EXPRESSION AND PURIFICATION OF THE REGULATORY DOMAIN OF HUMAN CARDIAC TROPONIN C (HCTNC): A PROTEIN METHOD OPTIMIZATION STUDY

by

Luis Oliveira

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Luis Oliveira

Greenville, NC

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Approved by:

Dr. Eric Anderson, Dr. Anne Spuches

(ECU Biology Department, Thomas Harriot College of Arts and Sciences)

(ECU Chemistry Department, Thomas Harriot College of Arts and Sciences)

ABSTRACT

The purpose of this experimentation was to optimize the purification methodology for Human Cardiac Troponin C (HcTnC). This was done by replacing the old methodology of the size exclusion column and ion exchange column, with a phenyl sepharose column. This increased the protein concentration from 0.095 mg/mL to 0.251 mg/mL. This is important because it allows HcTnC to be purified faster with a higher yield, so more experimentation can be conducted. Further experimentation should be done to optimize the elution gradient as well as optimize the protein induction.

INTRODUCTION

Essential metals play crucial roles in protein function whereas metals deemed both non-essential and toxic may impair these processes by mimicking essential metal ions. The goal of the Spuches lab is to understand the fundamental thermodynamic driving force between proteins and essential divalent metal ions such as Ca (II), and Zn (II), as well as toxic metal ions such as Cd (II) and Pb (II). We are specifically interested in the binding of these metals to human cardiac troponin C (hcTnC), an EF-hand-containing protein that uses the binding of Ca (II) to initiate heart muscle contraction in cardiomyocytes

There is a problem with toxic metals binding with proteins in the human body. Lead and Cadmium should not be binding to Human Cardiac Troponin C, but it is. Lead toxicity is bad for the human body. Prolonged exposure can lead to brain and nervous system damage, heart, and kidney disease, and high blood pressure, among other problems. Metal toxicity needs to be

further researched as metals are continuously being found in commonly ingested products such as spices and paint.

Human cardiac Troponin C (HcTnC) is a protein that resides in the troponin complex on actin thin filaments of muscle and is responsible for binding calcium to activate muscle contractions in the heart. HcTnC is comprised of two protein domains, N and C-domains, each containing a pair of EF-hand peptide units. The C-domain has two high-affinity calcium binding sites and is considered the structural domain. The N-domain has only one calcium binding site and is considered the regulatory domain. (Figure 1) In this study, the focus is only on the N-domain. The N-domain has two cysteine residues, one cysteine residue lies in the EF-hand and the other lies near the linker region.

