Purification of CLoG1 from *Physomitrella patens* through cleaving bond of Halo®Tag by PreScission Protease

A Major Qualifying Project

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By

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Physcomitrella patens (*P. patens*) is a eukaryotic species of moss that belongs to the family *Funariaceae*, from the subkingdom of *bryobiotina*. Since this plant performs homologous recombination efficiently, *P. patens* is an important resource to the genetic community. In 2018, a research article was published indicating the first discovery of CLoG1, a gene found in *P. patens*. The focus of our project was to determine if CLoG1 can be purified using Halo®-Link resin to receive a purified product. To break the bond between CLoG1 and the Halo®-Link resin, PreScission protease (PPX) was needed for cleavage. His-PPX and GST-Snc2 were both successfully purified via their respective tags, and gel electrophoresis confirmed these results. Once both were successfully purified, PPX effectively cleaved the bond between GST-Snc2 proving PPX works. After analyzing with a gel and immunoblot, the purification of CLoG1 was inconclusive. Research into the purification of CLoG1 and eGFP through Halo®-Link resin is ongoing.

Introduction

Physcomitrella patens (*P. patens)* is a eukaryotic species of moss that belongs to the family *Funariaceae*, from the subkingdom of *bryobiotina*. This family of plants consists of a pale green color, with larger leaves and red-brown stems to create a "mesh" of layers. *P. patens* specifically can be found around bodies of water in temperate climates and is indigenous to Australia and New Zealand (NZ Flora, 2019).

P. patens was chosen to be the model organism for non-seed plants and as such was the first of its kind to have its genome sequenced. Due to the dominant life cycle phase of *P. patens* being haploid, it makes the plant optimal for research in the genetic field (Cove, 2005). Moss has a large genome, with about 32,275 protein encoding genes, approximately 10,000 more genes than the human genome (Zimmer, 2013). Recombination can be described as the transfer of genetic material within chromosomes during prophase of meiosis 1 (O'Connor, 2008). Homologous recombination focuses around homologous chromosomes that participate in crossing over in order to have a random combination of genetics from the two different parents. *P. patens* performs homologous recombination efficiently when transgenes are integrated to probe the functions of genes. Homologous recombination is important for genetic transfers within plants because this type of recombination essentially results in a "knockout" of a gene to observe phenotypic changes. Since this process is important for genetic transfer, *P. patens* is an important resource to the genetic community (Ostrander, n.d.). Even though the *P. patens* genome has more genes than the human genome, it is considered less complex because it is a model plant. Therefore, when studying genetics it is easier and more cost efficient to test in plants, rather than humans and more specifically using homologous recombination strategies.

Temperature-dependent mutants are used to learn more about the function and growth of a gene. These cells can lose function or the mutations can become lethal at a certain temperature due to these mutants. Lesions can occur once the temperature threshold is reached which can cause damage such as in cell division, synthesis of protein, or disrupting the formation of the cell wall (Hartwell, 1967).

P. patens Conditional Loss of Growth 1 (*CLoG1*) is the gene of focus for this project. In 2018, a research article was published indicating the first discovery of *CLoG1*. This was discovered using the gene "knockout" method and determined that a loss of function occurred when removing the gene from the genome. Upon analysis, it was believed that observed

phenotypes indicated CLoG1 could potentially modulate or participate in microtubule growth, a polymer that provides strength to the cell and is a necessary element for cell division (Ding, 2018). Since this is the only publication about CLoG1, it was important to be able to purify the protein in order to understand its structure and function.

Pollen tubes allow sperm cells to travel to the ovule to begin fertilization (Helper, 2001). Stimuli from surrounding areas provide directional guidance in polarized growth. Within a pollen tip, there are microtubules which are a type of filament. Along with the filaments, there is myosin, kinesins, and dynein which are motor proteins that aid in directional growth. For a plant to grow, the cell walls and cytoskeleton must expand (Khurana, 2016). This is achieved by turgor pressure which provides the force needed for elongation of the pollen tip. CLoG1 has been found within the microtubule cytoskeleton of *P. patens*. The gene is thought to have capabilities that follow and monitor the depolymerization of microtubule ends. (Ding, 2018)

Protein purification can be achieved using multiple strategies. Affinity chromatography is typically used to purify recombinant proteins (Fujii, 2014). This requires an affinity tag system that aids in obtaining the target protein. One of the most common tags is the polyhistidine tag (His-tag). Due to how small the tag is, the tag will typically not change the function or shape of the protein it is purifying. The only downside to this tag is that it is less specific when binding to a resin charged with divalent nickel or cobalt. This makes it difficult to achieve a high purity level when looking for one specific protein (England, 2015). To combat this problem of low specificity, a new tag was created called the Halo®Tag. This tag is much more precise in yielding a pure sample after purification (England, 2015). This method is irreversible once the Halo®Tagged protein interacts with and binds to the Halo®-Link resin in the column. There are several steps such as the flowthrough, wash, and elution step which are needed to obtain a purified sample of the protein (Locatelli-Hoops, 2013). During the elution step, a protease recognizing a specific amino acid sequence comes in to cleave the covalent bond between the tag and the enzyme. In this project, PreScission protease was used due to the Halo®Tag cleavage site having this specific sequence. This allows for a precise cut and ultimately isolation of a pure product.

