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Preparative reversed-phase liquid chromatography of proteins from rabbit skeletal troponin, a multi-protein complex

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Abstract

A reversed-phase high-performance liquid chromatography protocol for purification of all proteins in a multi-protein (TnI, TnC, TnT, tropomyosin) complex from rabbit skeletal muscle has been developed, enabling efficient purification of sample amounts ranging from 43 mg of protein complex on a standard analytical column, to 1400 mg on a column of 21.2 mm I.D. and finally, to 5700 mg on a column of 50 mm I.D. Due to problems associated with scale-up procedures for these proteins (e.g. aggregation and/or solubility issues), an initial sample fractionation was devised whereby 50% of the TnC component was precipitated with acetonitrile prior to sample introduction on the RPLC column. By subsequently taking advantage of sample overload conditions to enhance the displacement effect between sample components, coupled with very slow gradient conditions (0.1% acetonitrile/min), we were able to achieve excellent protein separations at high yields of purified proteins.

Keywords: Preparative chromatography; Proteins

1. Introduction

Striated (skeletal) muscle contraction involves the interaction of the thick and thin filaments of myosin and actin, respectively, with Ca^{2+}-dependent regulation of vertebrate skeletal muscle requiring the regulatory proteins troponin (Tn) and tropomyosin (Tm) to be bound to the actin thin filament [1]. The troponin complex consists of troponin I (TnI), the inhibitory protein; troponin T (TnT), the tropomyosin binding protein; and troponin C (TnC), the Ca^{2+}-binding protein. A representation of the molecular arrangement of the thin filament proteins is shown in Fig. 1. Changes in the interactions between members of the Tn complex and Tm control muscle contraction in a Ca^{2+}-dependent manner. In order to understand at the molecular level how the binding of Ca^{2+} to TnC leads to the eventual movement of Tm proposed in the steric blocking model, extensive structure/function studies using a variety of techniques have been carried out to determine the structure, function and sites of interactions between each of the troponin components and their change in conformation upon binding calcium (reviews can be found in Refs. [2–4]). Our own approach, over many years, to try and understand muscle regulation at the molecular level is to determine the functionally active regions in these proteins, as well as determining the sites of interaction between these proteins in the presence and absence of calcium (Refs. [5–12] represent selected publications of our laboratory in this field of research). Thus, we have a continual and,

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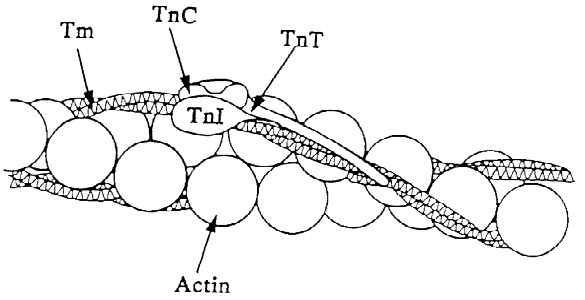


Fig. 1. Schematic representation of the molecular arrangement of skeletal muscle thin filament proteins. Two strands of the F-actin molecules (open circles) form the core of the structure. Tropomyosin (Tm) molecules are shown as coiled-coils which lie in the grooves of the actin filament. Each Tm molecule spans seven G-actin monomers on each of the two strands of the actin filament and interacts with one troponin (Tn) complex, comprising troponin T (TnT), troponin I (TnI) and troponin C (TnC). The Tm/Tn complex forms the basic regulatory unit of the thin filament.

indeed, growing requirement for isolating substantial amounts of the individual Tn subunits, from this multi-protein regulatory complex in as efficient a manner as possible.

In the present study, we describe the development of a high-performance preparative multistep protocol utilizing the minimum number of chromatographic modes (reversed-phase liquid chromatography, RPLC) and mobile-phase conditions to optimize resolution and yield in purifying sample amounts ranging from milligrams of protein complex to multigram levels. In addition, problems associated with scale-up procedures for these proteins (e.g. aggregation, poor solubility and/or complex formation) are discussed together with our approaches to overcoming such difficulties.

2. Experimental

2.1. Materials

HPLC-grade water and acetonitrile were obtained from BDH (Poole, UK). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI, USA). ACS-grade orthophosphoric acid and triethylamine (TEA, redistilled before use) were obtained from Anachemia (Toronto, Canada). Dithiothreitol (DTT) was purchased from ICN Biomedicals (Aurora, OH, USA). Ethylene diamino bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid (EDTA) and ammonium hydrogen carbonate were purchased from Sigma (St. Louis, MO, USA). Rabbit skeletal muscle was purchased from Pel-Freeze Biologicals (Rogers, AR, USA), followed by extraction and purification of troponin according to the protocol of Ebashi et al. [13]. Individual troponin components were purified as described by Ingraham and Hodges [14].

2.2. RPLC packing

The packing chosen for the scale-up procedure was a silica-based C_8 packing of 15–20 μm particle size and 300 Å pore size, specifically μBondapak C_8 material. Large particle materials (e.g. 10–15 μm, 15–20 μm, 20–30 μm) are generally used in large-scale purification since they are considerably less costly than small particle materials, result in lower column back-pressures and are easier to pack in large diameter columns [15–19].

2.3. Instrumentation

Analytical and some preparative RPLC runs were carried out on a Varian Vista Series 5000 chromatograph (Varian, Walnut Creek, CA, USA) coupled to a HP 1040A detection system (Hewlett-Packard, Avondale, PA, USA), HP9000 series 300 computer, HP9133 disc drive, HP2225A Thinkjet printer and HP7460A plotter. Large-scale (multigram samples) preparative RPLC runs were carried out on a Prochrom LC50 instrument equipped with a self-packing column using dynamic axial compression (DAC) technology [20]. Column packing was carried out either on a Shandon HPLC packing pump (Sewickley, PA, USA) or a Haskell Model 29426 column packing system (Burbank, CA, USA).

2.4. Column packing procedures

For the 4.6 mm I.D. and 21.2 mm I.D. columns, the silica packing was suspended in isopropanol to a slurry concentration of ~5% (w/v) and degassed under vacuum for 5 min. The packing was then allowed to settle and most of the isopropanol decanted off. The slurry was then packed downward and vertically at 5000 p.s.i. into a 250×4.6 mm I.D.

(Shandon pump) or 250×21.2 mm I.D. (Haskell pump) stainless steel HPLC column, with isopropanol as packing solvent and nitrogen as the pressurizing gas (1 p.s.i.=6894.76 Pa). The solvent flow was allowed to continue until 100 ml (4.6 mm I.D. column) or 1000 ml (21.2 mm I.D. column) of isopropanol had passed through the column.

