NON-CHROMATOGRAPHIC PROTEIN PURIFICATION VIA MINI-INTEIN CLEAVAGE

A Senior Scholars Thesis

by

NAJLA EUNICE VALDES

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Chemical Engineering

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Approved by:

Research Advisor: Zhilei Chen
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Non-Chromatographic Protein Purification via Mini-Intein Cleavage. (May 2012)

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A protease-free, column-free protein purification technology was developed through a combination of an engineered thio-responsive split-intein and a stimulus-responsive elastin-like polypeptide (ELP). The DnaE split intein from *Nostoc punctiforme* was engineered to catalyze C-terminal cleavage, rather than *trans*-splicing reaction under reducing condition at neutral pH. ELP is fused to the N-fragment of the split-intein and the protein of interest (POI) is fused to the C-fragment of the split-intein. Each fusion protein is expressed separately in E. Coli and after cell lysis, these proteins are mixed at pH8. After mixing, the N- and C- fragments of the split-intein associate with each other, linking the ELP to the POI. Salt is then added to the protein mixture to precipitate the ELP. The precipitant containing the ELP-intein-POI complex is then solubilized in a low-salt reducing buffer, inducing intein C-terminal cleavage reaction and separating POI from the intein and ELP. After intein reaction, the ELP-intein complex is precipitated again by the addition of high salt buffer, resulting in purified POI in the supernatant. Implementation of this technology in large scale applications can reduce the complexity, cost, and time required for protein purification.

DEDICATION

To my parents for their love, understanding, support, and encouragement

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Zhilei Chen for her support throughout the project. I would also like to thank all the members of Chen's group for their support, with a special thanks to Miguel Ramirez who collaborated with me in this project.

NOMENCLATURE

DsRed	Red Fluorescent Protein
Extein	External Protein
ELP	Elastin-Like Polypeptide
GFP	Green Fluorescent Protein
MBP	Maltose Binding Protein
NBT	Nitro Blue Tetrazolium Chloride
Npu	*Nostoc Punctiforme*
NpuC	C-Fragment of the *Npu* Split-Intein
NpuC*	Mutated NpuC
NpuN	N-Fragment of the *Npu* Split-Intein
PTDH	Phosphate Dehydrogenase
POI	Protein of Interest

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CHAPTER I

INTRODUCTION

Protein purification techniques continue to be improved over time as a response to demand for increased purity and decreased costs. Current methods include the use of expensive columns and long incubation periods and thus lack effectiveness when implemented in large scale purification. An alternative to column purification is provided by protein tags that do not require a stationary medium. In 2005, Mahmoud Banki and David Wood introduced a new method of protein purification using self-cleaving ELP tags in solution [1]. ELPs are a series of pentapeptides that have the ability to aggregate at high temperatures and high salt concentrations. Banki and Wood's purification procedure eliminated the use of affinity chromatography and proteolytic tag removal [1].

After further optimization of ELP aggregation, Dr. David Wood's lab reported optimized temperatures and salt concentrations [2]. Their study reported that ammonium sulfate salt is able to induce ELP aggregation at room temperature. Since changes in temperature can compromise the activity of certain proteins, the ability of ELP to aggregate at room temperature makes it a useful tag for protein purification.

This thesis follows the style of Protein Expression and Purification.

For ELP to be successfully used as a tag for protein purification, ELP must have the ability to attach to, and detach from the target protein. A controlled medium that connects an ELP tag to the target protein can be provided by inteins. An intein is a type of protein that is naturally inserted in an external protein (extein) and has the ability to self-excise and join the two halves of the exteins with a peptide. This process is referred to as *trans*-splicing and is depicted in Fig. 1A.