The object of this project was to successfully purify Halo-CLoG1 from *E. coli*, expressed in both *E. coli* and moss. To accomplish this, the first step is to express and purify His-tagged PreScission protease by IMAC and SEC. The second step is to express and purify GST-Snc2

from *E. coli* by GST affinity chromatography to create a substrate to test the PreScission protease. A PPX assay on Snc2 would be completed and analyzed with an SDS-PAGE gel. From there, Halo-CLoG1 could be purified using the protease made. To test the Halo®Tag with the PreScission protease, the expression and purification of Halo®Tag-GFP from moss would be performed. The design of the project is seen in Figure 1 below with each step explained in this section.

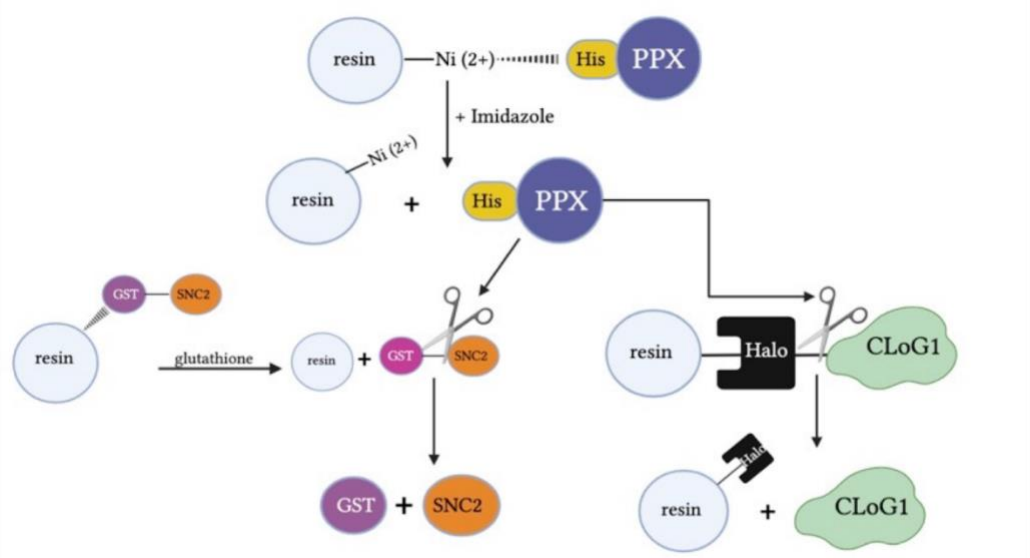

Figure 1. Design and flow of project. The first aspect is purifying PPX so that it can cleave the bonds between GST-SNC2 and Halo-CLoG1.

Materials & Methods

Purification of His-PreScission Protease

An immersion blender was used to lyse 12.5 mL of 1X His/GST Lysis Buffer (Appendix) and His-PPX pellets from 50 mL of IPTG-induced *E. coli* cultures. Once thoroughly lysed, the cell extract was centrifuged for 30 minutes at 4600 RPM to pellet debris. 12.5 uL of DNAse (NEB) was added to the supernatant to digest the chromosomal DNA to reduce viscosity. The supernatant was removed and placed into a clean tube, and 500 uL of crude extract was saved for analysis. 2 mL of 50% resin slurry was prepared by washing with 10 mL of 1X His/GST Lysis Buffer three times. The resin was then added to the crude extract and left to bind by rocking for 30 minutes at room temperature. The supernatant and resin were pipetted into the column and the

flowthrough was collected. The purification column was washed with 10 mL of His/GST Lysis Buffer. 8 mL of Imidazole Elution Buffer (Appendix) was added to the column, and eight 1mL elution fractions were collected.

Purification of GST-Snc2

An immersion blender was used to lyse 12.5 mL of 1X His/GST Lysis Buffer (Appendix) and GST-Snc2 pellets. Once thoroughly mixed, it was centrifuged for 30 minutes at 4600 RPM. The supernatant was removed and placed into a clean tube where 500 uL of crude extract was saved. 12.5 uL of DNAse (NEB) was added to the supernatant to digest the chromosomal DNA to reduce viscosity. 20 mL of 50% resin slurry was prepared by washing with 10 mL of 1X His/GST Lysis Buffer three times. The resin was then added to the crude extract and left to bind by rocking for 30 minutes at room temperature. The supernatant and resin were then pipetted into the column where the flowthrough was collected. The purification column was washed with 10 mL of His/GST Lysis Buffer. GST Elution buffer (Appendix) was made which contained 30.732g of reduced glutathione (10mM) into 10 mL of 1X His/GST Lysis Buffer. The elution was collected in eight 1 mL fractions.

