UNIVERSITY OF CALIFORNIA
RIVERSIDE

Identify Important SUMOylation Site of Influenza Virus Protein Using FRET Technology and New Strategy for Fluorescence Protein Expression

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Bioengineering

by

Jin Jiang

December 2017

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ACKNOWLEDGEMENTS

Firstly, I'd like to express my sincere gratitude to my advisor, Dr. Jiayu Liao, for giving me the opportunity to join his lab. Without his valuable guidance and instruction, it would be impossible for me to finish my project. I would also thank my thesis committee, Dr. Justin. W. Chartron and Dr. Xiaoping Hu, for guiding me and giving me their helpful instructions. The senior phD students, Zhehao Xiong, George Way and Vipul Madahar gave me lots of advices and taught me the various techniques in this lab. Also I am grateful to the other graduate students, Chitra Hariharan and Xuefeng Dong, for helping me trouble shooting problems gave me suggestions. What's more, I'd like to thank the undergraduate students Kevin D. Portacio, Kate An, Mary Nwangwu and Yiheng Huang for their assistance throughout my projects. I also got help from Dr. Jefferson Perry's lab in UCR Biochemistry Department for the instrument using and from Dr. Shasha Zheng's lab in California Baptist University for the lab resources and supplements. Lastly, this work is dedicated to my parents (Peng and Shanhong) and my best friend Danni, without your support and encouragement, this work won't be possible.

ABSTRACT OF THE THESIS

Identify Important SUMOylation Site of Influenza Virus Protein Using FRET Technology and New Strategy for Fluorescence Protein Expression

by

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Master of Science, Graduate Program in Bioengineering
University of California, Riverside, December 2017
Dr. Jiayu Liao, Chairperson

Influenza, which is also commonly known as "the flu", is an infectious disease caused by an influenza virus. The symptoms of influenza could be mild to severe, including fever, headache, sore throat, feeling tired, headache, coughing, and muscle pains. The influenza virus infects and kills over 100,000 people in the world every year. Due to the fast pace in which influenza virus mutates, the virus itself would develop resistances to the current FDA approved drugs. The influenza non-structural protein (NS1) is related to the SUMOylation pathway, a post-translational modification involved in various cellular processes.

Chapter 2 of this thesis is mainly focused on finding out the SUMOylation site of the NS1 influenza protein by using site-directed mutagenesis and Förster Resonance Energy Transfer (FRET) based technology. Since the SUMO protein is conjugated to the lysine residue of the NS1 protein, by mutating each lysine to Alanine and using FRET-based SUMOylation assay to test every mutant of NS1, the Lysine residue K131 was founded to be the SUMOylation site of NS1. This result was further proved by Plaque assay, which showed the mutation of Lysine 131 would affect the viral replication in MDCK cells.

Identification of essential SUMOylation site(s) of NS1 can provide supporting evidence to develop new medicine for treating Influenza virus.

Chapter 3 of this thesis is focused on designing a new strategy for fluorescence protein expression. Nowadays, using *E. coli* bacteria as the host for protein production is a very common strategy. However, there are several challenges that needs to be overcome by using *E. coli* protein production, such as the degradation of the target protein, the toxic of protein towards the host, the misfolding and instability of the protein, and the complex purification procedures. By expression the proteins in inclusion bodies (IBs) would help alleviating these problems. This thesis conducted a signal sequence TMAO-reductase (ssTorA) to drive the target protein Ypet-Uba2, into the inclusion bodies and overcame the degradation of the protein. Also, a buffer screening method was designed to purify the protein from inclusion bodies. As a result, trehalose was proved as a high efficient additive for the Ypet-Uba2 protein purification.

During the studies of my research, we have established a sensitive FRET methodology to identify the SUMOylation site(s) of Influenza virus protein and a new approach to express and purify SUMO E1 ligase subunit, Uba2, which was very difficult to be expressed as high purity protein.

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CHAPTER 1
Introduction

1.1 Influenza and Influenza A Virus

Influenza is an infectious disease caused by an influenza virus [1] (Fig 1). The symptoms of influenza could be mild to severe, including fever, headache, sore throat, feeling tired, headache, coughing, and muscle pains [2] (Fig 2). Influenza A virus belongs to the family of Orthomyxoviridae. It is an enveloped virus with a negative sense RNA segmented genome [4]. This virus has evolved a large number of mechanisms that enable it to invade host cells and subvert the host cell machinery for its own purpose, that is, for the sole production of more virus. Influenza A viruses are negative-sense, single-stranded, segmented RNA viruses. The several subtypes are labeled according to an H number (for the type of hemagglutinin) and an N number (for the type of neuraminidase). [5] Hemagglutinin is a protein that causes red blood cells to agglutinate, and neuraminidase is an enzyme that cleaves the glycosidic bonds of the monosaccharide [6]. There are 18 different known H antigens (H1 to H18) and 11 different known N antigens (N1 to N11). This thesis mainly focuses on the subtype Influenza A virus H1N1. Current H1N1 influenza virus vaccine works by exposing you to a small dose of the virus, which helps your body to develop immunity to the disease. There are several kinds of vaccines like injectable H1N1 vaccine and nasal spray H1N1 vaccine. These vaccines have common limitations, which is, H1N1 vaccine may not provide right immune protection in every year. Also, many people may not develop immune response to some vaccines. In other words, the current vaccines to H1N1 influenza requires development in both stability and universality.

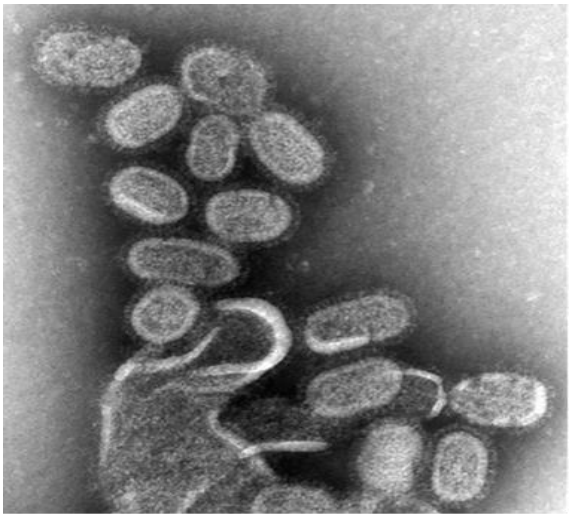


Figure 1. Influenza virus, magnified approximately 100,000 times[1]

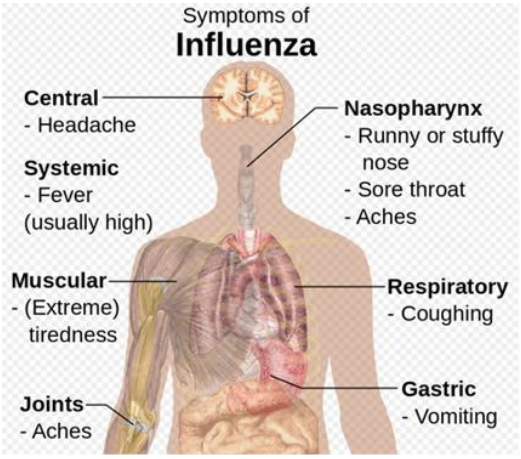


Figure 2. Symptoms of the influenza[4]

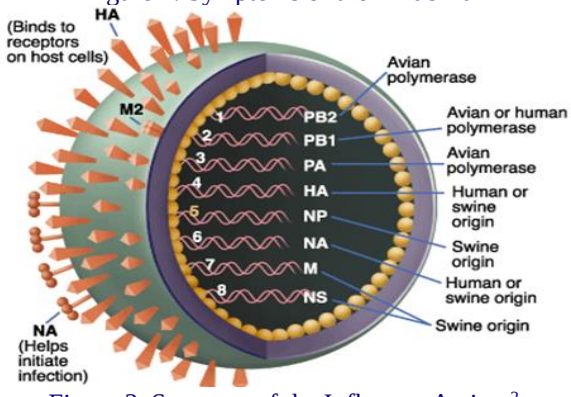


Figure 3. Structure of the Influenza A virus[2]

As for the drugs for H1N1 influenza, two classes of FDA- approved antiviral drugs called neuraminidase inhibitors (oseltamivir and zanamivir) and M2 protein inhibitors (amantadine derivatives) are used against the disease. Unfortunately, increasingly prevalent resistance to neuraminidase inhibitors has led to researchers to seek alternative antiviral drugs with different mechanisms of action. Neuraminidase inhibitors (NAIs) are a class of drugs which block the neuraminidase enzyme. They are commonly used as antiviral drugs because they block the function of viral neuraminidases of the influenza virus, by preventing its reproduction by budding from the host cell. [8,9] The antiviral drugs amantadine and rimantadine inhibit a viral ion channel [6] (M2 protein), thus inhibiting replication of the influenza A virus. However, measured resistance to M2 inhibitor increased dramatically in several influenza viruses (increase to 91% in H3N2). This high level of resistance may be due to the easy availability of amantadine as part of over-the-counter cold remedies in countries such as China and Russia, and their use to prevent outbreaks of influenza in farmed poultry[7]. As a result, Centers for Disease Control (CDC) recommended against using M2 inhibitors during the 2005–06 influenza season due to the resistance.