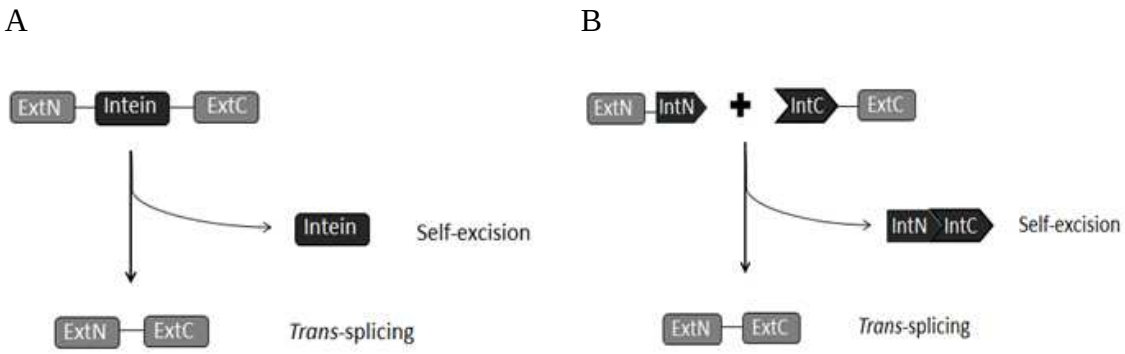

Fig. 1. Intein *trans*-splicing. (A) Continuous-intein *trans*-splicing. (B) Split-intein *trans*-splicing.

In nature some inteins have their catalytic residues split between two separate proteins. These inteins are called split-inteins. As seen in Fig. 1B, split-inteins also have the ability to *trans*-splice, linking two separate extein proteins. An example of naturally split-inteins is the DnaE intein from *Nostoc punctiforme* (*Npu*) [3]. In 2009, Zettler and Schütz characterized the kinetics and *trans*-splicing efficiency of multiple split-inteins and reported the *Npu* split-intein has the highest *trans*-splicing efficiency and reaction rate [4]. We recently engineered the C-fragment of the *Npu* split-intein (NpuC*) that

catalyzes C-terminal cleavage reaction instead of the *trans*-splicing reaction, without affecting the binding affinity between the N- and C-terminal of the split-intein. The schematic of the reaction catalyzed by NpuC* is depicted in Fig. 2. When NpuC* reacts with NpuN in the absence of a reducing agent, *trans*-splicing is prevented and the split-inten and its extein form a complex. After the NpuN-NpuC* complex has been formed, addition of a reducing agent induces cleavage and the extein at the C-fragment is cleaved from the complex.

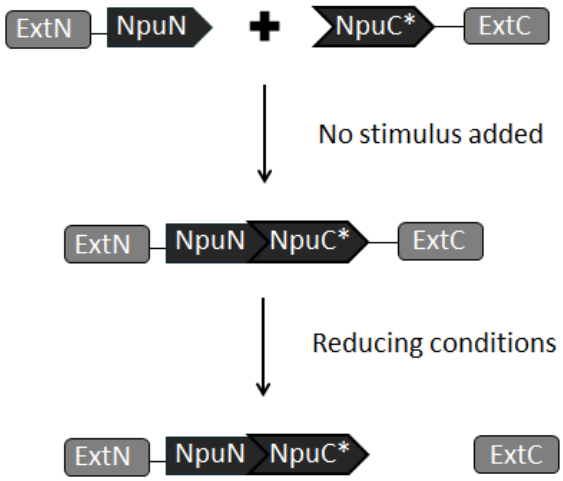

Fig. 2. Schematic of NpuC* reaction

The mechanism of NpuC* described in Fig. 2 provides the basis of the purification scheme shown in Fig. 3, where ExtN is replaced by ELP and ExtC is replaced by the

protein of interest (POI). It is expected that the fast reaction rate of *Npu* will have a positive impact on the time required to purify the POI.