Purification of Halo®-CLoG1

CLoG1 cells were used from *E. coli*. An immersion blender was used to mix 12.5 uL DNAse, 12.5 mL of Halo Lysis Buffer (Appendix) and Halo®-CLoG1 pellets. Once thoroughly mixed, it was centrifuged for 30 minutes at 4600 RPM. The supernatant was removed and placed into a clean tube where 500 uL of crude extract was saved. 20 mL of 50% resin slurry was prepared by washing with 10 mL of Halo Lysis Buffer three times. The resin was added to the crude extract where it was left to bind by shaking for 30 minutes at room temperature.

The supernatant and resin were then pipetted into the column where the flowthrough was collected. The purification column was washed with 10 mL of Halo® Lysis Buffer. The bottom of the column was capped, and 2 mL of Halo® Lysis Buffer mixed with 100 uL of His-PPX was pipetted into the column and left to sit overnight at 4C. The elution was then collected.

Purification of Halo®-eGFP (moss)

0.989 g of moss was added to 1.5 mL of Moss Extraction Buffer (Appendix) and centrifuged at 13.4 RPM at 4C for 10 minutes. After centrifugation, 200 uL of the supernatant was saved as the crude extract and the rest of the supernatant was transferred into a new microcentrifuge tube. 50 uL of Halo®Link resin was added to the microcentrifuge tube and incubated at 4C for 60 minutes on an orbital platform. The tube was then spun at 800 RPM for 1 minute and the supernatant was removed and saved as the flow through. The resin was then washed with 1 mL of Moss Wash Buffer (Appendix) containing Tween and spun again for 1 minute at 800 RPM. The supernatant was removed and saved as wash 1. This was repeated 4 more times for a total of 5 washes. 200 uL of Moss Wash Buffer without Tween and 50 uL of PreScission protease was added to the resin and was left to shake overnight at 4C. The next day, the tube was spun at 800 RPM for 1 minute and the supernatant was collected as the elute.

PPX Efficacy

Five dilutions were made to test the efficacy of PPX. 20 uL of GST + Snc2 was added to each of the five fractions. 0 uL of PPX was added into tube 1, 2 uL into tube 2, 4 uL into tube 3, 10 uL into tube 4, and 20 uL into tube 5. 1X His/GST Lysis Buffer (Appendix) was added into each tube to make the total volume 40 uL. The reactions were then analyzed via SDS-PAGE gel electrophoresis.

Gel Electrophoresis

SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed when analyzing the purifications of 10x polyhistidine tagged PreScission Protease (His-PPX), glutathione transferase tagged yeast Snc2 (GST-Snc2), and Halo® tagged CLoG1 (Halo-CLoG1) purifications. SDS-PAGE was also performed when testing for the efficacy of PPX via digestion of the GST-Snc2 substrate. Precast 4-20% Tris-glycine gradient gels were used under the Mini-PROTEAN TGX brand, with a catalog number of 4561095. 80uL of sample was mixed with 20uL 5X gel loading dye for each gel that was run, and 10 uL was loaded for each sample in the individual wells. 10 uL of protein size marker was used in the gel to compare to the samples (BLUeye Prestained Protein Ladder, Sigma 94964). The gels were run at 150V for ~60 minutes

in 1X standard Tris-glycine Running Buffer with SDS. The gels were stained for total protein with GelCode Blue Safe Protein Stain, Thermofisher catalog number 1860957.

Total Protein Assay

A Bradford assay was performed twice to analyze the purification of PPX, one before spinning down the protein and one after spinning down to pellet any precipitate. The reagent used was the Coomassie Plus Protein Assay Reagent from Thermofisher, catalog number 23238. 50 uL of RODI water was added to the reference wells and 50 uL of the 500 ug/mL of bovine serum albumin (BSA) standard was added to wells B1 and B2. 50 uL of sample was added to their respective wells in B3-B6. 50 uL of each sample was then diluted six times in its respective column in wells C-H. Absorbances were collected via a plate reader at 595nm.

Immunoblotting

CLoG1 immunoblot used a semi-dry method for transfer. The PVDF membrane was soaked in 100% MeOH and placed on top of the gel which had two filter papers underneath. Two more filter papers were placed on top and the cathode electrode plate was placed on top of the stack. The blot was run at 300-500 mA (10V for two hours). 15 uL of Anti-Halo® Tag Monoclonal Antibody was added to 15 mL of blocking solution and left to shake overnight at 4C. Washes were done with PBS-T three times and enough PBS was added to cover the membrane after the washes were completed. The membrane was then placed back on the shaker until ready to image.

