Rapid Large Scale Purification of Myofilament Proteins using a cleavable His<sub>6</sub>-tag

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Abstract

With the advent of high-throughput DNA sequencing, the number of identified cardiomyopathy causing mutations has increased tremendously. As the majority of these mutations affect myofilament proteins, there is a need to understand their functional consequence on contraction. Permeabilized myofilament preparations coupled with protein exchange protocols are a common method looking into contractile mechanics. However, producing large quantities of myofilament proteins can be time-consuming and require different approaches for each protein of interest. Here, we describe a unified automated method to produce troponin C, T and I, as well as myosin light chain 2 fused to a His(6)-tag followed by a tobacco etch virus (TEV) protease site. The TEV protease has the advantage of a relaxed P1’ cleavage site specificity, allowing for no residues left following proteolysis and preservation of the native sequence of the protein of interest. Following expression in E. coli, cells were lysed by sonication in an imidazole containing buffer. The His(6)-tagged protein is then purified using a HisTrap nickel metal affinity column and the His(6)-tag removed by His(6)-TEV protease digestion for 4hrs at 30°C. The protease is then removed using a HisTrap column, and complex assembly is performed via column-assisted sequential desalting. This mostly automated method allows for purification of protein in one day and can be adapted to most soluble proteins. It has the advantage of greatly increasing yield while reducing the time and cost of purification. Therefore, production and purification of mutant proteins can be accelerated and functional data collected in a faster, less expensive manner.

Key words: troponin; protein purification; His-tag; TEV protease;

Abbreviations: FPLC, Fast protein liquid chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; MLC2v, Myosin Light Chain 2 ventricular; TEV, Tobacco Etch Virus; Tn, Troponin;

New & Noteworthy: Large scale production of high quality myofilament proteins is a standard method for experiments exploring cardiac mechanics. Current approaches to produce and purify proteins are costly and labor intensive. Here, we describe extensive improvement to currents method that reduce time and increase yield.
Recent advances in gene screening have identified dozens of novel cardiomyopathy causing mutations in sarcomeric proteins such as the troponin complex (8). An effort to identify putative consequences of such mutations is currently underway (19; 24). However, functional studies measuring the contractile properties of myofilaments usually require large amount of recombinant protein (1; 4; 7; 18; 21). Traditionally, recombinant proteins are produced by expression in *E. coli* competent cells followed by purification using fast-protein liquid chromatography (FPLC) based on the biophysical properties of the protein of interest (1; 2; 11; 12; 16; 17; 20). For instance, in order to assemble a functional troponin complex, troponin T is purified by anion exchange on a DEAE fast flow column. Troponin C is purified on a DE52 column and Phenyl Sepharose based on anion exchange and calcium affinity, respectively, while troponin I is purified first using cation exchange on a CM Sepharose column, then on a custom troponin C capture column. These methods are very time-consuming (4+ days per protein) and low efficiency (1; 2; 11; 12; 16; 17; 20). In order to scale up experiments, a rapid method for sarcomeric protein purification is necessary.

One method to streamline production is to employ a tag to help in the purification process (25). Unfortunately, for most sarcomeric proteins, any tag or leftover amino acids on either N-terminal or C-terminal can potentially, but not necessarily, affect function. One exception is a myc-tag placed on the N-terminus of cardiac Troponin-T which has been shown to be benign (3). As a workaround, protease sites can be engineered to cleave off the tag, although until relatively recently, all proteases left one or more amino acids behind (25). Additionally, large quantities of highly active purified protease necessary are cost prohibitive. Recently, a solution to both problems has been found. Novel point-mutations to the tobacco etch virus (TEV) protease has greatly improved its activity while making it resistant to self-proteolysis. These advances have made the protease much more suitable and reproducible for protein purification. Additionally, the TEV protease can be itself His6-tagged to aid in its purification, as well as allowing it to be removed following digestion (23). His6-TEV protease cleaves at the amino acid sequence of ENLYFQ/G (23). Furthermore, the P1’ recognition site of the TEV protease is relatively flexible so that glycine can be substituted by methionine, the universal start codon of proteins, or by almost any other residue (except proline), thereby resulting in a ‘native’ protein sequence following cleavage (9). Indeed, most mammalian and bacterial proteins have their N-terminal processed by methionine amino peptidases (MAPs) that cleave the initial methionine (5). Therefore, care can be taken to have the sequence match the native protein. Conversely, charged residues can replaced the N’ amino acid to mimic N’ acetylation, such as in the case of tropomyosin (13; 14), without having to alter the purification approach.