1.2 Non-structural influenza protein

The non-structural (NS1) protein of influenza A virus is a non-essential virulence factor that has multiple accessory functions during viral infection. In recent years, the major role ascribed to NS1 has been its inhibition of host immune responses, especially the limitation of both interferon (IFN) production and the antiviral effects of IFN-induced proteins. NS1

might also inhibit splicing of pre-mRNA by binding to a stem-bulge region in U6 small nuclear RNA (snRNA)[12]. Although consisting of only approximately 230 amino acids, NS1 is able to interfere with several systems of the host viral defense. In addition, NS1 is probably able to suppress the interferon response in the virus-infected cell leading to unimpaired virus production.[11]

1.3 SUMO protein and SUMOylation pathway

SUMO (Small Ubiquitin-related MOdifier) are a family of small proteins that are covalently attached to and detached from other proteins in cells to modify their function. SUMOylation is the process whereby SUMO proteins attach themselves to the target proteins within cells and modify their function. [12] It is an important posttranslational protein modification with critical roles in multiple biological processes. SUMO proteins are similar to ubiquitin, and SUMOylation is directed by an enzymatic cascade analogous to that involved in ubiquitination. Before the reversible cascade begins, SUMO was synthesized as a precursor protein, then it was catalyzed by SUMO-specific proteases (SENPs) to become mature. As a result, the nascent SUMO has to be cleaved to expose the C-terminal glycine-glycine (GG) motif to form an isopeptide bond with ε-amino group of a Lys residue in its substrate. Posttranslational conjugation of mature SUMOs occurs through the cascade. Firstly, the mature SUMO protein activates its C-terminal glycine motif and Cys residue of heterodimer SUMO-specific E1 activating enzyme (Uba2/Aos1) to form a thioester bond. This reaction undergoes ATP-dependent. Next activated SUMO is transferred from E1 enzyme to a SUMO- conjugating enzyme E2 (Ubc9), forming a

thioester linkage between the catalytic Cys residue of Ubc9 and the C-terminal carboxy group of SUMO. Then, SUMO transfers to the substrate to form an isopeptide bond between SUMO C-terminal glycine residue and a lysine side chain of the substrate, usually together with specific SUMO E3 ligase. Last, SENPs will cleave SUMO from conjugated target substrate which release SUMO to free for the further cycles. [13]

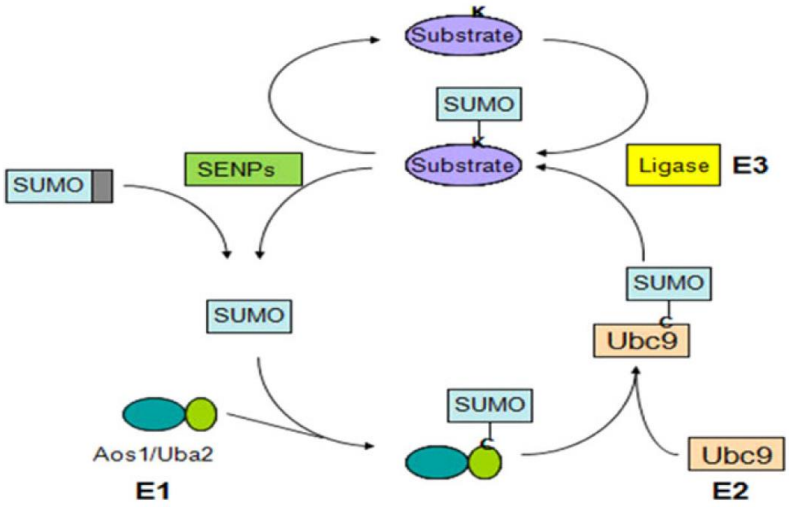


Figure 4. Multiple protein–protein interactions are involved in SUMOylation conjugation pathway[13]

The misregulation of SUMOylation pathway would lead to different kinds of diseases (Figure 5), including cancer, heart diseases, neurodegenerative diseases, and diabetes[15].

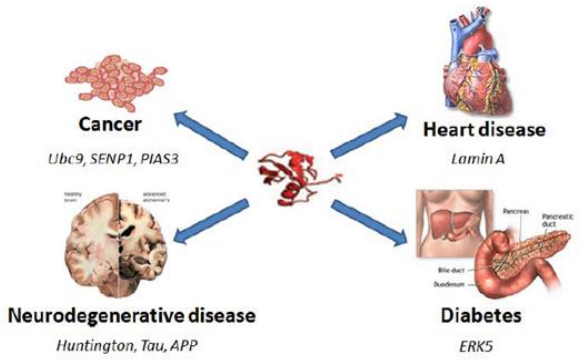


Figure 4. Misregulation of SUMOylation is related to different kinds of diseases

There are 3 kinds of enzymes involved in SUMOylation pathway, the SUMO-activating enzyme (E1), the conjugation enzyme and the SUMO ligases enzyme (E3), The E1 enzyme is a heterodimer and composed of two subunits, one is known as Aos1 located at the N-terminal and the other subunit is Uba2 which corresponds at the C-terminal contains the active site cysteine. Conjugation enzyme E2 is also known as Ubc9, which plays an important role in SUMOylation cascade. It is worth to mention that Ubc9 is the only SUMO-conjugating enzyme in yeast invertebrates and most of the vertebrates. SUMO ligases enzyme (E3) catalyze the transfer of proteins from E2 enzyme to a substrate. E3 also mediate and stabilize the interaction of the substrate with the E2. PIAS (protein inhibitor of activated STAT) is the most common type of the E3 ligase.

1.4 Forster Resonance Energy Transfer and its application

Förster resonance energy transfer (FRET) is a nonradioactive energy transfer between the excited chromophore to a proximal ground state chromophore, which is also known as the donor and the acceptor. Measurements of FRET efficiency can be used to determine if two fluorophores are within a certain distance of each other. Such measurements are used as a research tool in fields including biology and chemistry. It is an electrodynamic phenomenon which explained by using classical physics. When the donor and acceptor with favorable orientations are close to each other, 2-10nm, the energy evoke from the excited donor will transfer to the acceptor to induce emission.[19] The range in 2-10nm distance between donor and acceptor is comparable to the size of biomolecules or the distance of protein sites. Figure 6 shows the basic mechanism of FRET. Here in our lab,

we apply FRET technology into elucidating protein interactions in many cellular processes. To be more specific, in this thesis, a pair of engineered fluorescence protein called Cypet and Ypet is used to fused with the protein of interest. Also, FRET-based technology is used in many other biological researches, such as study protein folding, high-throughput screening for drug usage, and sensory of signaling pathway.[20]

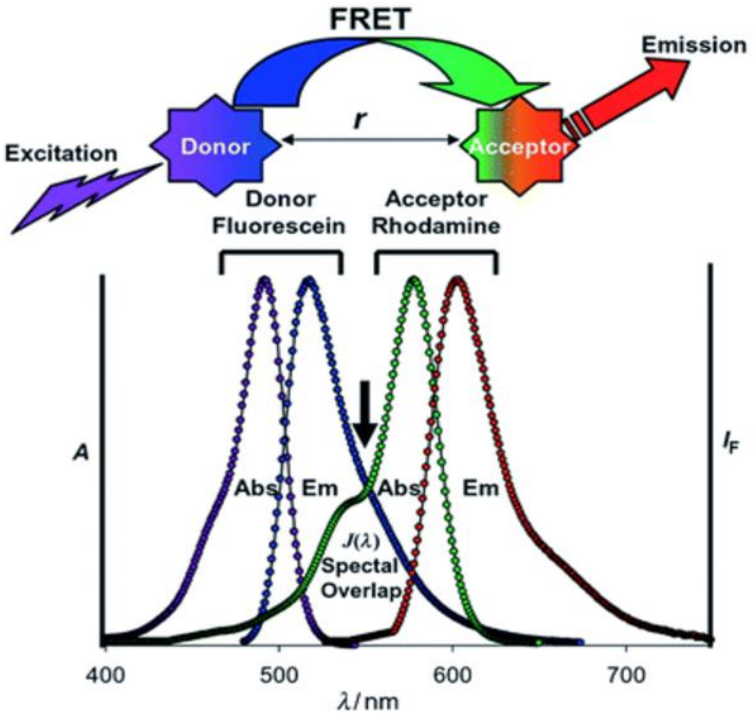


Figure 6. Schematic representation of FRET. An excited donor transfers energy to acceptor that emits it through fluorescence in a non-radiative fashion.

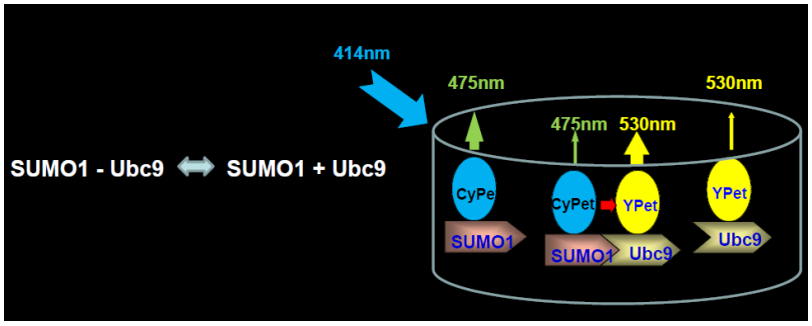


Figure 7. Diagram of using FRET for elucidating protein-protein interaction between Ubc9 and SUMO1[13]

1.5 Site-directed mutagenesis

Site-directed mutagenesis is a molecular biology method that is used to make specific and intentional changes to the DNA sequence of a gene and any gene products, it is widely used for investigating the structure and biological activity of DNA, RNA, and protein molecules, and for protein engineering.