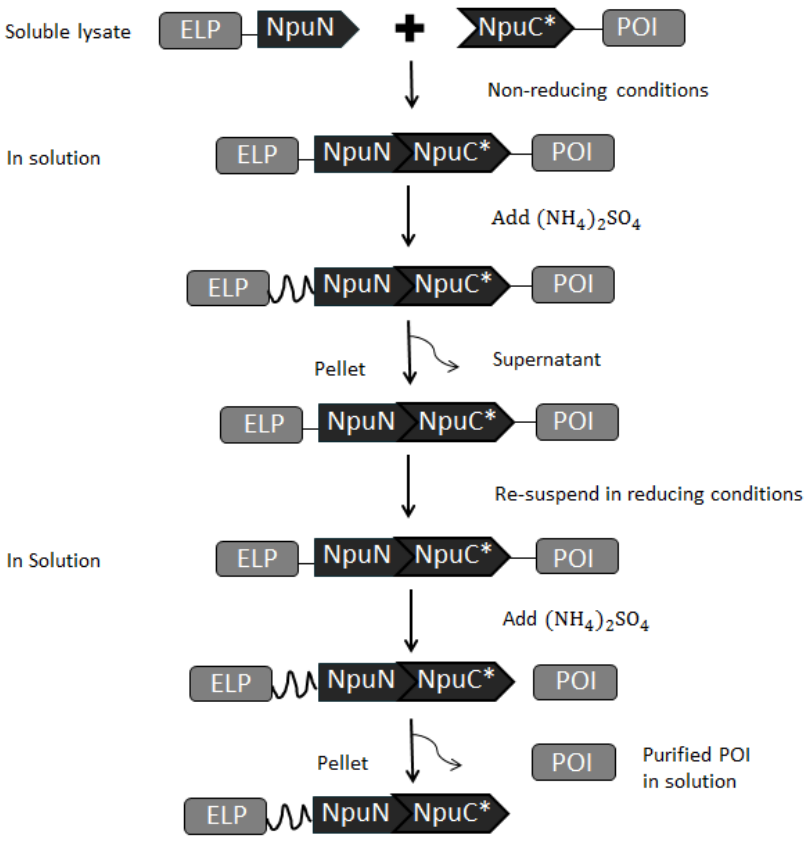

Fig. 3. Schematic of protein purification of POI using NpuC* and ELP

CHAPTER II

METHODS

Cloning

Cloning of all constructs was verified by sequencing.

pET-ELP-L-NpuN

Plasmid PET-ELP-I-MBP, a gift from Dr. David Wood (The Ohio State University), was digested with HindIII and EcoRI. The L-NpuN fragment was PCR amplified from pI-GFP-L-NpuN (construct previously cloned in our laboratory) using oligonucleotides obtained from Fisher. The PCR product was purified and inserted into the digested pET-30 vector.

pI-NpuC-PTDH*

Plasmid pI-NpuC-GFP was digested with XhoI and NdeI. PTDH was PCR amplified from HZ529-PTDH-E175A-pRW2, a gift from Dr. Zhao (University of Illinois), and linked to NpuC*. The fragment NpuC* contains mutation D118G, achieved through site-directed mutagenesis, and the NheI restriction site at its 5’ strand to simplify the cloning of the remaining constructs. NpuC*-PTDH was inserted into the digested pET-26 vector.

pI-NpuC-GFP*

Plasmid pI-NpuC*-PTDH was digested with XhoI and NheI. The GFP fragment was PCR amplified from pI-NpuC-GFP using oligonucleotides obtained from Fisher. The PCR product was purified and inserted into the digested pET-26 vector.

pI-NpuC-MBP*

Plasmid pI-NpuC*-PTDH was digested with XhoI and NheI. The MBP fragment was PCR amplified from pET-ELP-I-MBP, a gift from Dr. David Wood (The Ohio State University), using oligonucleotides obtained from Fisher. The PCR product was purified and inserted into the digested pET-26 vector.

pI-NpuC-DsRed*

Plasmid pI-NpuC*-PTDH was digested with XhoI and NheI. The DsRed fragment was PCR amplified from pI-NpuC-DsRed (construct previously cloned in our laboratory) using oligonucleotides obtained from Fisher. The PCR product was purified and inserted into the digested pET-26 vector.

Protein expression

Expression of all protein constructs used in the purification of POI begins with the transformation of E. Coli BLR (DE3) cells with a plasmid construct. Following heat-shock, cells were transferred to 1 mL of LB media inside a 14 mL culture tube and incubated at 37°C, shaking at 250 rpm for 1 h. After incubation, 100 μL of the cell

culture were transferred to an agarose + antibiotic plate and incubated overnight at 37°C. The construct pET-ELP-L-NpuN was incubated in a plate containing 5 µg/mL of Tetracycline and 100 µg/mL of Ampcillin for a period of 12-15 h. Constructs of the form pI-NpuC*-POI were incubated in a plate containing 5 µg/mL of Tetracycline and 50 µg/mL of Kanamycin for a period of 15-18 h.