Here, we describe a rapid method for purification of recombinant protein to make active troponin complex for exchange into cardiac myofilament preparations. This new approach allows for a simplified workflow to purify all three troponin proteins, as well as, myosin light chain 2v (MLC2v), protein kinase A, and others. Following expression in a bacterial system, the recombinant protein is initially purified using a nickel-sepharose affinity column followed by a sephadex desalting column to perform buffer exchange into the TEV digestion buffer. After incubation with TEV protease to cleave the His6-tag off, the protein is run again through the
affinity column to remove any undigested protein, as well as the protease itself, and then
trough the desalting column again to replace into the desired buffer (22). We show that time
and monetary cost is significantly decreased, while the yield of the target protein is increased at
the same time.

Materials and Methods

Transformation and Expression of Cleavable His6-tag Troponin Proteins in *E. coli*

DNA fragments of His6-Human cardiac troponin C, I and T-myc (His6-HcTnC, His6-HcTnI, His6-
myc-HcTnT, respectively) with a TEV protease cleavage site (ENLYFQ/X, where X is the
desired 1st amino acid of the native protein) at the N-terminal (Integrated DNA Technologies,
USA), were ligated into pET28a vector (Novagen, USA) which contains a T7 promoter and a
His6-tag coding sequence (Figure 1A). Given that both mammalian cells and E. coli have
methionine aminopeptidases (MAPs) that can cleave N' terminal methionines (5), care was
taken to have the appropriate first amino acid (Met for TnC, Glu for c-myc-TnT and Ala for TnI).
His6-HcTnC and His6-HcTnI were transformed into *BL21* (DE3) competent cells (Novagen, USA)
and plated on Luria agar plates with 40 µg/ml kanamycin while His6-HcTnT-myc was
transformed into *Rosetta* (DE3) competent cells (Novagen, USA) and grown on plates with 40
µg/ml kanamycin and 34 µg/ml chloramphenicol. Rosetta cells were chosen as they co-express
rare codons for mammalian proteins and can improve protein translation. Colonies were allowed
grow at 37°C incubator overnight. Up to 5 colonies per construct were selected and grown in
suspension in 4ml of Luria broth (LB) with selection antibiotics, at 37°C overnight. The colony is
grown overnight in 4ml LB, then split into 2 aliquots, one aliquot is kept un-induced (U) and the
other is induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (I). After 3 hours, both
bacterial growths are centrifuged, lysed and run on SDS-PAGE side by side to look at the
production of protein following induction (Figure 1B). The colony that resulted in the highest
expression level combined with the fastest growth is determined by looking at the relative
amount of protein produced and then selected for large scale protein production.

Large Scale Production and Purification

Optimal colonies were picked (Figure 1B) and grown overnight in 20 ml LB which is then used to
inoculate 1L LB with selection antibiotics. Bacteria are allowed to grow for 8 hours at 37°C until
the absorbance at 600nm, a common surrogate for bacterial growth in suspension, reaches 0.4.
Induction of protein expression is initiated with 1mM IPTG at 32°C overnight. Bacterial pellet is
collected by spinning down culture at 9,000 x g, 20 min, 4°C. Pellets are stored at -80°C until
needed.