The basic procedure of site-directed mutagenesis requires the synthesis of a short DNA primer (Figure 8). This synthetic primer contains the desired mutation and is complementary to the template DNA around the mutation site so it can hybridize with the DNA in the gene of interest. In this thesis, the gene of the NS1 influenza protein is mutated by site-directed mutagenesis strategy. To be more specific, every 12 lysines' codon(AAA/AAG) in NS1 gene is mutated to Alanine codon (GCA/GCG) one by one by site-directed mutagenesis methodology.

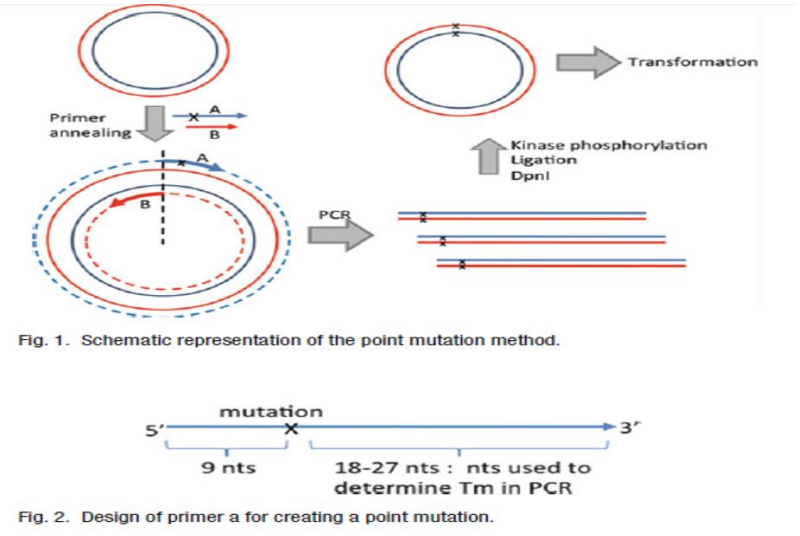


Figure 8. Principle of site-directed mutagenesis (Figure from Springer Protocols: Enzyme Engineering Methods and Protocols. James Samuelson. Human Press. 2013)

1.6 Recombinant protein expression and purification

Protein expression refers to the way in which proteins are synthesized, modified and regulated in living organisms. In protein research, the term can apply to either the object of study or the laboratory techniques required to manufacture proteins. Essentially, DNA encoding a target protein is cloned downstream of a promoter in an expression vector. This vector is then introduced into a host cell, and the cell's protein synthesis machinery produces the desired protein. There are several approaches of protein expression, like *E. coli* protein expression, yeast protein expression, cell-free protein expression, etc. Among all these approaches, protein expression in the bacterium E. coli has been the most popular means of producing recombinant proteins for over two decades. E. coli is a well-established host that offers short culturing time, easy genetic manipulation and lowcost media. Additionally, E. coli has a long history of being able to produce a wide variety of different types of proteins.

Protein purification is a series of processes intended to isolate one or a few proteins from a complex mixture, usually cells, tissues or whole organisms. Basically, there are 2 aims of protein purification: preparative purification and analytical purification. Preparative purification aims to produce a relatively large quantity of purified proteins for subsequent use. Analytical purification produces a relatively small amount of a protein for a variety of research or analytical purposes, including identification, quantification, and studies of the protein's structure, post-translational modifications and function. To purify protein from *E. coli*, several steps are required such as extraction, Precipitation and differential solubilization, ultracentrifugation.[21] As for the purification strategies, metal binding is used

in our lab. The principle of metal binding is to engineer a sequence of 6 to 8 histidines into the N- or C-terminal of the protein.[22] The polyhistidine binds strongly to divalent metal ions such as nickel and cobalt. The protein can be passed through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged proteins pass through the column. The protein can be eluted with imidazole, which competes with the polyhistidine tag for binding to the column, or by a decrease in pH, which decreases the affinity of the tag for the resin.[22]

1.7 Inclusion bodies

Inclusion bodies (IBs), sometimes called elementary bodies, are nuclear or cytoplasmic aggregates of stable substances, usually proteins. (Fig. 9). Many recombinant polypeptides are unable to fold properly within the cell and associate to form large protein aggregates. For long, IBs were considered to consist solely of unfolded or highly misfolded polypeptides. However, it now seems clear that, at least in specific cases, a significant part of IBs consists of properly folded and biologically active protein.[20] More importantly, there are several advantages of the expression in inclusion bodies. Firstly, proteins in IBs are largely resistant against degradation by host cell proteases and less likely to exert toxic effects. Moreover, IBs are easy to isolate from cell lysates by differential centrifugation.

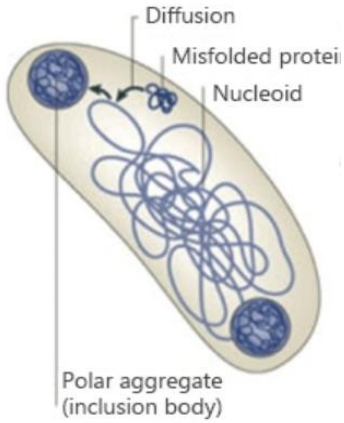


Figure 9. Structure of inclusion bodies[22]

1.8 *E. coli* TorA Signal Sequence

In order to drive the soluble protein into inclusion bodies, a 39amino acid signal sequence of E. coli TMAO reductase (ssTorA) is fused with our protein of interest (Figure 10). This ssTorA was founded to have the function to direct high-level expression of the human protein in inclusion bodies. This signal sequence is firstly observed by the scientists from VU University (Amsterdam, Netherlands). They applied this ssTorA to the expression of several human proteins hEGF, Pla2 and IL-3. Also, they found out that this sequence was able to mediate IB formation of the highly soluble *E. coli* proteins TrxA and MBP. The second part of this thesis is mainly focus on applying ssTorA to the SUMOylation E1 ligase Uba2. By driving this soluble protein into inclusion bodies and make it insoluble, it can overcome the degradation of the Uba2 protein when using the regular expression and purification method.

Fusion	Amino acid sequence	Mode of IM targeting/IM translocon
ssTorA/POI	mnnndlfqasrrrflaqlggltvagmlgpslltprrasa-*POI*	Posttranslational/Tat

Figure 10. Amino acid sequence of ssTorA[20]

1.9 Purify protein from inclusion bodies

After driving the protein into inclusion bodies (by fusing ssTorA with the gene of protein Ypet-Uba2), the ssTorA-Ypet-Uba2 needs to be purified from inclusion bodies, which means the regular way in our lab to purify soluble Ypet-Uba2 cannot be used to purify the ssTorA-Ypet-Uba2 protein. Here, a screening strategy was designed for purifying insoluble proteins. The goal of this screening methodology is to find out an optimal lysis conditions for the ssTorA proteins. More specifically, in order to solubilize the insoluble protein, several kinds of additives were added respectively to the basic lysis buffer. The cell pellet collected by centrifugation were resuspeneded in different lysis buffers.

1.10 FRET-based Kd Determination

Dissociation constant, Kd, is an important parameter for characterizing protein–protein interactions. For example, we have a general reaction $A_xB_y \Leftrightarrow xA + yB$, in which a complex A_xB_y breaks down into x A subunits and y B subunits, the dissociation constant is defined by the following equation:

$$Kd=[A]^x[B]^y \div [A_xB_y],$$

where [A], [B], and $[A_xB_y]$ are the concentrations of A, B, and the concentration of complex A_xB_y, respectively.

Based on the fact that FRET signal is proportional to the amount of bound FRET pair, we developed a FRET-based technology for Kd determination. FRET technology has been used for determining the dissociation constant for protein-protein interaction and cell-based high-throughput screening assay in SUMOylation cascade. To be more specific, this

technology is based on the energy transfer between Cypet and Ypet fused with our proteins of interest (in this thesis, Cypet is fused with Aos1 protein, and Ypet is fused with Uba2 protein. The Kd determined by FRET is compatible with those determined with other traditional approaches, such as isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR). To implement FRET-based technology for Kd determination, two key issues need to be solved. First, we need to differentiate and quantify fluorescence signal of FRET from other direct fluorescence signals of donor and acceptor at the excitation wavelength.[30] Second, we need to convert fluorescence signals to corresponding concentrations of bound partners, donor and acceptor proteins. We started with the general law of mass action for protein–protein interaction. The specific kd measurement would be introduced in the 'Materials and methods' part in the chapter 3 of this thesis.

CHAPTER 2

Determine Essential SUMOylation Site for the NS1 Protein in Influenza A Virus by Using FRET Technology and Site-directed Mutagenesis

2.1 Abstract

SUMO (Small Ubiquitin-related MOdifier) are a family of small proteins that are covalently attached to and detached from other proteins in cells to modify their function, this modification is called SUMOylation. To be more specific, SUMOylation is the process whereby SUMO proteins attach themselves to the target proteins within cells and modify their function. It is an important posttranslational protein modification with critical roles in multiple biological processes. It was reported that Influenza A virus interacts extensively with the cellular SUMOylation system during infection. To be more specifically, the influenza non-structural protein (NS1) is a bona fide SUMO target [14]. In SUMOylation pathway, nascent SUMO needs to be cleaved to expose the C-terminal glycine - glycine(GG) motif to form an isopeptide bond with ε-amino group of a Lys residue in its substrate. However, there are 12 lysine residues in NS1 protein, which lysine residue is related to SUMOylation is not clear yet. In order to figure out which lysine residue is the SUMOylation site, each lysine residue was mutated to alanine by site-directed mutagenesis technology. This thesis applied 3 different methods to create mutations in NS1 gene, including regular gene cloning, TA cloning and NEBuilder assembly strategy. After the 12 lysine sites were mutated respectively, mutated proteins were expressed and purified. By conducting a SUMOylation assay to all these mutated proteins, we figured out Lysine residue 131 is the essential SUMOylation site, which is contrast to the previous study

(which reported k70 and k219 are the NS1 SUMOylation site) [36]. In order to further prove the importance of k131, different NS1 mutations were cloned into pDZ vector and were tested in vivo. As a result, the viral growth of influenza virus was affected when k131 was mutated, which further proved that k131 is the essential SUMOylation site.

