Optimizing Expression and Purification of HaTRPA1

Wilhelm Hansson Wennersten

Degree Project in Biochemistry, 2024
Department of Chemistry
Lund University
Sweden

MSc, 60 hp

LUND
UNIVERSITY

Optimizing Expression and Purification of HaTRPA1

Wilhelm Hansson Wennersten

Degree Project in Biochemistry
2024
MSc, 60 hp

Supervisor:
Urban Johanson
Division of Biochemistry
and Structural Biology
Lund University
Sweden

Examiner:
Susanna Horsefield
Division of Biochemistry
and Structural Biology
Lund University
Sweden

Lund University
Department of Chemistry
Centre for Biochemistry and Structural Biology
P.O. Box 124
SE-221 00 Lund, Sweden

Popular Science Description

Have you ever put too much wasabi on a piece of sushi? The painful sensation you feel that causes instant regret is generated by the so-called Transient Receptor Potential Channel, subfamily A, member 1 (TRPA1). The protein TRPA1 plays an essential role in detecting irritants and noxious substances, like our dear friend wasabi. While wasabi may be harmless, there are many substances that can be highly damaging, and our bodies need a way to tell us to stay away from them. Therefore, once TRPA1 is activated by a noxious substance, a signal is sent to our nervous system to generate a painful sensation. While human TRPA1 has been the subject of many studies, it is also worth studying TRPA1 in other organisms.

Hylobius abietis, or the pine weevil, is a major pest in the commercial forest industry. It eats the bark of seedlings causing them to die. The pine weevil is responsible for millions of euros in damages each year. Currently, the fight against the pine weevil has involved plenty of harmful insecticides, and with a greater focus on environmentally friendly forestry, there is an interest in other alternatives. If the 3D structure of *H. abietis* TRPA1 was resolved, it would be possible to develop an activator of TRPA1 that would trigger a similar response in the pine weevil as our overindulgence in wasabi caused.

This study attempted different conditions and protocols to produce a substantial amount of TRPA1 from the pine weevil for structural studies. Two different constructs were studied: the full-length construct and a truncated version. Both constructs of the protein were tagged with a green fluorescent protein to enable tracking of the protein throughout the purification and expression. The protein was expressed in the yeast species *Pichia pastoris*. Afterwards, the protein was solubilized and purified under different conditions to find the optimal protocol.

Abstract

TRPA1, also known as the wasabi receptor, is a non-selective cation ion channel that plays an essential role in the detection of noxious substances and irritants. *Hylobius abietis*, the pine weevil, is a pest that causes substantial damage to the forestry industry. The structure of the pine weevils TRPA1 (HaTRPA1) is so far unknown. If the structure were to be determined, an agonist could be developed, which could be used as a repellent against the pine weevil. Membrane proteins are naturally expressed at lower levels and can be highly unstable in certain conditions. Heterologous expression and different protocols must often be attempted and evaluated to produce a high-yield, stable membrane protein sample. The aim of this study is to optimize and evaluate different protocols to express and purify HaTRPA1, including using a truncated version of HaTRPA1 and a cleavable green fluorescent protein tag.

HaTRPA1 was expressed through large-scale fermentation in *Pichia pastoris*. The cells were broken, and the membrane was isolated and washed. The protein was solubilized using Fos-choline 14, purified using Immobilized metal ion affinity chromatography (IMAC), cleavage with the tobacco etch virus protease, reverse IMAC, and separated using size exclusion chromatography. Steps throughout the expression and purification were evaluated using SDS-PAGE. The protocols were attempted for two constructs of HaTRPA1, the full-length construct and a truncated version labelled C2. The results showed that degradation was the major issue with the expression and purification of HaTRPA1. Both the full-length construct and the truncated C2 showed substantial degradation. A decrease in degradation was observed when the induction time during expression was reduced. Implementing a urea wash in the membrane preparation was beneficial in reducing the amount of degradation.

The conclusion of this paper was that there is little difference in stability between the two constructs of HaTRPA1. To prevent degradation, a shorter induction time should be implemented during fermentation.

Keywords: Degradation | Fermentation | GFP | TRPA1 |

Acknowledgment

I would like to thank my supervisor, Urban Johanson, for the opportunity to work in his lab and for the guidance and knowledge he has bestowed upon me. I also extend my gratitude to my co-supervisor, Balder Werin, for teaching me the techniques and methods used in this report. I am also thankful for the assistance and cooperation of Erikas Blazukas, who has been a great lab partner. Lastly, I would like to thank Susanna Horsefield for taking on the task of being my examiner.

Table of Contents

1 List of abbreviations

CMC	Critical Micelle Concentration
Cryo-EM	Cryogenic electron microscopy
CV	Column Volume
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced Green Fluorescent Protein
EGTA	Triethylene glycol diamine tetraacetic acid
FPLC	Fast Protein Liquid Chromatography
FL	Full Length
Ha	*Hylobius abietis*
HEPES	2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid
His	Histidine
IMAC	Immobilized Metal Ion Affinity Chromatography
MQ	MilliQ
Ni-NTA	Nickel-nitrilotriacetic Acid
NGC	Next-generation Chromatography
PBS	Phosphate-buffered Saline
PMSF	Phenylmethylsulfonyl Fluoride
PTM	Pichia Trace Metal
cOmp	cOmplete Protease Inhibitor Cocktail Tablets (Roche)

SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
TBS	Tris-buffered Saline
TEV	Tobacco Etch Virus
TRP	Transient Receptor Potential channel
TRPA1	Transient Receptor Potential channel, Subfamily A, Member 1
YPD	Yeast extract Peptone Dextrose

2 Introduction

2.1 Ion Channels

Ion channels are permeable membrane proteins that regulate the flow of ions across the membrane of the cell. They can be classified in many ways, for example, by their method of regulation, which ions they facilitate the transport of, or their structure. Their functions include establishing a resting membrane potential, regulating cell volume, and shaping electrical signals by controlling the flow of ions [1]. Like most membrane proteins, ion channels are naturally expressed at lower levels. These limitations have made it more difficult to conduct structural studies on ion channels since achieving a high yield of intact protein has proved challenging [2]. Therefore, evaluating a range of different protocols for expression and purification has been necessary.