The bacterial pellet is resuspended and lysed in a combined Lysis/Equilibration buffer (6 M
Ultra-pure Urea, 50 mM NaH2PO4, 300 mM NaCl, 0.05% (v/v) Tween 20, pH 8.0) at a 1:10 (w/v)
ratio, then sonicated for 15 min on ice (30 sec on, 30 sec off). The lysate is centrifuged at
23,000 x g for 45 min, 4°C and the clarified supernatant is loaded onto an automated FPLC
system (AKTÄ FPLC, GE Healthcare, USA).
The target protein is purified in three steps (Figure 2); 1) purification using the His$_6$-tag with a HisTrap nickel affinity column (GE Healthcare, USA) equilibrated with lysis/equilibration buffer followed by desalting on a HiPrep 26/10 (GE Healthcare, USA) equilibrated with the TEV digestion buffer (1.5 M NaCl, 50 mM Tris-HCl, 5 mM DTT, pH 8.0). First, the sample is loaded onto the HisTrap column and the column is washed with 4% elution buffer (6 M Ultra-pure Urea, 50 mM Ultra-pure Imidazole, 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 0.05% (v/v) Tween 20, pH 8.0) until the UV absorbance at 280nm reaches equilibrium, around 100ml. His$_6$-tagged proteins are eluted out with 50ml of 70% elution buffer, then their buffer exchanged with 53ml of TEV buffer using the desalting column. 2) The His$_6$-tag is cleaved by TEV protease at 0.3 mg protease/mg target protein at 4°C, overnight, or at 30°C for 4 hours. 3) Finally, any undigested protein and the protease itself is removed using the HisTrap nickel affinity column. At this step, however, the flowthrough is collected instead then desalted with 70ml of the desired buffer. In order to facilitate troponin complex formation, troponins are desalted into urea buffer (6 M Ultra-pure Urea, 1 M KCl, 3 mM MgCl$_2$, 0.5 mM DTT, 10 mM MOPS, pH 7.0) (1; 17), while other proteins, such as MLC2v, are desalted into a simplified relax buffer (10 mM EGTA, 100 mM BES, 66.32 mM KOH, 15 mM NaCl, 6.48 mM MgCl$_2$, 49.76 mM potassium propionate, pH 7.0)

Production of His$_6$-TEV protease

The TEV protease plasmid, pRK793, was a gift from David Waugh (Addgene plasmid # 8827) (10). His$_6$-TEV protease was expressed in Rosetta (DE3) competent cells as above and lysed in lysis buffer without urea (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 0.05% (v/v) Tween 20, pH 8.0). Purification was performed using a HisTrap nickel affinity column and the elution buffer replaced with 2xTEV digestion buffer (3 M NaCl, 100 mM Tris-HCl, 10 mM DTT, pH 8.0) using the desalting column. His$_6$-TEV protease was stored at -20°C in 50% glycerol to prevent freezing.

Formation and purification of troponin protein complex

Troponin complex was formed by sequential desalting mimicking the sequential dialysis procedure described before (1; 17). Briefly, equal molar quantities of each subunit are mixed and the buffer is slowly depleted of urea and salt which allows the denatured proteins to refold back into native conformation. The starting concentration of all three troponin subunits (T, I and C) is 60 µM in 1 ml urea buffer and 50 mM L-Arginine, 50 mM L-Glutamine are added to help prevent degradation and precipitation that can occur later. Four sequential desalting steps are then performed by FPLC. The first step is with 60ml of 2 M Urea, 1 M KCl, 3 mM MgCl$_2$, 0.5 mM DTT, 10 mM MOPS, pH 7.0, then with 60ml of 1 M KCl, 3 mM MgCl$_2$, 0.5 mM DTT, 10 mM MOPS, pH 7.0, and finally with 60ml of 0.15 M KCl, 3 mM MgCl$_2$, 0.5 mM DTT, 10 mM MOPS, pH 7.0. The collected peak from the 3rd desalting step, containing the now-assembled troponin complex is then loaded onto a ResourceQ column (GE Healthcare, USA) equilibrated with 0.15 M KCl, 3 mM MgCl$_2$, 0.5 mM DTT, 10 mM MOPS, pH 7.0. A gradient (1% per ml) of troponin elution buffer (1 M KCl, 3 mM MgCl$_2$, 0.5 mM DTT, 10 mM MOPS, pH 7.0) is then used to separate the formed complex from the troponin monomers. The peak fractions are then pooled and SDS-PAGE performed to validate troponin complex purity and concentrated using a centrifugal filter unit, or “spin column” with cutoff of 10 000 Da (Millipore, USA) at 3000 x g for 30
mins or until a concentration of ~2 mg/ml is reached, according to the manufacturer’s instructions.

