CLONING, EXPRESSION, PURIFICATION AND ENZYME KINETICS CHARACTERIZATION OF DEUBIQUITINASE USP30

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Chemistry and Biochemistry.

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ABSTRACT

Deubiquitinating enzymes (DUBs) are imperative participants in multiple cellular processes, and as a consequence the control of DUB function has become the subject of great interest due to its possible implications in neurodegenerative diseases, anti-viral/bacterial responses, and potential anti-cancer targets. Recently a new DUB, USP30, was shown to be localized in the mitochondria and identified as having a role in the regulation of mitochondrial mitophagy, whose dysfunction is associated with the neurodegenerative Parkinson's disease. The ubiquitin specific proteases (USPs), cysteine proteases, have a conserved catalytic core including cysteine, histidine and aspartate/asparagine. USP30 is very similar to other DUBs, except for the Asp/Asn conserved residue which is a serine. Our efforts were to understand how this unconventional catalytic residue in the catalytic core plays a role in the control of deubiquitination by first characterizing its steady state kinetics. A truncated USP30 was cloned into a pSUMO vector for expression in *E.coli* to obtain the wild-type (WT) turnover rate. Mutations in the catalytic triad were made to further understand the influence of the serine in the catalytic core. The WT k_{cat}/K_M was estimated to be 3.1 x 10^4 $M^{-1}s^{-1}$, while the C77A USP30 mutant had no detectable activity, as expected, and the S477A USP30 mutation had an estimated k_{cat}/K_M of 2.2 x10^2 $M^{-1}s^{-1}$. Our results give insight to the importance of the serine and its possible role in regulating the rate of the USP30.

Chapter 1

INTRODUCTION

Protein post-translational modifications are involved in many cellular processes including the protein degradation pathway. The degradation pathway of proteins relies on ubiquitin, a small 76 amino acid protein, to tag proteins for degradation by proteasome. Ubiquitin molecules are conjugated to the protein to form a poly-ubiquitin chain. This poly-ubiquitin chain signals the degradation of target proteins by proteasome. Figure 1-1 shows the ubiquitin-proteasome system that uses three enzymes E1, E2, and E3 to conjugate ubiquitin to the target protein and initiate the proteasomal degradation of the protein (Ikeda and Dikic 2008; Finley 2009; Komander 2009; Kulathu and Komander 2012; Meyer and Rape 2014). The ubiquitin molecules are removed from the protein before being processed by the proteasome. The removal of ubiquitin from the protein is accomplished by enzymes called deubiquitinases (DUBs) (Welchman, Gordon et al. 2005; Sacco, Coulson et al. 2010).

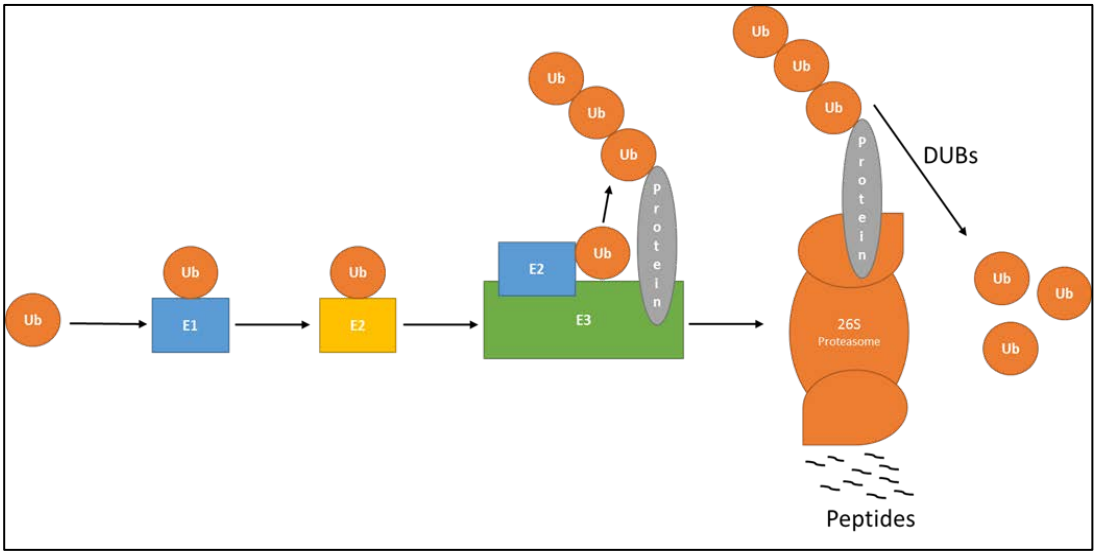

Figure 1.1. Ubiquitin conjugation and the ubiquitin-proteasome system. Ubiquitin is bound to E1 which is then transferred to E2 and then ligated to the protein using E3. Once the proteins' poly-ubiquitin tail is long enough, the proteasome is recruited for protein degradation and the deubiquitinases are recruited to recycle the ubiquitin.

Deubiquitinases are involved in several processes that not only include the recycling of ubiquitin from proteins targeted to the proteasome, but also the removal of ubiquitin to edit the length of poly-ubiquitin chains, and the removal of ubiquitin from proteins as a form of regulation, as is illustrated in Figure 1.2 (Kim, Park et al. 2003; Amerik and Hochstrasser 2004; Nijman, Luna-Vargas et al. 2005; Reyes-Turcu and Wilkinson 2009; Clague, Barsukov et al. 2013). In the family of human DUBs there are ~100 different DUBs that fall into 5 categories: ubiquitin specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Machado-Joseph disease (MJD) protein domain proteases and the Jab1/MPN/MOV34 metalloenzymes (JAMM) (Nijman, Luna-Vargas et al. 2005). Of the 5 classes of

deubiquitinases, USPs are the largest group containing ~60 proteases with their sizes ranging from 50-300 kDa. A key feature of these proteases is their conserved catalytic core which contains a cysteine, histidine, and aspartate (or asparagine) amino acid residues (Figure 1.3) (Pfoh, Lacdao et al. 2015). These residues are important because they carry out the enzymatic cleavage of ubiquitin from proteins. However, there is diversity among families including the USP family of deubiquitinases. There are USPs that conserve only the cysteine and histidine and vary in the third catalytic residue, like USP30, USP16 and USP45, which contain a serine instead of the typical Asp/Asn residue. This leads to the question: what purpose does this change serve in terms of catalytic activity or function of USPs? (Nijman, Luna-Vargas et al. 2005; Ye, Scheel et al. 2009).

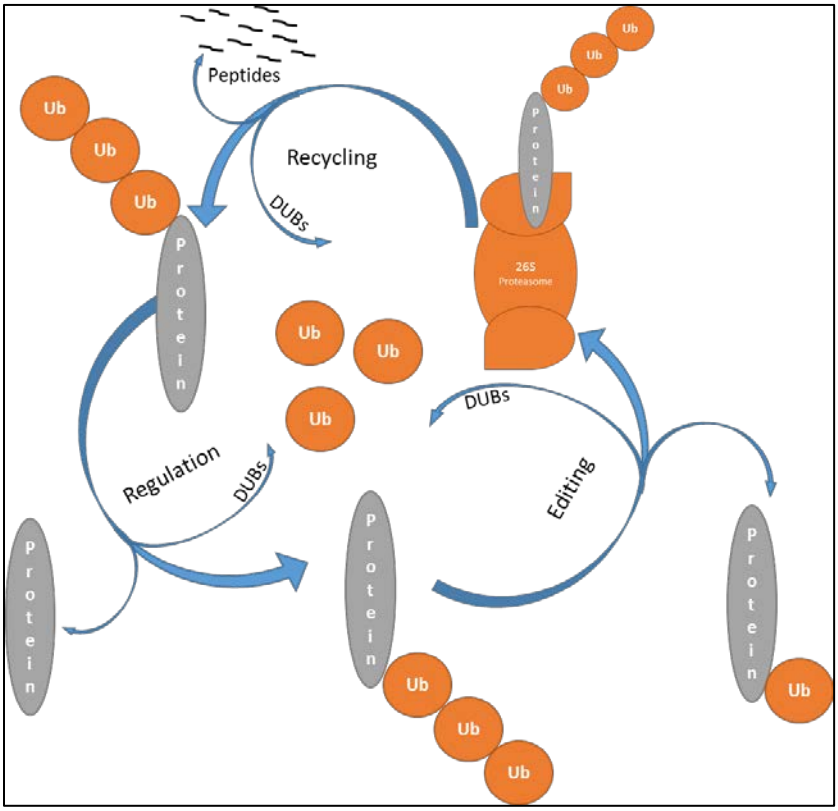

Figure 1.2. Reactions catalyzed by deubiquitinases (DUBs). Ubiquitin is regulated, edited and recycled.

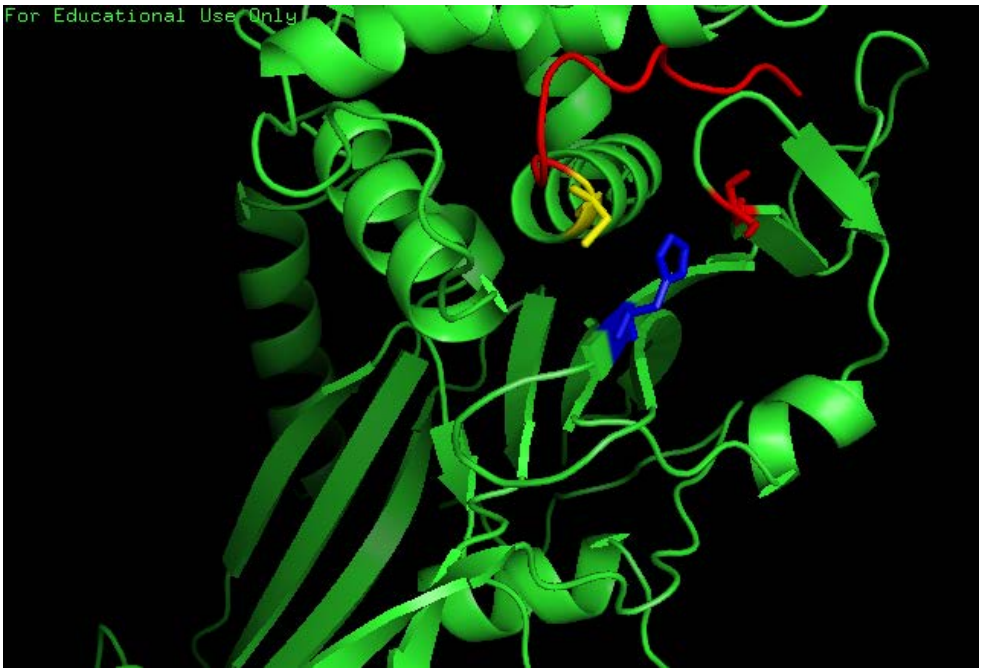

Figure 1.3. The catalytic core of USP30, using UCH21 as a template in the Phyre 2 program. The blue is histidine, the yellow is cysteine, the red is serine, and the red tail is the N-terminus up to the catalytic cysteine.

The DUB USP30 is particularly interesting due to its localization on the mitochondria and its involvement in Parkinson's disease. It is involved in the regulation of the mitochondrial morphology. Mitochondria are organelles whose main function is to produce ATP as an energy source for the cell. They contain their own DNA (mtDNA) and the capability to shrink and grow according to the needs of the cell. (Nakamura and Hirose 2008; Liang, Martinez et al. 2015; Scheibye-Knudsen, Fang et al. 2015). This ability is not only important for the needed ATP production, but also for cellular differentiation, mtDNA inheritance and apoptosis (Hales and

Fuller 1997; Nunnari, Marshall et al. 1997; Skulachev 2001; Smirnova, Griparic et al. 2001; Nakamura and Hirose 2008; Kasahara and Scorrano 2014).

The mitochondrial dynamics of growing and separating, also known as fusion and fission, are controlled by many proteins as can be seen in Figure 1.4. The mitochondrial fusion process involves the merging of two mitochondria. The mitochondrial outer membranes (MOM) are fused using the Mfn1 and Mfn2 GTPase mitofusins while the inner mitochondrial membrane is fused using the OPA1 GTPase protein (Figure 1.4) (Santel and Fuller 2001; Chen, Detmer et al. 2003; Olichon, Baricault et al. 2003; Cipolat, Martins de Brito et al. 2004; Chen, Chomyn et al. 2005; Chan 2006). Also involved in this process in the intermixing of the mitochondrial matrix and mtDNA, which is very important for the proper function of mitochondria. Dysfunction of some of these proteins, such as Mfn2 and OPA1, causes neurodegenerative diseases (Chan 2006).

The mitochondrial fission process is the division of a mitochondrion into two separate mitochondria. There are two main proteins involved in fission, i.e. Drp1, a cytosolic GTPase dynamin-related protein, and the MOM protein Fis1 (Frank, Gaume et al. 2001; Smirnova, Griparic et al. 2001; Lee, Jeong et al. 2004; Stojanovski, Koutsopoulos et al. 2004; Nakamura and Hirose 2008) (Figure 1.4).

The Drp1 protein is recruited by the Fis1 protein when there is change in membrane potential (Chan 2006). The accumulation of Drp1 around the mitochondrion constricts the membrane until it eventually separates into two parts (Chan 2006). It is important because it allows the mitochondrion to divide for transport, especially to

nerve cells, which require abundant amounts of ATP, but also to remove damaged portions of the mitochondrion preventing the debilitation of the entire network (Shaw and Nunnari 2002). It has been shown when Drp1 is altered it causes a variety of issues: from incorrect division of mitochondria in dividing cells to problems with apoptosis, and interruption of correct mitochondrial transport (Shaw and Nunnari 2002; Verstreken, Ly et al. 2005; Chen and Chan 2009; Han, Tomizawa et al. 2011). When the transportation of mitochondria is affected, in particular to nerve terminals, it also causes neurodegenerative diseases (Shaw and Nunnari 2002; Verstreken, Ly et al. 2005; Chen and Chan 2009; Han, Tomizawa et al. 2011). Therefore, when the fusion or fission involved proteins are impeded in any way, such as mutations or abnormalities, problematic disorders arise.

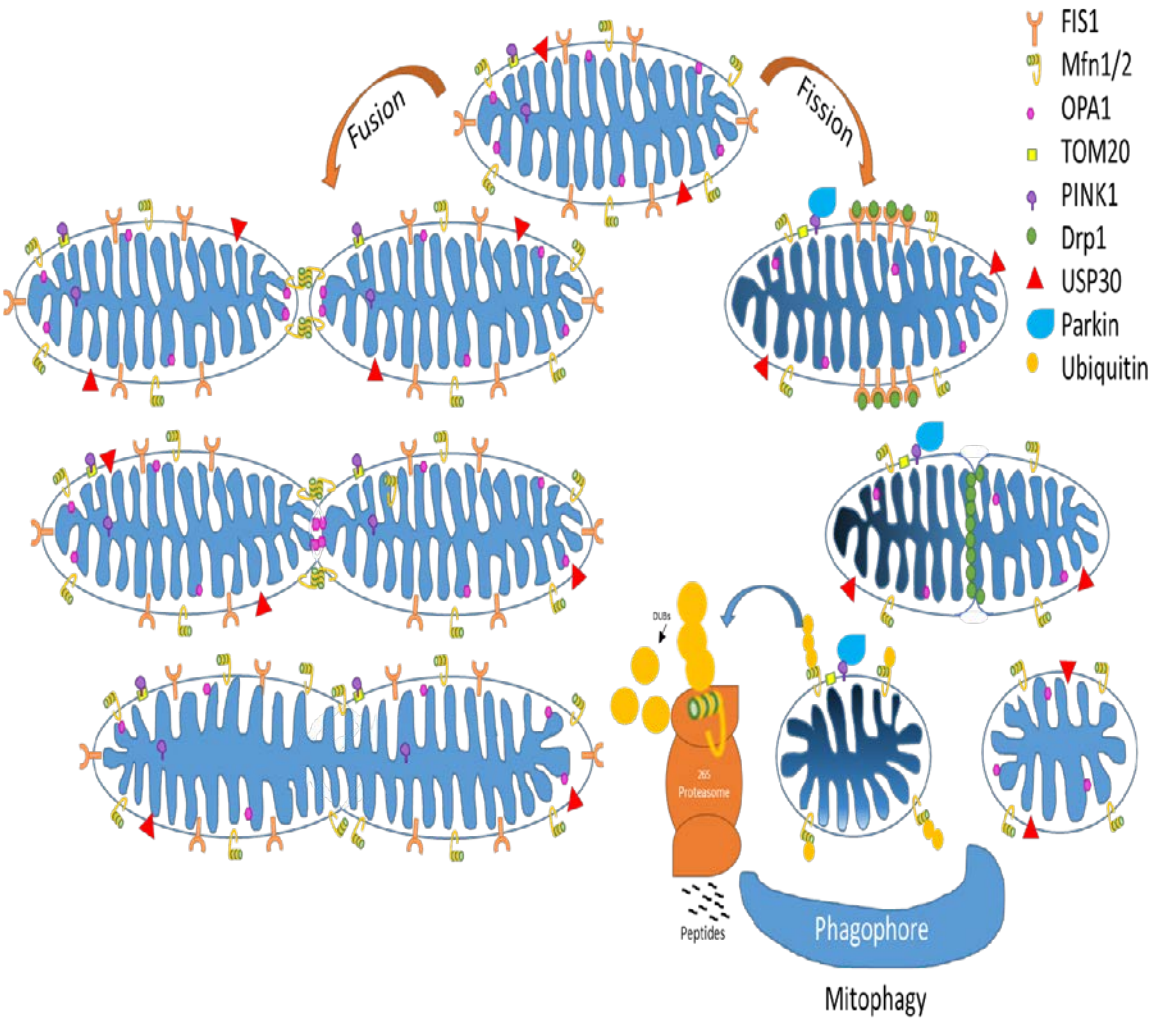

Figure 1.4. Mitochondrial fusion and fission. On the left is the fusion process which uses Mfn1 and Mfn2 as well as Opa1 to merge the outer and inner membranes, respectively. On the right is the fission process. Upon a change in membrane potential ($\Delta m\Psi$) (indicated by the color change) in the mitochondrial inner membrane, the PINK1 protein is localized to the outer membrane from the inner membrane of the mitochondria, which localizes Parkin. Parkin ubiquitinates the MOM proteins targeting them for degradation via the proteasome while the Fis1 protein binds the Drp1 proteins. Drp1 accumulates constricting the membrane until it pinches off and is degraded by the phagophore.