2.2 Introduction

Influenza, commonly known as "the flu", is an infectious disease caused by an influenza virus.[1] The symptoms of influenza can be mild to severe. Common symptoms include: a high fever, runny nose, sore throat, muscle pains, headache, coughing, and feeling tired. The outbreaks of Influenza can kill millions of people worldwide during pandemic years and hundreds to thousands during other years.[2] The first convincing record of an influenza pandemic was of an outbreak in 1580, which began in Russia and spread to Europe via Africa. In Rome, over 8,000 people were killed, and several Spanish cities were almost wiped out.[2] Pandemics continued sporadically throughout the 17th and 18th centuries, with the pandemic of 1830–1833 being particularly widespread; it infected approximately a quarter of the people exposed.

Influenza can be spread in three main ways: by direct transmission (when an infected person sneezes mucus directly into the eyes, nose or mouth of another person); the airborne route (when someone inhales the aerosols produced by an infected person coughing, sneezing or spitting) and through hand-to-eye, hand-to-nose, or hand-to-mouth transmission, either from surfaces or from direct personal contact such as a handshake.[3] Influenza infection and replication is a multi-step process[3] : First, the virus has to bind to

and enter the cell, then deliver its genome to a site where it can produce new copies of viral proteins and RNA, assemble these components into new viral particles, and, last, exit the host cell.

The entire Influenza A virus genome is 13,588 bases long and is contained on eight RNA segments that code for 11 proteins. Segment 1 encodes RNA polymerase subunit (PB2). Segment 2 encodes RNA polymerase subunit (PB1) and the PB1-F2 protein, which induces cell death, by using different reading frames from the same RNA segment. Segment 3 encodes RNA polymerase subunit (PA); an alternate form of this polymerase can sometimes be made with a change to the reading frame. Segment 4 encodes for HA (hemagglutinin). About 500 molecules of hemagglutinin are needed to make one virion. HA determines the extent and severity of a viral infection in a host organism. Segment 5 encodes NP, which is a nucleoprotein. Segment 6 encodes NA (neuraminidase). About 100 molecules of neuraminidase are needed to make one virion. Segment 7 encodes two matrix proteins (M1 and M2) by using different reading frames from the same RNA segment. About 3000 matrix protein molecules are needed to make one virion. Segment 8 encodes two distinct non-structural proteins (NS1 and NEP) by using different reading frames from the same RNA segment. This thesis is mainly focus on the non- structural (NS1) Influenza virus protein.

Among the 11 kinds of influenza proteins, non-structural (NS1) protein of influenza A viruses is a non-essential virulence factor that has multiple accessory functions during viral infection. In recent years, the major role ascribed to NS1 has been its inhibition of host immune responses, especially the limitation of both interferon (IFN) production and the

antiviral effects of IFN-induced proteins.[15] Also, NS1 acts directly to modulate other important aspects of the virus replication cycle, including viral RNA replication, viral protein synthesis, and general host-cell physiology. To be more specific, NS1 protein is associated with numerous roles during influenza infection, including: Modulation of viral RNA replication, general inhibition of the nuclear export of mRNAs carrying polyadenylated tails, the inhibition of the transcriptional elongation of host genes, the regulation of host and viral protein synthesis, and the neutralization of the initial signaling pathway which is leading to the synthesis and production of the type I Interferon.[14]

2.3 Materials and Methods

2.3.1 Plasmid constructs

Pet28 (b) Ypet-NS1 construct with different mutants

The open reading frames of the genes were amplified by PCR and the PCR products were cloned into pet28 (b) vector. To be more specific, pet28(b) Ypet wild type NS1 construct was made by the previous phD student in our lab. Before generating mutations on each lysine codons in NS1 gene, the amino acid sequence of NS1 was viewed by using CLC sequence viewer. From figure 11, there were 12 lysine residues in NS1 gene, including K20, K20, K41, K62, K70, K78, K108, K110, K126, K131, K175, K217, K219. Since from a previous paper, K70 and K219 were identified as possible SUMOylation site, so K70 was mutated firstly in the NS1 gene. Then the other lysines are mutated in the order shown in figure 12. The method of generating mutations was to introduce a codon substitution in the forward PCR primer. After the PCR reaction, the PCR product was

checked on a 0.8% Agarose gel. By purifying the PCR gel band, the mutant NS1 insert was then digested by the Restriction Enzymes (SalI and NotI). By ligation this digested insert to a digested pet28(b) vector, the recombinant plasmid was made. If both the digestion check and sequencing gave positive results, the mutant construct was ready for protein expression and purification. In total, all of the 12 mutants of the pet 28(b) Ypet-NS1 construct were made by this method.

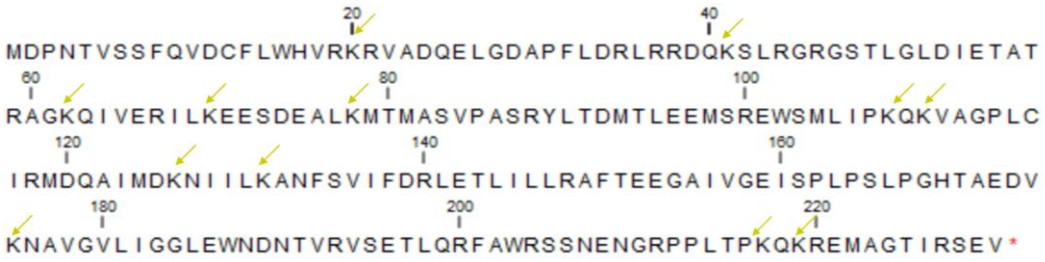


Figure 11. The amino acid sequence of NS1, every yellow arrow points to a lysine residue

NS1 Mutant	Lysine Residues mutated to Alanine
1	70
2	175
3	70, 175, 217
4*	70, **108**, 175, 217
5	**41**, 70, 108, 175, 217
6	41, **62**, 70, **126**, 108, 175, 217
7*	41, 62, **78**, 126, 108, 175, 217
8	**20**, 41, 62, 78, 126, 108, 175, 217
9	20, 41, 62, 78, 126, 108, **110**, 175, 217
10	20, 41, 62, 78, 108, 110, 126, **131**, 175, 217
11	20, 41, 62, 78, 108, 110, 126, 131, 175, 217, **219**
12	20, 41, 62, **70**, 78, 108, 110, 126, 131, 175, 217, 219

Figure 12. The order of the mutation sites

After the SUMOylation assay was conducted and k131 was found that would be the true SUMOylation site, which was incompatible with the previous study. Therefore, two other pet28(b) constructs were made. One of the two constructs were the pet28(b) Ypet-

NS1(K70A+K219), which was made by mutating k219 in the pet28(b) Ypet-NS1(K70A) construct. The other construct was made mutating K131 in the wild type NS1 construct.

Ypet-NS1 pDZ vector with different mutants

To test whether the K131 affect the viral growth, the pDZ vector is a kind of viral recovery vector, which is usually used in mammalian cell culture and many other kinds of *in vivo* experiments. Since K70 and K219 are the possible SUMOylation sites detected by previous paper, and K131 is the SUMOylation site we found by our in vitro SUMOylation assay., 2 kinds of pDZ Ypet NS1 mutants were made (K70A+K219A and K131A). To generate mutations in NS1 in pDZ vectors, we used a new cloning technology called NEBuilder Assembly Kit. This Kit allowed us to assemble more than 2 fragments at one time. Also, each insert fragment is not required to be digested by restriction enzymes, which saved a lot of time for the gene cloning. After mixing the fragments (PCR inserts and digested vector) with the NEB mastermix in a specific ratio, the mixture was incubated in 50 degrees for 1 hour. Then the incubated mixture was transformed into TOP10 competent cells by electroporation and shaked for 1 hour in 37 degrees. After the culture is plated in LB Agar with Ampicillin and incubated overnight, single colonies are inoculated for plasmid extraction. If both the digestion check and sequencing gave positive results. The pDZ vector could be used for transfection to mammalian cells.

2.3.2 Protein expression and purification of the mutant Ypet-NS1 proteins

After the 12 mutants pet28(b) NS1 constructs were made, all of them were transformed respectively to BL21(DE3) for protein purification. Single colonies were picked up and

inoculated in 5mL LB (K+) medium and were shaken overnight at 37 degrees. Then the LB culture was used to inoculate the 1L 2×YT medium, when the 2×YTs were shaken at 37 degrees, 180 rpm until the OD reached 0.5-0.6, 0.6mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added in order to induce the proteins. After a 16 degree overnight shaking at 150 rpm, the cells were collected by centrifugation. Then 35 mL of regular lysis buffer was added to the cell pellet, and the cell membrane was broken by sonication. After this, the sonicated mixture was centrifuged and the supernatant was collected. By adding the supernatant to Nickel Beads and wash the beads several times by the wash buffers, the protein was collected by elution buffer. After an overnight dialysis of the protein, the protein sample was checked by Bradford assay and 10% SDS PAGE gel, if the purity and the concentration are good, the proteins can be used for in vitro SUMOylation assay.