Functional comparison of recombinant troponin complex produced by traditional versus rapid method exchanged in human permeabilized myofilaments

The Institutional Review Board at Loyola University Chicago approved the protocol for the use of de-identified human donor samples. Permeabilized (skinned) myofilaments were prepared from healthy human myocardium as previously described (1) by incubating relaxing solution (in mM: 97.92 KOH, 6.24 ATP, 10 EGTA, 10 Na2CrP, 47.58 Kproprionate, 100 BES, and 6.54 MgCl2) with 1% Triton-X100 added for 10-15 minutes on a slowly rotating shaker. The triton is then removed and myofilament had their troponin complex exchanged with 2mg/ml recombinant complex overnight at 4°C. The myofilaments are then washed 3 times in relaxing solution and attached with glue to a force transducer and motor, exposed to free [Ca2+] ranging the activation of contraction at both short (SL=1.9 µm) and long (SL=2.3 µm) sarcomere length. Force data were fit to a modified Hill equation for each individual cell to obtain the nH. pCa50 was calculated from [Ca2+] required to achieve 50% force activation. Data are expressed as mean±SEM; statistical analysis was performed by 2-way ANOVA as appropriate (SigmaPlot).
Results

Purification of cleavable His<sub>6</sub>-tag protein produces native myofilament proteins. His<sub>6</sub>-tagged human cardiac troponin C, I and T (with myc-tag at N-terminal) with a TEV protease cleavage site were expressed in competent cells and purified by FPLC. (Figure 1B and 3) After purification, protein purity was assessed by SDS-PAGE. We show that our novel and rapid method results in purified native troponin C and troponin I (Figure 4A, 4B) while troponin T exhibited two degradation products (Figure 4C) that are less than 5% of the total protein and do not incorporate into the complex after purification, as they are absent from gels of assembled complex (Figure 5C). Additionally, we have successfully purified myosin light chain 2v (Figure 4D) which demonstrated <1% of non-intact protein for MLC2v. Yields per liter of bacterial growth are significantly higher using FPLC for the purification, with 14.8±0.5 mg/L of TnC, 13.0±0.7mg/L of TnT and 13.6±1.0mg/L of TnI compared to 2-4mg/L using traditional approaches. Importantly, the new FPLC method can easily accommodate several liters of medium in the same run to produce >50mg of protein per purification.

Recombinant human troponin complex (T, I and C) assembly and purification by FPLC.

Troponin T, I and C were purified as described above. Equal amounts of troponin T, I and C are mixed then desalted sequentially to decrease urea and salt concentration, thereby allowing the denatured proteins to slowly refold back into their native conformation and form a protein complex without precipitating (Figure 5A). After purification on a ResourceQ column, we collected all the fractions at 1 ml intervals (Figure 5B). Troponin I elutes out at 5-10% of elution buffer, and troponin C elutes out at about 20% of elution buffer. Troponin T typically precipitates during dialysis as the salt concentration and it solubility decreases. The complex elutes out at ~25% elution buffer. (Figure 5C) The eluted complex, then concentrated via spin column, does not contain any monomers or dimers of troponin C, I or T (Figure 5D). While the yield of complex is slightly lower that using the dialysis method (~23% lower, 3.0±0.1mg vs 3.9±0.2mg, p<0.05, Modified vs Traditional Method), the time savings of sequential desalting greatly make up for the loss of protein (Table 1).