2.2 TRP-family

Transient receptor potential channels (TRP) are a group of membrane proteins that facilitate the signalling of a range of physical and chemical stimuli. They are permeable to a range of different cations, including sodium, calcium, and magnesium. They generally share the same fold, containing six membrane-spanning helices with transmembrane helices 5 and 6 forming the channel pore [2]. The first TRP was discovered from a *Drosophila* mutant in 1969 [3]. Disruption in the gene for this TRP led to a transient receptor potential in the organism's photoreceptor, hence the name TRP. Since then, there has been a great pharmacological interest in the human TRP family members due to their role in inflammation and pain reception [4].

2.3 TRPA1

Transient receptor potential channel, subfamily A, member 1 (TRPA1), is a calcium ion-permeable channel that senses irritants and noxious substances. The subfamily A of the superfamily TRP is characterized by the ankyrin repeats present at the N-terminal, which appears to play some role in thermosensation [5][6]. However, the thermosensation of TRPA1

remains a controversial topic as it appears to be species-specific. TRPA1 is present in many different cells, including pain-detecting nerve cells, where TRPA1 regulates the flow of Ca^{2+} ions across the plasma membrane. TRPA1 is a homotetrameric ion channel with the membrane-spanning helices S5-S6 forming the channel pore. Some irritants that act as agonists for TRPA1 are electrophiles, which bind through covalent modification of cysteine residues in the cytoplasm in what is known as the coupling domain [7] [8]. However, studies suggest that TRPA1 is also activated by reactive oxygen species present during inflammation [9]. This has led to some interest in TRPA1 for potential treatment of conditions such as arthritis. At the C-terminal of TRPA1 is a calcium-binding site that can regulate the gating of the channel pore [10].

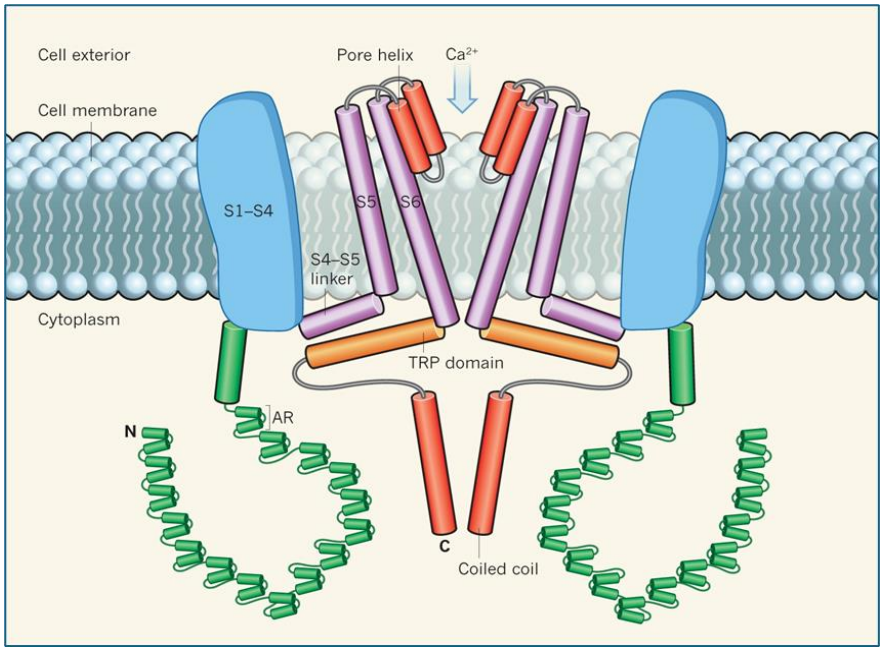


Figure 1: TRPA1 is shown as a dimer and displays the main features of the protein, including the S5-S6 channel pore and the 16 ankyrin repeats [11].

In this study, TRPA1 of *Hylobius abietis* (HaTRPA1) was the protein of interest. *H. abietis* (Pine weevil) is a major pest for commercial forestry, leading to millions of euros in damage each year [12]. Pine weevil causes this damage by eating the bark of newly planted seedlings, often causing what is known as ringbarking, where the bark is removed in a circular manner, leading to nutrients not being able to be transported between the roots and the foliage, causing inevitable death [12]. These damages have been mitigated using insecticides in the past, but in

2023, the last of the insecticides for pine weevil control were banned, only allowing protective coatings [13]. Therefore, investigating the structure of HaTRPA1 is of great interest in presenting an alternative to these pesticides. If the structure of HaTRPA1 is determined, it would be possible to develop a potent agonist that could be used to treat conifer seedlings and prevent further damage.

This study continued the work conducted by Robin Olsson and Oliwia Kolodziejczyk. Robin Olsson worked on making a construct of the HaTRPA1 gene with the pPICZA-eGFP plasmid [14]. Two constructs were created: one with HaTRPA1 FL opt_pPICZB to pPICZA-eGFP and the other with HaTRPA1 C2 opt_pPICZB to PICZA-eGFP. These constructs differ from the previous ones in that the enhanced green fluorescent protein tag is introduced. The new constructs will be referred to as the full-length construct, FL, and the truncated construct, C2. The truncated construct is mainly truncated at the ankyrin repeats region (see sequences in Appendix 8.1). The truncated construct was developed in the hopes that it would prove to be more stable and easier to express and purify in large amounts, as was shown for the human TRPA1 [15].

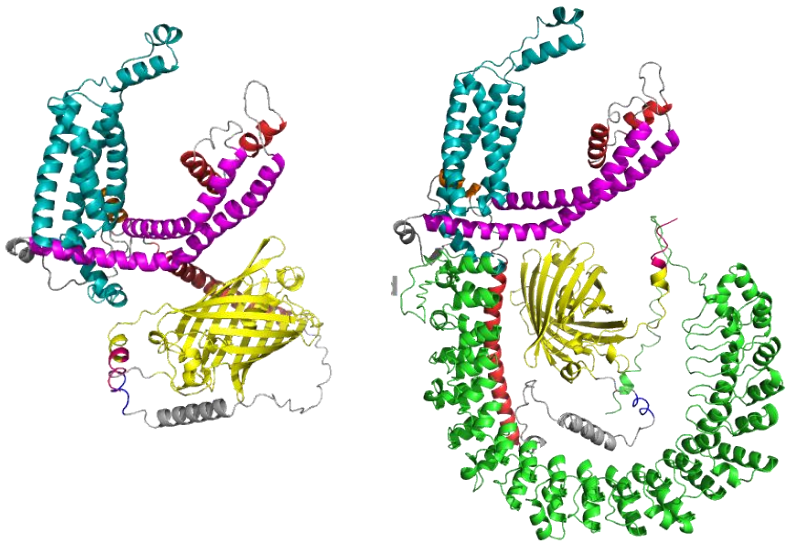


Figure 2: AlphaFold monomeric models of the Full-length HaTRPA1-eGFP (right) and the truncated C2 HaTRPA1-eGFP (left) using the sequences presented in Appendix 8.1 and presented with the same colour scheme as in Figure 1. The Ankyrin repeats in green, S1-S4 in teal, S5-S6 in magenta, TRP-domain in orange, coiled coil and pore helix in red, TEV-site in blue, GFP in yellow, and His-Tag in pink.