Following overnight incubation, a single colony was picked from the plate and transferred to 5 mL of LB media inside a 14 mL culture tube which contained 5 µL of either Ampcillin (100 mg/mL) or Kanamycin (50 mg/mL) and 5µL of Tetracycline (5 mg/mL). The colony in the media was incubated at 37°C shaking at 250 rpm until the cell density reached a value of 0.6. The cell culture was then transferred to 1 L of LB (containing 1 mL of 5 mg/mL of Tetracycline plus 1 mL of 50 mg/mL of Kanamycin or 100 mg/mL of Ampcillin) inside a 2.8 L flask. The cell culture was incubated at 37°C, shaking at 230-250 rpm until cell density reached 0.6, followed by a cooling period of 10 min in an ice-water bath. After the cooling period, 200 µL of 1M IPTG were added to the cell culture to induce protein expression, and the culture was incubated at 18°C shaking at 230-250 rpm for 15 h. Following overnight incubation, the cell culture was transferred to centrifuge tubes and centrifuged at 6000xg for 20 min at 4°C. The supernatant was discarded and the cell pellet was transferred to 14mL culture tubes in aliquots of 1 g of wet pellet and stored at -20°C overnight. All proteins used in our experiments were expressed as described above.

NpuC* activity test

Proteins pI-GFP-L-NpuN, pI-NpuC-GFP, and pI-NpuC*-GFP were purified using a Ni-NTA column to test the activity of NpuC*.

Ni-NTA purification

First, 100 µL of Ni-NTA agarose beads were transferred to a 5 mL column, followed by 3 mL of lysis buffer to equilibrate the column. After equilibration, 5 mL of cleared cell lysate were added to the column through a pipette, making sure that the beads were well mixed with the lysate. The column was washed with 3 mL of lysis buffer followed by a wash with wash buffer which contained 10% elution buffer (elution buffer composition: 150mM imidazole, 500mM NaCl, and 50mM tris-HCl). The protein was eluted with 600 µL of 100% elution buffer and collected inside a mini-centrifuge tube.

Activity test

Purified pI-GFP-L-NpuN and pI-NpuC-GFP were mixed in a 2:1 molar ratio with 1M DTT (final concentration of 10mM) in ddH20 inside a 1.5 mL mini-centrifuge tube. The procedure was repeated for the reaction between pI-GFP-L-NpuN and pI-NpuC*-GFP and for each of the individual proteins. All tubes were incubated overnight at room temperature. All samples were analyzed by SDS-PAGE.

Target protein purification

The following sections describe the methodology for protein purification of POI.

Cell lysis

A single aliquot of frozen cell pellet was thawed in ice and suspended in 10 mL of lysis buffer per gram of wet pellet (lysis buffer composition: 500 mM NaCl, 50 mM tris-HCl, pH8). The suspended pellet was sonicated in ice at the following conditions: 40 amps, 1 s pulse, 2 s pause, and 3 min of total sonication time. Following sonication, the sample was transferred to a centrifuge tube and centrifuged at 16,000xg for 20 min at 4°C. Cleared cell lysate was transferred to a clean centrifuge tube and stored at 4°C. After obtaining the soluble lysate for pET-ELP-L-NpuN and pI-NpuC*-GFP, pI-NpuC*-MBP, pI-NpuC*PTDH, or pI-NpuC*-DsRed, the purification of the POI was carried out.

Pre-purification of ELP-L-NpuN

To the cleared soluble lysate of ELP-L-NpuN, ammonium sulfate (0.4 M) was added and the mixture was incubated at room temperature for 10 min. The sample was centrifuged at 16,000xg for 20 min at 4°C. The supernatant was discarded and the pellet was re-suspended in the same volume of lysis buffer.

Purification of POI

All of the POIs were purified as described below for GFP. First, 2 mL of pre-purified pET-ELP-L-NpuN were mixed with 1 mL of pI-NpuC*-eGFP inside a centrifuge tube.