As mentioned previously, the fusion and fission dynamics of the mitochondria is heavily involved in the regulation of mitochondrial degradation, also known as mitophagy (Twig, Elorza et al. 2008; Youle and Narendra 2011; Nunnari and Suomalainen 2012). There are two main ways that mitophagy is controlled with the ubiquitin-proteasome system as explained in Figures 1.1 and 1.2 and also with the phagosome as depicted in Figure 1.4 (Rugarli and Langer 2012; Eiyama and Okamoto 2015; Scheibye-Knudsen, Fang et al. 2015). The mitophagy process uses various proteins to carry out these two pathways that includes PINK1, an intermembrane spaced kinase, and Parkin, a cytoplasmic ligase. In conjunction, the PINK1 is localized to the outer membrane when there is a change in membrane potential ($\Delta m\Psi$), yielding oxidative stress, which then recruits Parkin setting off a trigger for mitophagy via the phagophore (Greene, Whitworth et al. 2003; Clark, Dodson et al. 2006; Park, Lee et al. 2006; Narendra, Tanaka et al. 2008; Nunnari and Suomalainen 2012; Bingol, Tea et al. 2014). If this pathway is dysfunctional, and the "damaged" section of the mitochondrion is not removed, the entire network can be endangered leading to the cell death in extreme cases or compromised functioning mitochondria (Bingol, Tea et al. 2014). And as mitochondria are vital to many biological processes, it's logical that a malfunction in this organelle could create a multitude of disorders and diseases (Nakamura and Hirose 2008; Youle and Narendra 2011).

USP30 plays a role in the mitophagy pathway as it is part of the mitochondrial outer membrane. What makes it more interesting is its unique catalytic core and evidence of its regulation through inhibition of mitophagy (Nakamura and Hirose

2008; Youle and Narendra 2011). In the larger scheme of diseases, Parkinson's disease is directly affect by the mitophagy pathway and the inhibition of USP30 would allow proper removal of damaged mitochondria from the cell (Nunnari and Suomalainen 2012; Bingol, Tea et al. 2014). Therefore, the general aim of this project was to clone, express, and purify USP30 and to test its kinetic properties. We describe here details of cloning, sequencing, expression and purification of USP30 wild-type and mutants and their kinetic analysis. This study reveals that the cysteine is necessary for catalytic function, as is the serine. The WT k_{cat}/K_M was estimated to be 3.1 x 10^4 $M^{-1}s^{-1}$, while the C/A had no detectable activity, as expected, and the S/A mutation had an estimated k_{cat}/K_M of 2.2 x10^2 $M^{-1}s^{-1}$.

Chapter 2

METHODS

2.1 Cloning and Expression

Bacterial Glycerol Cell Stock: Under aseptic conditions, add 500 µL of overnight cell culture to 500 µL of 50% autoclaved glycerol. Tubes are placed into liquid nitrogen (N_2) for a few minutes in order to freeze the cells before storage in the -80°C freezer.
Cell Culture Growth (small): Under aseptic conditions, using 20 mL of autoclaved LB media, add appropriate antibiotic to correct concentration. Use a sterile pipette tip to select a single colony from an LB agar plate or to scrape the bacterial glycerol cell stock. Drop the tip into the LB media. Cap the tube and incubate bacterial culture at 37°C for 12-18hrs (overnight) in a shaking incubator. Ensure growth by observing clear to cloudy media change.

Cell Culture Growth (large): Under aseptic conditions using a sterile pipette tip, scrape your glycerol cell stock. Start 2 overnight cell culture growths in accordance to the Cell Culture Growth, small protocol. Under aseptic conditions, using 2 L autoclaved LB media, add appropriate antibiotic to correct concentration. Pour the 2 overnight cultures into the LB media. Cover the flask and incubate bacterial culture with shaking at 37°C until OD_{600} reaches ~0.4. Sample 1 mL of "un-induced culture" for SDS-PAGE analysis. Drop temperature to 17°C for 30 min. To culture add 400 µL of 1M IPTG for a final concentration of 0.2 mM IPTG. Continue culturing cells overnight for 15-17 hrs. Harvest 1 mL sample of "induced culture" for SDS-PAGE analysis. Harvest cells by following the Harvesting Cell Culture protocol (medium to large scale).

> SDS-PAGE sample preparations: Prepare all culture samples as described in Expression Testing protocol.

Colony PCR of pSUMO USP30ΔTM: under aseptic conditions, pick colony with pipette tip and place into a 1.5 mL tube containing 10 µL of ddH_2O. Resuspend colony well. Continue picking colonies to screen several colonies making a master agar plate of all the colonies selected as well.

> PCR Cocktail (50 µL)
> 10x Cloned Pfu Rxn Buffer 5 µL

25 mM $MgCl_2$	3 µL
dNTP's (0.2 mM each final conc)	1 µL
DNA template (Re-suspended colony)	5 µL
Forward Primer (?USP30fwdpET, 0.2 µM final)	1 µL
Reverse Primer (USP30revpETpGpF, 0.2 µM final)	1 µL
LongAmp *Taq* DNA Polymerase	2 µL
ddH_2O	33 µL

PCR Cycling conditions

1) 1 min @ 95°C (initial)
2) 30 sec @ 95°C (denaturing)
3) 45 sec @ 55°C (annealing)
4) 10 min @ 68°C (extension)
5) Go to #2, repeat 30x
6) 5 min @ 68°C (final extension)
7) Hold 4°C

Double Digest of USP30ΔTM and pSUMO: Mixed and incubated for 3 hrs at 37°C, 50 µL reaction.

DNA (3µg, each)	
Buffer 2 (NEB)	5 µL
EcoRI	0.5 µL
XhoI	0.5 µL
ddH_2O	

Used QIAquick Nucleotide Removal Kit using a Microcentrifuge Protocol for USP30ΔTM, and QIAquick Gel Extraction Kit using a Microcentrifuge Protocol for pSUMO.

Expression Testing (small scale): Under aseptic conditions, using 4x 20 mL autoclaved LB media, add appropriate antibiotic to correct concentration. Using a sterile pipette tip, select a single colony from your LB agar plate or scrape your glycerol cell stock. Drop the tip into the LB media. Cap the tube and incubate bacterial culture at 37°C until OD_{600} reaches ~0.5. Sample 1 mL "un-induced culture" for SDS-PAGE analysis. To each culture add 4 µL of 1 M IPTG for a final concentration of 0.2 mM IPTG. Continue culturing cells overnight. Harvest 1 mL sample of "induced culture" for SDS-PAGE analysis.

SDS-PAGE sample preparations: centrifuge all un-induced and induced culture samples for 5 min @ 4,000 rpm. Remove all supernatant with pipette tip. Add 100 µL of ddH2O to all samples. Re-suspend pellets with vortexing. To prepare SDS-PAGE sample use 10 µL of culture re-suspension, 10 µL

ddH2O, and 4 µL of 6x SDS loading dye. Boil for 5 min at 100°C and load all of sample onto a 12% SDS-PAGE gel.

QIAquick Gel Extraction Kit using a Microcentrifuge Protocol: Cut out the DNA fragment from the agarose gel using a clean razor blade. Weigh the slice of the agarose and add 3 volumes of Buffer QG to 1 volume of gel. Incubate at 50°C for 10 minutes. Ensure that the gel has completely dissolved. Add 1 gel volume of isopropanol to the sample and mix. Place a QIAquick spin column in a 2 mL tube. Place the sample into the column to bind the DNA and centrifuge for 1 minute at 13,000 rpm. Discard flow through. Add 0.5 mL of Buffer QG to the column and centrifuge for 1 minute. Add 0.75 mL of Buffer PE to wash column and centrifuge for 1 minute. Discard the flow through and centrifuge for 1 more minute. Place QIAquick column into a clean 1.5 mL tube. Add 30 µL of Buffer EB to the center of the membrane. Let stand for 1 min and then centrifuge for 1 min to elute the DNA. Store the clean digested vector at -20°C

Harvesting Cell Culture protocol (medium to large scale): Centrifuge cell culture at 5,000 rpm for 10 minutes. Scrape off pellet and weigh. Store at -80°C or resuspend in cold lysis buffer to continue with extraction/purification.

Ligation: use formula to calculate the amount of gene to use. After the reaction mix is combined, gently aspirate. Let sit at room temperature for 5-10 min. Use 10 µL for transformation into competent cells.

$$\frac{3*(50\text{ ng digested plasmid})(\text{gene length}\sim 1500\text{ n.t.})}{(\text{vector length }(\sim 5600\text{ n.t.}))(\text{Gene conc. }\sim 190.6\text{ ng/µL})}$$

Ligation reaction mix (20µL)

USP30 (XhoI/EcoRI, cleaned)	0.21 µL
2x Ligation Buffer	10 µL
50 ng Vector (pSUMO, actually 22 ng)	10 µL
Quick T4 DNA Ligase	1 µL

QIAquick Nucleotide Removal Kit using a Microcentrifuge Protocol: Add 10 volumes of Buffer PNI to 1 volume of digested sample and mix. Place a QIAquick spin column in a 2 mL tube. Place the sample into the column to bind the DNA and centrifuge for 1 min. at 6,000 rpm. Discard flow through. Add 0.75 mL Buffer PE to the QIAquick column to wash and centrifuge for 1 min at 6,000 rpm. Discard flow through. Centrifuge for 1min. at 13,000 rpm. Place QIAquick column into a clean 1.5 mL tube. Add 30 µL of Buffer EB to center of the QIAquick membrane to elute DNA. Let stand for 1 min. and then centrifuge for 1min. Store clean digested gene at -20°C.

QIAquick PCR Purification Kit using a Microcentrifuge Protocol: Remove PCR product from PCR tube and place into a 1.5 mL tube. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. Place a QIAquick spin column in a 2 mL tube. Place the sample into the column to bind the DNA and centrifuge for 1 min. at 13,000 rpm. Discard flow through. Add 0.75 mL Buffer PE to the QIAquick column to wash and centrifuge for 1 min. Discard flow through. Centrifuge for 1min. Place QIAquick column into a clean 1.5 mL tube. Add 50 µL of Buffer EB to center of the QIAquick membrane to elute DNA. Let stand for 1 min. and then centrifuge for 1 min. Store clean PCR product in -20°C.

QIAGEN Plasmid Purification Protocol: Grow cell culture as described in Cell Culture Growth, small protocol. Remove cell cultures from 37°C shaker and centrifuge using a table top swinging bucket rotor for 10 minutes at 4,000 rpm at room temperature. Pour off supernatant and resuspend pelleted bacterial cells in 250 µL of cold Buffer P1 (with RNaseA). Transfer to 1.5 mL tube. Add 250 µL of Buffer P2 and mix by inverting 6x. Add 350 µL of Buffer N3 and mix immediately 6x by inverting. Centrifuge for 10 min at 13,000 rpm in microfuge at room temperature. Remove supernatant and place into QIAprep Spin Column. Centrifuge for 1 min. Discard flow through. Wash QIAprep spin column with 0.75 mL Buffer PE. Centrifuge for 1 min. Discard flow through. Centrifuge for 1 min. to remove residual buffer. Elute DNA by placing the QIAprep spin column into a 1.5 mL tube. Add 50 µL of warmed Buffer EB to center spin column. Let stand for 1 min. and then centrifuge for 1 min. Store plasmid DNA in -20°C.

PCR Mix and cycler conditions for pDEST USP30

PCR Cocktail (50 µL)	
10x Cloned Pfu Rxn Buffer	5 µL
dNTP's (0.4 mM each final conc)	2 µL
DNA template (100 ng)	0.376 µL
Forward Primer (?USP30fwdpET, 0.2 µM final)	1 µL
Reverse Primer (USP30revpETpGpF, 0.2 µM final)	1 µL
PfuTurbo DNA Polymerase	2 µL
ddH_2O	38.62 µL

PCR Cycling conditions

1) 1 min. @ 95°C (initial)
2) 30 sec. @ 95°C (denaturing)
3) 45 sec. @ 55°C (annealing)
4) 10 min. @ 68°C (extension)
5) Go to #2, repeat 30x
6) 5 min. @68°C (final extension)
7) Hold 4°C

For large scale PCR product extraction, used 4x the PCR cocktail and QIAquick PCR Purification Kit using a Microcentrifuge Protocol.

Primer Preparation for Sequencing
For 1 reaction use 5 µL of 10 µM Primer and 5 µL ddH2O

Primer Stock 100 µM: obtain the nmoles value and multiply by 10. This value is the volume in µL required to produce a 100 µM stock. Dissolve the primer in the attained TE buffer volume.

Primer Stock 10 µM, 50 µL
Add 5 µL of 100 µM Stock and 45 µL of TE Buffer.

GeneWiz Sample Preparation
Combine Primer Preparation for Sequencing reaction with Template Preparation for Sequencing reaction and send. Send template alone only when primer is selected from GeneWiz in-house primer collection.

Site-Directed Mutagenesis Mix and cycler conditions for Mutants

PCR Cocktail (25 µL)	
5x Buffer (GC Buffer)	5 µL
dNTP's	0.8 µL
DNA template (50 ng)	1 µL
Forward Primer	1 µL
Reverse Primer	1 µL
Phusion DNA Polymerase	2 µL
ddH_2O	15.7 µL

PCR Cycling conditions
1) 1:30 min. @ 95°C (initial)
2) 30 sec. @ 95°C (denaturing)
3) 1:30 min. @ 55°C (annealing)
4) +0°C +0°C
5) R=3.0°C/s + 0°C
6) G=5°C
7) 10 min. @ 72°C (extension)
8) Go to #2, repeat 18x
9) 15 min. @72°C (final extension)
10) Hold 4°C

Template Preparation for Sequencing
For 1 reaction calculate 80 ng/uL in 20 µL and BTV with ddH2O

Transformation: Add 10 µL of a ligation reaction, or 1 µL of purified plasmid to competent cells. Flick tube gently 2x to mix. Let stand on ice for 30 minutes. Incubate at 42°C for 40 sec. Place on ice for 2 min. Add 400 µL of pre-warmed SOC media. Grow for 1 hour at 37°C in a shaker. Spin for 1 min @ 4,000 rpm. Under aseptic conditions, pour off supernatant, ~100µL remaining. Re-suspend pellet in remaining SOC media. Plate onto antibiotic appropriate LB agar. Spread in one area, then streak away from concentrated area to obtain individual colonies. Place plate upright in 37°C incubator. Flip plate upside down after 1 hour for overnight incubation. Remove plate after 12-18 hrs.