GFP immunoblot used a wet method for transfer. The PVDF membrane was activated in 100% MeOH and placed on top of the gel which had two filter papers and a sponge underneath. Two more filter papers were placed on top along with the top sponge and the sides were clamped. The membrane ran at 10V for two hours. Once the transfer was complete, the membrane was washed with PBS + Tween and the added 20 mL of block solution and 4 uL of the secondary antibody (goat anti mouse, HRP conjugate). This shook for 30 minutes and was washed three times with PBS-T.

Results

Purification of His-PreScission Protease

Gels were performed throughout this project to analyze the purifications that were executed. As seen in Figure 2, PPX was successfully purified. The band is seen across elutes 1-6; however, elute 2 showed most of our target protein in relation to other unwanted proteins. The elute that was taken for the PPX purification was elute 2 at ~20 kDa. The crude extract and flowthrough showed many proteins are present in large amounts. In fact, the elutes showed many bands present; however, the protein that was being observed showed the darkest and was used for further analyses.

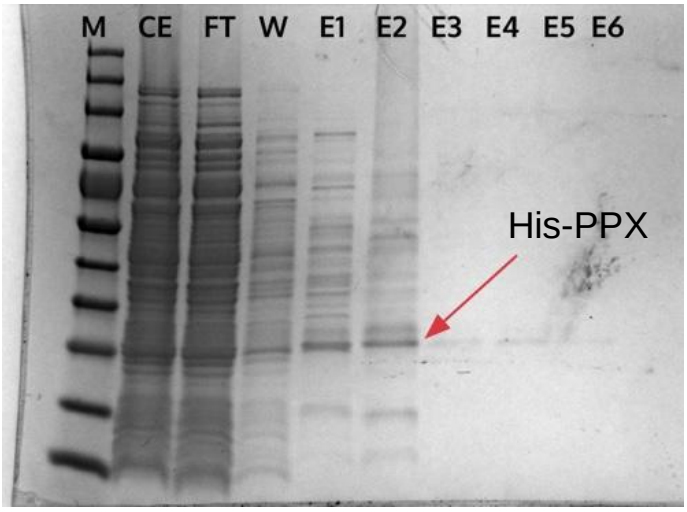

Figure 2. Gel stain for PPX with loading 10uL of sample and 10uL of protein size marker. The red arrow signifies the elute to be believed with the desired protein, His-PPX, in its most purified form (expected size 24.5 kDa).

Two Bradford assays were completed in order to gather quantitative data on the protein concentration. The first assay used the crude extract and elutes 1-3 when the precipitate was still part of the sample. The second assay included the crude extract and elutes 1-3 after centrifugation and taking the supernatant as the sample. Figure 3 displays the line of best fit before centrifugation. The crude extract had either 2.9 or 3.9 mg/mL of protein, E1 had 0.180 mg/mL, E2 had 0.137 mg/mL, and E3 had 0.067 mg/mL.

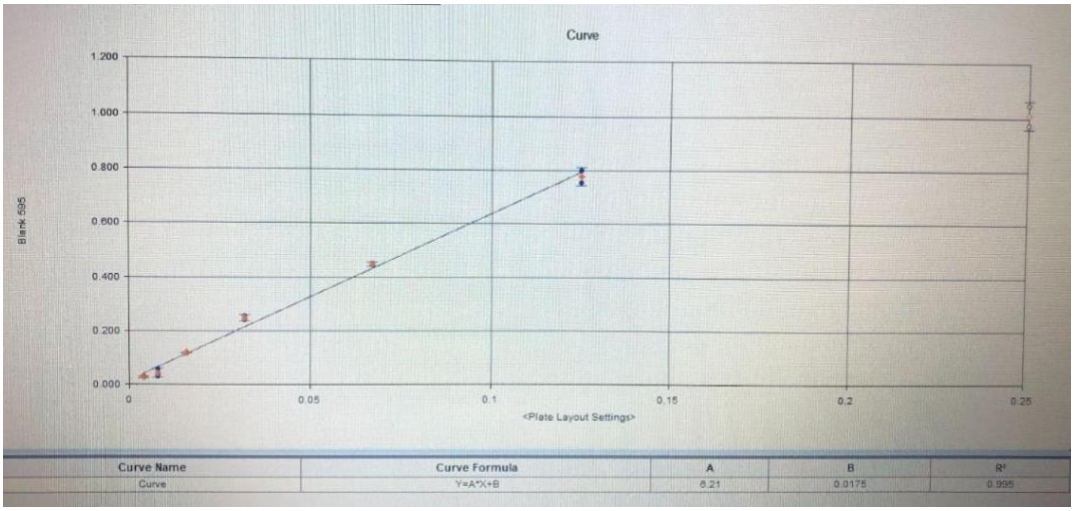

Figure 3. Adjusted Line of Best Fit for Bradford Concentrations Before Removing Precipitate from Centrifugation.