2.3.3 *In vitro* FRET based SUMOylation assay

All the proteins from 2.3.2 are used for the *in vitro* FRET based SUMOylation assay. Figure 13 shows the SUMOylation pathway of NS1. To identify the SUMO site of H1N1 NS1, all components of the SUMOylation assay including 1 μM CyPet-SUMO1, 0.2 μM Aos1, 0.2 μM Uba2, 0.5 μM Ubc9, 2 μM YPet-NS1 or its mutants were combined in a buffered solution containing 50 mM Tris–HCl (pH 7.4), 1 mM DTT and 4 mM MgCl2 in a total volume of 60 μL. The sample mixtures were incubated in a Greiner 384-well plate (Sigma-Aldrich) at 37 °C. After adding 1 mM ATP to the sample well the fluorescence emissions were measured in time course using Flexstation II 384 (Molecular Devices). The

emission intensities under three wavelength settings were obtained: 475 nm and 530 nm at an excitation wavelength of 414 nm, and 530 nm at an excitation wavelength of 475 nm. The absolute FRET signal was calculated by subtraction of direct contributions from donor-CyPet and acceptor-YPet, as described in (Y.Song, 2010). Different controls were set up in the absence of SUMO E1(Aos1/Uba2) or SUMO E2(Ubc9) or ATP under the same experimental condition. The amount of SUMOylated YPet-NS1 and its mutants from different samples were determined by western blot using anti-NS1 antibody.

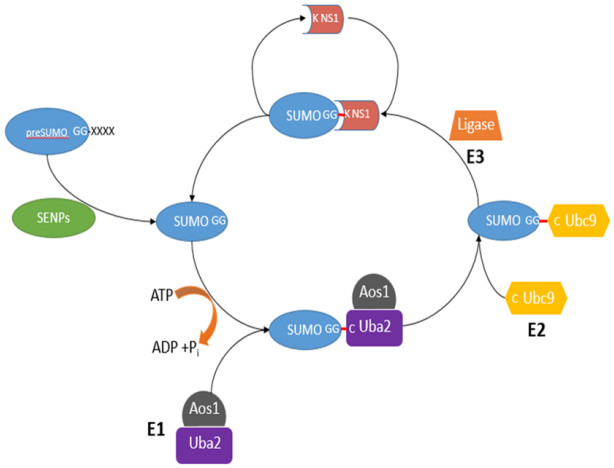


Figure 13. SUMOylation of NS1

2.3.4 *In vivo* plaque assay

Plaque is an area of cells in a monolayer which display a cytopathic effect, like appearing round and darker than other cells under the microscope, or as white spots when visualized by eye while the plaque center may lack cells due to virus-induced lysis. Plaque-based assays are the standard method used to determine virus concentration in terms of infectious dose[16]. MDCK cells have been successfully employed for the primary isolation of influenza A virus. Parallel titration of several clinical specimens showed that the inoculation into

MDCK cells followed by incubation in the presence of trypsin was an isolation procedure as sensitive as the amniotic inoculation into fertile eggs. Firstly, several pDZ vectors with different influenza viral proteins were transfected to MDCK cell lines, including the pDZ-NS1 (with different mutants), pDZ-M, pDZ-NA, pDZ-NP, pDZ-PA, pDZ-PB1, pDZ- PB2 and pDZ-HA. The virus is supposed to be recovered in MDCK cells. The infection of the MDCK cell lines was conducted in different titrations of the influenza viral particles.

2.4 Result

2.4.1. After NS1 K131 was mutated, the EmFRET signal decreased dramatically

According to the Figure.14 (a), after K131 was mutated (series 10), the EmFRET signal decreased dramatically, which means, after K131 was mutated to Alanine, the Ypet-NS1 cannot conjugated to Cypet-SUMO1 protein, and FRET was not occurring between these 2 proteins. What is worth mention is that, after K70 is mutated series 1), EmFRET signal was still detected. Therefore, these results gave us the evidence that K131 is the SUMOylation site of NS1.

NS1 Mutant	Lysine Residues mutated to Alanine
1	70
2	175
3	70, 175, 217
4*	70, **108**, 175, 217
5	**41**, 70, 108, 175, 217
6	41, **62**, 70, **126**, 108, 175, 217
7*	41, 62, **78**, 126, 108, 175, 217
8	**20**, 41, 62, 78, 126, 108, 175, 217
9	20, 41, 62, 78, 126, 108, **110**, 175, 217
10	20, 41, 62, 78, 108, 110, 126, **131**, 175, 217
11	20, 41, 62, 78, 108, 110, 126, 131, 175, 217, **219**
12	20, 41, 62, **70**, 78, 108, 110, 126, 131, 175, 217, 219

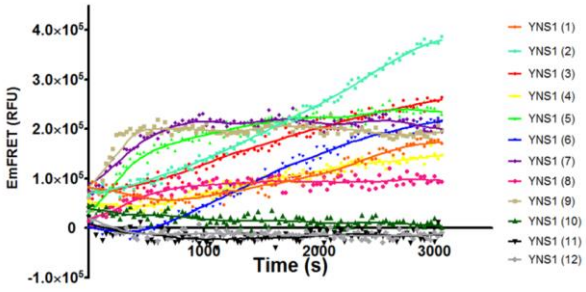


Figure 14. (a) Mutation order of the NS1 gene (b) Fluorescence spectrum of different NS1 mutants (Figure from Zhehao Xiong)

2.4.2 NS1 mutants with K219A+K70A shows FRET signals while the mutant with K131A killed the FRET signal

To compare the FRET signal between K219A+K70A mutants with the K131A mutants, a pet28 (b) construct with double mutations K219A and K70A was made. After the protein mutants are purified, the SUMOylation assay between Cypet-SUMO1 and Ypet-NS1 was conducted, figure15 shows the fluorescence spectrum of each SUMOylation pairs.

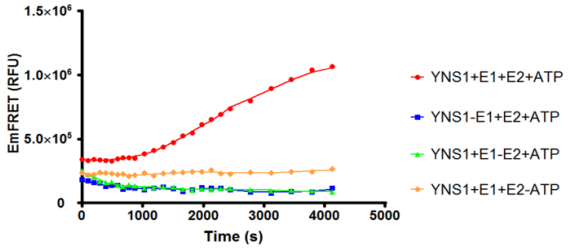


Figure 15 (a) Wild type NS1 shows FRET signal

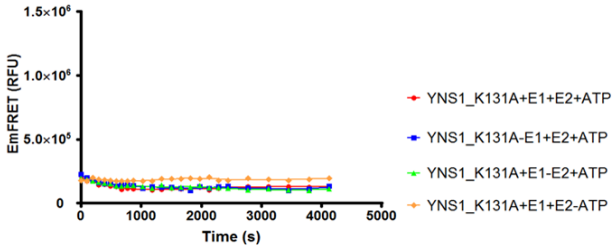


Figure 15 (b) NS1 with K131A shows no FRET signal

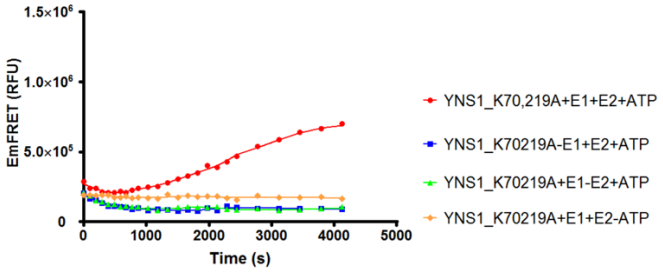


Figure 15 (c) NS1 with K70A and k219A still has FRET signal
(Figure from Zhehao Xiong)

According to the figures above, the mutation of the K219 and K70 still showed FRET signal, which is around the same level of the SUMOylation assay between WT-Ypet-NS1

and Cypet-SUMO1. On the contrary, the mutant K131A showed no FRET signal. The EmFRET remains the same with the control series. This result enhanced the conclusion that K131 is the SUMOylation lysine residue of NS1 protein.

2.4.3 Mutation of NS1 K131 affects the viral growth of the Influenza A Virus

Figure 16 shows the result of the plaque assay in MDCK cell. From the figure, by doing a series of dilution to the virus to infect the MDCK cell lines, virus with k70 mutation can still kill the cell under 10^{-6} dilution of the viral particle. On the other hand, the pDZ vector with K131A showed lower infection under 10^{-6} dilution, which gave us the evidence that, K131 of the NS1 protein highly affects the viral growth of the influenza A virus.