Oliwia Kolodziejczyk's work included finding the best clones for expressing the two HaTRPA1-eGFP constructs. These clones were used in this study to express and purify HaTRPA1. Her work also included detergent screens, which led to the conclusion that Fos-choline 14 was probably the best for the solubilization of HaTRPA1, which was used in this study.

2.4 GFP

Green fluorescent protein (GFP) is a fluorescent protein that is found naturally in the *Aequorea* genus. Shimomura et al. first discovered GFP as a companion protein to aequorin [16]. It emits green fluorescence when exposed to light in the blue to violet range, with an excitation peak at 395 nm and an emission peak at 509 nm [17]. It has seen broad usage as a marker protein, commonly fused to the C-terminus of membrane proteins, which allows for quantifying expression through fluorescence measurements. It also allows for tracking of the protein throughout purification by fluorescence scans of SDS-PAGES. The GFP construct utilized in this study is the so-called enhanced green fluorescent protein (eGFP). This mutant of GFP is characterized by an increased brightness in its fluorescence, which is highly useful when working with smaller amounts of protein [18].

2.5 Experimental Methods

2.5.1 IMAC

Immobilized metal ion affinity chromatography (IMAC) is a common method for purifying proteins. The technique is dependent on the protein's histidine content [19]. In many cases, a His-tag is used; however, it is possible to purify native proteins using IMAC, depending on their histidine content. The purification method is based on the interaction between histidine

and metal ions such as Ni^{2+}+, Cu^{2+}, and Zn^{2+}. Nickel IMAC uses Ni^{2+}, which binds to recombinant proteins with polyhistidine tags, with the most common histidine tag being the hexahistidine tag. Immobilized nickel resins are produced through the interaction of a chelator with the metal ions to form a coordination compound [20]. One of the most common chelators is nitrilotriacetic acid (Ni-NTA). Electrostatic interaction of the nickel beads can lead to unspecified binding, which is usually prevented by having NaCl present in the protein buffer and adding imidazole to prevent weak protein binding. In a similar manner, a wash buffer is usually applied to the Ni-NTA column to remove weakly bound proteins before eluting the protein of interest with a buffer of higher imidazole content.

2.5.3 Reverse IMAC

Reverse IMAC is a method based on traditional IMAC for removing a specific protein. The tobacco etch virus protease (TEV) is a protease which specifically recognizes the amino acid sequence ENLYFQG/S and cleaves the protein between the amino acids Q and G/S [21]. The constructs used in this study are equipped with a TEV cleavage site just after the eGFP and His-tag (see Appendix 8.1 for specific placement of TEV cleavage site). The TEV cleavage site makes it possible to remove GFP and the His-tag before the final purification step. To remove the His-tagged GFP reverse IMAC can be utilized. After cleavage with TEV, the solution can be incubated with Ni-NTA agarose to allow for binding of the His-tagged GFP. A standard IMAC procedure is then conducted, but instead of the elution being of interest it is the flow-through. The cleaved protein of interest will not bind to the column and therefore be collected in the flow-through and after the GFP and any uncleaved protein can be collected with the elution.

2.5.4 SDS-PAGE

Within biochemistry, one of the most common methods for protein analysis is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [22]. By denaturing proteins using anionic detergent, which also binds to them, all negative charges are given proportionally to their mass. Based on their mass, the proteins are then separated through electrophoresis across a polyacrylamide gel matrix.

2.5.5 SEC

Size exclusion chromatography (SEC) is a purification and analysis technique that separates proteins based on their size while retaining the structure of the protein complex [23]. Proteins are separated through a column of gel beads with pores for a specific size distribution. Molecules elute at different volumes depending on whether they enter these pores. Smaller proteins and molecules enter the pores and are slowed down, while larger particles cannot and pass more quickly through the matrix. To prevent proteins from separating on any factor besides size, it is imperative that a buffer with neutral pH and intermediate ionic strength is used.

3 Materials and Methods

3.1 Fermentation

Cells from a glycerol stock containing the relevant clone were streaked out on a YPD agar plate a few days before the start of the fermentation and stored at 30℃. An overnight culture with 100 mL YPD medium was started using the cells from the YPD agar plate. Fermentation was conducted in a 3 L bioreactor vessel containing 1.5 L basal salt medium (7 mM $CaSO_4$, 0.1 M K_2SO_4, 60 mM $MgSO_4$ x7 H_2O, 74 mM KOH, 4% glycerol, 2.3% H_3PO_4) supplemented with PTM trace metal salt (24 mM $CuSO_4$, 0.5 mM NaI, 18 mM $MnSO_4$ × H_2O, 0.8 mM Na_2MoO_4×2 H_2O, 0.3 mM H_3BO_4, 2 mM $CoCl_2$, 150 mM $ZnCl_2$, 234 mM $FeSO_4$×7 H_2O, 0.8 mM Biotin, 0.4% H_2SO_4). The bioreactor containing the basal salt medium was autoclaved, and the culture was inoculated with the overnight culture. Once the glycerol in the basal salt medium had been consumed, a feed of 50% glycerol with PTM was started. For the 20 and 45-hour inductions, the culture was fed 200 ml of 50% glycerol, while the 13-hour induction was fed around 300 ml of 50% glycerol. The culture was induced with a 100% methanol feed supplemented with PTM. Different induction timespans were conducted: 13 hours, 20 hours, and 45 hours. Throughout the entire fermentation, samples were taken to measure the O.D. 600. The temperature was kept at 30℃, the pH at five, and the D.O. at around 20-30% throughout the fermentation. The cells were harvested by centrifugation for 30 minutes at 6000 rpm (6900 rcf) in a JLA 8.1000 rotor. The cells were aliquoted into 100 g pieces and stored at -80℃.