2.2 Protein Purification

Initial De-sumoylation Assay Method: Use fresh non-frozen IMAC elution. Set-up three 50 µL reactions: control, pre, and post as follows below. The control reaction contains desumoylase ULP1, the sumo cleaving enzyme only. The pre reaction contains the SUMO-USP30 only, and the post contains both substrate SUMO-USP30 and enzyme ULP1. After combining, mix and incubate at room temperature for 30 min. For SDS-PAGE analysis, take 20 µL of each reaction and mix with 4 µL of 6x SDS loading dye. Boil for 5 min at 100°C, load all of sample onto 12% SDS-PAGE gel.

	Control	Pre	Post
1 M DTT (5 mM final)	0.25 µL	0.25 µL	0.25 µL
5x Reaction Buffer (1x final)	10 µL	10 µL	10 µL
100 µM ULP1 (4 µM final)	2 µL	---	2 µL
8 µM SUMO USP30 (4 µM final)	---	25 µL	25 µL
ddH_2O	38 µL	13 µL	13 µL

ULP-1 De-sumoylation Ratio Determination Assay Method: Set up reactions as detailed below in duplicate. Place all samples in 4°C. Remove one set of samples after 4 hrs and prepare SDS-PAGE samples as described in the Initial De-sumoylation Assay Method. Continue to incubate the remaining samples overnight at 4°C. Prepare overnight SDS-PAGE samples as described above.

Table 2.1: ULP-1 De-sumoylation Ratio Table

	Control	Pre	1:1	1:10	1:25	1:50	1:75	1:100
ULP1:pSUMO USP30	2uM	2uM	2uM:2uM	0.2uM:2uM	0.08uM:2uM	0.04uM:2uM	0.0267uM:2uM	0.02uM:2uM
1M DTT (5mM Final)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
100uM/10uM ULP1	2		2	10	4	2	1.36	1
pSUMO USP30 ΔTM		11.1	11.1	11.1	11.1	11.1	11.1	11.1
ddH2O	47.75	38.65	36.65	28.65	34.65	36.65	37.29	37.65
Total Volume	50	50	50	50	50	50	50	50

ULP-1 De-sumoylation Assay Method: Determine the concentration of the IMAC-E sample via Bradford Assay. Prepare the sample with final concentrations of 5 mM DTT, 1 µM ULP1, and 100 µM SUMO USP30. Mix sample and place into dialysis cassette MWCO 10,000 Da. Incubate at 4°C with stirring for 4-9 hrs in Dialysis buffer.

Initial IEX Chromatography after De-sumoylation Method pH 7.0 or 8.5: Using the NaCl Re-IMAC Elution sample and an Amicon Ultra-15 MWCO 10,000 Da, buffer exchange into IEX Buffer A (20 mM Tris, pH 7.0 or 8.5 containing 5 mM DTT). Load ~3 mL of buffer exchanged sample into a 6 mL loop. Inject over an equilibrated 1 mL pre-packed GE HiTrap Q FF column at 2 mL/min. Collect the Flow Through (FT) when UV Absorbance increases. Perform a linear NaCl gradient with IEX Buffer B (20 mM Tris, pH 7.0 or 8.5 containing 5 mM DTT and 250 mM NaCl) from 0% to 100% B over 5 minutes and collect elution fractions of 0.5 mL when UV absorbance increases.

> SDS-PAGE sample preparations: Mix each fraction well. Take 20 µL of each fraction and mix with 4 µL of 6x SDS loading dye. Boil for 5 min. at 100°C, load all of sample on 12% SDS-PAGE gel.

IEX Chromatography of De-sumoylated IMAC-Elution Method: Used the IMAC-Elution after de-sumoylation and dialysis sample. Buffer exchange 15x into IEX Buffer A (20 mM Tris, pH 7.0 containing 5 mM DTT) using an Amicon Ultra-15 MWCO 10,000 Da. Connect a Q Sepharose FF 1 mL column to an AKTA Chromatography system. Load buffer exchanged sample over an equilibrated 1 mL pre-packed GE HiTrap Q FF column at 2 mL/min. Collect flow through when UV absorbance increases. Perform linear NaCl gradient with IEX Buffer B (20 mM Tris, pH 7.0 containing 5 mM DTT and 250 mM NaCl) from 0% to 100% B over 10 min at 2 mL/min to separate proteins. Collect 0.75 mL fractions when UV absorbance increases. Prepare SDS-PAGE analysis samples as described in the Initial IEX Chromatography after De-sumoylation Method.

IEX after IMAC protocol (initial): Prepare the IMAC-Elution SUMO-tagged sample by diluting at 20x minimum (≤ 10 mM Imidazole) with Initial IEX Buffer A. Connect a Q Sepharose FF 1 mL column to an AKTA Chromatography system. Equilibrate the column with Initial IEX Buffer A for 10 CV at 2 mL/min. Load entire volume of diluted IMAE-sample at 2 mL/min. Collect flow through when UV absorbance increases. Wash with 20 CV of Buffer A.* Perform 100 mM NaCl incremental step gradient starting at 100 mM NaCl until at 100 % B. Collect fractions and prepare SDS-PAGE analysis samples as described in the Initial IEX Chromatography after De-sumoylation Method.

IEX after IMAC protocol (Proof of Concept): Follow IEX after IMAC protocol (initial) up until the asterisk. Use Proof of concept Buffers for elution with 250 mM NaCl. Collect elution and prepare SDS-PAGE analysis samples as described in Initial IEX Chromatography after De-sumoylation Method.

IMAC Chromatography after De-sumoylation Protocol: Load sample onto column containing equilibrated Ni- or Co- IMAC resin. Rinse with 5-10 CV of Wash buffer. Elute with 5 CV of Elution Buffer, collecting entire volume.

IMAC-Elution Target Stability Study Protocol: Prepared the IMAC-Elution sample using the IMAC Proof of Concept Buffers. Split the IMAC-elution and dialyzed ½ of the target (in a 10 kDa Dialysis cassette) into Dialysis Buffer for Stability Study. The other ½ of the IMAC-Elution was stored overnight at 4°C.

IMAC Preparation and Chromatography Protocol: Add 10x volumes of lysis buffer (from IMAC Buffers) to cell weight. Resuspend cells in buffer. Sonicate on ice 30x using sonicator at 50% duty, pulse, 30 sec on, 30 sec off. Centrifuge entire lysate for 30 min at 15,000 rpm, separate supernatant from pellet. Prepare SDS-PAGE samples as described in the Solubility Testing protocol. The supernatant (or sample)* is either incubated with 1-2 mL of resin /1-3 g of cells for 1-2 hrs or directly loaded onto a gravity flow column. Collect flow through and then wash with 5-10 column volumes (CV) of wash buffer. (Gradients were washed with 4.5 CV per gradient fraction). Use 5 CV of elution buffer and collect 4-5 CV of elution.

Solubility Testing: Use the Cell Culture Growth (small protocol) to start 2 overnight cell culture growths. Under aseptic conditions, use 1 L of autoclaved LB media and add appropriate antibiotic to correct concentration. Pour the overnight culture into the LB media. Cover the flask and incubate bacterial culture with shaking at 37°C until OD_{600} reaches ~0.4. Sample 1 mL "un-induced culture" for SDS-PAGE analysis. Drop temperature to 17°C for 30 min. To culture add 200 µL of 1 M IPTG for a final concentration of 0.2 mM IPTG. Continue culturing cells overnight for 15-17 hrs. Harvest 1 mL sample of "induced culture" for SDS-PAGE analysis. Use Harvesting Cell Culture protocol (medium to large scale) to harvest cells and add 10 volumes of

lysis buffer to cell weight. Re-suspend cells. Sonicate on ice 30x using 50% duty, pulsed, 30 sec. on, 30 sec. off settings. Sample 100 µL of lysate. Centrifuge entire lysate for 30 min. at 15,000 rpm. Separate supernatant and pellet. Sample 100 µL of supernatant and pellet (scape and resuspend into 100 µL of ddH2O).

SDS-PAGE sample preparations: Prepare all culture samples as described in Expression Testing protocol. Prepare Lysate, Supernatant and pellet by using 1 µL of each, 19 µL ddH2O, and 4 µL of 6x SDS loading dye. Boil for 5 min. at 100°C, load all of sample on 12% SDS-PAGE gel.

2.3 Enzyme Kinetics

Enzyme Kinetics Method: Using the concentration determined by the Edelhoch Method, prepare 100 µL stocks at 500 nM concentration of the WT USP30 and the mutants with Buffer A. Incubate on ice for 30 min. Use the table below to prepare reaction mix at room temperature. Measure enzyme kinetics reaction using a 50 µL, 10 mm pathlength, Quartz Fluorometer with sub-micro and Fluoromax-4 fluorescence spectrophotometer. Following addition of enzyme, the fluorescence emission at 440 nm was recorded with excitation at 335 nm. Used the 120 sec method to obtain CPS values (initial rate in nM/sec) and Final CPS values (fully turned over rate). Create a standard calibration curve using the Final CPS values vs. substrate concentrations and obtain the slope. Using the slope, convert the CPS values to enzymatic kinetics rates for the WT and the mutants. Repeat the Ub-AMC substrate concentrations at a minimum in duplicate.

Table 2.2: Enzyme Kinetics Buffer Table

	Buffer A (Incubation Mix)	Buffer B (Reaction Mix)
H2O	3.6 uL	156 uL
5x Reaction Buffer	80 uL	800 uL
BSA (10 mg/mL)	16 uL	40 uL
DTT (1M)	0.4 uL	4 uL
total volume	100 uL	1000 uL

2.4 Additional Methods

Concentration determination via Edelhoch Method: For blank, mix 100 µL of 8 M Urea with 25 µL of buffer (Q Buffer A with spiked NaCl- 50 mM HEPES, pH 7.2,

10% Glycerol, 1 mM DTT, and 50 mM NaCl). Use a 120 µL, 1 cm path length Quartz Cuvette (Science Outlet) along with a Dioarray Spectrophotometer with Zenith Data Systems general scanning software. Measure blank. Prepare USP30 and mutants by mixing 100 µL of 8 M Urea and 25 µL of protein sample. Measure spectrum and obtain A280 value. Use the A280 value, the dilution (5), and the calculated extinction coefficient (53400 $M^{-1}cm^{-1}$) to obtain the concentration in µM.

Coomassie Staining/Destaining Protocol: After gel has finished running, remove SDS-PAGE gel from glass, place in a container and rinse in ddH_2O for 15 minutes to remove SDS. Remove all the water from the gel container and add enough Coomassie Brilliant Blue R-250 Stain to completely cover the gel. Microwave on high power for 30 seconds or until the stain boils. Incubate the gel in the stain for 30 minutes. Pour off the stain. Rinse the container with Destain solution to remove stain from the container. Add Destain solution to container to cover the gel. Microwave on high power for 30 seconds or until the solution boils. Incubate the gel in the Destain solution for 1-2 hrs with rocking. Incubate a second time for 10 minutes to overnight with rocking.

IMAC-E Mass Spectrometry Sample Preparation Method: Freeze/thaw IMAC-E sample 5x's to degrade target in preparation for abundant low molecular weight impurity to be analyzed by Mass Spectrometry.

SDS-PAGE Power Conditions: Lower the Inner Chamber Assembly with gel(s) into the Mini Tank. Fill the inner chamber completely with running buffer. Add ~200 mL of running buffer to the Mini Tank (lower buffer chamber). Load samples into the wells. Place the lid on the Mini Tank ensuring correct color alignment. Insert electrical leads into the PowerPac Basic Power Supply with the proper polarity. Turn power on. Select milliamperes as the constant parameter. Enter desired mA (20 mA/gel). Program a timed run for 1 hr. 15 min. Start the run. When run is complete, press stop key, then turn off power and disconnect the electrical leads. Remove the tank lid and carefully lift out the Inner Chamber Assembly. Pour off and discard the running buffer.

Chapter 3

RESULTS

3.1 Cloning

3.1.1 Cloning of USP30 into SUMO vector

The plasmid, pDEST USP30, obtained from Addgene in DH5α cells was cultured using the Cell Culture Growth (small) protocol, purified using the QIAGEN Plasmid Purification Kit protocol, and then transformed into TOP10 cells using the transformation protocol. Amplified and purified the plasmid from TOP10 cells using the same methods as above. Used primers USP30+ and USP30-fwd2 to verify the sequence via GeneWiz of USP30. The gene sequence was confirmed as missing the N-terminal nine amino acids, and no mutations.

A construct expressing residues 57-517 was cloned into a pET-derived vector with an N-terminal 6x His tag. Used ?USP30fwdpET and USP30revpETpGpF primers to amplify USP30ΔTM. PCR Mix and cycler conditions for pDEST USP30 Protocol was employed, following PCR purification using the QIAquik PCR Purification Kit Protocol. Figure 3.1.A shows the PCR amplification that generated the expected 1,438 nucleotide size band. Purified USP30ΔTM PCR product and pSUMO vector followed the Double Digest protocol in preparation for ligation.

Figure 3.1.B shows the pSUMO double digest with the correct expected size band at ~5,600 nucleotides.

The USP30 gene was cloned into the multiple-cloning site using the restriction enzymes XhoI, and EcoRI. The USP30 PCR and pSUMO double digests were cleaned by following the QIAGEN Nucleotide removal kit protocol and the QIAGEN Gel Extraction protocol, respectively. Cleaned USP30ΔTM PCR EcoRI/XhoI product and pSUMO EcoRI/XhoI product were ligated using the Ligation protocol. The ligation was used for transformation into TOP10 cells by following the Transformation protocol to obtain many colonies. The colonies were then screened using the Colony PCR for pSUMO-USP30ΔTM protocol as can be seen in Figure 3.1.C. Two of the positive gene containing colonies were cultured for plasmid amplification and purification. The two pSUMO-USP30ΔTM vectors were sequenced using T7fwd (GeneWiz in-house) and USP30-fwd2 primers. Only colony #2 contained the correct sequence. Colony #2 of pSUMO-USP30ΔTM was transformed into bacterial T7 expression *E.coli* strain Rosetta (DE3) using the Transformation Protocol and numerous colonies were obtained. Cell culture growth was performed for the generation of glycerol cell stocks and for initial expression testing.

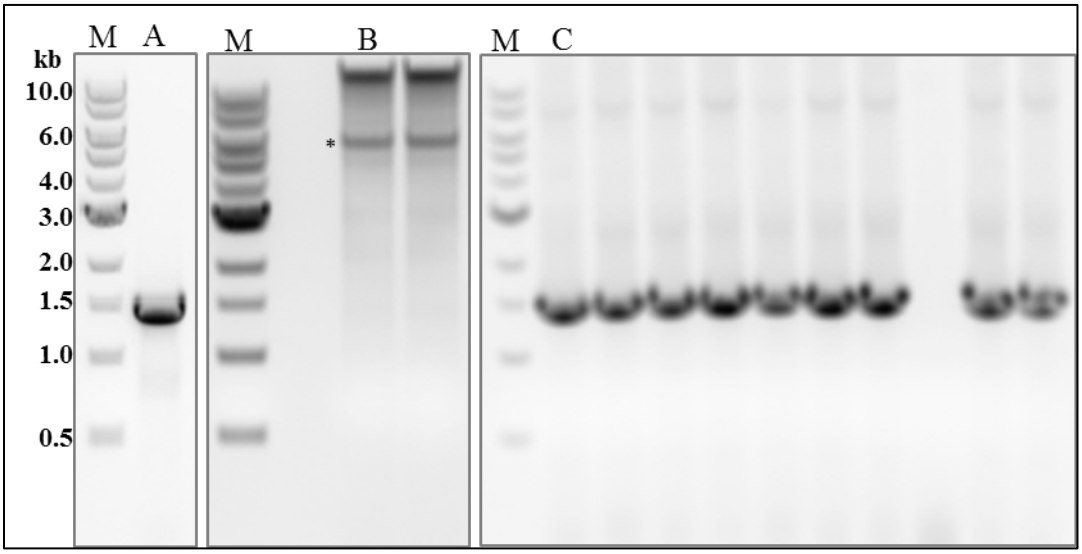

Figure **3.1.** Cloning of USP30ΔTM with ethidium bromide staining after 0.8% agarose gel electrophoresis. **A:** PCR product of USP30ΔTM (1438 b.p) **B:** EcoRI/XhoI double digest of pSUMO vector, shown by the asterisk (~5600 b.p.) **C:** Positive representation of colony PCR product. M, electrophoresis marker, 1kb DNA ladder.