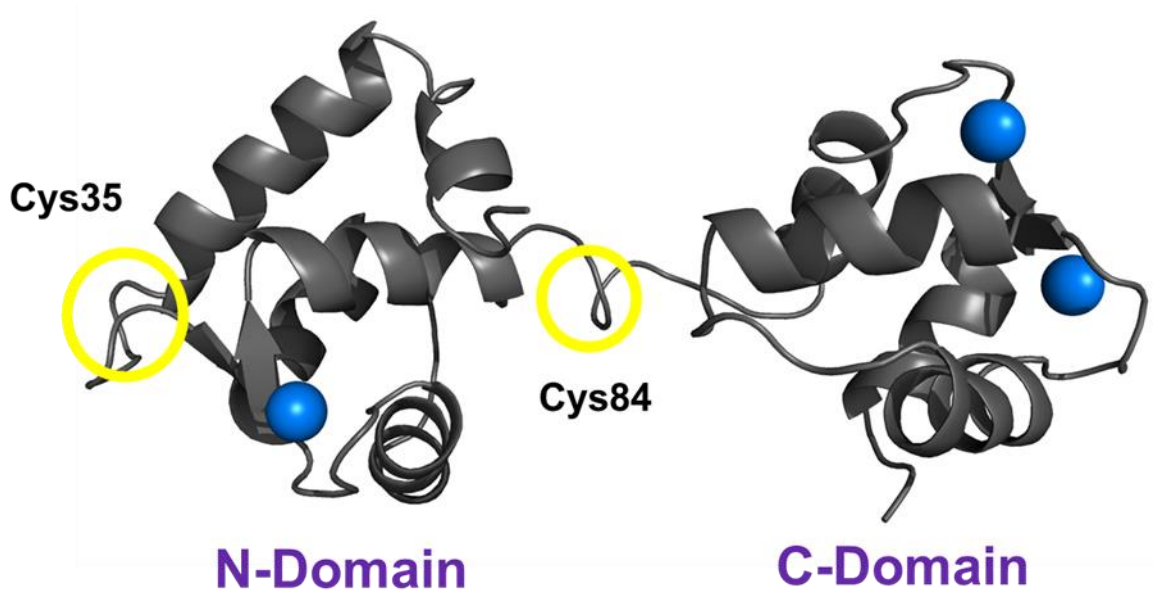

Figure 1: HcTnC Solution Structure in complex with 3 Ca (II) ions bound (1AJ4). Cys positions are highlighted in yellow circles.

The Spuches Lab is interested in understanding the fundamental thermodynamic and kinetic driving forces between proteins and essential divalent metal ions such as Ca (II), and Zn (II), as well as toxic metal ions such as Cd (II) and Pb (II). Specifically, we are interested in the binding of these metals to HcTnC an EF-hand-containing protein that uses the binding of Ca (II) to initiate heart muscle contraction in cardiomyocytes. Since studies often include utilizing large quantities of biologically active protein, we would like to find a purification method that will produce higher yields than currently obtained Size Exclusion Chromatography. [1,2]

METHODOLOGY

The HcTnC protein was expressed using BL21 de3 pLysS Vector. The methodology for cell culture expression is shown below.

Cell Cultures

To grow proteins for purification, 4 Fernbach flasks with 900 mL LB broth (900 mL H2O, 22.5 g LB-Miller Broth powder) and 1 Fernbach flask with 400 mL LB broth (400 mL H2O and 10 g LB broth powder) were autoclaved on a liquid cycle at 121 °C sterilization temperature and 20-minute sterilization time (Setting to 3/3). It was covered with foil with a piece of autoclave tape on top before it was placed in the autoclave. Once the 400 mL culture flask cooled to room temperature, both 400 µL ampicillin stock in water (100 mg/mL; 1000x) and 400 µL Chloramphenicol in ethanol (35 mg/mL; 1000x) were added to the broth. Utilizing a sterile technique, one loop of frozen cell stock was added to the culture. Once inoculated, the culture flask was incubated overnight at 37 °C shaking at 225 RPM.

A small amount of LB broth was saved so that nanodrop could be blanked before taking absorbances the next morning, 900 µL of ampicillin (100 mg/mL; 1000x) was added to each of the four culture flasks and the 400 mL starter culture was divided among them (100 mL to each culture). The four cultures were then incubated at 37 °C while shaking at 225 RPM for approximately 1 hour.

Optical Density measurements were taken every 0.5 hours (make sure to correct absorbance).

After ~1 hour (or when OD reached approximately 0.6 to 0.8), 1000 µL of IPTG stock (0.4 M, for a 0.4 mM final concentration) was added to each stock and was allowed to continue to shake and incubate for an additional 3-4 hours.

Once the incubation period was over, each culture was spun down at 5000 RPM (Spuches Lab Swinging Bucket Rotor) for 20 to 30 mins minutes at 4 °C.

The pellets were then resuspended in 10 to 15 mL Lysis buffer/Liter of culture and placed in 50 mL conical tubes (2 pellets/L per tube). The pellets were stored in the -80 °C freezer until further use.

The buffer solutions used can be seen in Figure 2.

Lysis Buffer	Equilibrium Buffer	Wash Buffer	Elution Buffer
1 mM EDTA	1 M NaCl	1 M NaCl	5 mM EDTA
50 mM Tris/HCl	1 mM $CaCl_2$	0.2 mM $CaCl_2$	20 mM Tris/HCl
pH 8.0	50 Mm Tris/HCl	50 mM Tris/HCl	pH 8.0
	pH 8.0	pH 8.0	

Figure 2: Buffer system used for the purification of HcTnC via fast-performance liquid chromatography (FPLC)

Cell Lysis Technique

1 tube (2 pellets in ~30 to 35 mLs of Lysis buffer) of resuspended cells from -80 °C Freezer were obtained and thawed at room temperature.

To this solution, 0.6 mL of a 50 mM PMSF stock was added to ~30 mL of resuspended cells. This amounts to a final PMSF concentration of 1 mM.

The solution was then frenched pressed on ice five times to ensure adequate lysing of cells.