For the self-packing 50 mm I.D. column, the slurry was prepared as above and then packed as described in Ref. [20].

3. Results

3.1. Analytical RPLC of muscle proteins

The excellent resolving power of RPLC has made it the predominant HPLC technique for peptide separations [21–23]. Indeed, not only is RPLC usually superior to other modes of HPLC with respect to both speed and efficiency, but it also offers the widest scope for manipulation of mobile phase conditions to optimize separations. In addition, the availability of volatile mobile phases makes it ideal for both analytical and preparative separations, either as the sole HPLC mode or as the final purification/desalting step of a multi-step purification protocol [24]. Although not as commonly employed for protein separations as it is for small peptides, frequently due to concerns of protein denaturation by the hydrophobic stationary phases and non-polar organic modifiers characteristic of RPLC [21–23], this technique has still often proven to be useful for preparative purification of proteins (Ref. [25] and references cited therein). Indeed, in the present study, concern about denaturation is not an issue, for the following reasons: (i) as noted previously, complete dissociation of the Tm/Tn regulatory complex (as well as dissociation of the two polypeptide chains of Tm), in addition to complete unfolding of the Tn subunits, is ideal for simple and effective purification of the four proteins; and (ii) previous work has shown that denaturation of the troponin complex by high urea concentrations or denaturation that is effected by RPLC was reversible when the protein subunits were reconstituted [9,14].

Fig. 2 shows an analytical elution profile of crude rabbit skeletal Tn at pH 2.0, under run conditions (linear gradient of acetonitrile in aq. TFA mobile phase) frequently employed for RPLC of polypeptides [21,23]. Apart from the three Tn subunits, some Tm is also present. It was necessary for the separation to be carried out in the presence of EDTA in order to ensure the absence of metal ions since, with

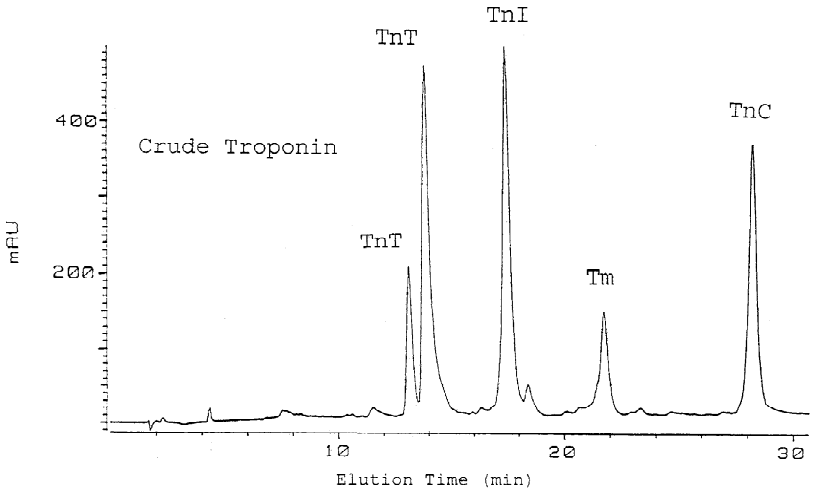


Fig. 2. Analytical RPLC of crude rabbit skeletal troponin. Column: Zorbax SB300 C_8 (150×4.6 mm I.D.; 5 μm particle size, 300 Å pore size) from Agilent Technologies. Conditions: linear A–B gradient (1% B/min starting from 25% acetonitrile) at a flow-rate of 1 ml/min, where eluent A is 0.05% aq. TFA and eluent B is 0.05% TFA in acetonitrile; 26 °C. The sample (~2 mg/ml) was dissolved in 0.5% aq. TFA containing 0.5 m*M* EDTA and 1 m*M* DTT. TnT, TnI, Tm and TnC denote troponin T, troponin I, tropomyosin and troponin C, respectively.

Ca^{2+} present, the troponin I–troponin C interaction is extremely stable, e.g. the I–C complex will not dissociate even during PAGE in the presence of 6 *M* urea. In addition, the presence of reducing agent (DTT) prevents disulphide bond formation (TnC, TnI and Tm contain one, three and one cysteine residue(s), respectively). Finally, in order to ensure denaturation of all proteins in the protein mixture, the crude Tn sample was dissolved in 25% acetonitrile in aq. TFA (pH 2.0) prior to injection, thus minimizing the possibility of multiple protein conformers due to protein–protein interactions or only partial denaturation by the hydrophobic stationary phase. From Fig. 2, it can be seen that all four proteins are well separated under these conditions. Interestingly, TnT is eluted as a doublet, with subsequent amino acid analysis determining this to be a result of different TnT isoforms.

At first sight, the excellent analytical separation shown in Fig. 2 would suggest a relatively straightforward scale-up to milligram amounts of protein sample and, the ultimate goal, to multigram-scale separations and the latter was now attempted. However, as shown below, certain complications immediately became apparent when attempting to increase sample concentration of the crude Tn for subsequent preparative scale-up.

3.2. Effect of Tn concentration on solubility of Tn subunits

Whole crude Tn was now dissolved in 0.05% aq. TFA at concentrations ranging from 0.2 to 14 mg/ml. The solutions were then centrifuged (1000 *g*) and aliquots of the supernatants were subsequently analyzed by RPLC under the same conditions as shown in Fig. 2. Fig. 3 now shows the levels of TnI and TnC in the supernatants over the whole range of sample concentrations attempted. Assuming that the subunits are certainly completely solubilized at the lowest sample concentration (0.2 mg/ml), it can be seen that TnI maintained good solubility at pH 2 throughout the entire sample concentration range. Similar profiles were obtained for TnT, as well as for Tm, although these results are omitted for the sake of clarity. In contrast, the amount of TnC in the supernatant decreased substantially with increasing sample concentration, reaching a level of only ~50% from that detected at low sample concentration of troponin. With each increase in the amount of crude complex we were attempting to dissolve, there was also a concomitant increase in the size of the pellets formed after centrifugation. Subsequent solubilization of these pellets (in 10 m*M* aq. ammonium hydrogen carbonate), followed by analytical RPLC as shown previously (Fig. 2), revealed these pellets to be almost entirely comprised of the missing TnC. The bars above the plots in Fig. 3 denote acceptable and unacceptable Tn concentrations in terms of loss of subunit (particularly TnC) yield as sample concentration is raised. As noted previously, efficient preparative scale-up is best achieved when maximizing sample concentration and, hence, minimizing sample volume. Clearly, from Fig. 3, the preferred sample concentration for preparative chromatography (at least 10 mg/ml, preferably more, would appear reasonable) is not even close to being achieved under these conditions.