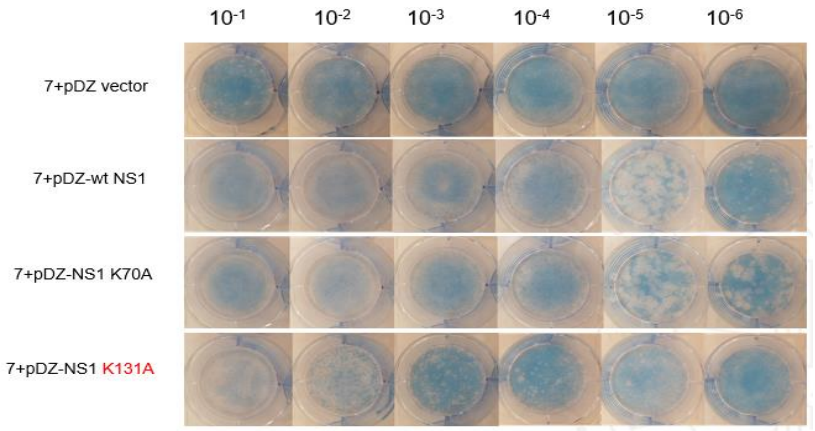


Figure 16. Comparison of the viral growth in MDCK Cell lines of the different NS1 mutants, the number of the plaques was much lower in the same dilution of the viral particles when K131 was mutated. These results gave us the evidences that the mutation of K131 can affect the viral growth of the virus. (Figure from Zhehao Xiong)

2.5 Discussion

Here, we have reported a methodology to determine the SUMOylation site of the NS1 influenza protein. By conducting the site-directed mutagenesis and FRET-based SUMoylation assay, lysine residue 131 was determined to be an essential SUMOylation site of NS1 protein. Since this result is incompatible with the previous studies, which

reported that K70 and K219 are the SUMOylation sites. To prove the result from our assay, both mutants (K70A+k219A and K131A) were tested by *in vitro* SUMOylation assay and *in vivo* plaque assay. Results from both assays have shown that K131A affects both the SUMOylation of the NS1 and the viral growth of influenza A virus in MDCK cell lines. Also, both the results from the *in vitro* and *in vivo* assays proved that the previous studies were wrong.

Future work of this project would be finding out other SUMOylation sites in other influenza A virus proteins. Previous studies showed that M2, NS2, PB1, PB2, NP and many other influenza A virus proteins are efficiently SUMOylated. It is necessary for us to find out if there's any SUMOylation site which is more essential than the K131 of NS1. Also, the identification of essential SUMOylation site(s) can provide us more supporting evidences to develop new kinds of inhibitive drugs for treating the Influenza virus.

CHAPTER 3

Develop a New Methodology to Express Difficult Proteins for FRET-based Assay

3.1 Abstract

Nowadays, using *E. coli* bacteria as the host for protein production is a very common strategy. However, there are several challenges that needs to be overcome by using *E. coli* protein production, such as the degradation of the target protein, the toxic of protein towards the host, the misfolding and instability of the protein, and the complex purification procedures.[26] By expression the proteins in inclusion bodies (IBs) would help alleviating these problems. This thesis conducted a signal sequence TMAO-reductase (ssTorA) to drive the target protein Ypet-Uba2, into the inclusion bodies and overcame the degradation of the protein. Also, a buffer screening method was designed to purify the protein from inclusion bodies. As a result, trehalose and zwittergent were proved to be a high efficient additive for the Ypet-Uba2 protein purification. This protein expression and purification strategy gave us a new direction of harvesting high quality proteins, including the reduction the degradation of the proteins and improving the yield of the protein products.

3.2 Introduction

The current protein expression and purification methods in our lab are mostly dealing with the soluble proteins. After the transformed BL21(DE3) strain was inoculated in 2xYT medium and induced by IPTG, the cell was collected and treated with a regular lysis buffer.

By using the sonication to break the cell membrane, the protein would be solubilized by the regular lysis buffer. The reality is, by using this method to purify several proteins in the SUMOylation pathway, like UBA2, this method was causing a degradation of the target protein. The unwanted degradation of the target protein may be caused by the proteases in *E. coli* host, which can be overcome by driving the protein into the inclusion bodies. Inclusion bodies can give the protection of both our protein and the *E. coli* host. Since the protein is highly aggregated, on the one hand, it can protect the protein from degradation. On the other hand, it can protect the host from the toxicity of the target protein.

However, the propensity of proteins to form IBs is variable and difficult to predict[32,33], which means we need to find out a way to give our protein a high propensity to form inclusion bodies. This can be achieved by the fusion of a signal sequence called ssTorA. The sequence was introduced detailedly in Chapter 1 of this thesis.

After proving the protein was driven into the IBs, the next step would be the purification of the inclusion bodies. This thesis conducted a lysis buffer selection method, which is, using different lysis buffers to solubilize the inclusion bodies and compare the solubilities of each buffer.[32] The additives in the buffers contains 0.5% Zwittergent, 0.5M Trehalose, 1M Guanidine HCL, Tween-20. At a result, zwittergent and trehalose showed that both of them can solubilize 93% of the inclusion bodies. After purified the inclusion bodies from the supernatant, ssTorA-Ypet-Uba2 showed a higher purity and higher yield than the regular Ypet-Uba2 proteins.

3.3Materials and Methods

3.3.1 Plasmid construct

SsTorA protein Construct

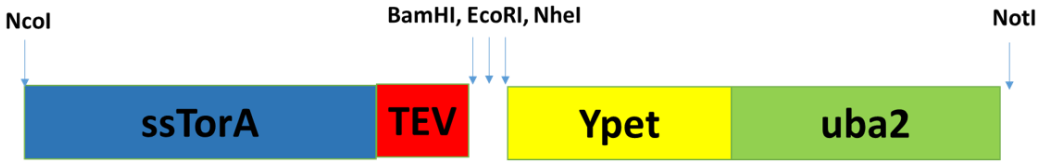


Figure 17. Construct design of the ssTorA-Ypet-Uba2 gene

The Construct encoding Ypet-Uba2 was cloned into the pET28(b) vector, engineered with ssTorA and TEV sequence as described previously. Also, several cutting sites of the restriction enzyme were cloned in this construct, such as NcoI (upstream of ssTorA), BamHI and EcoRI (between ssTorA and Ypet), SalI (between Ypet and Uba2), and NotI (at the downstream of Uba2). After this construct was made, it can be used to express other proteins. By using EcoRI and NotI to release the Ypet-Uba2 insert, other protein gene can ligate to this plasmid construct.

Currently, there are several proteins which cannot be purified very well such as Ypet-PDL1, Ypet-PA, Ypet- PB1, Ypet- PIAS1, etc.The Ypet-Uba2 in the ssTorA plasmid construct can be replaced by all these protein gene.

3.3.2 Protein expression and purification for Regular Ypet-Uba2

Escherichia coli cells of strain BL21 (DE3) were transformed with pET28 vectors encoding Ypet-Uba2 (without ssTorA). The transformed bacterial cells were plated onto LB agar plates containing 50 mg/ml kanamycin, and a single clone for the protein Ypet-Uba2 was picked up for starter culture and inoculated in 10 mL LB overnight at 37°C. After the

overnight innoculation, the 10mL culture was transferred to 1 L 2XYT medium and grown at 37°C for 3hrs until the optical density of the culture reached 0.5-0.6. The expression of Regular Ypet-Uba2 protein is achieved by the induction with 0.6mM Isopropyl b-D-1 thiogalactopyranoside (IPTG) at 16°C overnight. The Ypet-Uba2 protein were purified from bacterial lysates with nickel agarose affinity choromatography (Qiagen, Valencia, CA, USA), after several rounds of wash buffers, proteins were eluted by elution buffer (20mM Tris HCl, pH 7.4, 20mM NaCl, 150mM imidazole). After an overnight dialysis in the dialysis buffer (20 mM Tris-HCL, pH 7.4, 50 mM NaCL, 1mM DTT), protein was collected in an eppendorf tube and examined by SDS-PAGE. The concentrations of the proteins were determined by the Bradford assay (Thermo Fisher Scientific, USA).

3.3.3 Lysis buffer selection for protein purification

Currently the regular lysis buffer for the soluble proteins in our lab is made of 20mM Tris HCL, pH 7.4, 0.5M NaCl, and 5 mM Imidazole. This buffer worked well towards most of the soluble proteins in our lab.

In order to solubilize the ssTorA -Ypet -Uba2 protein aggregations, different additives were added to a basic buffer. The 2L Basic lysis buffer was made of: 50 mM Tris–HCl, 500 mM NaCl with 10 % glycerol, pH 8.0, 1mM DTT, 1mM EDTA. Then 1L basic lysis buffer was aliquoted into 5X 200mL buffers and 4 kinds of different additives are added respectively into each basic lysis buffer. The additives include: (a) 1 M guanidine HCl, (b) 0.5 % Tween 20, (c) 0.5 % n -tetradecyl- N, N -dimethyl-3-ammonio-1- propanesulfonate (Zwittergent

3–14), (d) 0.5 M trehalose. The rest 200 mL basic buffer and the regular lysis buffer (used for resuspending soluble protein) were also used as controls.

3.3.4 Protein expression and purification of ssTorA-Yet-Uba2

The expression steps of protein ssTorA-Ypet-Uba2 is the same as the expression of Ypet-Uba2. After the 16°C overnight IPTG induction of 1L LB culture, cell pellet is collected after 8000 rpm centrifugation at 4°C. Then the cell pellet is suspended in 6 different lysis buffers (the regular lysis buffer, the basic lysis buffer, the basic lysis buffer with 1M Guanidine HCL, the basic lysis buffer with 0.5% Tween 20, the basic lysis buffer with 0.5% Zwittergent 3-14 and the basic lysis buffer with addition of 0.5M Trehalose). After Resuspension, 6 samples were treated by sonication (65A, 5s on/5s off, 5min process time), the cell membrane was disrupted and the contents were released. After the centrifugation at 25000 g, 30min, 4degree, supernatants were collected.

3.3.5 Fluorescence measurement of the supernatants after buffer resuspension

Different supernatants were purified by the same purification method for regular Ypet-Uba2 protein. Before purification, 100ul of each sample was collected. By measuring the fluorescence of each sample, the solubility of different additives can be figured out. The Fluorescence measurement was done by Flexstation II 384 Multi-Mode Micro- Plate Reader, by using the reader to excite the ssTorA-Ypet-Uba2 at 475nm, and detect the fluorescence intensity at 530 nm, we could compare the fluorescence signals in different supernatants.

