek, Z. (1978) Comp. Biochem. Physiol. 606, 1–6.

[14] Gopalakrishna, R. and Anderson, W.B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836.
[15] Van Eerd, J.P. and Takahashi, K. (1976) Biochemistry 15, 1171–1180.
[16] Burtnick, L.D., McCubbin, W.D. and Kay, C.M. (1975) Can. J. Biochem. 53, 15–20.
[17] Brzeska, H., Venyaminov, S., Grabarek, Z. and Drabikowski, W. (1983) FEBS Lett. 153, 169–173.
[18] Hincke, M.T., McCubbin, W.D. and Kay, C.M. (1978) Can. J. Biochem. 56, 384–395.
[19] Leavis, P.C., Rosenfeld, S.S., Gergely, J., Grabarek, Z. and Drabikowski, W. (1978) J. Biol. Chem. 253, 5452–5459.