The solution was then spun down in 50 mL conical tubes at 14,000 RPM (fixed angle rotor) for 30 to 40 minutes and the supernatant solution was saved.

Purification Technique

A phenyl Sepharose column was utilized and connected to a Fast Performance Liquid Chromatography (FPLC) instrument.

The column was prepared by first washing the column (flow rate 2 mL/ min) with 5 Column Volumes (CV) of water (18 MΩ) prior to equilibration. This was done to ensure the removal of 20% Ethanol in 18 MΩ water prior to the introduction of buffer and minimize alt precipitation.

This was followed by 4 CV of Equilibrium Buffer at a 2 mL/ min flow rate.

The lysed cell solution was then connected to the instrument using a 60 mL syringe. The solution was then injected into the pre-equilibrated phenyl sepharose column using the inject parameters shown in figure 3.

Inject Parameters	
Alarm pressure enabled	0.5 mPa
Flow	2.0 mL/min
Gradient	0%, 0
Flow path	Inject
Manual Fractions	None
Pause timer, volume,	70 mL

Figure 3: The inject parameters for loading protein onto the column.

The instrument was then set to wash under the parameters shown in Figure 4.

Wash Parameters	
Alarm Pressure enabled	0.5 mPa
Flow	2.0 mL/min
Gradient	0%, 0
Manual Fractions	None
Pause timer, volume	250 mL

Figure 4: The wash parameters for protein purification

The equilibrium buffer was then replaced with the wash buffer on pump line A and the elution buffer was added to pump line B. Both lines were purged and pumped/washed. A linear gradient (200 mL) was run to elute the protein, the gradient started with 100% wash buffer (line A) / 0% elution buffer (pump line b) and ended with 0% wash buffer / 100% elution buffer. The gradient elution parameters are shown in Figure 5.

Gradient Elution Parameters	
Alarm Pressure enabled	0.5 mPa
Flow	2.0 mL/min
Gradient	100%, 200 minutes
Manual Fractions	18 mm, 12 mL, Next Tube
Pause timer, volume	200 mL

Figure 5: Gradient elution Parameters are shown for FPLC

The column was then rinsed using the rinse parameters shown in Figure 6.

Rinse Parameters	
Alarm Pressure enabled	0.5 mPa
Flow	2.0 mL/min
Gradient	0%, 0 minutes
Manual Fractions	None
Pause timer, volume	100 mL

Figure 6: Rinse parameters are shown for FPLC

An SDS-page gel was used to analyze the fractions. The fractions were combined, large-sized proteins were removed via a 30 kDa cut-off centrifuge column, and small-sized proteins were removed via a 5 kDa cut-off centrifuge column.

The final sample was lyophilized and resuspended. Following resuspension, the concentration of the protein was found using a UV 280 nm nano spectrophotometer, where the size and extinction coefficient was inputted to measure the specific concentration of the terminal domain of HcTnC obtained.

RESULTS

Phenyl Sepharose (50 mL C.V)	Equilibration Parameters	Inject Parameters	Wash Parameters	Gradient Elution Parameters	Column Rinse Parameters
Flow (mL/min)	2.0	2.0	2.0	2.0	2.0
Run Volume (mL)	300	50	250	200	100
Buffer Used	Equilibration	Equilibration	Equilibration	Wash to Elution	18 MΩ Water