Figure 4 displays the line of best fit before centrifugation. Elution 1 had 0.105 mg/mL of protein, E2 had 0.073 mg/mL, and E3 had 0.018 mg/mL.

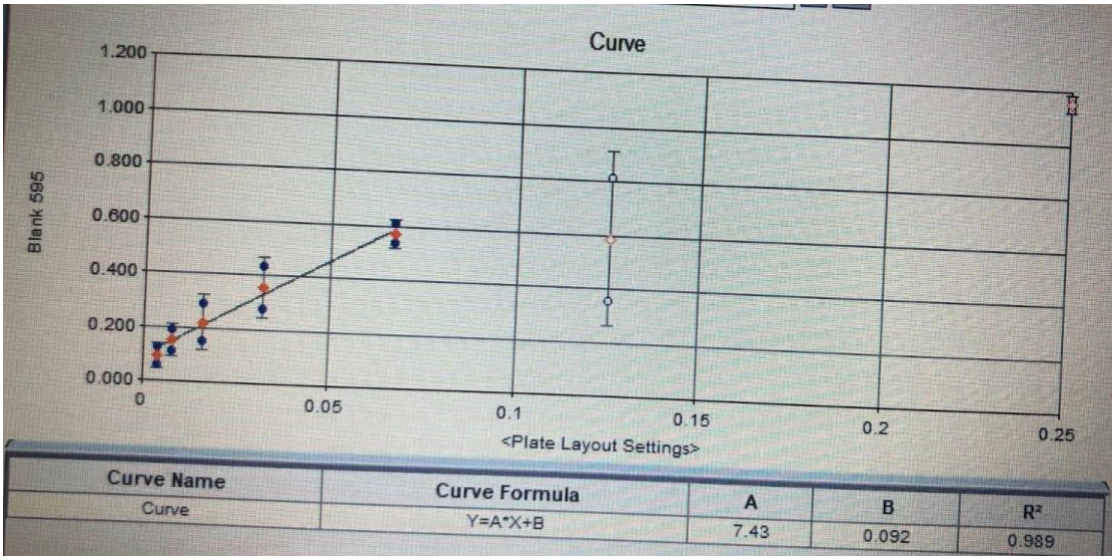

Figure 4. Adjusted Line of Best Fit for Bradford Concentrations After Removing Precipitate from Centrifugation

Once PPX was purified, it was tested for efficacy with GST-Snc2, as seen in Figure 5. The elute that was taken for the SNC_2 purification was elute 6 at ~35 kDa. The band is seen across elutes 2-7 however elute 6 was the most purified before the protein tapered off. Both GST and Snc2 were found in the elutes of the gel. The purest form was elute 6 where the only two prominent bands are the GST and Snc2. PPX successfully cleaved GST from Snc2. It was most prevalent when 20 uL of PPX was used. The expected size of the GST-Snc2 fusion protein was 38 kDa together (GST 25 kDa and Snc2 13 kDa).

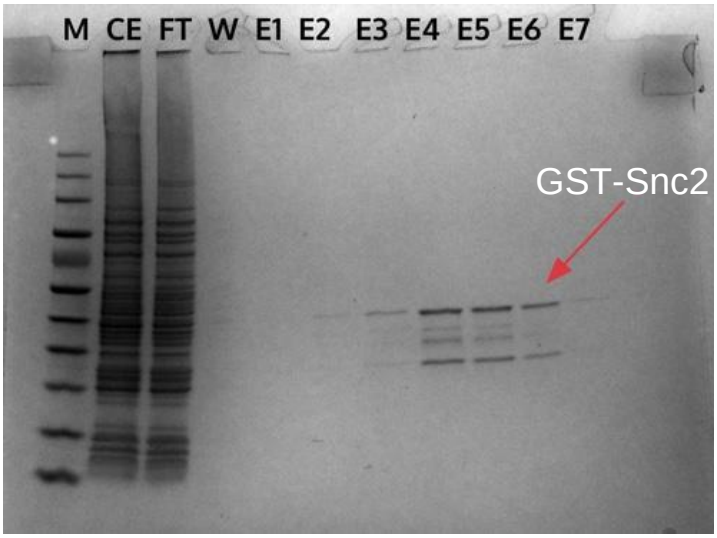

Figure 5. Gel stain for SNC_2 purification gel. The red arrow signifies the elute to be believed with the GST-Snc2 protein in its most purified form.