3.2 Membrane Preparation

Approximately 100 g of cells were thawed in 180 mL breaking buffer (50 mM NaH_2PO_4, 1 mM EDTA, 5% Glycerol, 1 mM PMSF, pH 7.4). Leupeptin and pepstatin were also added to the breaking buffer in later attempts. Cells were then lysed in an ice-cold bead beater for 12 minutes with a 30-second cooldown pause for every 30 seconds it was active (24 minutes in total) in a cold room. The cell suspension was transferred from the bead beater vessel to JA 25.50 centrifuge tubes. Unbroken cells and cell debris were pelleted by centrifugation in JA 25.50 rotor at 9.500 rpm (7.386 rcf) for 30 minutes at 4℃. The supernatant was transferred to

Ti-45 tubes and centrifuged for 1 hour at 4℃ in a Ti-45 rotor at 45,000 rpm (158.024 rcf). A 1 ml sample, labelled supernatant 1, was taken from the supernatant and stored at -20℃ before the supernatant was discarded. The pellets were resuspended in 5 ml Buffer A (20 mM HEPES, 0.5 M NaCl, 10% Glycerol, 2 mM β-mercaptoethanol, 1 mM PMSF, pH 7.8) using a potter homogenizer. Likewise, leupeptin and pepstatin were added to buffer A in subsequent attempts. A 100 µl sample of the resuspended pellet, labelled pellet 1, was saved and stored at -20℃. The homogenized pellets were transferred to Ti-45 tubes filled with ice-cold urea (4 M Urea, 5 mM Tris base, 2 mM EDTA, 2 mM EGTA, pH 9.4) and incubated on ice for 20 minutes. The urea pellet wash was centrifuged at 4℃ in a Ti-45 rotor for 1.5 hours at 45,000 rpm. 1 ml of the supernatant, labelled supernatant 2, was saved and stored at -20℃. The pellet was resuspended with Buffer A in the same manner as described before. A 100 µl sample of the resuspended pellet, labelled pellet 2, was saved and stored at -20℃. The homogenized pellets were transferred to Ti-45 tubes and filled with buffer A. The buffer A pellet wash was centrifuged for 1.5 hours at 45,000 rpm in a Ti-45 rotor at 4℃. 1 ml of the supernatant, labelled supernatant 3, was saved and stored at -20℃. The supernatant was discarded, the pellet resuspended in 2 ml buffer A per gram membrane, and snap frozen in liquid nitrogen. A 100 µl sample of the resuspended pellet, labelled pellet 3, was saved and stored at -20℃. All membrane preps were stored at -80℃. The membrane prep was also attempted without the urea wash, and the membrane was directly washed with buffer A.

3.3 Solubilization and Purification

3.3.1 Solubilization

A 100 mL Buffer A solution supplemented with two EDTA-free cOmplete tablets, 2 mM B-mercaptoethanol, and 1 mM PMSF was prepared. An aliquoted membrane prep was mixed with the buffer A solution to create a 35 mL membrane solution. 0.7 g of Fos-choline 14 was dissolved in 35 mL of the buffer A solution to create a 2% Fos-choline 14 solution. The Fos-choline 14 solution was added dropwise to the membrane solution and incubated for 3 hours at room temperature. The 1% Fos-choline 14 membrane solution was transferred to Ti-45 tubes and centrifuged for 30 minutes in a Ti-45 rotor at 30,000 rpm.

3.3.2 IMAC

A column was filled with 1 ml Ni-NTA agarose bed solution, and the ethanol was removed through gravity flow. The Ni-agarose resin was equilibrated by allowing 10 ml of buffer B (20 mM HEPES, 0.3 M NaCl, 10% glycerol, 2 mM β-mercaptoethanol, 1 mM PMSF, pH 7.8, 3xCMC Fos-choline 14) to pass through by gravity flow. The supernatant from the centrifugation was incubated with the equilibrated Ni-agarose overnight in the cold room. 10 mM imidazole was added to prevent unspecified binding. The next day, the column was packed with the Ni-agarose resin solubilization solution. The flow-through was saved and the column was washed with 10 ml of buffer B with 30 mM imidazole. To elute the protein, 1 ml of buffer A with 300 mM imidazole was added and incubated for 5 minutes. This step was repeated six times, resulting in six elution fractions, 1 ml each. For each IMAC fraction, a 100 µl sample was saved for SDS-PAGE analysis. The protein concentration of each fraction was measured using nanodrop, and the relevant fractions were pooled for buffer exchange. The fluorescence throughout the purification could be observed using a blue light table which helped keep track of the protein.

3.3.3 Buffer exchange and TEV cleavage

A PD-10 column was equilibrated with 25 ml of TEV buffer (25 mM Tris, 200 mM NaCl, 2 mM EDTA, 2 mM β-mercaptoethanol, 3×GDN, pH 8.0) before adding 2.5 ml of the relevant IMAC fractions. The flow-through was discarded, and 3.5 ml of TEV buffer was added to the column. The eluate was collected, and the protein concentration was measured using nanodrop. TEV stock (1.4 mg/ml) was added to the eluate, so there was a 1:70 mass ratio of TEV:HaTRPA1 and incubated in the cold room overnight. A PD-10 column was equilibrated the next day with 25 ml TEV removal TBS buffer (20 mM Tris, 150 mM NaCl, 3xCMC GDN, pH 7.5). 2.5 ml of the TEV product was added, and the flow-through was discarded. 3.5 ml of TEV removal buffer was added, and the eluate was collected. The protein concentration was measured using nanodrop. The extinction coefficients used for the different constructs were HaTRPA1-FL-eGFP: 127 115 $M^{-1}cm^{-1}$, HaTRPA1-C2-eGFP: 84 690 $M^{-1}cm^{-1}$, HaTRPA1-FL (cleaved): 103 600 $M^{-1}cm^{-1}$

3.3.4 Rev IMAC

A Ni-NTA column with a bed volume of 1 ml was prepared, and the ethanol was removed through gravity flow. The nickel agarose was equilibrated by adding 10 ml of TBS buffer. The nickel agarose was transferred to the falcon tube containing the TEV removal eluate and incubated in the cold room for 1 hour with slight agitation. The column was packed with the incubated solution, and the flow-through was collected. The protein bound to the column was eluted with 5 ml of TBS buffer containing 300 mM imidazole. the protein concentration of the flow-through and the eluate was measured with nanodrop. The flow-through was concentrated to approximately 600 µl using a Vivaspin 6 100 kDa MWCO concentrator, and the protein concentration was measured with nanodrop. The concentrated sample was filtrated using a spin-x 0.45 µm.