The sample was incubated inside a tube turner for 30 min at room temperature (22°C). After the 30 min incubation, ammonium sulfate was added to a final concentration of 0.4 M and the sample was incubated an additional 10 min at room temperature. The sample was then centrifuged at 14000xg for 10 min at room temperature. Following centrifugation, the supernatant was discarded and the pellet was suspended in 1 mL of lysis buffer. C-terminated cleavage was induced by adding DTT to a final concentration of 50 mM. The sample was transferred to a clean 1.5 mL mini-centrifuge tube and incubated at room temperature for 3 h or for 20 h after which ammonium sulfate was added to a final concentration of 0.4 M. The samples were centrifuged at 14000xg for 10 min at room temperature and the purified POI was recovered in the supernatant. All samples were analyzed by SDS-PAGE.

SDS-PAGE analysis

All samples for SDS-PAGE analysis were prepared by mixing 50 µL of each sample with 50 µL of 2X SDS Buffer inside a 1.5 mL mini-centrifuge tube. Samples were heated at 95˚C for 5 min and 10 µL of each sample was loaded into different lanes of a 10% polyacrylamide gel. The gel was run at 120 mV for 50 min.

PTDH activity assay

Small scale purification was performed to carry out the PTDH activity assay. Inside seven 1.5 mL mini-centrifuge tubes, 500 µL of pET-ELP-L-NpuN and 100 µL of pI-NpuC*-PTDH were mixed. One tube was saved to be the sample for mixed cell lysates

and to the remaining 6 tubes, ammonium sulfate was added to a final concentration of 0.4 M. One tube was separated and the remaining 5 tubes were incubated at room temperature for 10 min and centrifuged at 14,000xg for 10 min at room temperature. One tube was set aside to be the sample of the precipitation of the entire ELP-L-NpuN-NpuC*-PTDH complex and the remaining 4 tubes were subjected to the remaining purification procedure, setting aside a sample after each subsequent step. Once samples of all purification steps were separated, 1 µL of Nitro Blue Tetrazolium Chloride (NBT) assay mix was added per 10 µL of purification sample. The purpose of the assay is to visualize the presence of PTDH in solution (PTDH catalyzes the reduction of NBT characterized by a change in color from yellow to dark blue).

CHAPTER III

RESULTS

The activity test of NpuC* was performed as described in Chapter II and results are displayed in Fig.4. It is observed that when GFP-L-NpuN reacts with wild-type NpuC-GFP, NpuC-GFP disappears and a *trans*-splicing product appears (Lane 4). Unlike NpuC wild-type, when NpuC* reacts with NpuN, NpuC* disappears and a cleavage product appears (Lane 5) instead of a *trans*-splicing product. At pH8 and under the presence of DTT, NpuC* looses *trans*-splicing activity and achieves a high percentage of cleavage efficiency.

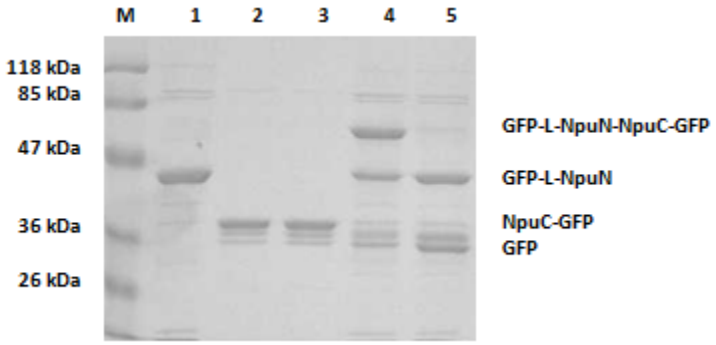

Fig. 4. Activity test of NpuC*. Lanes: M: molecular weight marker, 1: pI-GFP-L-NpuN, 2: pI-NpuC-GFP, 3: pI-NpuC*-GFP, 4: reaction with wild-type NpuC, 5: reaction with mutant NpuC