To cleave the covalent bond between CLoG1 and the Halo®Tag, a protease is needed. For this project, PreScission protease was chosen due to the specific amino acid sequence that lines up with the cleavage site, as seen in Figure 6. The function of PreScission protease was tested before using it on CLoG1 to verify the purified product works correctly.

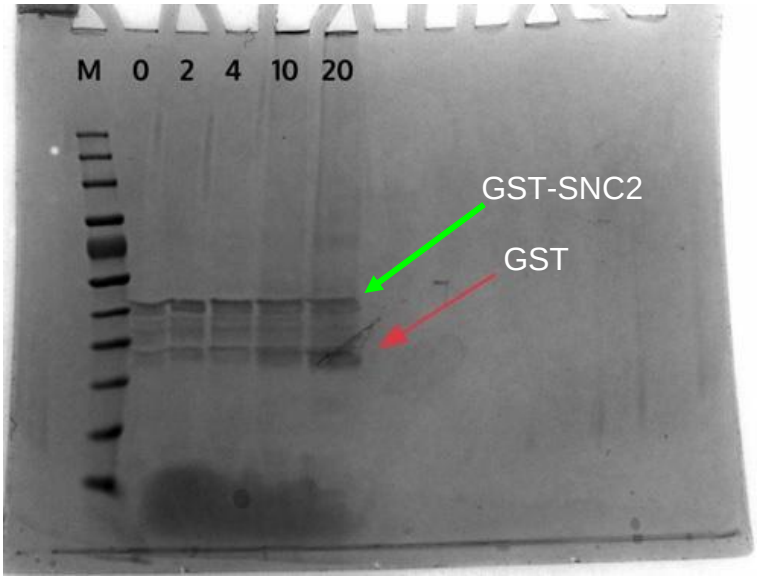

Figure 6. Gel stain for the efficacy of PPX. The green arrow indicates the intact GST-Snc2, and the red arrow indicates one of the digestion products. The labels represent the microliters of the enzyme that was used in each reaction.

Since the purification of GST-SNC2 was confirmed from the gels in Figures 5 and 6, which meant that PPX was functioning, CLoG1 was then attempted to be purified. The gel for this purification is seen in Figure 7. The research objective was to purify Halo-CLoG1 from *E.coli*; however, no band in the elute was noticed on the gel. This is most likely due to the number of cells that were present in the original pelleted culture. An immunoblot was done to see if any specific protein could be found.

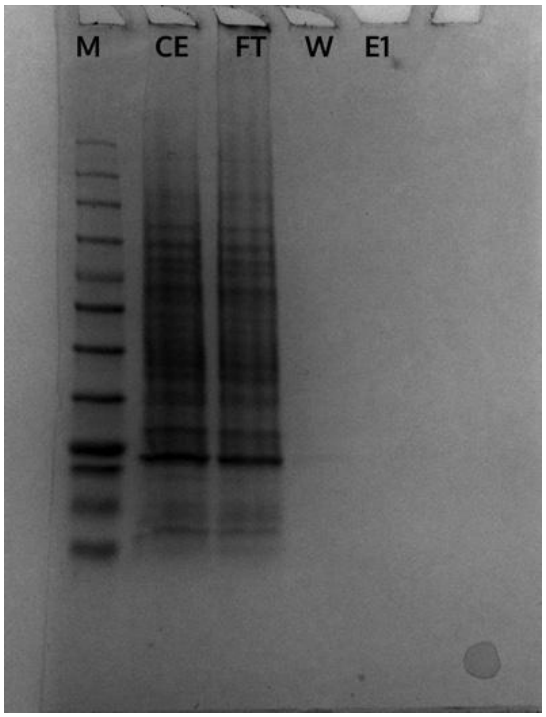

Figure 7. Gel stain for CLoG1 purification gel. Crude extract and flow through contain multiple proteins. The wash appears to have a small amount of protein present, and the elute has no bands. Due to the Halo®Link resin binding tightly and specifically, there are no unwanted bands present in the wash. Other purification wash methods have numerous proteins present because binding to the resin is less specific.

Immunoblots were performed throughout the experiments because they are able to detect specific proteins via antibodies. Figure 8 shows results from the immunoblot for CLoG1 and were deemed as inconclusive due to markers typically being clear after running a western blot. It is unclear whether the band is the marker showing up or if it is the protein. There could have been a possibility that one of the bands in the protein standard was picked up by the antibodies used. This was checked through the manufacturer and was determined to not be the case. Therefore, these results suggest that the band was CLoG1 found in the crude extract.

Figure 8. CLoG1 Immunoblot membrane. A band was found that is predicted to be CLoG1 but cannot be verified completely.