3.3.5 SEC

SEC was carried out using an FPLC-NGC system and a Superose 6 column 300/10 with a column volume of 23.56 ml. The column was equilibrated with 1.5 CVs of degassed, sterile filtrated SEC buffer (20 mM Tris, 150 mM NaCl, pH 7.5, 3×CMC GDN). Approximately 500 µl of the concentrated sample was injected using a 1 ml loop. The protein was eluted in fractions of 500 µl. Relevant fractions were analyzed with SDS-PAGE, and the protein concentration was measured using nanodrop.

3.3.6 SDS-PAGE

Samples for SDS-PAGE were prepared by mixing 20 µl of the protein sample of interest with 10 µl SDS-loading buffer (0.375 M Tris, 200 mM SDS, 30% glycerol, pH 6.8, 0.1% bromophenol blue, 7.5 mM β-mercaptoethanol). The samples were incubated for approximately 20 minutes at room temperature before being loaded onto a 10% precast polyacrylamide gel (MiniPROTEAN TGX, Bio-Rad). The gel vessel was filled with running buffer (25 mM Tris, 0.19 mM glycine, 3.5 mM SDS, pH 8.3) and ran for 5-10 minutes at 100 V before the voltage was increased to 200 V. Fluorescent images were taken of the gels on a ChemiDoc Imaging System (BioRad, Hercules California USA) before they were stained using Coomassie.

4 Results and Discussions

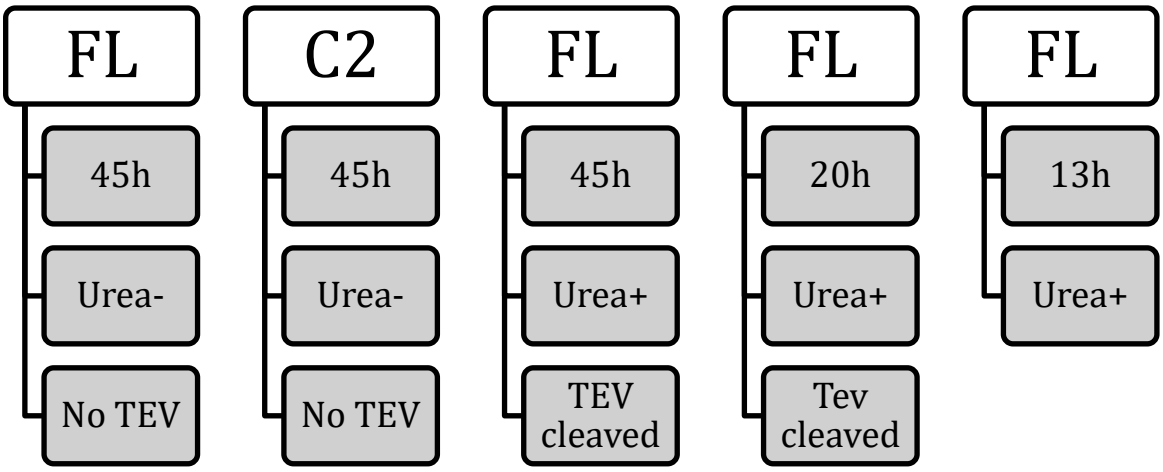


Figure 3: Schematic showing the properties of the different samples which are compared in this study. First showing if the sample was the full-length (FL) or the truncated construct (C2), second showing how many hours the induction was, third whether urea wash was implemented in the membrane prep, lastly whether the eGFP tag was cleaved with TEV or if SEC was run directly after IMAC. The 13-hour FL sample was not solubilized due to time constraints.

4.1 FL vs C2

One of the aims of this study was to assess whether the truncated C2 construct might be preferable to work with compared to the full-length protein. C2 was hypothesized to be less prone to degradation and produce a higher yield. To investigate this, Identical protocols were used to compare the two different constructs. For the comparison between the different constructs, there was no TEV-cleavage or reverse IMAC. Instead, the samples were concentrated directly after traditional IMAC, and were then run on FPLC-SEC. For both constructs, an induction time of about 45 hours was used. Neither had a urea wash in their membrane prep, and the additional protease inhibitors had not yet been implemented.