3.3. Effect of pH on solubility of Tn subunits

It was now decided to investigate whether there was a pH value where, not only were the three Tn subunits satisfactorily soluble, but also where the proteins may be applied at this optimum pH to preparative RPLC runs. Thus, aliquots (100 μl) of solutions (~1 mg/ml) of the individual proteins in water were made up to a 1 ml volume with buffers of different pH values (pH 2–7), centrifuged at 1000 *g* and identical aliquots of the supernatants subsequently applied to RPLC under the same conditions shown in Fig. 2. In addition, any protein pellets apparent after centrifugation were dissolved in 0.05% aq. TFA (except for TnC) and aliquots subsequently applied to RPLC. Fig. 4 shows the effect of pH on the solubility of TnI and TnT. Both the neutral (in terms of isoelectric point (p*I*); Table 1) TnT and basic TnI show good solubility over the range of pH 2–5, but show a rapid decrease in solubility as the pH is raised further. In contrast, under these low concentrations, the acidic TnC subunit (pI=4; Table 1) showed good solubility over the entire pH range investigated (data not shown). Interestingly, 10 m*M* ammonium hydrogen carbonate was the most favorable sample solvent for TnC in terms of symmetrical peak shape when subsequently chromatographed.

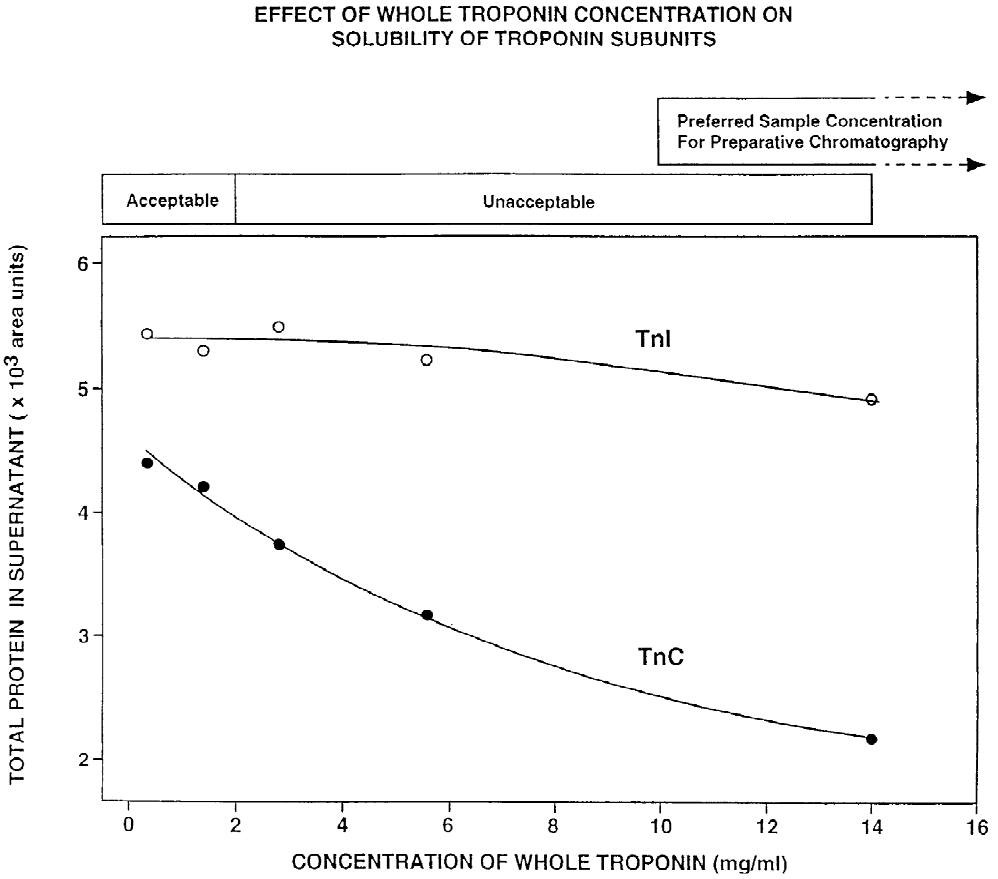


Fig. 3. Solubility of troponin subunits at various concentrations of the troponin complex. Experimental details are described in Section 3.2. TnI and TnC denote troponin I and troponin C, respectively.

From Fig. 4, pH 4–5 appeared to be a possible compromise for RPLC of crude Tn if all three subunits could be successfully chromatographed at these pH levels, in addition to whether the solubility of TnC was improved at these more elevated pH values. However, before the latter was investigated, it was deemed necessary to determine whether all three subunits could, indeed, be successfully chromatographed in the pH 4–5 range. Thus, aliquots (100 μl) of aqueous solution (~1 mg/ml) of the individual proteins were made up to a 1 ml volume in 20 m*M* aq. triethylammonium phosphate (TEAP) (pH 3, 4, 5) and applied to RPLC using the same linear acetonitrile gradient as previously (Fig. 2), but under pH 3, pH 4 or pH 5 run conditions. All three subunits chromatographed satisfactorily at pH 3 (indeed, TnC had previously been found to exhibit a satisfactory elution profile over the entire pH 2–7 range; data not shown). However, somewhat unexpectedly, neither TnI nor TnT could be eluted from the RPLC column at either pH 4 or 5, despite their solubility in aqueous solution at both pH values (Fig. 4).

To sum up: (i) preparative purification of TnC at low pH is impractical due to its tendency to precipitate at relatively low Tn sample concentrations; (ii) TnI and TnT are insoluble above pH 5.0; and (iii) even though they are soluble in aqueous medium at pH 4–5, TnI and TnT were not successfully chromatographed under pH 4 or pH 5 run conditions. To overcome this dilemma, it was now decided to approach a potential scale-up protocol by introducing an initial fractionation step, i.e. removal of at least one protein from the crude Tn sample prior to RPLC.