3.1.2 Site-directed mutagenesis

We also created two USP30ΔTM gene mutants, USP30 C77A, and USP30 S477A. The pSUMO-USP30ΔTM plasmid was used in conjunction with primers USP30 C146A F/USP30 C146A R and USP30 S546A F/USP30 S546A R and the Site Directed Mutagenesis protocol to generate the mutants. Figure 3.2. shows the correct size band at the expected 7.1 Kb after PCR. The same primers were used for pSUMO USP30ΔTM sequence verification to verify the mutants. The sequences were correct.

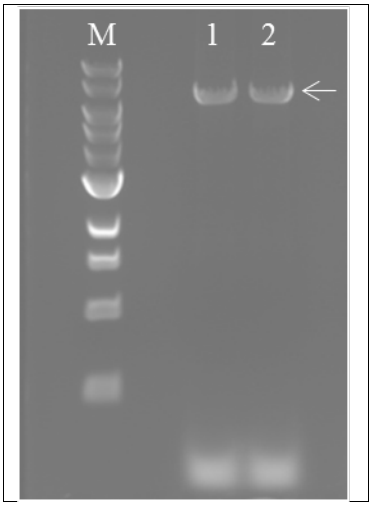

Figure **3.2.** Representative Site Directed Mutagenesis PCR agarose gel stained with ethidium bromide. C77A Performed at two different annealing temperatures 1, 50°C, and 2, 60°C. M, electrophoresis marker, 1kb DNA ladder. Arrow indicates the correct size band at ~7.1 Kb.

3.2 Expression and Solubility Testing

3.2.1 Testing expression of USP30

Using the Expression Testing protocol, the expression levels were studied with 0.2 mM IPTG induction overnight at 37°C. The induced and un-induced culture samples were analyzed in 12% SDS PAGE. Figure 3.3. gel result confirms target expression after induction.

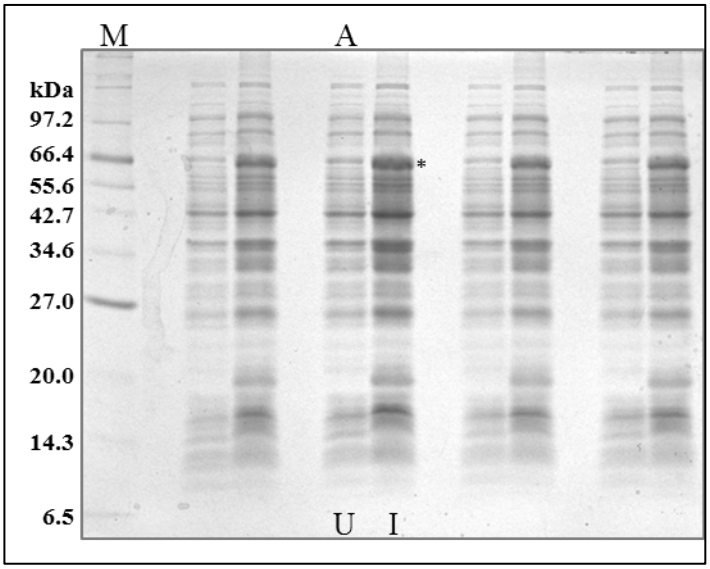

Figure **3.3**. Expression testing SDS-PAGE analysis of pSUMO USP30ΔTM in *E.coli* with Coomassie Staining **A:** U, un-induced bacterial cell culture. I, induced bacterial cell culture after OD_{600} 0.5 with 0.2 mM IPTG, shown next to asterisk. M, protein gel electrophoresis marker, 2-212 kDa. Asterisk indicates expression of target pSUMO-USP30 at ~66.7 kDa.

3.2.2 Testing solubility of USP30

The solubility testing protocol which included these modifications was used to determine solubility of SUMO-USP30ΔTM. Figure 3.4. shows the extracted soluble protein in the supernatant fraction. While there was considerable amount of target in the pellet fraction, the extracted soluble fraction was more than sufficient for kinetic studies.

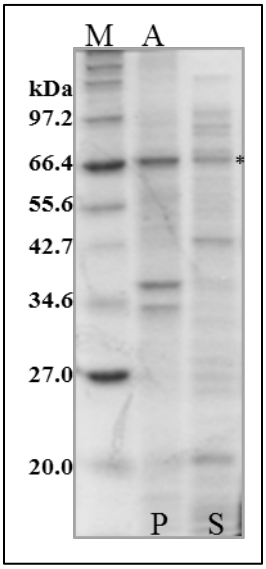

Figure **3.4**. SDS-PAGE analysis of pSUMO USP30ΔTM Solubility in *E.coli* with Coomassie staining. **A:** P, Pellet of lysate after extraction with sonication and centrifugation. S, supernatant of lysate after extraction with sonication and centrifugation, USP30ΔTM MW ~66.7 kDa is shown next to asterisk. M, protein gel electrophoresis marker, 2-212 kDa.

A substantial amount of cells were prepared for purification development by following the Cell Culture Growth, (large) protocol. Prior to harvesting the cells, all media was combined and then harvested. The pSUMO USP30ΔTM yielded ~18 g cells/6 L media (3g/L). The pSUMO USP30ΔTM C77A yielded and the pSUMO USP30ΔTM S477A yielded similar yields as the WT.

3.3 Purification Development

3.3.1 IMAC process development

3.3.1.1 Sodium chloride and imidazole gradients

A NaCl gradient was performed using the IMAC NaCl Gradient Buffers and the IMAC Preparation and Chromatography protocol to help produce a less impure product of SUMO-USP30ΔTM during IMAC Chromatography. The NaCl step gradient was composed of 100 mM NaCl increments from 100 mM to 1000 mM. The target was then eluted with 500 mM Imidazole. The NaCl gradient did not yield any extra impurity removal (data not shown). Therefore, the addition of NaCl to the Wash buffer was eliminated.

An imidazole gradient was executed using the IMAC Imidazole Buffers and IMAC Preparation and Chromatography protocol to identify the appropriate concentration for a possible imidazole wash step and the imidazole needed to elute the target. The elution gradient had increments of 50 mM imidazole starting with 50 mM up to 500 mM. Figure 3.5.A shows that the target elutes between 50 mM and 250 mM imidazole. Because the target eluted at relatively low imidazole concentrations, an imidazole wash was not implemented. Future experiments should elute with a maximum of 250 mM imidazole to ensure complete target recovery.

Performed one proof of concept IMAC purification to verify the first two gradient results, using no NaCl in the wash and 250 mM imidazole for elution. The

250 mM imidazole elution worked as expected, but shows 2-3 major impurities as can be seen in Figure 3.5.B.

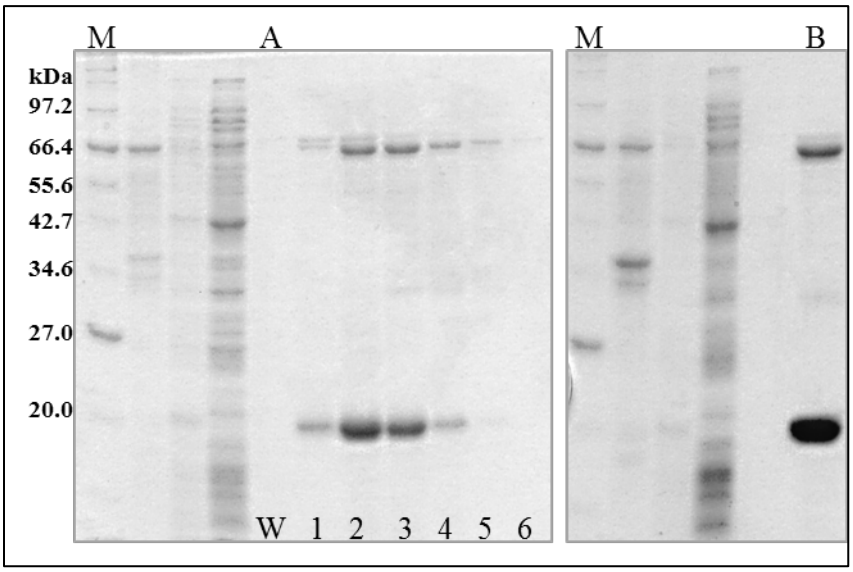

Figure **3.5.** Coomassie stained SDS-PAGE analysis of Ni-NTA process development. **A:** Imidazole gradient. W, wash, 1, 50 mM imidazole, 2, 100 mM imidazole, 3, 150 mM imidazole, 4, 200 mM imidazole, 5, 250 mM imidazole, 6, 300 mM imidazole, 350-500 mM imidazole gradient steps did not contain target and not shown. **B:** Separate purification with single step 250 mM imidazole elution. M, protein gel electrophoresis marker, 2-212 kDa.

3.3.1.2 Target stability

In order to prepare the IMAC-Elution suitable for a polishing purification step on ion exchange resin, the imidazole had to be removed or made negligible via dialysis or dilution, and the stability under these conditions was to be explored. Used the IMAC-Elution Target Stability Study protocol and fresh IMAC-Elution prepared using the IMAC Preparation and Chromatography protocol with the IMAC Proof of Concept Buffers. The observations of the overnight dialysis showed formation of white chunky

precipitate and the 4°C overnight hold had small actin-like fibril formations. Sampled the supernatants and pellets for both conditions and ran SDS-PAGE analysis following the SDS-PAGE Power Conditions and the Coomassie Staining/Destaining protocols. The overnight dialysis at 4°C of the IMAC-eluate showed significant target loss in comparison to the overnight hold sample as can be seen in Figure 3.6. Thus, overnight dialysis of the IMAC-elution during purification is not recommended.

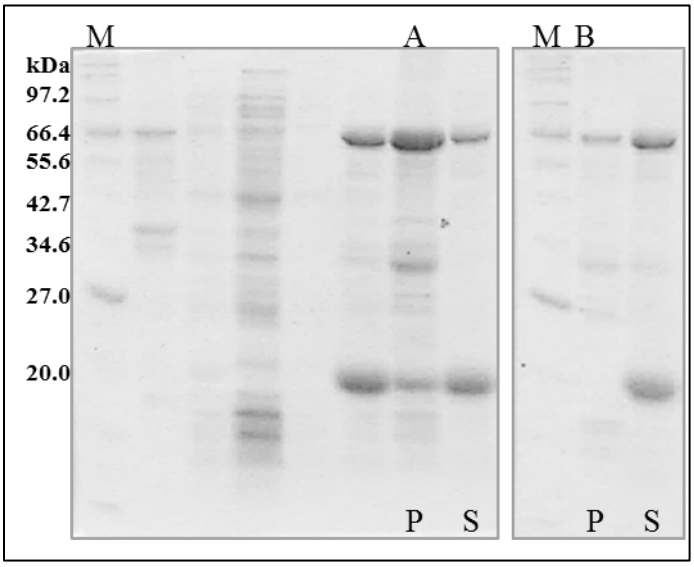

Figure **3.6.** Coomassie stained SDS-PAGE analysis of Dialysis vs. Overnight Hold. **A:** IMAC-elution with overnight dialysis, P, pellet, S, supernatant. **B:** IMAC-elution held overnight at 4°C before supernatant diluted for next IEX chromatography step. M, protein gel electrophoresis marker, 2-212 kDa.

3.3.1.3 IMAC resin scouting

Another method of yielding purer USP30, which was needed for kinetic analysis, was to scout IMAC resins. Nickel versus a cobalt immobilized metal charge on the resin was explored as both have affinity to his-tagged proteins. Figure 3.7. shows the

cobalt resin (B) with similar yields to the nickel resin (A) but with a higher purity. Thus cobalt resin was used in future purifications.

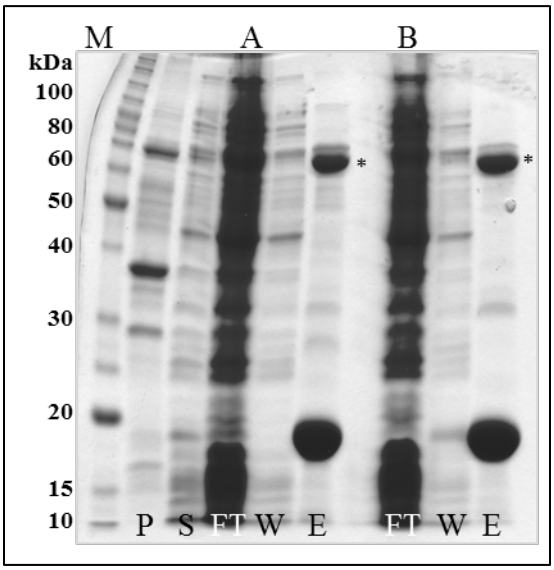

Figure **3.7.** Coomassie stained SDS-PAGE analysis of Nickel vs. Cobalt IMAC. P, pellet, S, supernatant from sonicated lysate **A:** Ni-IMAC-Flow Through (FT), Wash (W) and Elution (E) chromatography steps. **B:** Co-IMAC-Flow Through (FT), Wash (W) and Elution chromatography steps. M, protein gel electrophoresis marker, 10-220 kDa. Asterisk is target SUMO USP30ΔTM, MW ~66.7 kDa.

3.3.2 De-sumoylation assay development

3.3.2.1 After IMAC and after IEX chromatography

To investigate whether the IMAC purified SUMO-USP30ΔTM protein would precipitate upon removal of the SUMO tag, fresh Ni-IMAC-Elution was prepared using the IMAC proof concept buffers, the IMAC Preparation and Chromatography protocol, and the Initial De-sumoylation Assay Method. The assay used an initial ratio of enzyme to substrate at 1:1 with incubation for 30 min at room temperature. Figure

3.8.A SDS-PAGE analysis shows the de-sumoylation worked as expected. Note interestingly that the abundant lower impurity was also cleaved by the ULP1 enzyme indicating that it may be a truncated SUMO-USP30 protein subject to de-sumoylation.

3.3.2.2 ULP1 ratio determination

In order to determine the lowest ratio of ULP1 with complete cleavage to use at 4°C while buffer exchanging SUMO-USP30, the reaction was tested with ratios ranging from 1:1 to 1:100 and two time points, one at 4 hours and the other one overnight at 4°C. Used a stored 4°C IMAC-E sample containing the abundant lower impurity, which had shown to be to be susceptible to cleavage by ULP1, as the target SUMO-USP30 had already been degraded. Figure 3.8.B SDS-PAGE shows the de-sumoylation assay after 4hrs completely cleaved the SUMO portion at the 1:1, 1:10, and 1:25 ratios. At 4 hours, ratios of 1:50, 1:75, and 1:100 had not cleaved the SUMO portion completely. Overnight incubation at all dilutions yielded complete de-sumoylation. No precipitation formed after the SUMO was cleaved with 4 hours or overnight incubation. Note that the band with the asterisk in Figure 3.8.B is not SUMO-USP30 but the very similar ~70 kDa impurity.

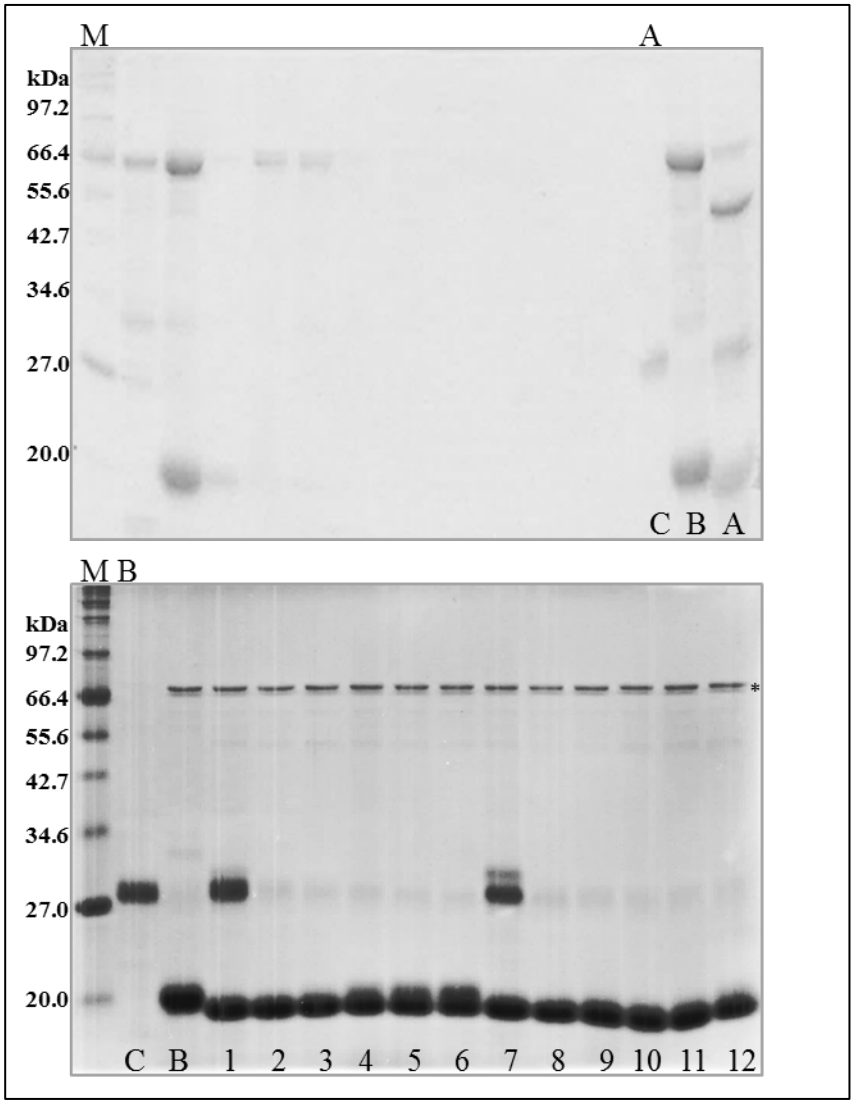

Figure **3.8.** SDS-PAGE Coomassie Stain analysis of De-sumoylation Assay. **A:** Initial De-sumoylation Assay results. C, ULP1 control with MW of 28 kDa, B, before ULP1 addition, SUMO-USP30 control with MW of 67 kDa, A, after ULP1 incubation with SUMO-USP30, with cleaved USP30, MW of 53 kDa. **B:** Ratio determination of ULP1 to SUMO USP30 for complete cleavage. Lanes 1-6 were incubated for 4 hrs at 4°C with the following ratios of 1:1, 1:10, 1:25, 1:50, 1:75, and 1:100 of ULP1 to SUMO USP30. Lanes 7-12 have the same ratios as Lanes 1-6 but were incubated at 4°C overnight. * Impurity that runs just above the target. M, protein gel electrophoresis marker, 2-212 kDa.