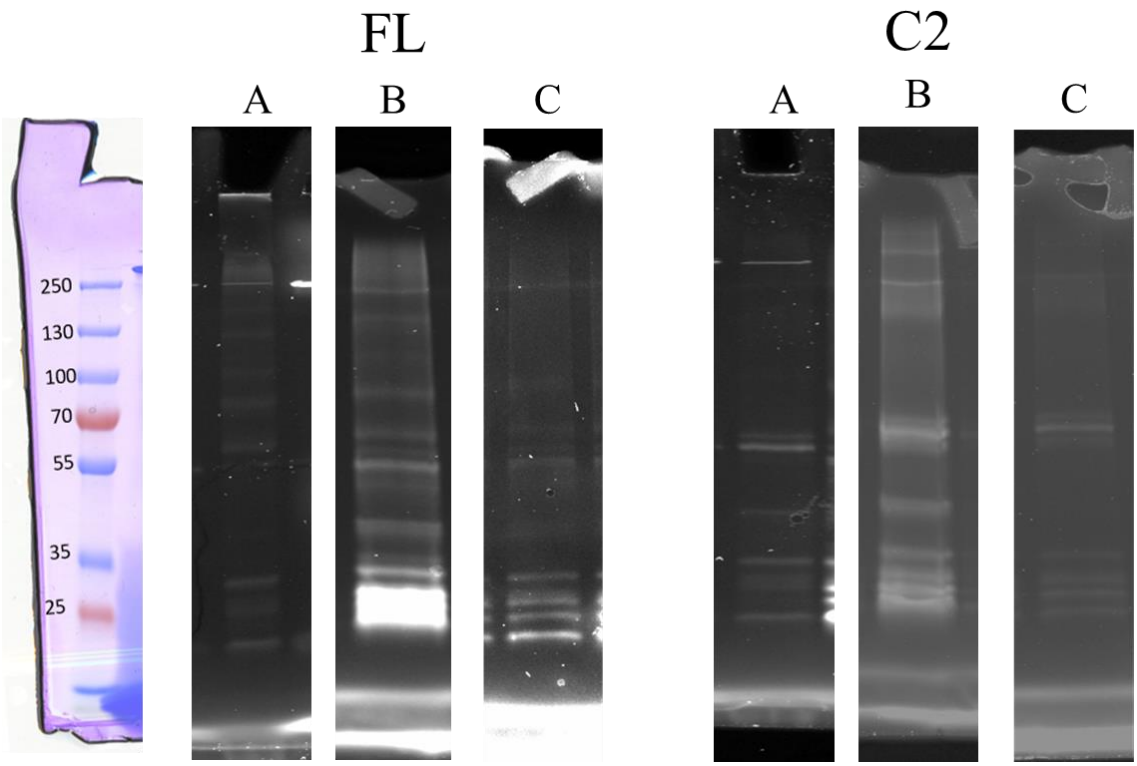


Figure 4: Fluorescence scans of SDS-PAGE gels comparing the degradation between constructs FL and C2 throughout the purification protocol. A is pellet 3 of the membrane prep corresponding to the final washed membrane. B is relevant IMAC fractions pooled and concentrated. C is the SEC fraction that corresponds to the peak in the chromatogram. See Appendix 8.2.1-8.2.5 for uncut gels.

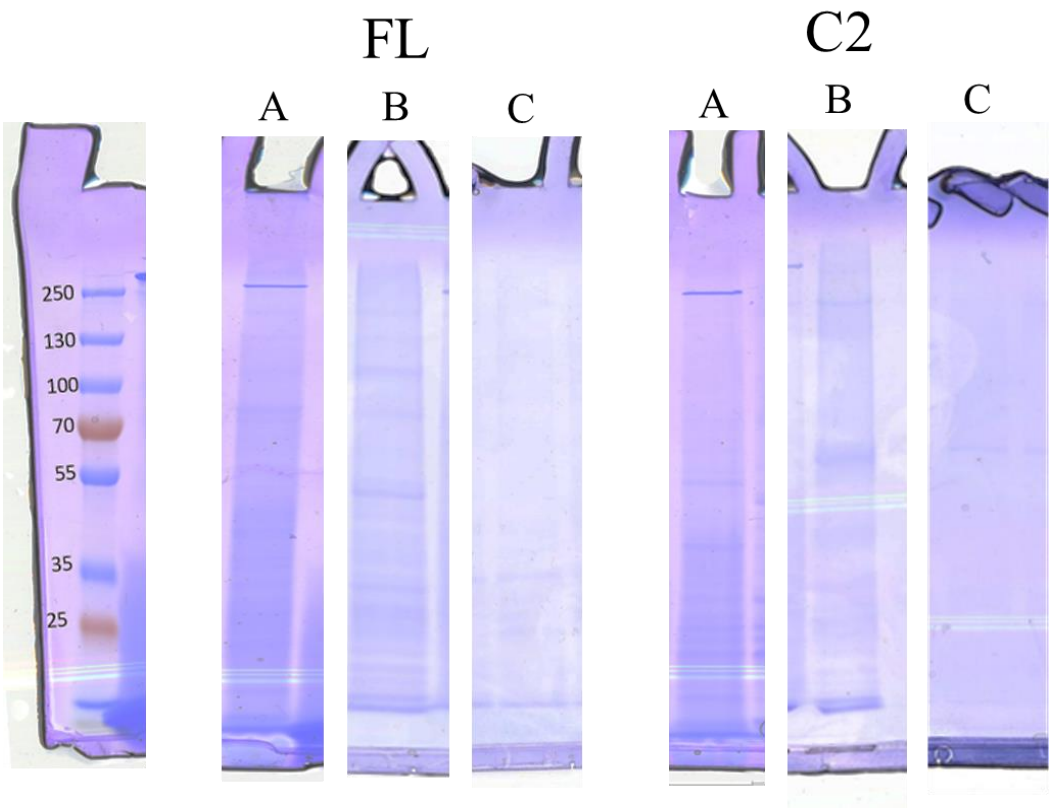


Figure 5: Scans of Coomassie stained SDS-PAGE gels comparing the degradation between constructs FL and C2 throughout the purification protocol. A is pellet 3 of the membrane prep corresponding to the final washed membrane. B is relevant IMAC fractions pooled and concentrated. C is the SEC fraction corresponding to the chromatogram peak. See Appendix 8.2.1-8.2.5 for uncut gels.

SDS-PAGEs in Figures 4 and 5 show little to no difference in the amount of degradation between the two constructs. These results discount the hypothesis of the C2 construct being less prone to degradation. Therefore, it was determined that it was not worth the time and effort to continue with C2 currently. While there are visible bands corresponding to the sizes of the undegraded constructs (160.8 kDa for FL-eGFP and 81.8 kDa for C2-eGFP), the intensity between these bands and the degradation products are very similar, or in some cases, the degradation products exhibit a greater intensity. Observing the lane titled A in Figures 4 and 5, the degradation appears to occur in the early stages of the protocol, as both constructs exhibit substantial degradation in the membrane prep. Therefore, the degradation has already occurred in the fermentation or membrane prep. To determine in which step the protein is

degraded, we must compare the amount present at the beginning of the membrane prep compared to the final membrane.