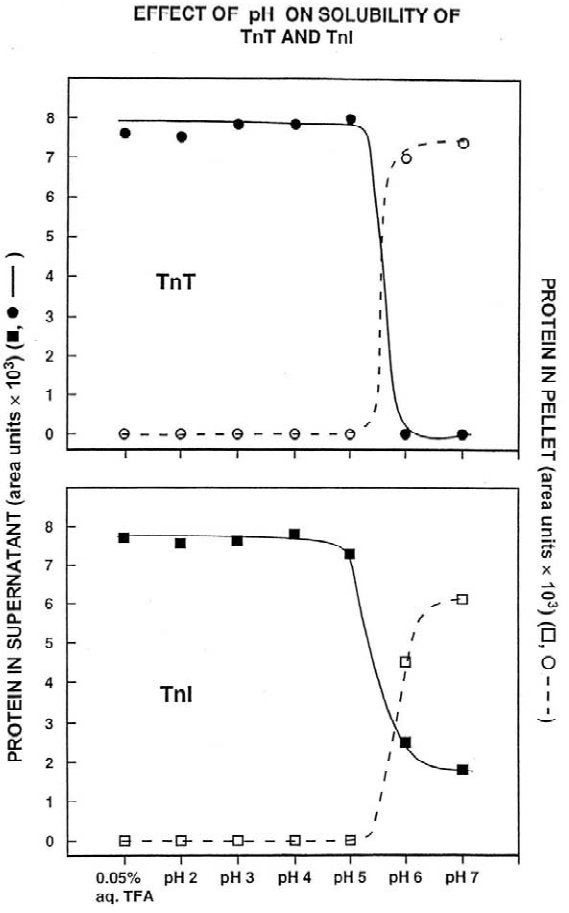


Fig. 4. Effect of pH on solubility of troponin T (TnT) and troponin I (TnI). Experimental details are described in Section 3.3. Buffers: 0.05% aq. TFA (pH 2.0); pH 2–pH 7 buffers were prepared by adding distilled triethylamine to 20 m*M* aq. orthophosphoric acid until the desired pH value had been obtained.

Table 1
Properties of rabbit skeletal muscle proteins

Protein	Number of residues	M_r	p*I*	Cys residues
Troponin C	159	17,965	4	1
Troponin I	178	20,700	10	3
Troponin T	259	30,500	7	0
Tropomyosin[a]	284	66,000	4.6	1

[a] Tm contains two identical α-helical chains (284 residues each) of M_r 33,000.

3.4. Precipitation of TnC with acetonitrile at pH 2.0

Selective protein precipitation with organic solvents is a very traditional approach to initial fractionation of proteins in solution. Such solvents promote the precipitation of proteins due to the decrease in water activity in the solution as the water is replaced by organic solvent [26]. The potential of this approach was now examined by adding increasing levels of acetonitrile to a solution (14 mg/ml) of crude Tn dissolved in 0.05% aq. TFA. As shown in Fig. 3, at this sample concentration, about 50% of TnC in the sample had already been precipitated from the sample, hence the smaller peak size for TnC in Fig. 5A compared to Fig. 2 where sample concentration was only ~2 mg/ml. It was now hoped that subsequent addition of acetonitrile to the sample, already containing depleted TnC, would enhance further the precipitation of this protein. The effects of acetonitrile levels of 10–60% on the crude Tn sample were determined by centrifugation of the sample (1000 *g*) at each solvent concentration, followed by RPLC of identical aliquots of the supernatant and solution (in 1 ml 10 m*M* aq. ammonium hydrogen carbonate) of the centrifuged pellet. Fig. 5A illustrates the RPLC elution profile of the Tm/Tn sample prior to addition of acetonitrile. Under these conditions, only 50% of the TnC remained in the supernatant applied to the column, the remainder having been precipitated prior to chromatography (Fig. 3). The profile shown in Fig. 5B now represents the composition of the sample following addition of 50% acetonitrile, i.e. only 7.6% of the TnC in the original crude sample remains, the bulk (92.4%) having precipitated at this acetonitrile concentration. Increasing the concentration of the solvent to 60% produced negligible further precipitation of the TnC. In addition, although most of the TnC was successfully precipitated at a 50% level of acetonitrile, the remainder of the sample proteins were little affected.

It should be noted that the RPLC elution profiles shown in Fig. 5 were (unlike the profile shown in Fig. 2) obtained on an analytical RPLC column containing a preparative C_8 packing of 15–20 μm particle size, hence the lack of resolution of the different TnT isoforms previously observed (Fig. 2;