Since CLoG1 could not be successfully purified, a Halo®-tagged green fluorescent protein (eGFP) was attempted to be purified. This was because eGFP is a fluorescent protein, and it can be visually detected as the purification progressed. The eGFP protein was found to be successfully purified on the gel stain detection found in Figure 9. The band on the elute is faint, so a Western blot was performed to confirm that the protein that was purified is eGFP.

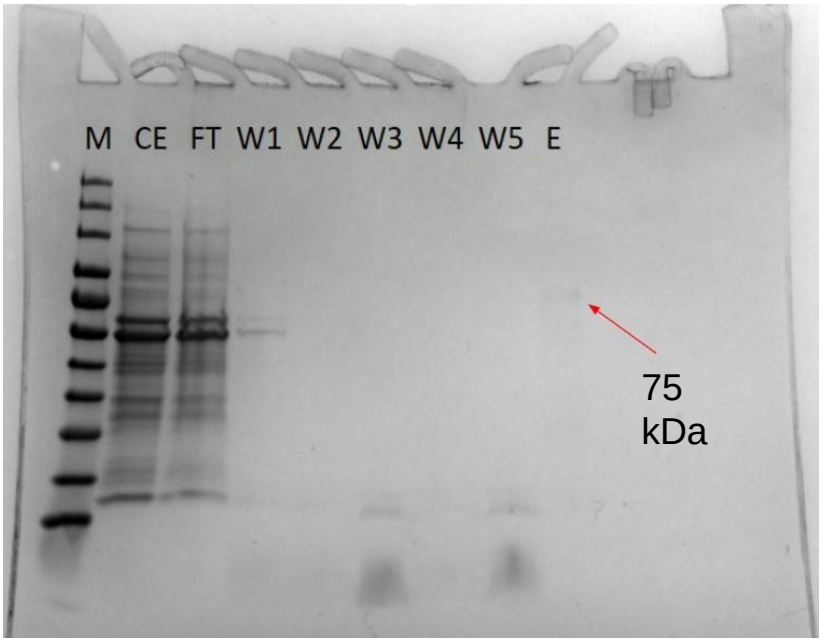

Figure 9. Gel analysis of eGFP purification. The molecular weight of the elute was 75 kDa. The expected elute size should have been 27 kDa, so the band appears not to be eGFP.

Figure 10 shows a western blot was also performed on the GFP gel, but there were no bands present. This could have been due to an error within the protocol or an issue with the antibodies used.

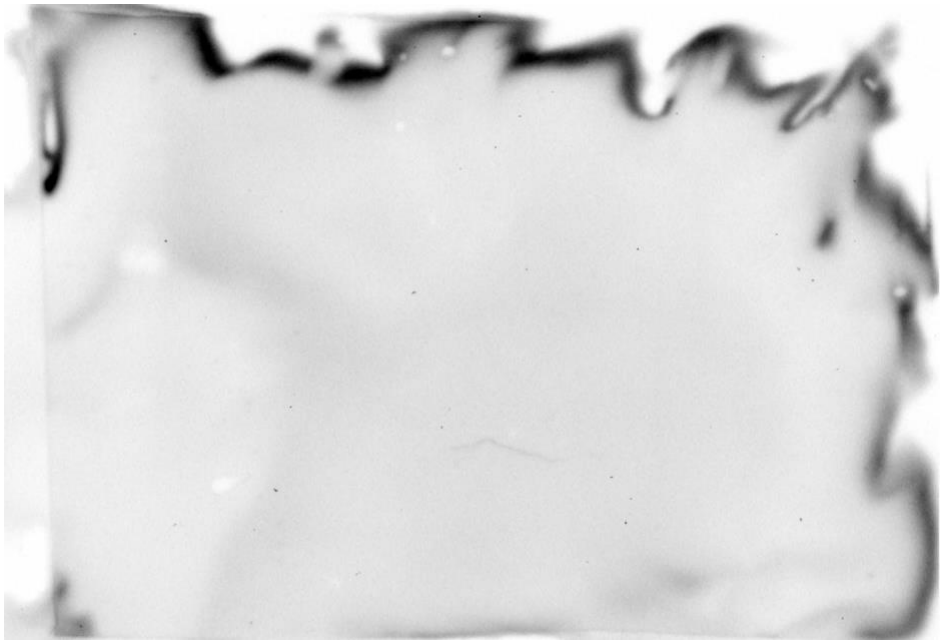

Figure 10. Immunoblot of eGFP. There are no bands evident.

Discussion

The overall research objective of this project was to be able to purify CLoG1 via PreScission Protease (PPX) to further explore and understand the protein's structure and function. To successfully purify CLoG1, a protease is needed to cleave the bond between the Halo®Tag and the protein. PPX was also used to cleave the bond of GST and Snc2 to verify the efficiency of PPX before using it on CLoG1. eGFP was used to test binding via the Halo®Tag and efficacy of PPX on a Halo®Tag to verify the sequences lined up correctly during cleavage. Overall, our hypothesis was not supported since there was no verification that CLoG1 was successfully purified via gel. GFP had mixed results with a band being present in the gel but not in the immunoblot. Due to the project only being completed over one semester, the research objectives have not been met. Further research could continue to attempt to successfully meet these objectives.