3.3.2.3 Additional Data

To determine if the largest impurity band (~20 kDa) was a truncated SUMO USP30 product/degradant, the IMAC-E Mass Spectrometry Sample Preparation Method in conjunction with Mass Spectrometry (MS) analysis yielded a mass spectrum highlighting 2 main mass-to-charge ratio values. Figure 3.9. shows the two main sizes of the truncations are 16297 and 16476. An assessment of possible truncated SUMO USP30 sizes reveals the 16297 is 144 amino acids located on the N-terminus with the removal of the start Methionine (2-145aa). The inconclusive mass-to-ratio 16476 is somewhere in between 145 and 146 amino acids. Amino acid 146 is the catalytic cysteine of our target. The MS investigation exposed that more than half of the truncated SUMO USP30 contains the important catalytic cysteine residue.

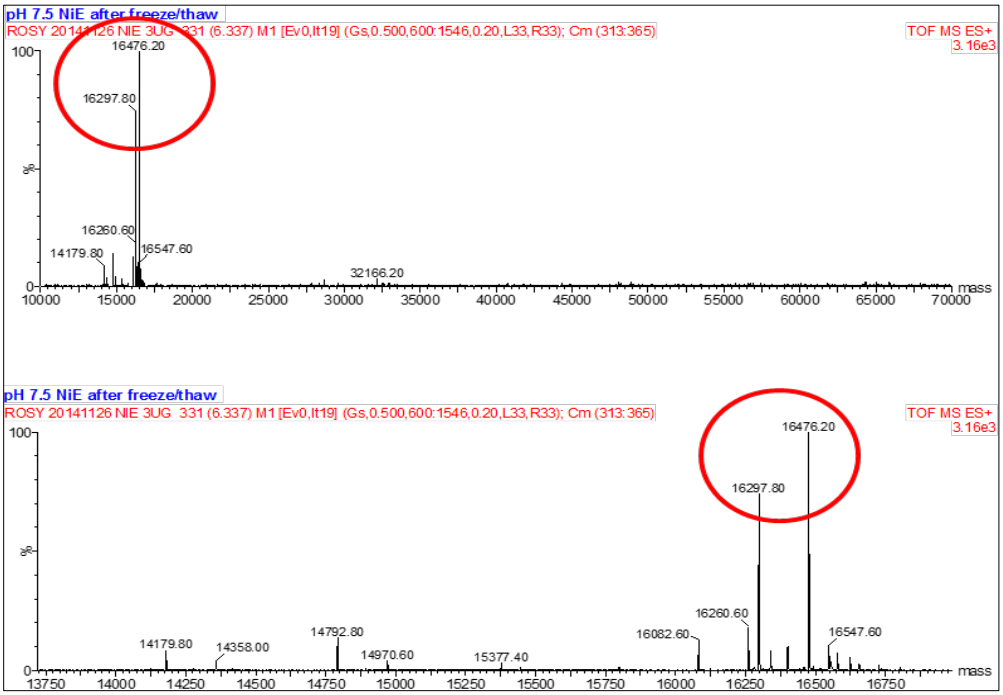

Figure **3.9.** Mass Spectrometry Analysis of abundant low molecular weight impurity. Most dominant peaks are 16476 and 16297 m-to-z ratio.

3.3.3 Selection of polishing column

3.3.3.1 IMAC after de-sumoylation

The IMAC or IEX elution sample was de-sumoylated according to the ULP-1 De-sumoylation Assay Method and was to be further purified by IMAC. Used the IMAC Proof of Concept Buffers and IMAC Preparation and Chromatography protocol starting from the asterisk. The initial pH of 7.5 was tested, however, the majority of the de-sumoylated USP30 was found in the elution fraction along with the cleaved 6x-His SUMO (~14 kDa) protein and not in the flow through as was expected and is

shown in Figure 3.10.B lanes 7 and 9. Two other pHs were evaluated at 7.0 and 8.0 and exhibited the same results as can be seen in Figure 3.10.A and C. In an additional attempt to purify desumoylated-USP30, we switched to IMAC Tris Buffers at pH 7.0 with 500 mM NaCl to prevent non-specific binding of target, but we were not successful as illustrated in Figure 3.10.D.

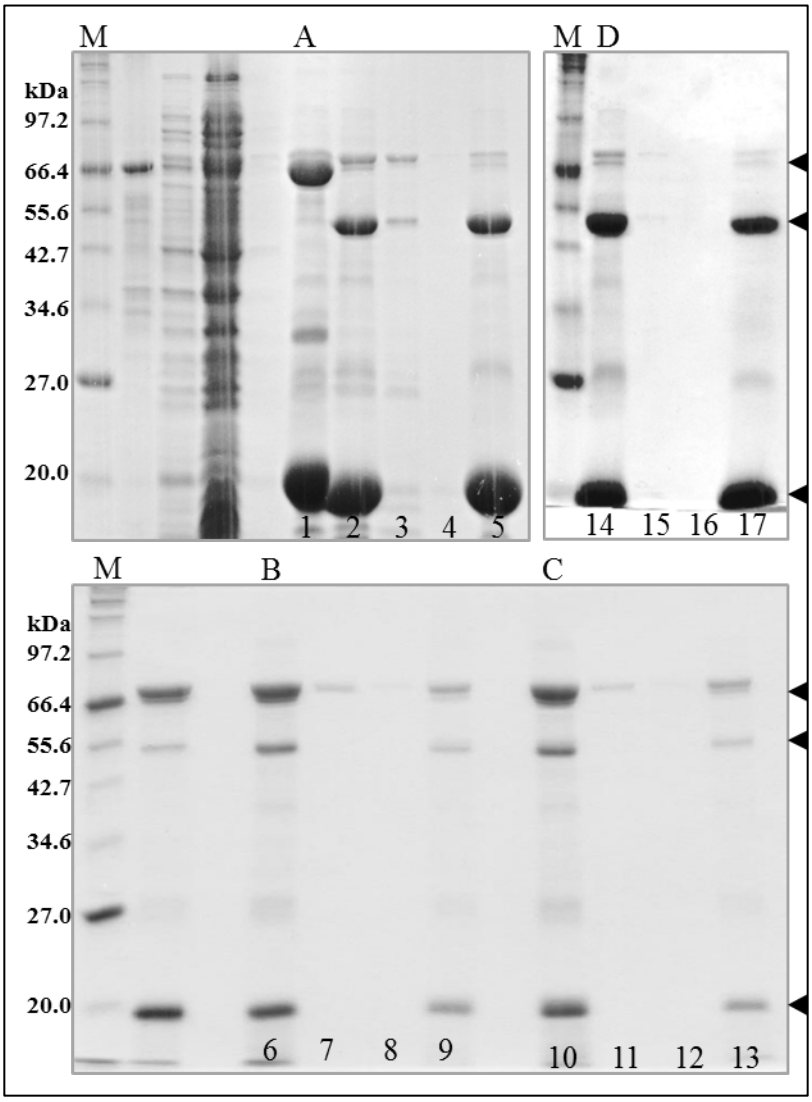

Figure **3.10.** SDS-PAGE Coomassie Stain analysis of Re-IMAC after De-sumoylation Assay. **A:** Re-IMAC of de-sumolyated USP30 at pH 7.0. **B:** Re-IMAC of de-sumolyated USP30 at pH 7.5. **C:** Re-IMAC of de-sumolyated USP30 at pH 8.0. **D:** Re-IMAC of de-sumolyated USP30 at pH 7.0 with 0.5 M NaCl in all buffers. M, protein gel electrophoresis marker, 2-212 kDa, Lane 1, IMAC-Elution after sonication at pH 7.5, Lanes 2, 6, 10, and 14 are product after desumolyation assay and dialysis, Lanes 3,7,11 and 15 are Re-IMAC-Flow through, Lanes 4,8, 12 and 16 are Re-IMAC wash, Lanes 5, 9, 13 and 17 are Re-IMAC Elution (250 mM imidazole).

In a final effort to purify de-sumoylated USP30, we utilized the Cobalt charged resin instead of the Nickel resin for Re-IMAC. Used the Cobalt Re-IMAC Buffers and the IMAC Preparation and Chromatography protocol starting from the asterisk, which performed a Cobalt IMAC imidazole gradient. The flow through contained ~1/2 of the target with the abundant lower MW impurity. The imidazole step gradient showed the target co-eluting with the impurity. Figure 3.11. shows the co-elution problem, thereby exhausting the possibility of removing the SUMO tag after de-sumoylation via another IMAC chromatography step.

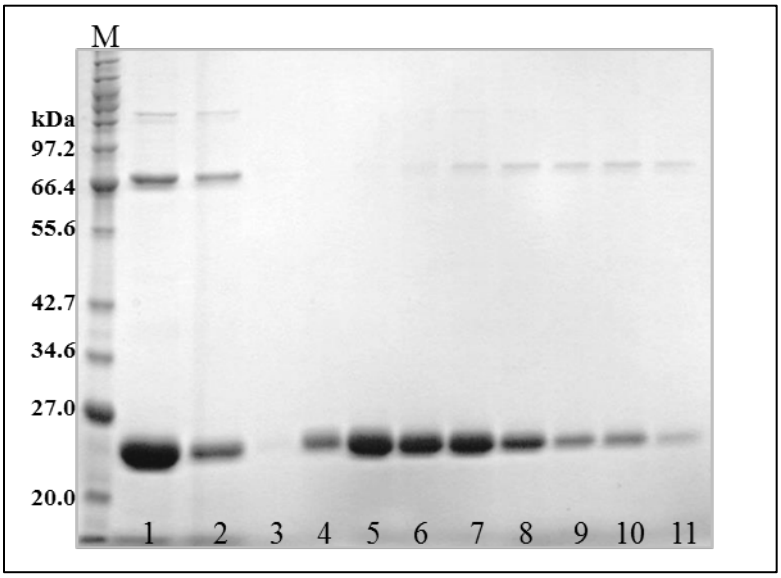

Figure **3.11.** SDS-PAGE Coomassie Stain analysis of Cobalt-IMAC after De-sumoylation Assay. M, protein gel electrophoresis marker, 2-212 kDa, Lane 1, IMAC-Elution after de-sumoylation/dialysis reaction, Lane 2, Co2+ Re-IMAC Flow through, Lane 3, Wash, Lane 4-11, Imidazole gradient starting at 30 mM with 30 mM increments to 250 mM Imidazole concentration.

3.3.3.2 IEX after IMAC

Due to the unsuccessful attempts to further purify de-sumoylated USP30 via IMAC, we used IEX after IMAC protocol (initial), Initial IEX Buffers and IMAC-Elution as a preliminary screen to further purify the SUMO-USP30. A NaCl step gradient with 100 mM increments was performed on 1 mL Q Sepharose FF column. Figure 3.12.A shows the majority of the target in the 100 and 200 mM NaCl fractions. The largest impurity appears in the flow through fraction, thus greatly improving the purity.

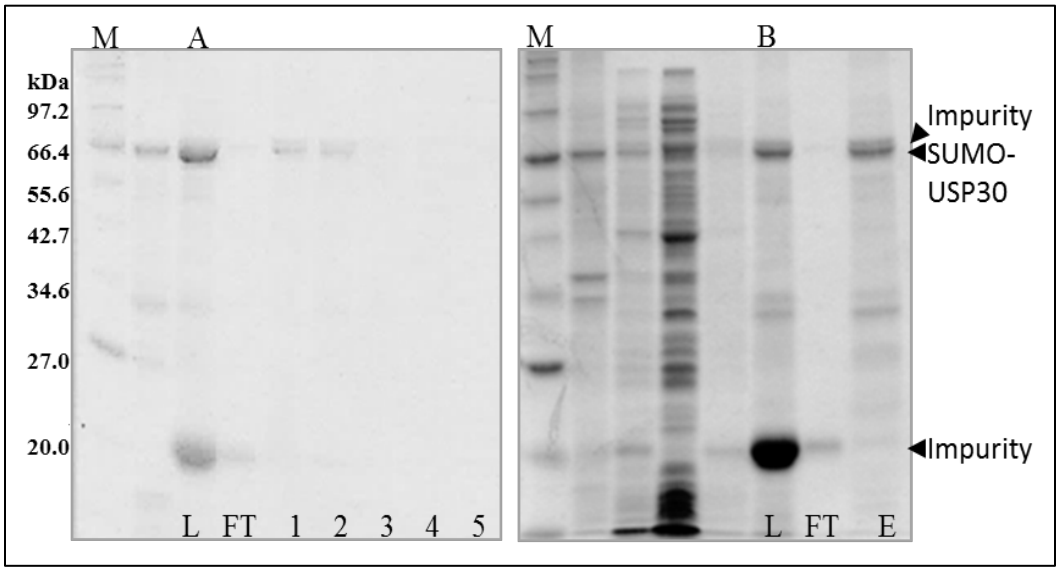

Figure **3.12.** SDS-PAGE Analysis of Q chromatography. **A:** IEX step gradient. L, load, FT, flow through, Lanes 1-5 are a NaCl gradient starting at 100 mM with 100 mM increments to 500 mM NaCl concentration. **B**: IEX proof of concept. E, 250 mM NaCl Elution. M, protein gel electrophoresis marker, 2-212 kDa.

Repeated by following the IEX after IMAC protocol (proof of concept), and Proof of Concept Buffers and an IMAC-Elution sample. The IEX column was eluted

with 250 mM NaCl. The chromatography results in Figure 3.12.B show the major impurity in the flow through (FT) and the target in the 250 mM elution fraction, but with another similar sized impurity.

3.3.3.3 IEX after de-sumoylation

In another effort to purify the de-sumoylated USP30, IEX chromatography was employed. Executed the Initial IEX Chromatography after De-sumoylation Method at pH 7.0 or 8.5, which performed a linear gradient from 0-100% B (250 mM NaCl) in 5 minutes and collected fractions to assess the de-sumoylated USP30 binding/elution properties. SDS-PAGE analysis of the flow through (FT) and NaCl gradient fractions revealed some protein properties. Figure 3.13.B shows that the IEX at pH 8.5 bound the target, but also co-eluted with the impurities offering no further separation. Figure 3.13.A of pH 7.0 shows some promising separation with the lower NaCl concentrations.