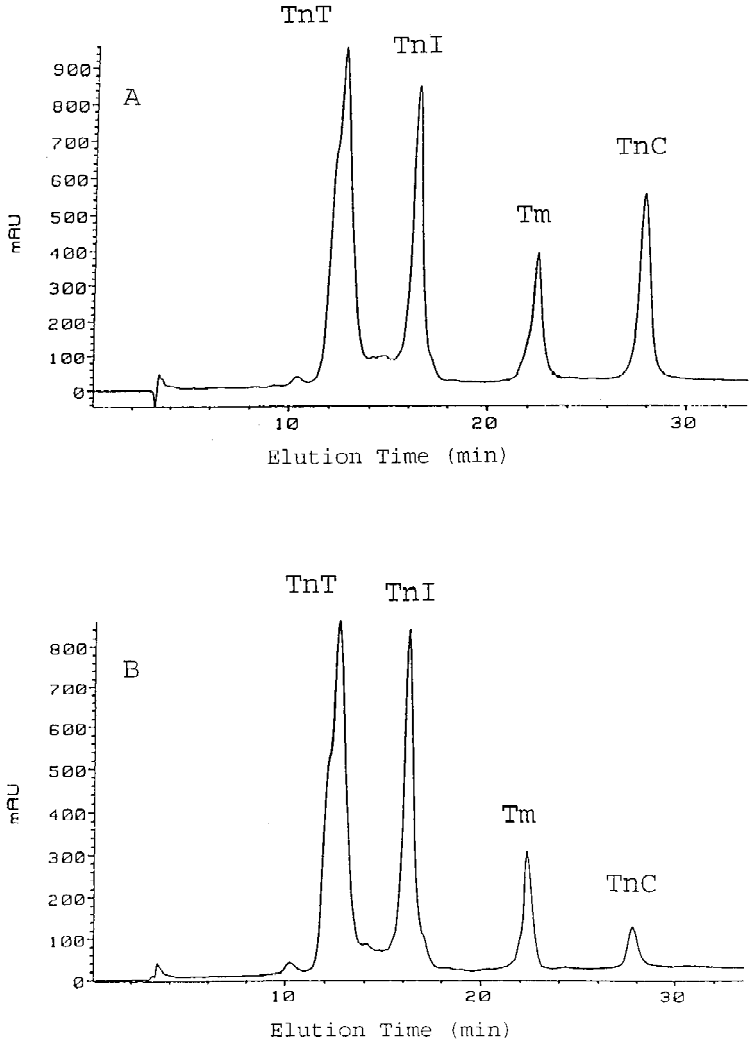


Fig. 5. Analytical RPLC of (A) crude rabbit skeletal troponin and (B) crude troponin following addition of 50% acetonitrile to aqueous solution of sample. Column as in Fig. 2. Conditions: linear A–B gradient (1% acetonitrile/min, starting from 25% acetonitrile) at a flow-rate of 1 ml/min, where eluent A is 0.05% aq. TFA and eluent B is 0.05% TFA in acetonitrile; 26 °C. The sample (14 mg/ml; sample volume, 30 μl) was dissolved in 0.05% aq. TFA (A) or 0.05% aq. TFA containing 50% acetonitrile (B). For both panels A and B, the sample solution also contained 0.5 m*M* EDTA and 1 mm DTT. For nomenclature, see Fig. 2.

5 μm particle size packing). For reasons explained below, this preparative C_8 packing was our packing of choice for our scale-up protocol.

3.5. Preparative RPLC of crude Tn

3.5.1. Protocol for preparative purification of crude Tn by RPLC

Based on the results discussed above and summarized in Figs. 2–5, the following procedure was chosen for preparative purification of the crude Tn complex by RPLC.

(i) Dissolve crude sample in 0.05% aq. TFA, containing 0.5 m*M* EDTA and 1 m*M* DTT, to a concentration of 12–15 mg/ml.
(ii) Add 0.05% TFA in acetonitrile to a final concentration of 50% (v/v acetonitrile) to precipitate TnC.

(iii) Centrifuge at 40,000 *g* for 1 h.
(iv) Decant supernatant; remove acetonitrile by rotary-evaporation.
(v) Apply remaining 100% aq. sample to RPLC column.
(vi) Redissolve pellet in 10 m*M* aq. ammonium hydrogen carbonate; analyze for TnC content and purify further if necessary.

Our final goal was to carry out a preparative run on a column of 50 mm I.D. In order to save sample material and chromatography eluents, it was decided to carry out an initial sample load study on an analytical (4.6 mm I.D.) column, followed by a preparative run on a column of intermediate size (21.1 mm I.D.). Following the latter run, load and run conditions could then be fine-tuned for the final large-scale purification. During this scale-up optimization strategy, it was important to use the same reversed-phase packing material at each preparative level for an accurate assessment of the necessary optimization steps.

3.5.2. Choice of RPLC packing

It has been demonstrated that both peptide [16] and protein [19] selectivity and retention are similar over a range of RPLC packings of different particle size, the major difference being that peak widths increase with increasing particle size, causing some resolution loss. However, for preparative separations, it is common to employ ''overload'' conditions (i.e. injecting crude samples at levels greater than sample capacity defined by optimum resolution [15–19,27–30] in order to maximize throughput). Under such conditions, large particle packings generally perform nearly as well as small particle packings [16,19]. Interestingly, it has also been demonstrated that 6.5 μm and 15–20 μm reversed-phase packings, when slurry packed with a HPLC pump, produced very similar (and very satisfactory) elution profiles of 10-residue peptide standards to those produced by these stationary phases when slurry packed by a commercial high-pressure column packer, underlying the relative ease with which the 15–20 μm particle materials may be packed to produce columns with the necessary efficiency [16]. Finally, a pore size of 300 Å is also recognized as a good compromise when purchasing reversed-phase materials for separations of polypeptides ranging from small peptides to larger peptides and proteins [22,31,32].

3.5.3. Preparative purification on RPLC column of dimensions 250×4.6 mm I.D.

Several preparative runs on this analytical column were carried out with increasing sample loads, with the largest (43 mg; Fig. 6, top) clearly illustrating the principles behind our scale-up strategy. Thus, following introduction of the sample, and a rapid gradient rise to 25% acetonitrile, the subsequent very shallow rising gradient (0.1%/min) effected a very efficient separation of TnT and TnI. This shallow gradient approach under overload conditions has been reported previously as achieving efficient separations, at high product yield and purity, for peptide mixtures [16,27,29]. Indeed, such an approach takes advantage of the overall loading capacity of the reversed-phase column, unlike smaller and analytical sample loads. In addition, a displacement effect can be observed for TnT, as illustrated by the higher concentration of this protein in its early stages of elution, such an effect being a well-recognized phenomenon in absorption chromatography techniques such as RPLC and ion-exchange chromatography at high sample load [16–18,27–29,33–37]. A further rapid rise in gradient rate (2.5%/min for 12 min) from 35% to 65% acetonitrile, followed by a period of isocratic elution at this highest acetonitrile level, served to elute any TnC off the column. As seen in Fig. 6a, top, this procedure also resulted in efficient resolution of Tm in the sample from both TnI and TnC.

3.5.4. Preparative purification on RPLC column of dimensions 250×21.2 mm I.D.

For the intermediate scale purification, a sample load of 1400 mg was now applied to the 21.2 mm I.D. column, a disproportionate scale-up compared to the smaller column (Fig. 6a) (a load of only 915 mg would have maintained the same relative sample load). This increased relative sample load was intended, not only to take advantage of the full adsorption capacity of the column, but also to accentuate the displacement effect of TnT, i.e. with as much pure TnT as possible displaced from the column during sample introduction. The results illustrated in Fig. 6a, middle, exhibit a generally