After analyzing the activity of NpuC*, the purification scheme was performed on different target proteins as described in Chapter II. Fig. 5 demonstrates the step-by-step purification of PTDH and DsRed. Protein purification begins with the mixture of the

lysates of ELP-L-NpuN and NpuC*-POI. As indicated in Fig. 5(A), the cell lysate mixture turned blue due to the presence of PTDH in solution. Following salt addition and centrifugation, the solution remained yellow confirming that NpuC*-PTDH was pulled down by ELP-L-NpuN. After the complex was re-suspended in low-salt buffer and DTT was added to induce cleavage reaction, the solution turned blue upon the addition of NBT, indicating that PTDH was again present in solution. In the final step after salt was added and the sample was centrifuged, it is observed that the ELP complex was precipitated and the cleaved PTDH was present in solution. In Fig. 5(B), the color red indicates the presence of DsRed. Results from Fig. 5(B) are similar to those of Fig. 5(A) indicating that both PTDH and DsRed were purified.

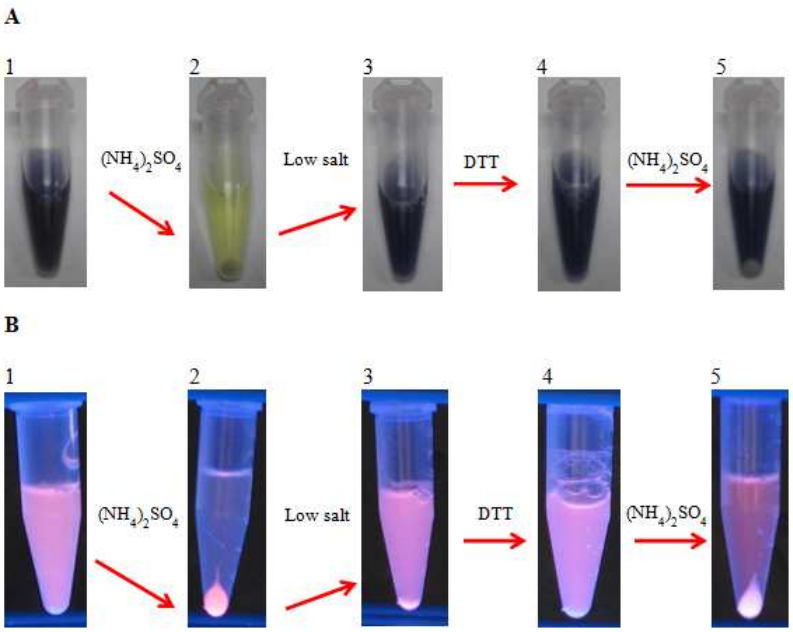

Fig. 5. Purification of (A) PTDH and (B) DsRed. (1) Cell lysate mixture, (2) pull-down of intein complex after salt addition and centrifugation, (3) re-suspension of intein complex, (4) induction of cleavage reaction, (5) removal of ELP complex from purified POI.

Fig. 6 below shows SDS-PAGE gels obtained from the purification of different target proteins including PTDH, GFP, MBP, and DsRed.