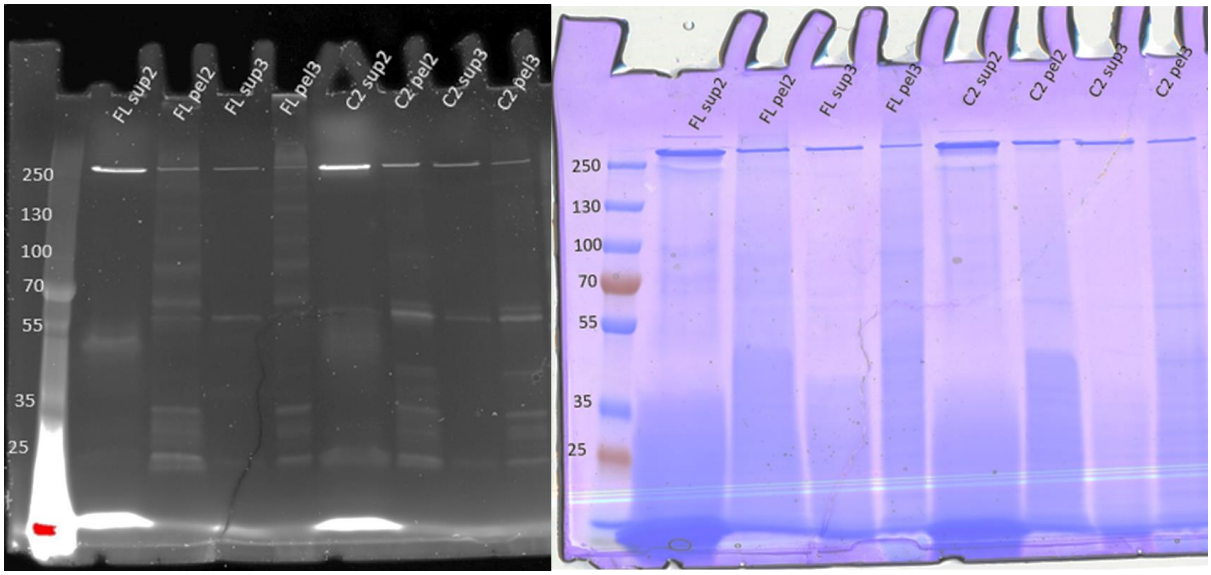


Figure 6: Fluorescent and Coomassie scans showing the different stages of the membrane prep for the FL and C2 construct.

In Figure 6, the degradation is already present in the early parts of the membrane prep in an almost identical pattern and intensity as is seen in the final pellet. Therefore, there is reason to believe that most of the degradation does not occur in the membrane prep but in the fermentation.

While there is little difference between the two constructs regarding the amount of degradation, one substantial difference is observed between them. The main difference observed is the molar amount of protein present.

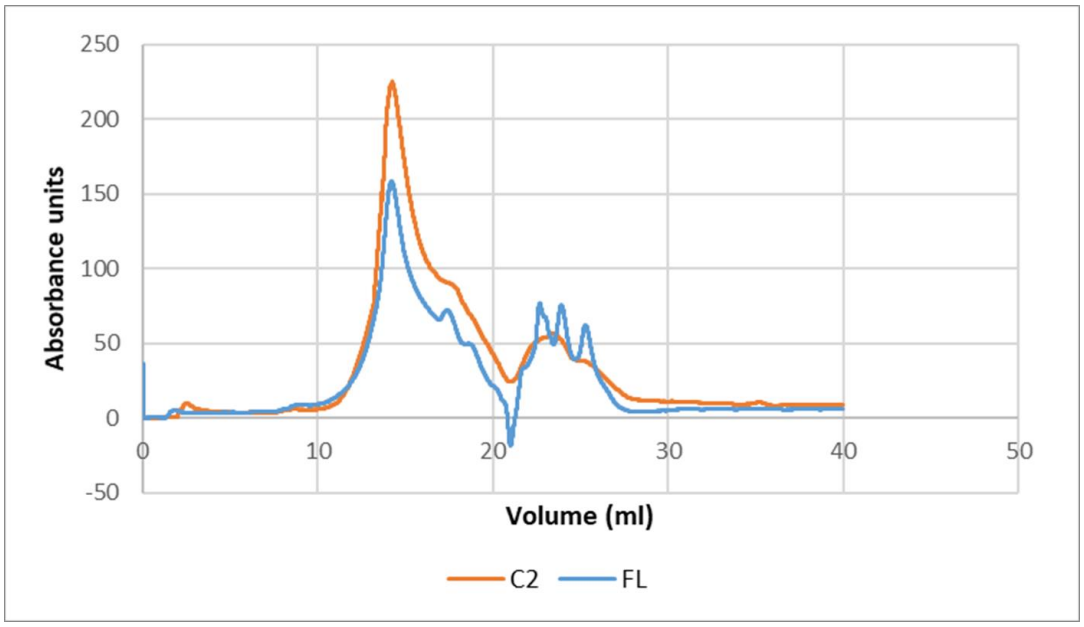


Figure 7: Comparison between the chromatogram peaks for the two different constructs obtained from SEC with the 280 nm absorbance shown on the y-axis and the volume shown on the x-axis.

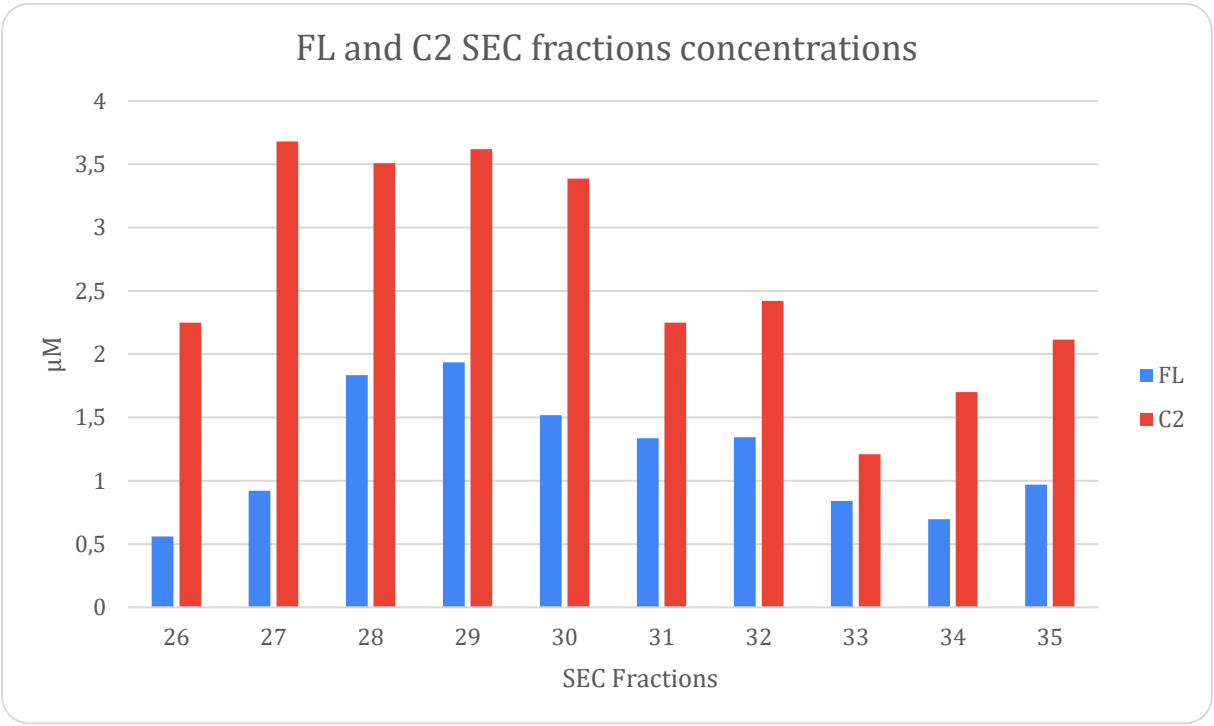


Figure 8: The SEC fractions' molar concentration corresponding to the chromatogram's peaks. Molar concentration was calculated from data obtained from nanodrop measurements.