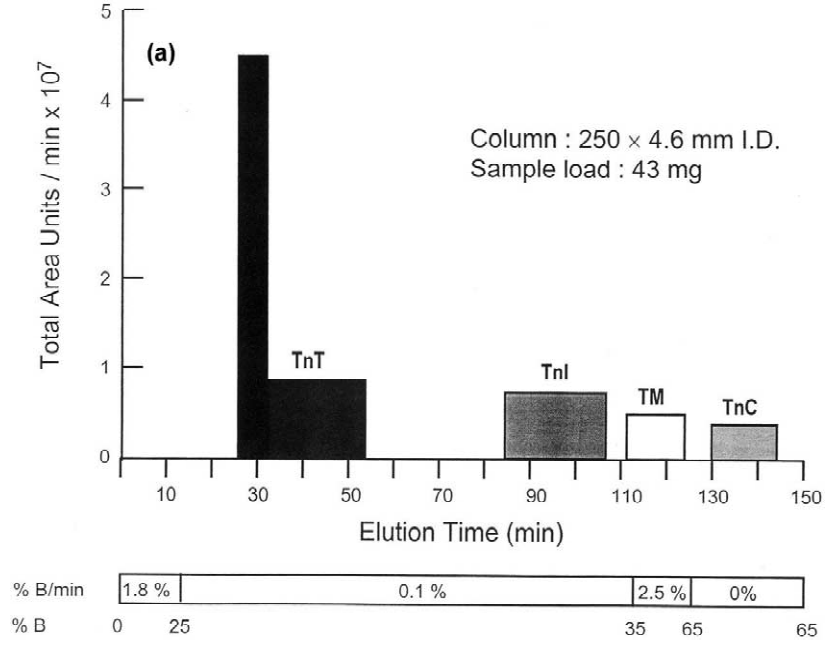
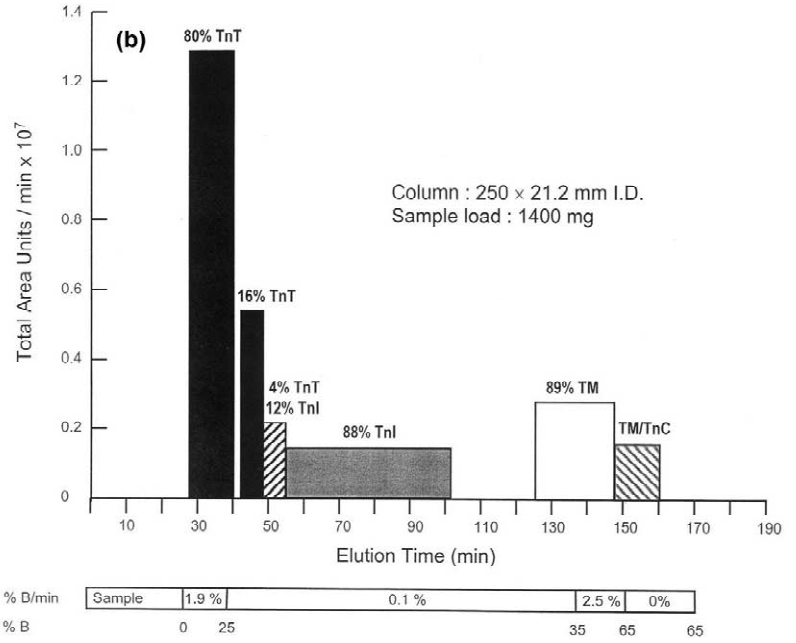


Fig. 6. Preparative RPLC of crude troponin. Columns: μBondapak C_8 packing (15 μm particle size, 300 Å pore size) in columns of dimensions 250×4.6 mm I.D. (a), 250×21.2 mm I.D. (b) and 280×50 mm I.D. (c). Conditions: (a) sample load, 43 mg in 3.5 ml 0.05% aq. TFA (~12 mg/ml); linear A–B gradient (1.8% acetonitrile/min) up to 25% acetonitrile, followed by 0.1% acetonitrile/min up to 35% acetonitrile, then 2.5% acetonitrile/min up to 65% acetonitrile and, finally, an isocratic hold of 65% acetonitrile; flow-rate, 1 ml/min (linear flow, 6.0 cm/min). b: sample load, 1400 mg in 100 ml 0.05% aq. TFA (14 mg/ml); following sample loading at 5 ml/min, a linear A–B gradient (1.9% acetonitrile/min) up to 25% acetonitrile, followed by 0.1% acetonitrile/min up to 35% acetonitrile, then 2.5% acetonitrile/min up to 65% acetonitrile and, finally an isocratic hold of 65% acetonitrile; flow-rate 10 ml/min (linear flow, 2.8 cm/min). c: sample load, 5700 mg in 440 ml 0.05% aq. TFA (~13 mg/ml); following sample loading at 22 ml/min, a 10-min isocratic hold with 0.05% aq. TFA, followed by a linear A–B gradient (1.7% acetonitrile/min) up to 25% acetonitrile, then 0.1% acetonitrile/min up to 35% acetonitrile and, finally, 0.5% acetonitrile/min up to 55% acetonitrile; flow-rate, 57 ml/min (linear flow, 2.9 cm/min). Summaries of these run conditions are also shown below the respective separations. For all three runs, eluent A is 0.05% aq. TFA and eluent B is 0.05% TFA in acetonitrile; temperature, 26 °C. Sample solutions for all three runs also contained 0.5 m*M* EDTA and 1 m*M* DTT. Prior to column loading, the majority of the TnC in the crude Tn had been precipitated with 50% acetonitrile and centrifuged down. The acetonitrile had then been removed by rotary-evaporation (for experimental details, see Section 3.5.1). For all three runs, 1-min fractions were collected and subsequently analyzed by analytical RPLC on the same type of packing material and under the same conditions as Fig. 2. The positions of the individual protein components identified following fraction analysis are denoted as histograms with the numbers above the histograms (runs b and c) representing the % yield of purified protein component compared to total yield of that component. The hatched histograms in the middle panel represent pooled overlap (i.e. contaminated) fractions, with 4% TnI of total yield of TnI contaminated with TnT and 11% of total yield of Tm contaminated with TnC. For nomenclature, see Fig. 2.

successful separation with 80% of the total yield of TnT eluted in purified form following 80% displacement from the column during sample loading. However, it can be seen that there is some overlap between the TnI and TnT fractions eluted from the column, due to the rapid rise to 25% acetonitrile (1.9%/min) being introduced too early following sample introduction at this high sample load. Hence, 4% of TnT was contaminated with TnI, also leading to a yield of just 88% pure TnI of total TnI yield. Similarly, the rapid rise in acetonitrile concentration (2.5%/min) towards the end of the run was introduced too early and/or was too steep a gradient, resulting in overlap in the Tm fractions (89% of total yield represented pure Tm) and 11% of the Tm was contaminated with the TnC. While still a good result, some fine-tuning was clearly required to maximize the potential yields of purified proteins.