Figure 7: Overall parameters for FPLC for this experimentation are shown

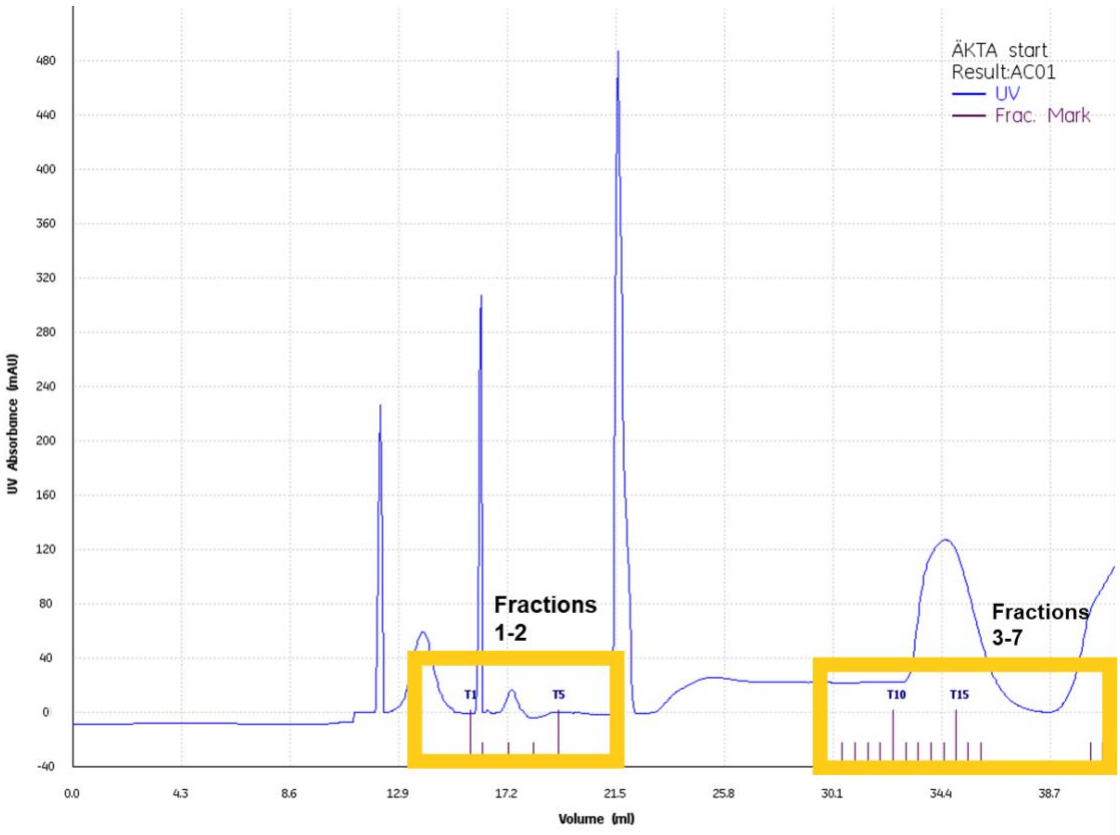

Figure 8: Elution chart of the gradient elution portion of purification. Tick marks represent the fractions collected and peaks represent the presence of protein elution.

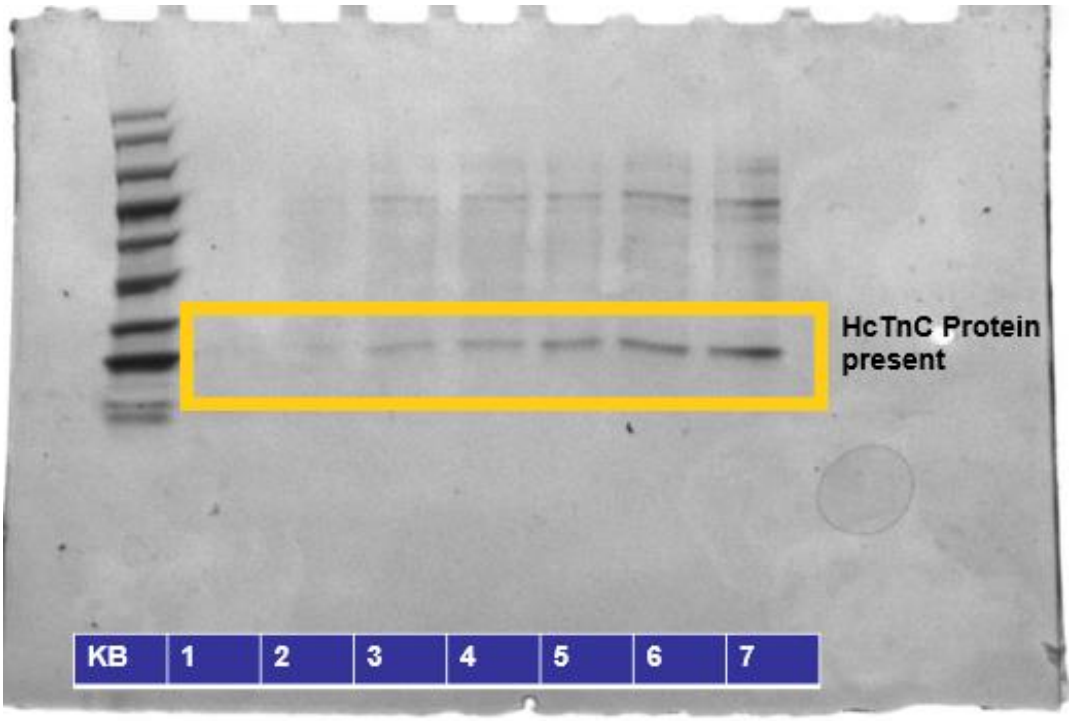

Figure 9: Collected fraction SDS Page gel showing HcTnC present in each of the seven fractions collected