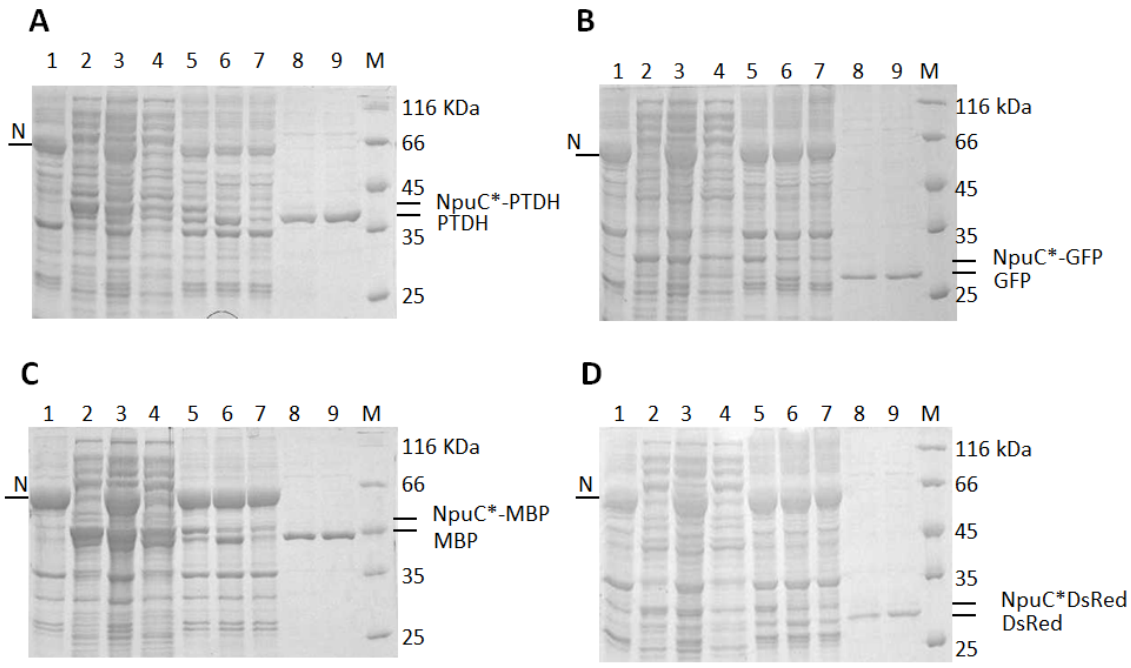

Fig. 6. SDS-PAGE analysis of target purification of (A) PTDH, (B) GFP, (C) MBP, and (D) DsRed. Lanes: M: molecular weight marker, 1: pre-purified ELP-L-NpuN, 2: NpuC*-POI lysate, 3: mixture of lysates, 4: supernatant after precipitation with $(NH_4)_2SO_4$, 5: pellet after precipitation with $(NH_4)_2SO_4$, 6: intein mixture following cleavage reaction, 7: pellet after precipitation following cleavage reaction, 8: purified POI after 3 h of cleavage reaction, 9: purified POI after 20 h of cleavage reaction.

Lanes 1-3 represent step 1 of Fig. 5. The presence of some of the POI in the supernatant as seen in Lane 4 of Fig. 6 indicates that not all of the NpuC*-POI gets pulled down. Despite the incomplete pull-down, the band corresponding to NpuC*-POI in Lane 5 has greater intensity than in Lane 4, indicating that a greater percentage of NpuC*-POI precipitates along with the ELP. The result of the cleavage reaction can be observed by

the transition from Lane 5 to Lane 6. The transition consists of the disappearance of the band corresponding to NpuC*-POI (Lane 5) and the appearance of the band corresponding to the POI (Lane 6). As seen in Lanes 8-9, the recovered POI is of high purity and the reaction is complete after 3 h since there is no visible change in the intensity of the band from Lane 8 to Lane 9. Based on the SDS-PAGE results and the pictures taken throughout the target protein purification, four different target proteins, PTDH, GFP, MBP, and DsRed were successfully purified.

CHAPTER IV

CONCLUSIONS

The C-fragment of the *Npu* split-intein was mutated to induce C-terminated cleavage upon addition of DTT. This mutation did not affect the affinity between the N- and C-fragment of the split-intein and allowed the cleavage reaction to be controlled. However, upon precipitation of the ELP-L-NpuN and NpuC*-POI mixture, an incomplete pull-down of NpuC*-POI was observed which is possibly due to steric hindrance between the POI and *Npu*.

The time required for the cleavage reaction to reach completion confirmed the original hypothesis that using *Npu* as the split-intein would positively affect the kinetics of the reaction. The time required for the cleavage reaction was determined to be 3 h at room temperature. These conditions resulted in the successful purification of four different proteins of multiple structures. These proteins are not processed for large periods of time and they are not subjected to temperature changes.

Although the purification scheme has the ability to purify proteins of multiple structures in a short period of time, the scheme is limited to the purification of soluble proteins. Future work will consist on the addition of a solubility enhancing tag to one of the fusion proteins to attempt to purify insoluble proteins. We will also focus on obtaining

quantitative measurements for the percentage of protein recovery and for the activity of the protein after purification.

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