Recombinant troponin complex behaves similarly using both purification methods.

To determine whether the modifications to the production and purification of the troponin complex affects its function compared to traditional methods, we performed exchange experiments in a frozen human left ventricle myocardium sample and measured force-pCa response. (Figure 6 A-F) Both complexes exchanged to similar extent into the permeabilized myofilament preparations (<90%, data not shown). We see that there is no difference in any parameter measured between complex produced by the new method compared to complex purified using traditional methods.
Discussion

Here, we describe a rapid large scale purification of myofilament proteins by adapting common techniques used in other fields to simplify the current standard methods. We demonstrate that, using cleavable tags to aid purification, we can produce large quantities of fully functional troponins that can assemble into a complex, and are no different from troponins produced in the traditional ways. Further, we show that assembling the complex on a desalting column speeds the process up even further and does not substantially affect yield.

The modifications that we have made to the traditional method have tremendously reduced the time needed to produce fully functional troponin complex (Table 1). While traditionally, it would require up to two weeks to go from a plasmid to fully assembled complex using the traditional methods, our improvements allows us to cut the time by half, with fully purified and assembled complex available within 4 to 7 days, with most of the time used for bacterial growth and protein expression (3 to 5 days), while the protein purification and complex assembly can be done in 1-2 days.

One major obstacle to increasing protein yield is precipitation. As the amount of protein per ml of solution is increased, we also increased the protein lost to precipitate. As a workaround, we adopted two strategies. The first consisted in keeping all the volumes large and the proteins diluted until the very last step of purification, as well as complex assembly. Second, we added two charged amino acids, L-Glu and L-Arg at a relatively high concentration (50 mM each) and dramatically increased the maximum amount of soluble protein (6). These amino acids are then removed at the same time as the concentration step, by using a spin column with a cutoff of 10000 Da. These two steps have allowed us to increase our yield per run by 3 to 5 times our traditional purification procedures while maintaining the time savings.

Further improvements to the technique can be achieved, such as using a gel filtration column to increase purity, or using an on-column digest with immobilized TEV protease. A more advanced FPLC system could also theoretically combine all three steps of the purification into a single method, with several holding loops and column valves. However, the time gained from such techniques would not out-weigh the additional costs of the purification, as buffer switching in an FPLC uses large volumes of buffer and significant amount of time. We believe we have identified a good compromise that increases efficiency and yield while also decreasing cost.

Conclusions

The method described above borrows from different protein purification methods to result in a simple, rapid, high yield procedure to produce large amounts of troponin complex. This greatly improves current techniques and should save investigators time and allow experiments with several different mutations in troponins to be carried simultaneously with little extra effort.

Acknowledgments

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Disclosures

None
Figure Legends.

Figure 1. Construct and Expression of mammalian protein in E. coli. A. Schematic of the pET 28a vector with TEV insertion for expression of myofilament proteins. Note that the C’ terminal His(6)-tag occurs after the stop codon and is not incorporated into the protein. B. Transformation and induction of His(6)-tagged recombinant human cardiac troponin T-myc (50 kDa) expression using IPTG in E. coli. Following an overnight growth phase, protein expression was induced for 3 hours then the bacteria lysed and expression verified by SDS-PAGE followed by coomassie staining. U, uninduced. I, induced using IPTG. M, marker lane. Each pair of U and I were grown from a single colony. Denoted with a red arrow is the colony that exhibited the highest expression of protein and was chosen for further purification.

Figure 2. Flowchart of the purification of protein using a cleavable His(6)-tag. Starting from bacteria, proteins are purified using two successive FPLC runs. Proteins can then be frozen, or used to form the troponin complex which is further purified by FPLC.