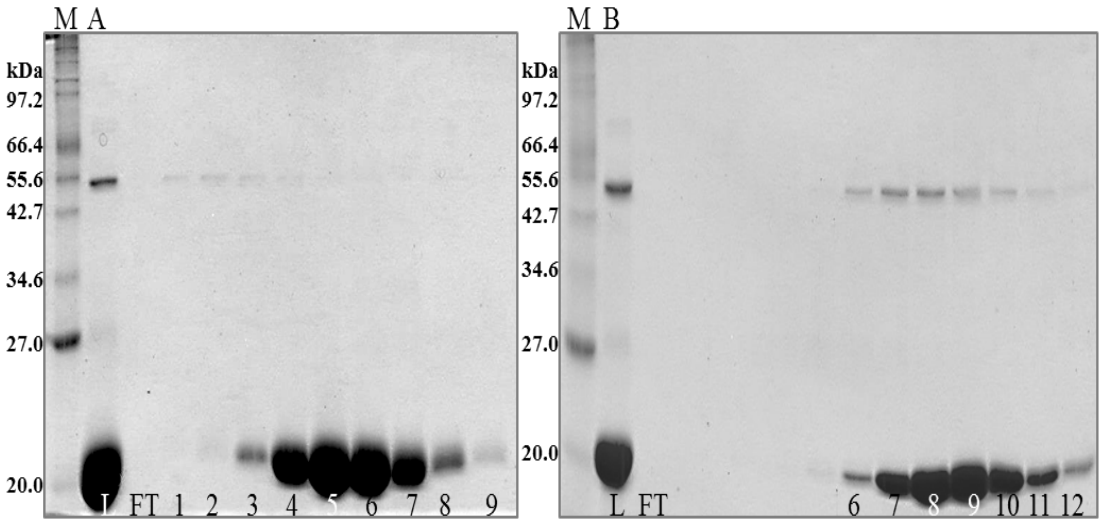

Figure **3.13.** SDS-PAGE Analysis of Q FF chromatography after de-sumolyation. **A:** IEX NaCl linear gradient at pH 7.0. M, protein gel electrophoresis marker, 2-212 kDa, L, load, FT, flow through, Lane 1, 145-155 mM NaCl, Lane 2, 170-180 mM NaCl, Lane 3, 195-205 mM NaCl, Lane 4, 220-230 mM NaCl, Lane 5, 245-250 mM NaCl, Lanes 6-9, 250 mM NaCl. **B:** IEX NaCl linear gradient at pH 8.5. Lane 6, 230-240 mM NaCl, Lanes 7-12, 250 mM NaCl. De-sumoylated target ~53 kDa.

IEX was performed again using a shallower NaCl gradient allowing further separation of the target from the impurity, as can be seen in Figure 3.14. The SUMO-USP30 similar sized molecular weight impurity ~70 kDa was eluted at higher NaCl concentrations. Pure target fractions should be pooled for enzyme kinetic studies.

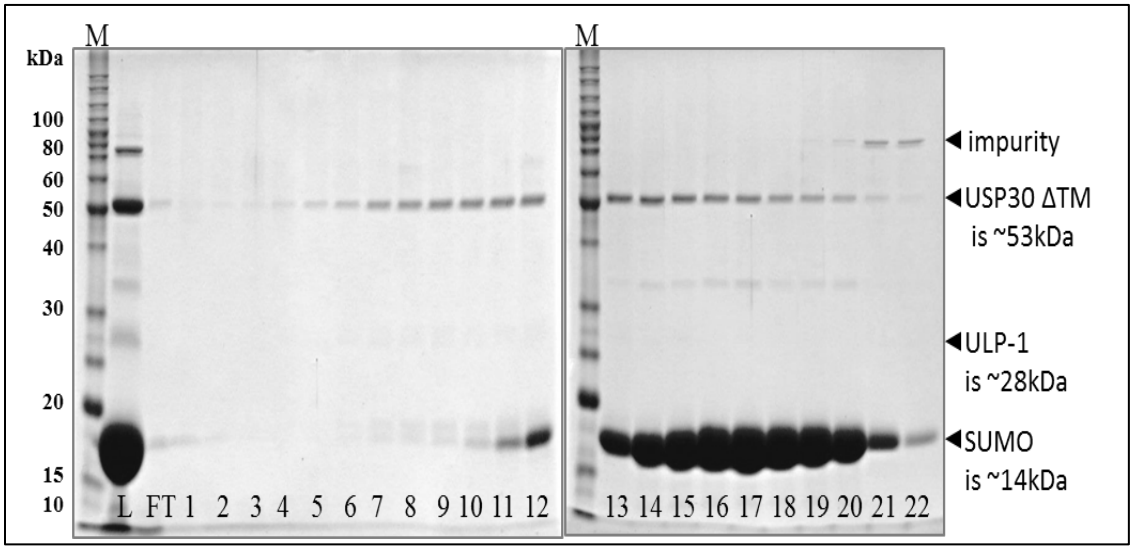

Figure **3.14.** SDS-PAGE Analysis of Q FF chromatography after de-sumoylation. IEX NaCl linear gradient at pH 7.0. M, protein gel electrophoresis marker, 10-220 kDa, L, load, FT, flow through, Lane 1, 41-50 mM NaCl, Lane 2, 50-60 mM NaCl, Lane 3, 60-70 mM NaCl, Lane 4 70-78 mM NaCl, Lane 5, 78-88 mM NaCl, Lane 6, 88-97 mM NaCl, Lane 7, 97-106 mM NaCl, Lane 8 106-116 mM NaCl, Lane 9 116-125 mM NaCl, Lane 10 125-135, Lane 11 135-144, Lane 12 144-153, Lane 13 154-163, Lane 14 163-172 mM NaCl, Lane 15 172-181 mM NaCl, Lane 16 181-190 mM NaCl, Lane 17 190-200, Lane 18 200-210 mM NaCl, Lane 19 210-220 mM NaCl, Lane 20 220-229 mM NaCl, Lane 21-22 250 mM NaCl.

3.3.4 Final Purification

Used the purification developed by Cunningham *et al* that consisted of a capture step on Nickel IMAC and a polishing step on Q FF resin. Purified 22.5 µM of WT, 12.3 µM of the C77A mutant, and 12 µM of S477A mutant. Figure 3.15. illustrates the purification of each construct.

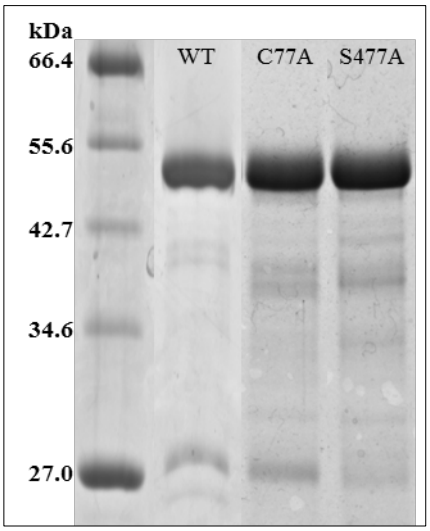

Figure **3.15.** Final protein purification of WT and mutants.

3.4 AMC Cleavage Assay

Widely used to assay DUB activity, the Ub-AMC is a reagent fused at its c-terminus to a molecule 7-amino-4-methyl-coumarin (Ub-AMC). Upon hydrolysis by the DUB, the free AMC reporter molecule produces a fluorescent signal that allows for a direct read-out of activity (Faesen, Luna-Vargas et al. 2011). The Ub-AMC was synthesized and purified in house. To determine k_{cat}/K_M for each protein, 10 nM USP30 or its mutants, USP30 S477A, and USP30 C77A were incubated with varying substrate concentrations of Ub-AMC at room temperature following the Enzyme Kinetics Method. Before adding the Ub-AMC, the mix was read in a fluorometer to verify no activity. Ub-AMC was added to the cuvette, mixed and measured in counts per second (CPS). The WT mutant had an estimated k_{cat}/K_M of 3.1 x 10^4 $M^{-1}s^{-1}$. The

C/A mutant had no detectable fluorescence, and the S/A mutation had an estimated k_{cat}/K_M of 2.2 x10^2 $M^{-1}s^{-1}$. Figure 3.16. shows the WT and mutant activity plotted.

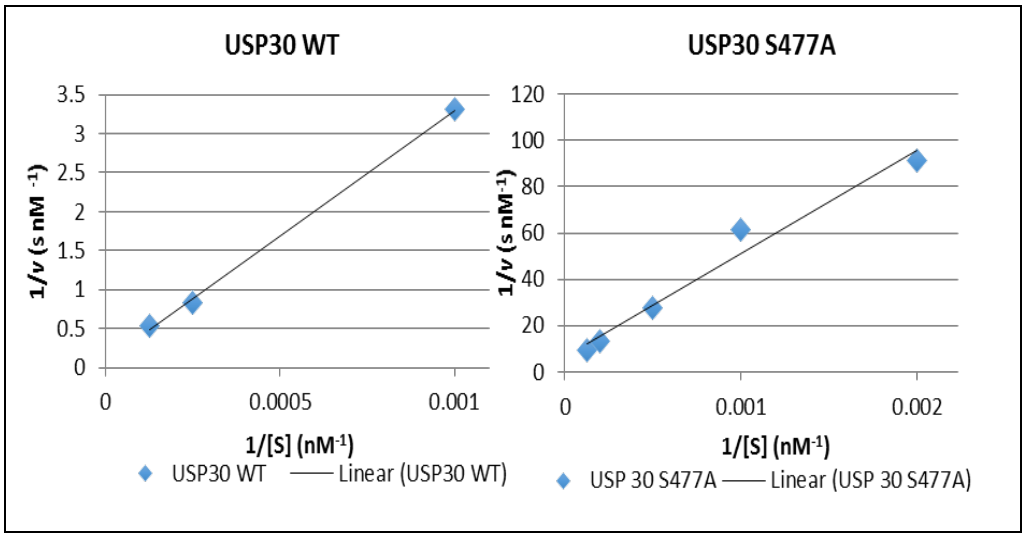

Figure **3.16.** Lineweaver-Burk analysis of WT USP30, obtained by measuring the initial rates at varying Ub-AMC concentrations. Left shows the USP30 WT analysis. Right shows the USP30 S477A mutant analysis.

Chapter 4

DISCUSSION

In recent years, many studies presented evidence on mitochondrial dysfunction and its involvement in the pathogenesis of neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease. Human USP30 is a deubiquitinating enzyme located on the mitochondrial outer membrane that works in conjunction with the ubiquitin proteasome system and the mitophagy pathway to control the degradation of Mfn1, Mfn2, Drp1, and Fis1 proteins and the signaling of autophagy (Tanaka, Cleland et al. 2010; Chan, Salazar et al. 2011; Wang, Song et al. 2011; Zhang, Wu et al. 2012). Familial Parkinson's disease (genetic/heredity form) can be caused by mutations in the PINK1 and parkin proteins whose involvement in the PINK1/parkin mediated mitophagy is opposed by deubiquitinase USP30 (Valente, Abou-Sleiman et al. 2004; Vives-Bauza, Zhou et al. 2010; Narendra and Youle 2011). Parkinson's disease affects the central nervous system (CNS) and causes issues with motion such as shaking, rigidity, and lethargic movements as well as the disruption of other functions such has sleep, lack of sensory (like feeling and smell), and mental awareness (Parkinson 2002). The pathophysiology is an abnormal accumulation of α-synuclein protein in brain neurons located in the substantia nigra of the midbrain, which further develops into Lewy bodies eventually causing nerve cell death (Moore, West et al. 2005; Hauser and Hastings 2013). Mitochondria in these neurons play an

important role in regulating Ca^{2+} which affects the neuronal malleability, neuron formation and neurotransmitter discharge, as well as supplying an abundant amount of ATP (Baughman, Perocchi et al. 2011; De Stefani, Raffaello et al. 2011). Dysfunctional mitochondrial dynamics not only affect the neurons and their transmitting properties but also mitochondrial transport to these neurons thereby potentially causing neurodegenerative problems.

In this work, we cloned, sequenced, process developed, purified and characterized the USP30 protein in order to understand the purpose of the atypical serine in the catalytic core and its influence on mitochondrial dynamics. The pDEST USP30 plasmid, was purchased from Addgene. It contained the antibiotic resistance gene for ampicillin and needed sequence verification before continuing forward. The sequence analysis showed that the USP30 from Addgene was composed of 508 amino acids (1524 n.t.) with the first 9 amino acids from the N-terminus removed and no mutagenesis (a.a 10-517). The abcam company produces a human USP30 full length protein for sale on the market that is also composed of the same 508 amino acids. While the 9 amino acids are part of the topological domain (1-35), they are not part of the USP domain thereby not affecting the catalytic activity.

In order to express and purify the target for kinetic analysis the USP30 was cloned into the pET28-a vector. Since the transmembrane domain is not part of the USP domain, we decided to clone the USP30 without this region (57-517 a.a.). A quick search found that Sino Biological Inc. had successfully purified human USP30ΔTM (Thr48-Glu508) fused to a SUMO tag at the N-terminus in insect cells.

We decided to use the SUMO fusion tag in order to help with initial protein folding during expression as Sino Biological Inc. had. For cloning we designed the forward primer to fit into the pET28a-SUMO vector containing an EcoRI restriction site and a methionine start codon beginning at the USP domain. The reverse primer was designed with an XhoI restriction site to fit not only into the pET28a-SUMO (*E.coli*, bacterial cell, with kanamycin resistance), but also the pGEX-4T-3 (*E.coli*, bacterial cell), and pFastBac HT A (*S.frugiperda*, insect cell) vectors as well. *E.coli* cells named TOP10 were chosen for transformation because of their plasmid stabilizing properties and high transformation efficiency. A double digestion, ligation and transformation of the USP30ΔTM gene insert and the pSUMO vector into TOP10 cells was successful and yielded many colonies. For the expression of the target, the Rosetta (DE3) expression *E.coli* competent cell strain was selected for its T7 promoter controlled expression capabilities, its supply of 6 rare tRNA codons, which facilitates the expression of eukaryotic proteins, and for its chloramphenicol resistance. Correct plasmid sequencing results from TOP10 cells allowed for the effective transformation of pSUMO-USP30ΔTM into the expression Rosetta (DE3) cells, thus permitting the continuation towards expression and purification of our protein.

The expression and solubility were tested to confirm target expression and solubility before moving towards large scale protein production. The induced cells produced an abundance of protein, especially at the molecular weight of 67 kDa, as expected. By the same token, testing the solubility of the target would confirm if it was somewhat soluble, otherwise we would have to return to cloning or develop

tougher purification regimens to purify SUMO-USP30ΔTM. In order to test the solubility of the target, we used Nakamura *et al*'s membrane extraction assay as a starting point. Consequently, we incorporated similar Triton X100 conditions with sonication. This preliminary screen showed a positive proof of concept and while there was still a considerable amount of target in the pellet fraction, the supernatant contained enough target for kinetic study's.

A fused 6x His and SUMO tag at the N-terminus of the pSUMO-USP30ΔTM vector allowed purification based on immobilized metal affinity chromatography (IMAC). The technique separates proteins on the basis of a reversible interaction between the His-tagged protein and the metal charged ligand coupled to the agarose matrix. In this case, the target protein binds to a nickel or cobalt charged matrix through the 6x-His tag and is released by the addition of a higher affinity chemical structure called imidazole. The optimal wash and elution conditions were studied to obtain good recoveries of bound target along with decent purity. The imidazole gradient began to elute the target at 50 mM imidazole and was mostly eluted by 200 mM imidazole thus leading to a modification of the original elution condition to 250 mM imidazole. Note, that by reducing the imidazole concentration, buffer exchange/dialysis would be less timely, thereby allowing us to move onto other purification steps such as IEX Chromatography or De-sumoylation.

We also tested two different stability scenarios: 1) Dialysis overnight, and 2) overnight storage at 4°C. The results suggest that the target is very unstable during overnight dialysis and slightly unstable during overnight hold. Therefore, we

recommended a continuous purification without overnight hold if possible and dialysis for only short periods of time.

For the purification of SUMO-USP30, nickel vs. cobalt resin was compared as well. The cobalt resin purified SUMO-USP30 in similar quantities, however with slightly less impurities, suggesting that the cobalt resin has a lower affinity for binding non-specific proteins in comparison to the nickel resin. Unfortunately, we came to test this out midway through the project, which is why the majority of the experiments conducted used nickel resin.

In order to further purify USP30, the SUMO-tag must be removed and therefore, de-sumoylation assays were set-up and performed. De-sumoylation assays can be performed using a 1:25 ratio for a total incubation of 4 hr. Interestingly, the abundant lower molecular weight impurity was also cleaved. Could this possibly be a truncated form of USP30? If this was true, then we could possibly engineer the vector to limit the cleavage/truncation, thereby yielding a more intact protein. However, we needed to investigate the portion of USP30 that was being cleaved, in case the area for alteration was too close to the active site, which might interfere with the enzyme kinetic studies. The 20 kDa protein sample was prepared and submitted for Mass Spectrometry (M.S.) analysis. The M.S. data shown in Figure 3.9. had 2 main mass-to-charge ratio values of 16297 and 16476. The 16297 was a truncated/degraded form of USP30 starting at the N-Terminus, minus the methionine, up to the threonine right before our catalytic cysteine. The 16746 mass-to charge ratio was inconclusive but

near the catalytic cysteine. Therefore, the possibility to reduce/limit the formation of the truncated/degraded 20 kDa protein via re-cloning was not a feasible option.