The design and flow of the experiments are seen in Figure 1. The first step refers to the purification of PPX where a band was found in the SDS-PAGE gel during analysis which confirms our hypothesis. The next step required a purified product of PPX to cleave the bond between the Halo®Tag and CLoG1. Before using the PPX as an elute during the purification of CLoG1, a less expensive purification was used to determine if the purified PPX cleaves at the correct site. To test this, Figure 1 demonstrates how GST tagged Snc2 was used as the control to verify the efficacy of the protease. The gel showed two distinct bands proving the hypothesis that the PreScission protease cleaves the bond between the tag and the enzyme successfully. Since both the purification and efficacy tests were successful, there would not be a need to change any variables in future research.

For the specific step for the purification of CLoG1 from *E. coli*, the hypothesis was inconclusive. The gel did not show evidence of elution of the target protein, so an immunoblot was performed to specifically detect the target protein. The immunoblot had a small band which could have been the protein of interest; however, it could not be verified since the protein marker could not be properly lined up with the band. Therefore, there was no verification of the purification of CLoG1. Future changes within this step would be to increase the yield of cells present when purifying the sample. It could be very possible that there were not enough *E. coli* cells present or the expression level was too low, which led to a lack of bands on the gel and only a faint band on the immunoblot. Another change could be creating an antibody that is specific for

CLoG1. This would allow for the protein to show up more visibly on the immunoblot which would lead to verification of the purification. However, this suggestion could be expensive and possibly not practical in the short term.

The purification of eGFP was inconclusive because a faint band was present in the SDS-PAGE gel during analysis; however, the expected size of eGFP was 27 kDa, but the size of the band that was obtained was 75 kDa. Therefore, the hypothesis was neither supported nor unsupported that the PreScission protease successfully cleaved the bond between a Halo®Tag and eGFP. In the future, using more moss or resin may be beneficial to obtain a more accurate band size. Since the band was faint, a western blot was performed to determine that the band present was the specific protein, eGFP. There was no band present in the immunoblot. This could be due to human error, or the antibodies used could have not been able to detect eGFP. For future research, a specific antibody could be obtained to guarantee a clear band would appear if eGFP had been effectively purified.

To continue this research, a microtubule stabilization assay would be the next step to further determine the function of CLoG1. This was originally an objective of this project, but a fully pure form of CLoG1 is needed to complete the microtubule assay. Another test would be to do a pulldown with Halo®-tagged CLoG1 to identify putative binding partners.

Overall, the results showed there are more factors and variables that need to be tested to attain a fully pure form of CLoG1. Once that is achieved, further tests can be run to continue to bring light to the structure and function of CLoG1 in moss.

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Appendix

- 2X His/GST Lysis Buffer:
    - Na phosphate: 50mL (100mM)
    - NaCl: 15mL (300mM)
    - DTT: 200uL (2mM)
    - 34.8mL of H2O
- 1X His/GST Lysis Buffer:
    - 50mL of 2X His/GST Lysis Buffer
    - 50mL of H2O
- Imidazole Elution Buffer: Stock: 2M; Final: 0.25M
    - Final recipe:
        - 5mL 1X Lysis Buffer
        - 1.25mL of imidazole
        - 3.75mL of H2O
- GST Elution Buffer: 30.732g of glutathione reduced into 10 mL of 1X Buffer. Try to do this at the last possible minute before starting the column.
    - Measured 0.034 mg of glutathione and added it to a 15 mL conical tube. Added 10 mL of 1X Buffer right after the wash and right before starting the elution.
- Halo Lysis Buffer:
    - 100 mM TRIS pH 7.9
    - 150 nM NaCl
    - 0.05% IGEPAL of 50 mL
    - 400 uL of PMSF
    - 40 uL of DTT

	Stock	Lysis Buffer	Volume
TRIS:	1.0M	0.1M	5mL
NaCl:	2.0M	0.15M	3.75mL
IGEPAL:		0.05%	25µL

- Moss Wash Buffer - NO TWEEN
    - 2.5 mL HEPES, pH 7.6 (Final 50 mM)
    - 5 mL KCl (Final 100 mM)
    - 42 mL RO H2O
- Moss Extraction Buffer
    - 50 mL of the above Wash Buffer – NO TWEEN
    - 500 uL of protease inhibitors (Viali Lab uses Roche tablets made at 50x; MQP used Halt Protease Inhibitor Cocktail from ThermoScientific 87786 which is a 100x liquid)
        - Separated 10 mL out to add Tween to 0.05%