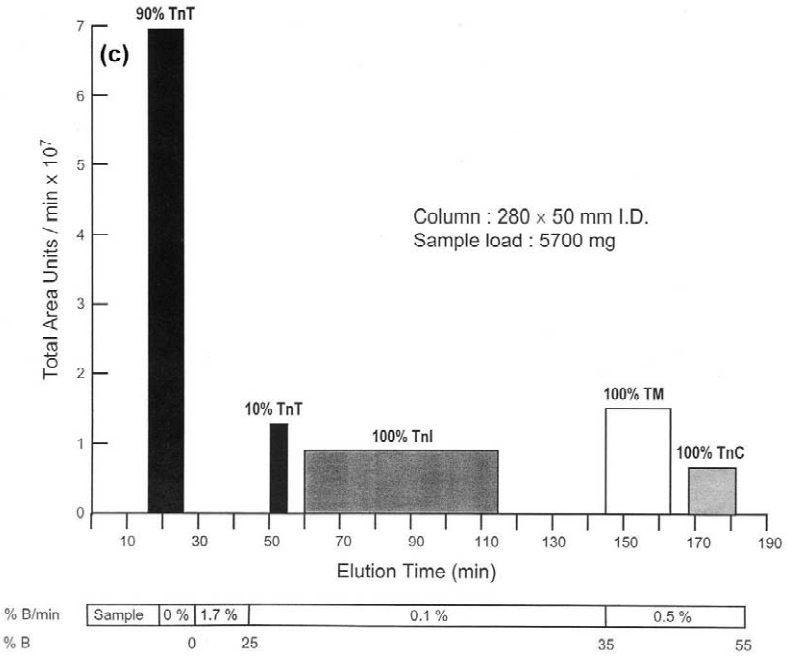


Fig. 6. (*continued*)

3.5.5. Preparative purification on RPLC column of dimensions 280×50 mm I.D.

Fig. 6c illustrates the results of our final scale-up protocol when applying a sample load of 5700 mg to the 50 mm I.D. column (a proportionate scale-up compared to the analytical column; Fig. 6a). Based on the results of the intermediate column (21.2 mm I.D.; Fig. 6b), a 10-min isocratic hold of 100% eluent A (0.1% aq. TFA) was introduced prior to the rapid rise (1.7% B/min) to 25% acetonitrile. This isocratic elution was designed to avoid the TnT/TnI overlap seen in fractions eluted from the intermediate column (Fig. 6b), as well as to maximize the displacement effect in 100% aqueous solvent. In addition, the subsequent rapid gradient rise from 35% to 65% acetonitrile (2.5%/min), followed by an isocratic elution of 65% aq. acetonitrile, towards the end of the separation (Fig. 6a and b) were replaced by a relatively shallow gradient (0.5%/min) from 35% acetonitrile throughout the remainder of the run. This change was intended to avoid the Tm/TnC overlap seen previously (Fig. 6b). The excellent results presented in Fig. 6c show the success of the scale-up protocol, with each purified sample component being obtained at a total yield of 100% and 90% of the TnT displaced from the column during sample introduction and the subsequent isocratic elution in 0.1% aq. TFA. TnT remaining on the column is now well resolved from TnI, as is Tm from TnC. In addition, the entire separation has been achieved in just 3 h.

The results of RPLC analysis of representative pooled fractions from this large-scale run are shown in Fig. 7A–D. These analytical runs were carried out on the same analytical C_8 column employed to produce the crude sample profile shown in Fig. 2. The high level of purity of the displaced TnT fraction (panel A) and the eluted TnI (panel B), TnC (panel C), and Tm (panel D) proteins is quite clear (note also the TnT doublet in panel A from different TnT isoforms, seen previously following analysis of the crude sample on this column (Fig. 2)). Following

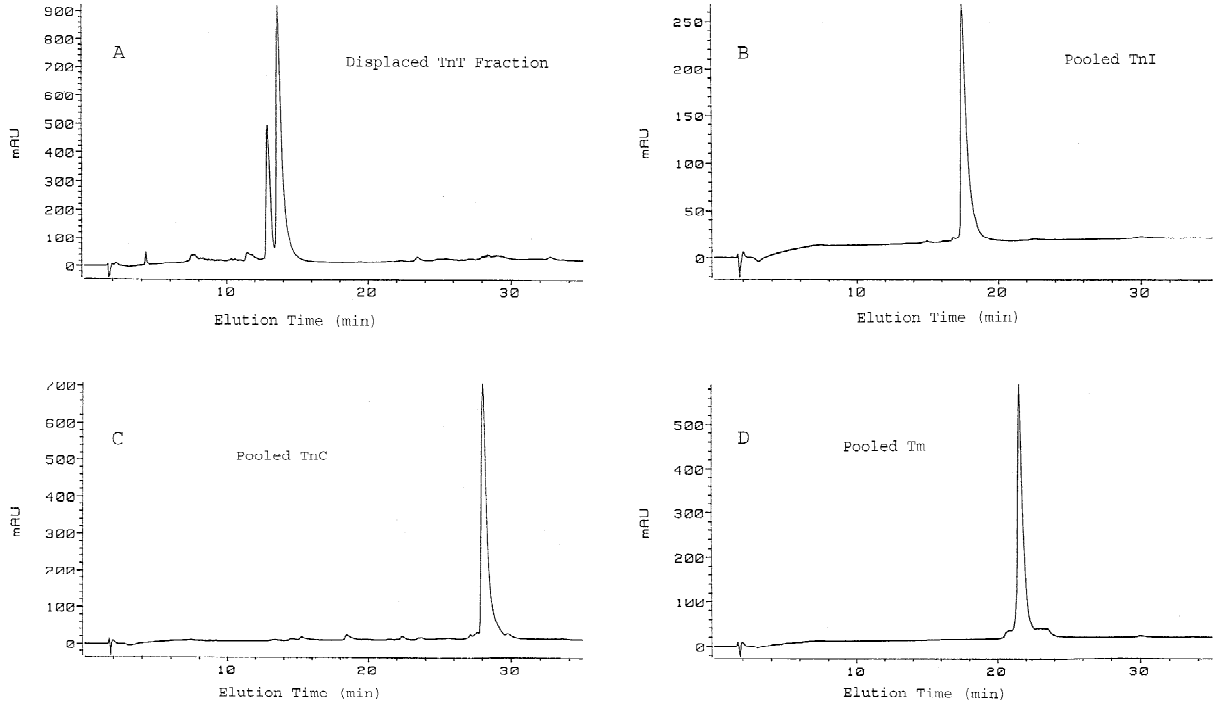


Fig. 7. Analytical RPLC of pooled purified fractions following large-scale (280×50 mm I.D. column; Fig. 6c) preparative purification of crude rabbit skeletal troponin. Column and elution conditions as in Fig. 2. (A) Troponin T displaced from column during sample loading and subsequent isocratic hold in 0.05% aq. TFA; (B) troponin I; (C) troponin C; (D) tropomyosin.