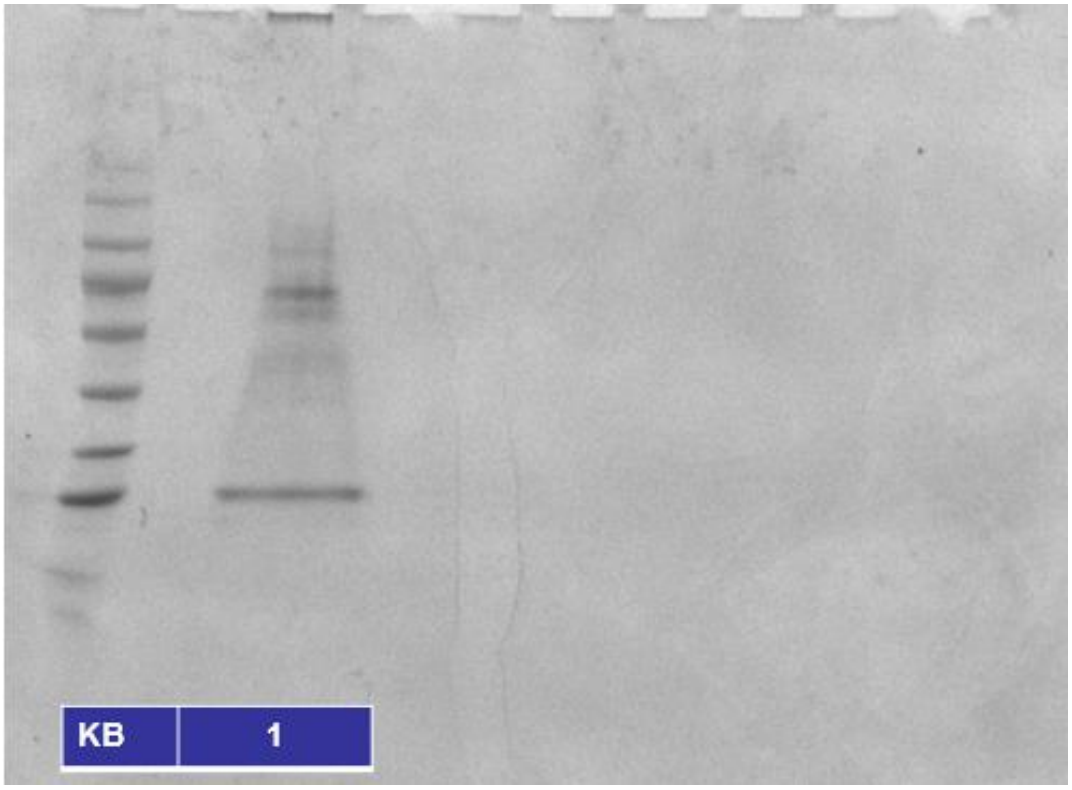

Figure 10: SDS-page gel of fraction after spin-down concentration. Lane 1 shows the KB ladder and lane 2 shows the concentrated fraction.

DISCUSSION

The final concentration of the protein was found to be 0.251 mg/mL. Previous runs using size exclusion chromatography yielded a concentration of 0.095 mg/mL. This shows that by utilizing a phenyl sepharose column instead of a size exclusion column, the concentration of protein will increase. Improvements going forward need to be made to change the elution gradient to collect a smaller number of fractions as well as further optimize the time when protein induction occurs.

REFERENCES

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[2] Stevens, C. M., Rayani, K., Singh, G., Lotfalisalmasi, B., Tieleman, D. P., and Tibbits, G. F. (2017) Changes in the dynamics of the cardiac troponin C molecule explain the effects of Ca2+-sensitizing mutations. J. Biol. Chem. 292, 11915–11926.