3.3.6 Kd measurement of Cypet-Aos1 and Ypet-Uba2

The definition of dissociation constant was introduced in Chaper 1.

Kd measurement was conducted by the following steps: 1. Mix recombinant CyPet–Aos1 and YPet–Uba2 and dilute them with phosphate buffered saline (PBS) to a total volume of 30 uL. The final concentration of CyPet– Aos1 was fixed to 1 uM and the final concentration of YPet– Uba2 varied from 0 to 4 uM. 2.Transfer the mixtures into a 384-well plate (Falcon) and measure the fluorescence emission spectrum of each well with a fluorescence multi-well plate reader (Molecular Devices, Flexstation II 384). Two excitation wavelengths were used: 414 nm to excite CyPet, and 475 nm to excite YPet. 3. Excite CyPet at 414 nm. We can observe an emission peak at 475 nm (FL_{DD}). With FRET, we can observe another emission peak at 530 nm (Em_{total}) which results from the energy transferred from CyPet to YPet. 4. Excite the mixture at 475 nm, we can observe an emission peak at 530 nm (FL_{AA}) which is from the direct excitation of YPet but not CyPet. 5. Excite the mixture of CyPet–Aos 1 and YPet–Uba 2 recombinant proteins at 414 nm. After the fluorescence data was collected, EmFRET was calculated by a formula, after the EmFRET is calculated, Prism 5 was used to process the data, calculate the kd and plot the curve graph.

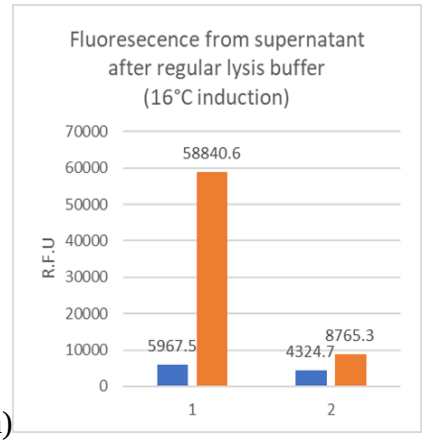
3.4 Results

3.4.1 SsTorA can drive Ypet-Uba2 protein into inclusion bodies

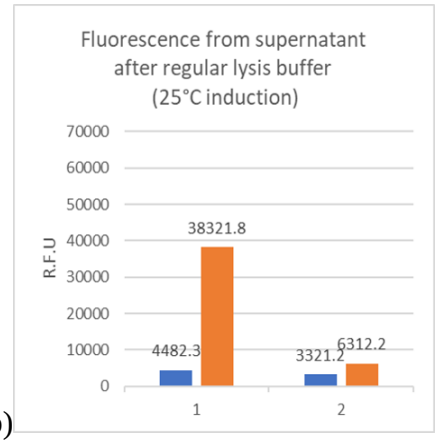
By comparing the fluorescence in the supernatant after regular lysis buffer treatment, we can detect a difference in the induced samples from ssTorA-Ypet-Uba2 construct and the regular Ypet-Uba2 construct. By comparing the fluorescence in figure 17, the fluorescence

of the induced regular Ypet-Uba2 showed is much higher than the induced ssTorA-Ypet-Uba2. Since the regular Ypet-Uba2 can be highly solubilized by regula lysis buffer, this gave us the evidence that the protein might be drove into inclusion bodies.

a)



b)



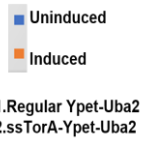


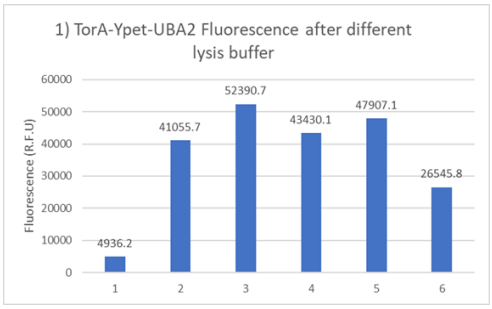
Figure 18. Fluorescence from supernatant after regular lysis buffer in different induction temperatures. Compare the fluorescence of supernatant-1 of series 1 and series 2, Yuba2 protein with the fusion of SsTorA showed a much lower fluorescence than the regular yu2 protein, which gave us the clue that the fluorescent protein was no longer in the supernatant Also, the induction under 16°C showed a higher fluorescence than the induction under 25°C

3.4.2 0.5M Trehalose and 0.1%Zwittergent showed better solubilization of inclusion bodies than other additives

To test the solubility of the different additives of the inclusion bodies, regular buffer was replaced by basic buffer with different additives. After the centrifugation, we can detect the fluorescence in the supernatant which showed in Figure 18. Among all the different lysis buffers, the buffer with the addition of 0.5% zwittergent showed the highest fluorescence than the other buffers. The basic buffer with the addition of 0.5M Trehalose also showed a high fluorescence in the supernatant. This result showed that with the addition of Trehalose and Zwittergent, the inclusion bodies can be highly solubilized, compared to the resuspension of regular lysis buffer.

After the supernatant was collected, the pellet after the 1st resuspension was treated with 6M Guanidine HCL and shook overnight at 4 degrees. By comparing the fluorescence in the supernatant after the 2nd resuspension, we can figure out how much protein was left in the pellet after the 1st resuspension. The result in figure 18 (b) showed that buffers with Trehalose and zwittergent can solubilize more inclusion bodies than the other lysis buffers.

a)



b)

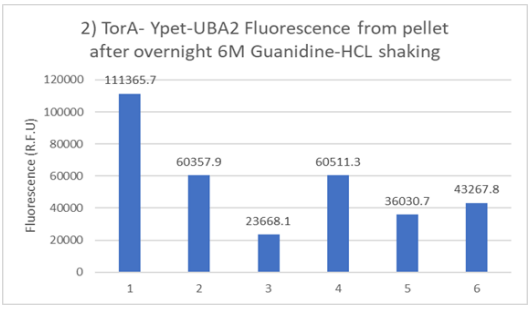
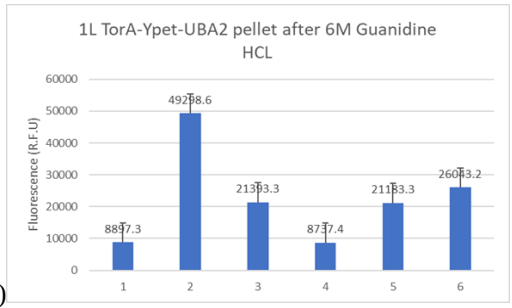


Figure 19. Fluorescence measurement of the supernatants after different lysis buffer treatments: a) The supernatant of ssTorA-Ypet-Uba2 after 1. regular lysis buffer; 2. Basic lysis buffer; 3. Basic lysis buffer+0.5% zwittergent; 4. Basic lysis buffer+1M Guanidine HCL; 5. Basic lysis buffer+ 0.5M Trehalose; 6. Basic lysis buffer+0.5% Tween-20. b) The pellet from a) are treated with 6M Guanidine HCL, and the Fluorescence of supernatants of each series after 6M guanidine HCL 1. Pellet from 1. regular lysis buffer; 2. basic lysis buffer; 3. basic lysis buffer+0.5% zwittergent; 4. Basic lysis buffer+1M Guanidine HCL; 5. P Basic lysis buffer+ -0.5M Trehalose; 6. Basic lysis buffer+ 0.5% Tween-20

The experiment was duplicated in 1L volume, which showed similar results in Figure 18. This time the supernatant and the pellet of regular Ypet-Uba2 was also tested by the 2 rounds of the lysis buffer treatment. From figure 19 (b), the supernatant of the regular Ypet-Uba2 after 6M Guanidine treatment showed a same level fluorescence. Which means the content of the proteins in the pellets are about the same level. Figure 20 showed a larger scale fluorescence testing and same result was shown as Figure 19.

a)



b)

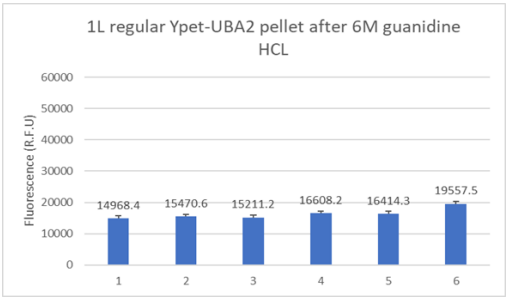


Figure 20. Fluorescence measurement of the supernatant-2 after 6M Guanidine HCL treatment.
a)1L TorA-Ypet-Uba2 pellet after 6M Guanidine HCL, the pellets are from: 1. Basic buffer +Trehalose; 2. Regular buffer; 3. Basic buffer + Tween-20 basic buffer; 4. Basic buffer + zwittergent; 5. Basic buffer+1M Guanidine HCL; 6. Basic buffer.

3.4.3 Distribution of the protein fluorescence in ssTorA-Ypet-Uba2

Pie chart was made based on the comparison of fluorescence in the supernatants after 1st lysis buffer resuspension and the 2nd lysis buffer resuspension. After the treatment of the basic buffer with no additives, 74% of the protein was solubilized. Basic buffers with the additive of zwittergent or trehalose can both solubilize 93% of the protein. Regular buffer can only solubilize 18% of the protein, which enhanced the evidence that the protein was formed into Inclusion bodies with the fusion of ssTorA.