Figure 3. Representative traces of the purification of TnC by FPLC. A. Initial purification of TnC using the His(6)-tag. B. Removal of undigested proteins and the protease from the purified TnC. In both panels, the upper graph shows protein concentration measured as absorbance at 280nm is plotted in blue against elution volume through the FPLC columns, while salt concentration measured as conductivity is plotted concurrently in black. The bottom graph shows Eluent concentration as % of total buffer is plotted in red.

Figure 4. Purification of the myofilament proteins troponin C, I, and T (A-C), and human myosin light chain 2 ventricular isoform (MLC2v) (D). For troponin T, we show that there is very little degradation products when purified properly when compared to traditional methods (Panel C, right). All results are screened by 15% SDS-PAGE and visualized by Coomassie staining. M: molecular weight standards (kDa). Lane 1: Clarified E. coli lysate. Lane 2: Pooled peak fraction after 1st run though HisTap and HiPrep Desalting columns. Lane 3: After overnight TEV protease digestion. Lane 4: Pooled peak fraction after 2nd run through HisTrap and HiPrep Desalting columns.

Figure 5. On-column forming via desalting and purification of the troponin complex by FPLC. A. Serial desalting by FPLC to re-fold the troponins and allow them to form a complex. B. Purification of recombinant human troponin complex on a ResourceQ column with increasing concentration of KCl. Samples were run on a 12% SDS-PAGE and visualized by coomassie staining. C. Consecutive 5ml fractions from the 3rd FPLC run depicted in panel B are then assessed via SDS-PAGE. Supporting the labels in panel B, fraction 1 displays a band with MW consistent with TnI, fractions 3 and 4 with TnC, and the complex eluting out in fractions 5 to 11. Finally, a TnT-TnI dimer elutes out in fraction 12. M: molecular weight standards (kDa). D. The complex purity following concentration by spin-column was verified using size-exclusion chromatography by FPLC. Only a single peak of protein elutes out of the size-exclusion column at a volume consistent with the size of the troponin complex.

Figure 6. Troponin complex produced by sequential desalting behaves identically to troponin complex produced by traditional methods. Force-pCa curves at short (A) and long
sarcomere lengths (SL) show no differences between methods (n=5 traditional method, n=8 modified method from one human donor sample, p=NS). Similarly, normalized curves at short (C) and long (D) SL, pCa50 (E), and hill coefficient (F) are similar.
Table 1. Approximate Cost and Speed Comparison between the traditional method and our modified method.

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<th>Traditional Method</th>
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<tr>
<td></td>
<td>Cost Per run</td>
<td>Time (hrs)</td>
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<tr>
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the unique N-terminal extension of cardiac troponin I. *Biochim Biophys Acta* 1833: 823-832, 2013.


Figure 1.

A. Diagram of the pET28a+ vector with T7 promoter and flanking restriction sites.

B. Gel electrophoresis showing bands labeled HcTnT-myc with molecular weight markers (50 and 37 kDa).
Transform and grow bacteria

Lysis bacterial pellet

Load on HisTrap column and elute His-tagged protein

Perform buffer exchange with desalting column

Digestion buffer

Mix TnT, Tnl and TnC in equal amounts

Fold proteins into complex by sequential dialysis

Relax buffer

Load on ResourceQ column collect peak fractions eluting past 0.4 M KCl

Concentrate using spin column to 2mg/ml

Store proteins in 6M Urea at -80ºC for further use

1st FPLC run

Load on HisTrap and collect flowthrough

2nd FPLC run

Perform buffer exchange on flowthrough 6M Urea

3rd FPLC run

Perform buffer exchange with desalting column

Digest His-tag with TEV Protease

6M Urea

Concentrate using spin column to 2mg/ml

Store proteins in 6M Urea at -80ºC for further use

1st FPLC run
Figure 3.
Figure 4.

A) Troponin C

B) Troponin I

C) Troponin T

D) MLC2
Figure 5.
Figure 6.

A

B

C

D

E

F

Traditional

Modified

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