Since the re-cloning idea was discarded the purification process development after IMAC continued forward with de-sumoylation. Theoretically if we use the enzyme that cleaves off the SUMO-tag, known as ULP1, then after de-sumoylation, the His-SUMO protein portion should bind to the IMAC resin, and the target should flow through generating a refined purer target. However, this was not the case with the de-sumoylated target as it bound the resin under several different pH conditions and NaCl spiked buffers and always co-eluted with the His-SUMO protein portion amassing the same impure product. These results taken together exemplify the issue of the target being inseparable from the impurities using IMAC.

Another route explored to further purify SUMO-USP30ΔTM was Ion Exchange chromatography (IEX) following IMAC chromatography before de-sumoylation. Figure 3.12.B shows the success of the experiment using 250 mM NaCl for IEX Elution. There was however another impurity that sits slightly above SUMO-USP30 ~70 kDa. IEX was also tried with Tris buffers at pH 7.5. However, the difference in buffers greatly affected the fractionation of the largest impurity yielding a co-elution of target with impurities.

A route considered for purification of USP30 after de-sumolyation besides IMAC chromatography was also IEX chromatography. Using Q Sepharose FF, several pH's were studied to understand how de-sumoylated USP30 interacted with the resin under different conditions. Figure 3.14. showed IEX at pH 7.0 with a good

separation of USP30 (~53 kDa) from the major impurity (~20 kDa) and the minor impurity (~70 kDa, which elutes with a higher concentration of NaCl).

The process development for IMAC chromatography resulted: in the selection of cobalt resin over nickel, a sufficient elution with 250 mM imidazole, and an understanding of SUMO-USP30 stability under various conditions. The de-sumoylation assay showed ratio assessment, proof of concept, and mass spectroscopy data verifying the impurity to be a form of degraded/truncated USP30 protein. The ion exchange chromatography showed two possible ways to purify SUMO-USP30 and USP30, before or after de-sumoylation. Unfortunately, Cunningham *et al* developed a purification process and we switched to their method of purification for both the WT and mutants, which contained an IMAC, dialysis with de-sumoylation, and IEX steps.

Mutations in the catalytic core were generated to better understand USP30's catalytic activity and serine's role. Sequence alignments confirmed USP30's catalytic core residues as C77, H452 and S477 (Nijman, Luna-Vargas et al. 2005). Because the serine is an anomaly in the catalytic triad, we decided to mutate it to an alanine (S477A), thus allowing us to determine if the serine's hydroxyl group was involved in the enzyme activity and to what extent. We also mutated cysteine 77 to an alanine (C77A) as well. The sequencing results of the mutations verified the correct sequences. Comparable expression and solubility of the mutants to the WT prompted large scale target production of WT and mutants.

After purification of the WT and mutants, the enzymatic kinetic assays were performed in order to have a better understanding of the catalytic core properties. The WT USP30 kinetic analysis estimated a k_{cat}/K_M of 3.1 x 10^4 $M^{-1}s^{-1}$, obtained from the slope of the Lineweaver-Burk analysis, but the Vmax was not obtained due to the K_M values being above the solubility limit for Ub-AMC. These values are similar with two previous studies where a k_{cat}/K_M of 1.3 x 10^4 $M^{-1}s^{-1}$ and 7 x 10^3 $M^{-1}s^{-1}$ were reported (Faesen, Luna-Vargas et al. 2011; Cunningham, Baughman et al. 2015). The C77A mutant lost its enzymatic properties, which has been shown with many other USP family members, such as USP2 and USP1 (Zhang, Sulea et al. 2011; Villamil, Chen et al. 2012). The S477A mutant had no enzymatic activity at a USP30 concentration of 10 nM but at 100 nM there was a very slight turnover and yielded a k_{cat}/K_M of 2.2 x10^2 $M^{-1}s^{-1}$. These results suggest that while the serine is an uncommon amino acid for the typical USP catalytic core, it plays an important role in deubiquitination. Another mutation to consider would be the S477D to further study the serine's significance. The Asp residue is common in most USP's. If the catalytic core was mutated to incorporate the amino acid Aspartate (D), several scenarios could develop: 1) the S477D mutation would have the same turnover values as the WT, implying the serine's role is interchangeable, and can perform the same functions as the Asp in the catalytic core, 2) the S477D mutation causes a greater turnover rate than the WT, indicating that the less active serine is influencing the deubiquitination reaction and 3) the S477D mutation yields a much lower turnover rate than the WT, suggesting the serine's role is not interchangeable and necessary for the proper

turnover rate of deubiquitination. In future work, other mutations can also be explored to gain further insight into the catalytic properties.

The aim of this project was to clone, express, purify and characterize USP30's enzyme kinetics of the catalytic core. USP30's location on the mitochondrial outer membrane has opened many questions about its role in mitochondrial morphology and organelle maintenance as mitochondrial dysfunction is involved in the pathogenesis of neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease (Han, Tomizawa et al. 2011; Nunnari and Suomalainen 2012). The USP30 cloning into the pSUMO vector was successful as was the site-directed mutagenesis. The expression of SUMO-USP30 was positive thereby permitting the solubility testing. The amount of soluble protein was sufficient for the purification process development and kinetic assays. While the SUMO-USP30 came close to having an in-house complete purification method, another group published a USP30 purification in the same time frame. Proteins were purified according to Cunningham *et al* and prepared for the characterization of the catalytic core. The k_{cat}/K_M values obtained were similar to others reported. The most interesting result was that the serine in the catalytic core was participating in the deubiquitinating activity. It's turn-over rate is medium in comparison to USP7 or USP8, which can indicate more of a regulatory function (Faesen, Luna-Vargas et al. 2011).

In understanding the USP30 mechanisms and how it ultimately affects more than one realm of disorders caused by mitochondrial dysfunction, the possibility for therapies, vaccines, and inhibitors can be further researched to treat and possibly

prevent these diseases. The mitochondrial dynamics (fusion and fission) are important in not only maintaining correct mitochondrial morphology, which is dependent on what the cell requires, but also in maintaining mobility, which is important for neuron function (Chan 2006). It is known that aging is caused by oxidative stress (reactive oxidative species (ROS)) which can promote mtDNA mutations and deletions over time leading to impairment of the Electron Transport Chain thereby reducing ATP production yielding more ROS accumulation (Chan 2006; Chandel 2010; Seo, Joseph et al. 2010; Youle and van der Bliek 2012; Hauser and Hastings 2013). Increased ROS can also promote aging and fibrosis of the heart, thereby promoting organ damage (Ikeda, Sciarretta et al. 2014). Note that centennials have elongated mitochondria allowing them the benefits of seniority (Sgarbi, Matarrese et al. 2014).

Other USP's involved with the mitochondria are USP15 and USP8. While USP15 has been shown to perform similar functions as USP30 by deubiquitinating mitochondrial proteins thereby preventing mitophagy, USP8 has been shown to deubiquitinate parkin, thereby promoting mitophagy (Eiyama and Okamoto 2015). Some USP's, such as USP15, can be involved in pathways that promote oncogenesis, however this area needs to be studied in more depth (Cornelissen, Haddad et al. 2014). If some of these USP's are involved in cancer or other diseases, having inhibitors to prevent their functions could provide some needed assistance in the therapy department as many drugs are building resistance. USP30, USP7, and USP14 are among some of the USP's with known inhibitors. Other inhibitors are too broad and cause more side effects, like the ubiquitin-proteasome inhibitor Velcade used as an

anti-cancer treatment (Guedat and Colland 2007). DUBs can be a new route of therapeutic agents providing less toxicity than current therapies. (Chen, Liu et al. 2011). And finally, vaccines are also an options as Parkinson's disease causes Lewy Bodies and in clinical trials is the PD01 vaccine candidate which binds to α-Synuclein aggregates, thus promoting the body to remove and them allowing the neurons to survive and live (Schneeberger, Mandler et al. 2012).

The key message is that by understanding the kinetic properties of USP30 and serine's importance as a member of the catalytic triad, we can further explore therapies for Parkinson's disease, other neurodegenerative diseases that also have dysfunctional mitochondria, and cancers that have shown an increase of USP30. Testing agents, like 15-oxospiramilactone (S3), that inhibit USP30 could restore the pathway leading towards proper function (Yue, Chen et al. 2014). Studies of USP30 will open up a new area of drug development and testing for the treatment of Parkinson's disease and other neurodegeneration diseases.

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Appendix A

MATERIALS

A.1 Organisms

Bacterial strains-*E.coli*

- Rosetta (DE3) Competent Cells- Novagen, Catalog# 70954
  genotype: F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE (Cam^R)
  Contains the transcription T7 RNA Polymerase promoter, which can be induced and regulated by the addition and concentration of IPTG. Designed to enhance the expression of eukaryotic proteins that contain 6 codons rarely used in *E.coli* (i.e. allows expression of human protein USP30) as well as conferring resistance to chloramphenicol.

- One Shot TOP10 Chemically Competent *E.coli* –Invitrogen, Catalog# C4040
  genotype: F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
  Used for high-efficiency cloning and plasmid propagation allowing stable replication of high-copy number plasmids.

A.2 Nucleic Acids

Vectors

- Flag-HA-USP30 – addgene, catalog #22578
  (Backbone: pDEST_LTR_N_FLAG_HA_IRES_puro, 6521bp, Harper_Lab; Growth in Bacteria: Amp^R, 37°C, DH5α, high copy; Gene insert: USP30, H.Sapiens (human), GenBank ID: BC004868.2)
- pET-28a DNA Vector, 5369bp, Kanamycin, Novagen, Catalog#69864
- pET28a-SUMO, 5633bp, Kanamycin, Rozovsky Lab, University of Delaware

Constructs

- Wild Type (WT) USP30 minus the transmembrane region (56-517)
- C77A (in pSUMO amino acid # C146)
- S477A (in pSUMO amino acid # S546)

Primers

usp30 seq + (used to verify sequence from pDEST vector sent from addgene) 5’ CAGCCCTCACTCCTTCTCTAGG 3’

Usp30-fwd2 (used to verify sequence from pDEST vector sent from addgene) 5’ CATGGAAGACTCACTAG 3’

?USP30fwdpET (used to clone USP30 out of pDEST into pET-SUMO vector) 5’ GCAGCAGGAATATATGTTATTTGGGAATTCATGACAGA AAGAAAGAAG 3’

USP30revpETpGpF (used to clone USP30 out of pDEST into pET-SUMO) 5’ CTTTTGTACAAGAACTCGAGTTATTCTTCAGACTTGCACTCCT G 3’

USP30 S546A F (used to mutate S477, S546 is the a.a.# with the SUMO tag) 5’ CTAGCAATCAGTGGCTGTGGGTCGCCGATGACACTGTCCG 3’

USP30 S546A R (used to mutate S477, S546 is the a.a.# with the SUMO tag) 5’ CGGACAGTGTCATCGGCGACCCACAGCCACTGATTGCTAG 3’

USP30 C146A F (used to mutate C77, S146 is the a.a.# with the SUMO tag) 5’ CTTGTTAATTTAGGGAACACCGCCTTCATGAACTCCCTGC 3’

USP30 C146A R (used to mutate C77, S146 is the a.a.# with the SUMO tag) 5’ GCAGGGAGTTCATGAAGGCGGTGTTCCCTAAATTAACAA G 3’

A.3 DNA Sequencing and Amino Acid Sequences

pDEST USP30 DNA sequencing confirmation (received from Addgene)

TGGNCTACANGGATGACGATGACAAGCTCGATGGAGGATACCCATACGATGTTCCAGATTA
CGCTGCTCGAGGTTATCAAACAAGTTTGTACAAAAAAGTTGGCATGACCGCGGCCGACA
GGGCCATCCAGCGCTTCCTGCGGACCGGGGCGGCCGTCAGATATAAAGTCATGAAGAACTG
GGGAGTTATAGGTGGAATTGCTGCTGCTCTTGCAGCAGGAATATATGTTATTTGGGGTC
CCATTACAGAAAGAAAGAAGCGTAGAAAAGGGCTTGTGCCTGGCCTTGTTAATTTAGGGAA
CACCTGCTTCATGAACTCCCTGCTACAAGGCCTGTCTGCCTGTCCTGCTTTCATCAGGT
GGCTGGAAGAGTTCACCTCCCAGTACTCCAGGGATCAGAAGGAGCCCCCCTCACACCAGTA
TTTATCCTTAACACTCTTGCACCTTCTGAAAGCCTTGTCCTGCCAAGAAGTTACTGATG
ATGAGGTCTTAGATGCAAGCTGCTTGTTGGATGTCTTAAGAATGTACAGATGGCAGATCTCA
TCATTTGAAGAACAGGATGCTCACGAATTATTCCATGTCATTACCTCGTCATTGGAAG

ATGAGCGAGACCGCCAGCCTCGGGTCACACATTTGTTTGATGTGCATTCCCTGGAGCAGCAG
TCAGAAATAACTCCCAAACAAATTACCTGCCGCACAAGAGGGTCACCTCACCCCACAT
CCAATCACTGGAAGTCTCAACATCCTTTTCATGGAAGACTCACTAGTAATATGGTCTGCAAA
CACTGTGAACACCAGAGTCCTGTTCGATTTGATACCTTTGATAGCCTTTCACTAAGTA
TTCCAGCCGCCACATGGGGTCACCCATTGACCCTGGACCACTGCCTTCACCACTTCATCTCAT
CAGAATCAGTGCGGGATGTTGTGTGTGACAACTGTACAAAGATTGAAGCCAAGGGAA
CGTTGAACGGGGAAAAGGTGGAACACCAGAGGACCACTTTTGTTAAACAGTTAAAACTAGG
GAAGCTCCCTCAGTGTCTCTGCATCCACCTACAGCGGCTGAGCTGGTCCAGCCACGGCA
CGCCTCTGAAGCGGCATGAGCACGTGCAGTTCAATGAGTTCCTGATGATGGACATTTACAAG
TACCACCTCCTTGGACATAAACCTAGTCAACACAACCCTAAACTGAACAAGAACCCAG
GGCCTACACTGGAGCTGCAGGATGGGCCGGGAGCCCCCACACCAGTTCTGAATCAGCCAGG
GGCCCCCAAAACACAGATTTTTATGAATGGCGCCTGCTCCCCATCTTTATTGCCAACGC
TGTCAGCGCCGATGCCCTTCCCTCTCCCAGTTGTTCCCGACTACAGCTCCTCCACATACCTCT
TCCGGCTGATGGCAGTTGTCGTCCACCATGGAGACATGCACTCTGGACACTTTGTCA
CTTACCGACGGTCCCCACCTTCTGCCAGGAACCCTCTCTCAACTAGCAATCAGTGGCTGTGG
GTCTCCGATGACACTGTCCGCAAGGCCAGCCTGCAGGAGGTCCTGTCCTCCAGCGCCT
ACCTGCTGTTCTACGAGCGCGTCCTTTCCAGGATGCAGCACCAGAGCCAGGAGTGCAAGTCT
GAAGAATGCCCAACTTTCTTGTACAAAGTGGTTCGATAACGAATTCCGCCCCCCCCCC
CTAACGTTACTGGCCGAAGCCGCTTNNNTAAGGCCGGTGTGCGTTTGTCTATATGTTATTTTC
CACCATANNGCCGTCTTTTGGCAATGTGAGNNCCNGNAANCTGNNCCTGTCTTCNTG
ACNANCATTCNAGGGNNNTTNCNNNTCTCNCCNAAGGAANGCANGNCTGNTGAATGNNCNT
GAAGGAAGCANTNCCTCTGGANNCT