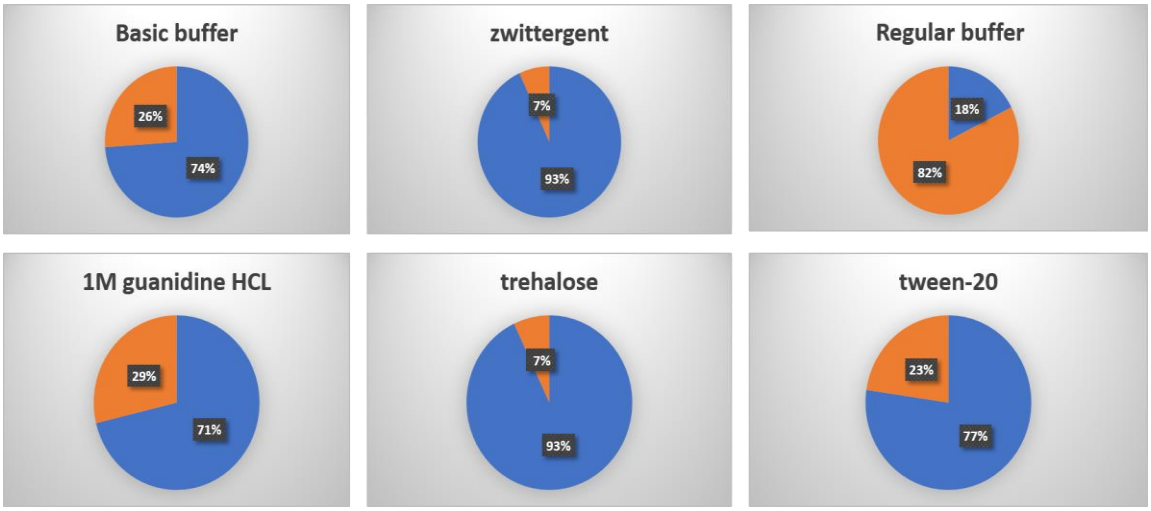


Figure 21. This Pie chart shows the distribution of the protein based on the Fluorescence in the supernatant after the 1st lysis buffer and the 2nd lysis buffer. Blue part of the pie chart showed the percentage of the fluorescence tested in the supernatant after the first buffer selection. Orange part of the pie chart showed the percentage of the fluorescence tested in the supernatant after 6M Guanidine HCL.

3.4.4 Purified ssTorA-Ypet-Uba2 showed a higher yield than regular Ypet-Uba2

Since lysis buffer with zwittergent and trehalose showed better solubility than other lysis buffers, the supernatant were collected and purified. Figure 21 showed the purified proteins on a SDS-PAGE gel. Each well was added by 5ug of the target protein.

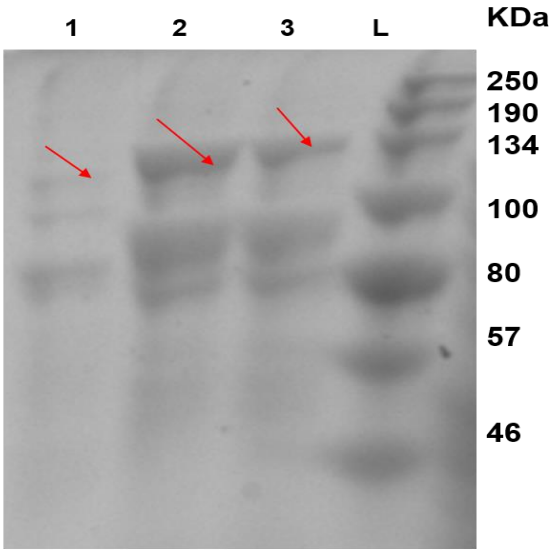


Figure 22. Purified Ypet-Uba2 protein on a SDS PAGE gel. 1.Regular Ypet-Uba2 purified by regular method; 2. ssTorA-Ypet-Uba2 protein resuspended by lysis buffer with Trehalose; 3. ssTorA Ypet-Uba2 protein purified from supernatant after basic buffer +zwittergent resuspension.

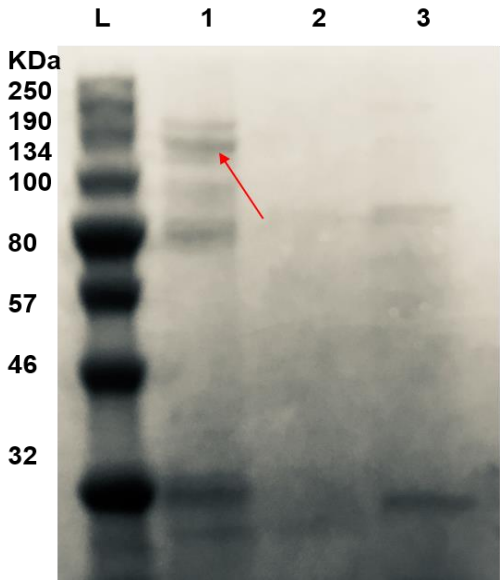


Figure 23. Purified Ypet-Uba2 protein on a SDS PAGE gel

From figure 22, by running the same amount of the proteins on a SDS-PAGE gel, the Ypet-Uba2 proteins purified from Trehalose and Zwittergent showed stronger band than the regular Ypet-Uba2 protein purified by the regular protein purification method. As a result, purify the protein from the inclusion bodies helped to increase the yield of the proteins. However, degradation was still detected in both Zwittergent and Trehalose treatments. Therefore, this method needs to be developed in overcoming the degradation of the target proteins.

3.4.4 Kd measurement of Cypet-Aos1 and Ypet-Uba2

1	Kd Determination	
2	Best-fit values	
3	EmFRETmax	2.710e+006
4	Kd	1.436
5	A	= 1.000
6	Std. Error	
7	EmFRETmax	153223
8	Kd	0.2206
9	95% Confidence Intervals	
10	EmFRETmax	2.397e+006 to 3.023e+006
11	Kd	0.9858 to 1.886
12	Goodness of Fit	
13	Degrees of Freedom	31
14	R2	0.9836
15	Absolute Sum of Squares	2.098e+011
16	Sy.x	82263
17	Constraints	
18	EmFRETmax	EmFRETmax > 0.0
19	Kd	Kd > 0.0
20	A	A = 1.000
21	Number of points	
22	Analyzed	33

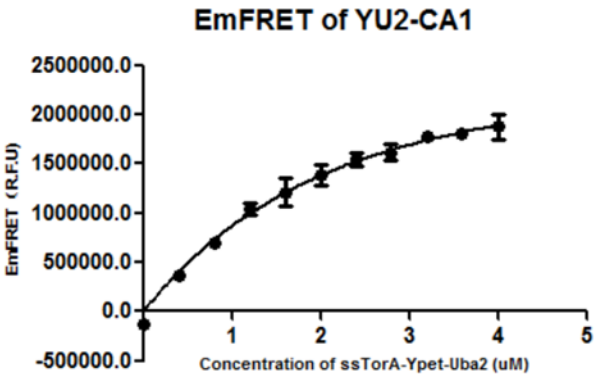


Figure 24. (a) Kd of ssTorA-Ypet-Uba2 from the supernatant after lysis buffer+Trehalose
(b) Curve graph of the EmFRET

Kd measurement	
Best-fit values	
EmFRETmax	2.638e+006
Kd	1.252
A	= 1.000
Std. Error	
EmFRETmax	168628
Kd	0.2323
95% Confidence Intervals	
EmFRETmax	2.294e+006 to 2.982e+006
Kd	0.7781 to 1.726
Goodness of Fit	
Degrees of Freedom	31
R2	0.9760
Absolute Sum of Squares	3.197e+011
Sy.x	101547
Constraints	
EmFRETmax	EmFRETmax > 0.0
Kd	Kd > 0.0
A	A = 1.000
Number of points	
Analyzed	33

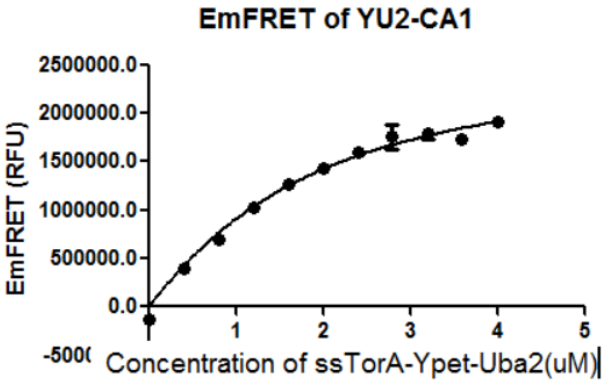


Figure 25. (a) Kd of the ssTorA-Ypet-Uba2 from the supernatant after treatment of lysis buffer+zwittergent
(b) Curve graph of the EmFRET (processed by Zhehao Xiong)

3.5 Discussion:

Here we report a methodology to purify difficult proteins for our FRET assay. By fusing a signal sequence with our protein of interest, the protein would form inclusion bodies and protect itself from degradation by the host. This thesis also applied a buffer selection method to solubilize the inclusion bodies and tried different methods to renature and purify the protein from inclusion bodies. By comparing the protein purified by this method and the regular method, the yield of the protein was better than but the purity of the protein still needs improvement.

Future work of this project would be addressed in following directions. Firstly, the protein purification method after the different buffer treatment needs to be developed. Secondly, the lysis buffer itself can be optimized by minimizing the concentration of the additives. Moreover, ssTorA should be tested in other proteins to see if the signal sequence can drive those proteins into inclusion bodies and overcome the degradation and reduce the toxicity of the target proteins.

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