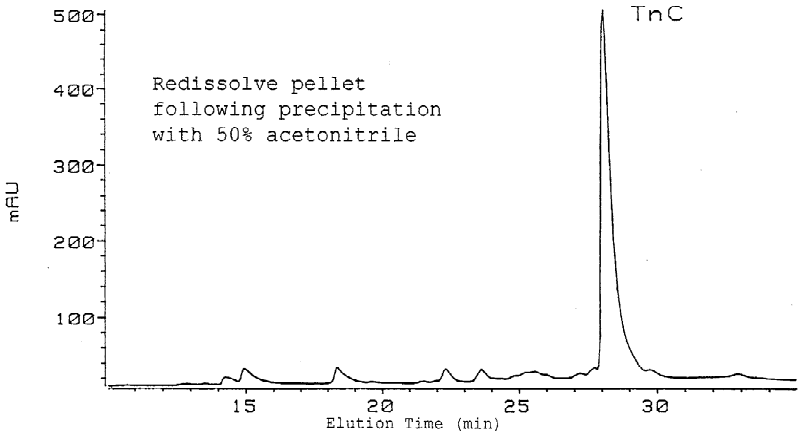


Fig. 8. Analytical RPLC of troponin C precipitated from crude whole troponin with 50% acetonitrile. Column and conditions as in Fig. 2. The troponin C pellet, centrifuged down following precipitation of the protein, was redissolved in 10 m*M* aq. ammonium hydrogen carbonate prior to analysis.

solvation in 10 m*M* aq. ammonium hydrogen carbonate of the precipitated TnC pellet obtained from fractionation of crude Tn with 50% acetonitrile, the RPLC analytical profile shown in Fig. 8 was obtained. Again, the protein has been obtained in satisfactory purity for most purposes, although, if required, it would be a straightforward matter to purify it further considering its good resolution from the small amounts of contaminants.

4. Discussion

4.1. Scale-up issues for purifying muscle proteins

Important scale-up issues for protein purification involve optimization of a number of sample parameters to ensure maximum efficiency in terms of speed, efficiency and product(s) yield, including solubility (maximize), pH (optimize), sample concentration (maximize) and sample volume (minimize). In addition, sample (i.e. protein) conformation can also be an important factor. Thus, it is essential to have the protein(s) in one form, either native (or folded) or completely denatured (unfolded) in order to eliminate potential problems in interpreting protein elution profiles due to the presence of multiple protein conformers. As noted above, prior to scale-up, it is important to understand as much about the nature of the protein(s) to be purified as possible.

4.2. Properties of muscle proteins and traditional purification approaches

Properties of the proteins pertinent to development of an efficient preparative protocol are summarized in Table 1. It should be noted that traditional extraction processes to isolate the Tn complex alone from muscle tissue generally results in the added presence of Tm to a greater or lesser extent, hence its inclusion in the development of the current preparative protocol. It can be seen that the three troponin proteins have a range of different properties including size and, particularly, p*I*, the three subunits ranging from acidic (TnC; pI=4) to neutral (TnT; pI=7) to basic (TnI; pI=10). Despite the presence of cysteine residues in TnC and TnI, there are no disulphide bridges formed between subunits, i.e. the three proteins of the Tn complex (and, indeed, the Tn/Tm regulatory complex) associate solely through non-covalent interactions. Note that, unlike the single polypeptide chain nature of the three troponin subunits, Tm is a dimer of two identical amphipathic α-helical polypeptide chains which form a two-stranded α-helical coiled-coil, now recognized as one of nature's favorite ways of creating a dimerization motif (Ref. [38] and references cited therein).

The aforementioned wide range of p*I* values of the Tn proteins has generally dictated traditional approaches to the purification of individual proteins in the complex. Such approaches have mainly involved

various combinations of cation- and anion-exchange chromatography (in order to take advantage of the wide p*I* range of the proteins) on traditional soft gels in open columns in the presence of high urea concentrations to dissociate individual subunits and to ensure their complete denaturation, followed by dialysis to remove salt [6,7,9,39–41]. On occasion, our laboratory would carry out a soft gel size-exclusion step prior to ion-exchange chromatography for an initial protein fractionation. However, despite the overall satisfactory effectiveness of such traditional approaches, these methods are time-consuming and, due to the use of open-column chromatography, the derived products are dispersed in large volumes of column eluent. Polyacrylamide gel electrophoresis (PAGE), generally in the presence of sodium dodecylsulphate (SDS), has also proved effective in separating structural muscle proteins, including troponin subunits (Ref. [42] and references cited therein) although, of course, SDS–PAGE remains basically an analytical technique as opposed to a preparative one, particularly where large (milligram to multigram) amounts of protein are being processed.

Cachia et al. [43] and Cachia and Hodges [44] took advantage of high-performance technology by separating both bovine cardiac and rabbit skeletal muscle troponin units on high-performance anion- and cation-exchange chromatography columns. Although the separation protocol developed by these researchers was quite successful, the major feature of the resultant elution profiles was the presence of multiple peaks on both the anion-exchange and cation-exchange columns. Specifically, rabbit skeletal TnI and TnT were both resolved as two peaks from the cation-exchange column (TnC was not retained by the column) and TnT exhibited three peaks on the anion-exchange column (TnI was not retained and TnC was eluted as a single peak), indicating multiple forms (isoforms) of TnI and TnT.

In the initial stages of a large-scale preparative process, it is always advantageous to minimize the number of fractions (peaks) to collect, particularly in the early stages of a purification protocol. As described above, if ion-exchange chromatography was used as a first step in the purification of Tn, one would be faced with multiple forms of two of the components. It is more logical to minimize the number of components as a first step, then separate multiple forms later, if desired. In addition, proteins separated by ion-exchange chromatography would still require desalting prior to using them for further applications.

5. Conclusions

We have developed an efficient large-scale preparative reversed-phase protocol for purification of proteins from a multi-protein complex, i.e. troponin from rabbit skeletal muscle. The scale-up procedure indicated a series of problems as a result of protein aggregation, solubility and/or complex formation which resulted in an initial fractionation with 50% acetonitrile to precipitate the majority of one component (TnC), followed by separation of the remaining proteins in the sample under overload conditions combining sample displacement with a slow linear gradient of acetonitrile. Excellent protein separations at high yields of purified proteins from sample loads as high as 5700 mg were achieved by the reported protocol.

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