pDEST USP30 confirmed Amino Acid Sequence

XXXYXDDDDKLDGGYPYDVPDYAARGYQTSLYKKVGMTAADRAIQRFLRTGAAVRYKVMK
NWGVIGGIAAALAAGIYVIWGPITERKKRRKGLVPGLVNLGNTCFMNSLLQGLSACPAFI
RWLEEFTSQYSRDQKEPPSHQYLSLTLLHLLKALSCQEVTDDEVLDASCLLDVLRMYRWQ
ISSFEEQDAHELFHVITSSLEDERDRQPRVTHLFDVHSLEQQSEITPKQITCRTRGSPHP
TSNHWKSQHPFHGRLTSNMVCKHCEHQSPVRFDTFDSLSLSIPAATWGHPLTLDHCLHHF
ISSESVRDVVCDNCTKIEAKGTLNGEKVEHQRTTFVKQLKLGKLPQCLCIHLQRLSWSSH
GTPLKRHEHVQFNEFLMMDIYKYHLLGHKPSQHNPKLNKNPGPTLELQDGPGAPTPVLNQ
PGAPKTQIFMNGACSPSLLPTLSAPMPFPLPVVPDYSSSTYLFRLMAVVVHHGDMHSGHF
VTYRRSPPSARNPLSTSNQWLWVSDDTVRKASLQEVLSSSAYLLFYERVLSRMQHQSQEC
KSEECPTFLYKVVR-RIPPPPPNVTGRSRXXKAGVRLSICYFPPXXRLLAM

pSUMO USP30ΔTM DNA sequencing confirmation

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATA
TGGCTAGCATGTCGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGT
CAAGCCTGAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGA
TCAAAAAGACCACTCCTTTAAGAAGGCTGATGGAAGCGTTCGCTAAAAGACAGGGTAAGGA
AATGGACTCCTTAAGATTCTTGTACGACGGTATTAGAATCCAAGCTGATCAGACCCCTGAAG
ATTTGGACATGGAGGATAACGATATTATTGAGGCTCACAGAGAACAGATTGGTGGATCCGA
ATTCATGACAGAAAGAAAGAAGCGTAGAAAAGGGCTTGTGCCTGGCCTTGTTAATTTAGGG
AACACCTGCTTCATGAACTCCCTGCTACAAGGCCTGTCTGCCTGTCCTGCTTTCATCAGGTGG
CTGGAAGAGTTCACCTCCCAGTACTCCAGGGATCAGAAGGAGCCCCCCTCACACCAGTATTT
ATCCTTAACACTCTTGCACCTTCTGAAAGCCTTGTCCTGCCAAGAAGTTACTGATGATGAGG
TCTTAGATGCAAGCTGCTTGTTGGATGTCTTAAGAATGTACAGATGGCAGATCTCATCATTT
GAAGAACAGGATGCTCACGAATTATTCCATGTCATTACCTCGTCATTGGAAGATGAGCGAG
ACCGCCAGCCTCGGGTCACACATTTGTTTGATGTGCATTCCCTGGAGCAGCAGTCAGAAATA
ACTCCCAAACAAATTACCTGCCGCACAAGAGGGTCACCTCACCCCACATCCAATCACTGGA
AGTCTCAACATCCTTTTCATGGAAGACTCACTAGTAATATGGTCTGCAAACACTGTGAACAC
CAGAGTCCTGTTCGATTTGATACCTTTGATAGCCTTTCACTAAGTATTCCAGCCGCCACATGG
GGTCACCCATTGACCCTGGACCACTGCCTTCACCACTTCATCTCATCAGAATCAGTGCGGGA
TGTTGTGTGTGACAACTGTACAAAGATTGAAGCCAAGGGAACGTTGAACGGGGAAAAGGTG
GAACACCAGAGGACCACTTTTGTTAAACAGTTAAAACTAGGGAAGCTCCCTCAGTGTCTCTG
CATCCACCTACAGCGGCTGAGCTGGTCCAGCCACGGCACGCCTCTGAAGCGGCATGAGCAC
GTGCAGTTCAATGAGTTCCTGATGATGGACATTTACAAGTACCACCTCCTTGGACATAAACC

TAGTCAACACAACCCTAAACTGAACAAGAACCCAGGGCCTACACTGGAGCTGCAGGATGGG
CCGGGAGCCCCCACACCAGTTCTGAATCAGCCAGGGGCCCCCAAAACACAGATTTTTATGA
ATGGCGCCTGCTCCCCATCTTTATTGCCAACGCTGTCAGCGCCGATGCCCTTCCCTCTCCCAG
TTGTTCCCGACTACAGCTCCTCCACATACCTCTTCCGGCTGATGGCAGTTGTCGTCCACCATG
GAGACATGCACTCTGGACACTTTGTCACTTACCGACGGTCCCCACCTTCTGCCAGGAACCCT
CTCTCAACTAGCAATCAGTGGCTGTGGGTCTCCGATGACACTGTCCGCAAGGCCAGCCTGCA
GGAGGTCCTGTCCTCCAGCGCCTACCTGCTGTTCTACGAGCGCGTCCTTTCCAGGATGCAGC
ACCAGAGCCAGGAGTGCAAGTCTGAAGAATAA

pSUMO USP30ΔTM confirmed Amino Acid Sequence

MGSSHHHHHHSSGLVPRGSHMASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFF
KIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIG
GSEFMTERKKRRKGLVPGLVNLGNTCFMNSLLQGLSACPAFIRWLEEFTSQYSRDQKEPP
SHQYLSLTLLHLLKALSCQEVTDDEVLDASCLLDVLRMYRWQISSFEEQDAHELFHVITS
SLEDERDRQPRVTHLFDVHSLEQQSEITPKQITCRTRGSPHPTSNHWKSQHPFHGRLTSN
MVCKHCEHQSPVRFDTFDSLSLSIPAATWGHPLTLDHCLHHFISSESVRDVVCDNCTKIE
AKGTLNGEKVEHQRTTFVKQLKLGKLPQCLCIHLQRLSWSSHGTPLKRHEHVQFNEFLMM
DIYKYHLLGHKPSQHNPKLNKNPGPTLELQDGPGAPTPVLNQPGAPKTQIFMNGACSPSL
LPTLSAPMPFPLPVVPDYSSSTYLFRLMAVVVHHGDMHSGHFVTYRRSPPSARNPLSTSN
QWLWVSDDTVRKASLQEVLSSSAYLLFYERVLSRMQHQSQECKSEE-

A.4Chemicals and consumables

Agar

LB Agar: 10 g Tryptone, 10 g NaCl, 5 g yeast extract, 15 g agar. Dissolve the mixture into 1 L ddH_2O and autoclave the solution for 20 min. Allow the solution to cool to ~55°C and add antibiotic if needed. Under aseptic conditions, pour the agar into Petri dishes, let harden and then invert and store at 4°C.

Agarose gel

0.8% Agarose gel (small): 0.4 g Agarose. Add to 50 mL of 1xTAE buffer and microwave until agarose is dissolved. Add 5 uL of Ethidium Bromide and mix. Pour agarose mixture into gel caster and add the comb. Let solidify. Use fresh or store in ziplock bag with some TAE buffer at 4°C.

Antibiotics

- Ampicillin 100 mg/mL stock: 1 g Ampicillin. Dissolve into 10 mL ddH_2O. Under aseptic conditions, filter sterilize (0.2 µm) into a sterile 15 mL conical tube. Aliquot and store at -20°C.

- Chloramphenicol 34 mg/mL stock: 0.34 g Chloramphenicol. Dissolve into 10 mL of 100% Ethanol. Under aseptic conditions, filter sterilize (0.2 µm) into a sterile 15 mL conical tube. Aliquot and store at -20°C.

- Kanamycin 50 mg/mL stock: 0.5 g Kanamycin. Dissolve into 10 mL of ddH_2O. Under aseptic conditions, filter sterilize (0.2 µm) into a sterile 15 mL conical tube. Aliquot and store at -20°C.

Buffers and Solutions

40% (w/v) Acrylamide/bis (Solution 37.5:1) VWR, Catalog# 0254

Ammonium persulfate (APS), BioRad, Catalog# 1610700

10% APS (Ammonium Persulfate) stock: 1 g APS dissolved in 10 mL of ddH_2O. Aliquot and store at -20°C.

6x Blue Loading Dye, NEB, Catalog# B7021S

Broad Range Protein Ladder, unstained, 2-212 kDa, NEB, Catalog# P7703 (Discontinued)

Bovine Serum Albumin (BSA), Lyophillized powder, Sigma, Catalog# A2153

BSA (Bovine Serum Albumin) 10 mg/mL stock: 0.1 g BSA dissolved in 10 mL of ddH_2O. Filter (0.2 µm) and store at 4°C.

Coomassie Brilliant Blue R-250, Biorad, Catalog# 161-0400

Coomassie Brilliant Blue R-250 Stain (100 mL) Solution: 90 mL of 50% Methanol (1:1, MeOH:ddH_2O), 10 mL Glacial Acetic Acid, 0.25 g Coomassie Brilliant Blue R-250, combined, filter and store at room temperature.

Destain Solution (100mL): 90 mL 50% Methanol, 10 mL Glacial Acetic Acid

1 kb DNA Ladder, NEB, Catalog# N3232

1 kb DNA Ladder for agarose gel (6 µL)
1 µL 1kb DNA ladder
1 µL BLD (6x Blue Loading Dye)
4 µL ddH_2O

Deoxynucleotide (dNTP) Solution Mix, 10 mM each nucleotide, NEB, Catalog # N0447

Dialysis Buffer for Stability Study: 1x PBS containing 5 mM DTT, pH 7.5

Ditthiothreitol (DTT), Fisher, Catalog# AC165681000

1 M DTT (Dithiothreitol) (10 mL) Stock: 1.5 g DTT dissolved into 8 mL ddH_2O, BTV, aliquot and store at -20°C

Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), Fisher, Catalog# S311

0.5 M EDTA, pH 8.0 (1 L) (ethylenediaminetetraacetic acid disodium salt dihydrate) Stock: 186.1 g EDTA added to 800 mL ddH_2O. Dissolve by adjusting the pH to 8.0 with 10 M NaOH. BTV, filter and store at room temperature.

Ethidium Bromide, 10 mg/mL, Invitrogen, Catalog# 15585011

Glacial Acetic Acid, Fisher, Catalog# 2401

Glycerol, ≥99.5%, Fisher, Catalog# BP229

50% Glycerol Solution: 250 mL Glycerol, 250 mL ddH_2O, Autoclave the solution for 20 min.

IEX Buffers

Initial IEX Buffers
Buffer A: 1x PBS containing 5 mM DTT, pH 7.5
Buffer B: 1x PBS containing 5 mM DTT, 1 M NaCl, pH 7.5

Proof of Concept Buffers:
Buffer A: 1x PBS containing 5 mM DTT, pH 7.5
Buffer B: 1x PBS containing 5 mM DTT, 250 mM NaCl pH 7.5

IMAC Buffers

Imidazole Gradient Buffers:
Lysis Buffer: Same as for Solubility testing
Wash Buffer: 1x PBS containing 10 mM β-Me, pH 7.5
Elution buffers: Wash buffer containing 50 mM and 100 mM increments of imidazole up to 500 mM

Sodium Chloride Gradient Buffers:
Lysis Buffer: Same as for Solubility testing
Wash Buffer: 1x PBS containing 10 mM β-Me, pH 7.5

NaCl Wash buffer: Wash buffer containing 100 mM NaCl increments to 1 M
Elution buffer: Wash buffer containing 500 mM Imidazole

IMAC Proof of Concept Buffers
Lysis Buffer: Same as for Solubility testing
Wash Buffer: 1x PBS containing 10 mM β-Me, pH 7.5
Elution buffer: Wash buffer containing 250 mM of Imidazole

IMAC Tris Buffers
Binding/Wash Buffer: 20 mM Tris, 500 mM NaCl, pH 7.0 containing 10 mM β-Mercaptoethanol
Elution Buffer: 20 mM Tris, 500 mM NaCl, pH 7.0 containing 10 mM β-Me, and 250 mM of Imidazole

Cobalt Re-IMAC Buffers
Dialysis/Binding Buffer: 50 mM Tris, 10 mM Imidazole, 500 mM NaCl, 10 mM β-Me, pH 7.5
Wash Buffer: 50 mM Tris, 10 mM β-Me, pH 7.5
Elution Buffers: 50 mM Tris, 10 mM β-Me containing 30 mM increments of imidazole up to 250 mM

4x Incubation buffer with BSA and DTT (1 mL) (also known as Buffer B)
800 µL 5x Reaction buffer
40 µL 10 mg/mL BSA solution (0.4 mg/mL)
40 µL 1 M DTT (40 mM)
80 µL 5 M NaCl (400 mM NaCl)
40 µL ddH_2O

Isopropyl-beta-D-thiogalactoside (IPTG), Invitrogen, Catalog# 15529019

1 M IPTG (isopropyl-beta-D-thiogalactoside) (10 mL) Stock: 2.38 g IPTG dissolved into 9 mL ddH_2O. BTV, filter (0.2µm), aliquot and store at -20°C.

Lysis buffer for Solubility testing: 1x PBS containing 0.1% Triton X-100, 10% Glycerol, 10 mM β-Mercaptoethanol, 10 mM Imidazole, pH 7.5.

LB Media: 10 g Tryptone, 10 g NaCl, 5 g yeast extract. Dissolve the mixture into 1 L ddH_2O and autoclave the solution for 20 min. Allow the solution to cool to ~55°C and add antibiotic if needed.

Methanol, Fisher, Catalog# A454

50% Methanol: Mix 500 mL 100% Methanol with 500 mL ddH_2O.

4x Reaction buffer with BSA and DTT (1 mL) (also known as Buffer A)

800 µL 5x Reaction buffer
40 µL 10 mg/mL BSA solution (0.4 mg/mL)
4 µL 1M DTT (4 mM)
80 µL 5M NaCl (400 mM NaCl)
76 µL ddH_2O

5x Reaction buffer

250 mM HEPES
2.5 mM EDTA
pH 7.8

SOC Media

0.5% Yeast Extract
2% Tryptone
10 mM NaCl
2.2 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
Autoclave, let cool, add 20 mM Glucose, filter (0.2 µm), store at -20°C

Sodium Chloride (NaCl), Fisher, Catalog# S641

5M NaCl (50 mL) Stock: 14.6 g NaCl. Dissolve into 30mL of ddH_2O. BTV and store at room temperature.

50x TAE (1 L)

2 M Tris-Base (242 g)
1 M Acetic Acid (57.1 mL Glacial Acetic Acid (17.5 M)
0.05 M EDTA (100mL 0.5 M EDTA, pH 8.0)

1x TAE (1 L): Mix 20 mL 50x TAE (final conc. 40 mM Tris, 20 mM Acetate, 1 mM EDTA), and 980 mL ddH_2O

TE Buffer pH 7.4

10 mM Tris
1 mM EDTA

Tris-base, Fisher, Catalog# BP152

Urea, Fisher, Catalog# U15

8M Urea (50 mL) Stock: 24 g Urea. Add 20 mL of warmed ddH_2O. Dissolve, BTV and store at room temperature.

Enzymes
Restriction Enzymes: EcoRI, XhoI, DpnI (NEB, Catalog# R0101, R0146, R0176)
Ligase: Quick T4 DNA Ligase (NEB, Catalog# M2200)
Polymerases: *PfuTurbo* DNA Polymerase, LongAmp *Taq* DNA Polymerase (Agilent Technologies, Catalog# 600250, NEB, Catalog# E5200S)

Kits
QIAprep Spin Miniprep Kit (QIAGEN, Catalog# 27106)

Resins
IMAC-Immobilized Metal Affinity Chromatography
Ni-NTA Agarose, Invitrogen, Catalog# R901-15
TALON Metal Affinity Resin, Clontech Laboratories, Catalog# 635502
IEX- Ion Exchange Chromatography
HiTrap Q Sepharose FF, GE, Catalog# 17-5053-01

A.5 Equipment and applications

AKTA Purifier 100
DNA gel electrophoresis equipment:
Mini-Sub Cell GT System, Bio-Rad, Catalog# 1704406

Column Hardware: Econo-Column Chromatography column, 1.5 x 10 cm, BioRad, Catalog# 7371512

Electrophoresis power supply:
PowerPac Basic Power Supply, Bio-Rad Catalog# 1645050

Protein gel electrophoresis equipment:
Mini-PROTEAN 3 Electrophoresis System, Bio Rad, Catalog# 165-3301
Mini-PROTEAN 3 Spacer plates with 1.5 mm spacers, BioRad, Catalog#165-3312

Fluorometry: 50µL Sub-micro Fluorometer Cell Type 16 F with a 10mm path length (Starna Cells cat # 16.50F-Q-10/Z15) and Fluormax-4 fluorescence spectrophotometer (Horiba Irvine, CA)

NanoDrop 2000c spectrophotometer

UV-Vis: 120 µL, 1 cm path length Quartz Cuvette (Science Outlet) along with an HP8452A Dioarray Spectrophotometer with Zenith Data Systems Z-Select 100 XE and HP89532A general scanning software