The SEC chromatogram (Figure 7) and the observed concentration of the SEC fractions (Figure 8) show that C2 exhibited about double the molar concentration as FL. This could be

due to the size difference between FL and C2. If the only determining factor between the two constructs concentration is the productional capacity, then C2 should be produced in a higher amount. However, there are many different factors which would influence to amount of protein produced such as the copy number which we do not know. From the SEC chromatogram, we can observe that the two constructs eluted at similar volumes. This is unexpected due to the theoretical elution volumes for the two constructs being quite different, with the FL construct having a theoretical elution volume of 13.75 ml and the C2 construct having a theoretical elution volume of 14.58 ml. The two constructs' degradation may have contributed to the elution volume change.

4.2 Induction time

Since the investigation into the differences between the FL and C2 constructs determined that the protein was already highly degraded at the start of the membrane prep, it was decided that the fermentation protocol would have to be revisited. However, additional protease inhibitors were also added to the membrane prep protocol to prevent further degradation. There was reason to believe that a shorter induction might be beneficial in providing a more intact protein sample due to work conducted by Oliwia Kolodziejczyk. Therefore, two more fermentations were conducted with shorter induction timespans, resulting in a comparison of three different timespans: 13, 20, and 45 hours.

The amount of glycerol consumed between the different fermentations is largely the same except for the shortest fermentation, which was fed more glycerol due to scheduling issues with conducting such a short induction. However, this should only affect the cell mass from the fermentation. Since the membrane preps used approximately the same amount of cells across all the different induction times, the amount of glycerol fed to the culture should not factor into the results obtained from the membrane prep. It is also difficult to differentiate how the induction time factors affect the results from the membrane prep compared to the additional protease inhibitors used in the 13 and 20-hour samples, which were not used for the earlier 45-hour sample.

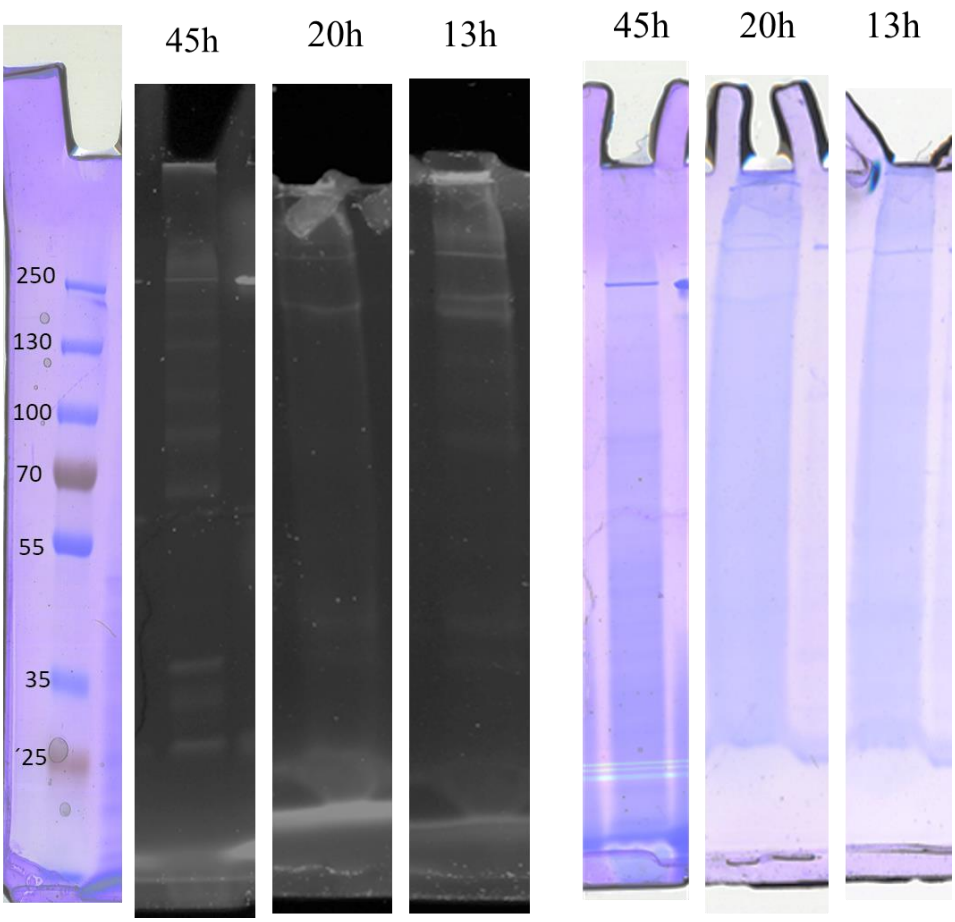


Figure 9: Fluorescence and Coomassie scans from the membrane preps of the different induction timespans. See Appendix 8.2.1, 8.2.8, and 8.2.9 for uncut gels.

When analyzing Figure 9, we can observe that shorter inductions seem to provide a less degraded sample. The 45-hour sample exhibited a lot of degradation, while the 20-hour and the 13-hour seem to be more intact. Comparing the 20-hour and the 13-hour sample bands corresponding to the intact size of the protein, the 13-hour sample has a greater intensity. This difference cannot be explained by the difference in the glycerol fed to the cultures or the condition changes in the membrane prep. While the 13-hour sample was fed more glycerol and provided a greater cell mass, an equal number of cells were used across all induction timespans for the membrane prep. When the 13-hour and the 20-hour cells were broken, additional protease inhibitors were implemented, so the difference observed between these two could not be explained by the difference in membrane prep protocol. The induction time is the most likely explanation for the difference observed between the 13-hour- and 20-hour samples.

4.3 Membrane prep

The protocol of the membrane prep has several different factors to evaluate. One of these factors is the use of a urea wash to rid the sample of proteases. The urea wash was not used for long induction samples of FL and C2 as it was believed too harsh. To determine whether the urea wash was beneficial or detrimental, the membrane prep was attempted with and without it using the same cell batch.

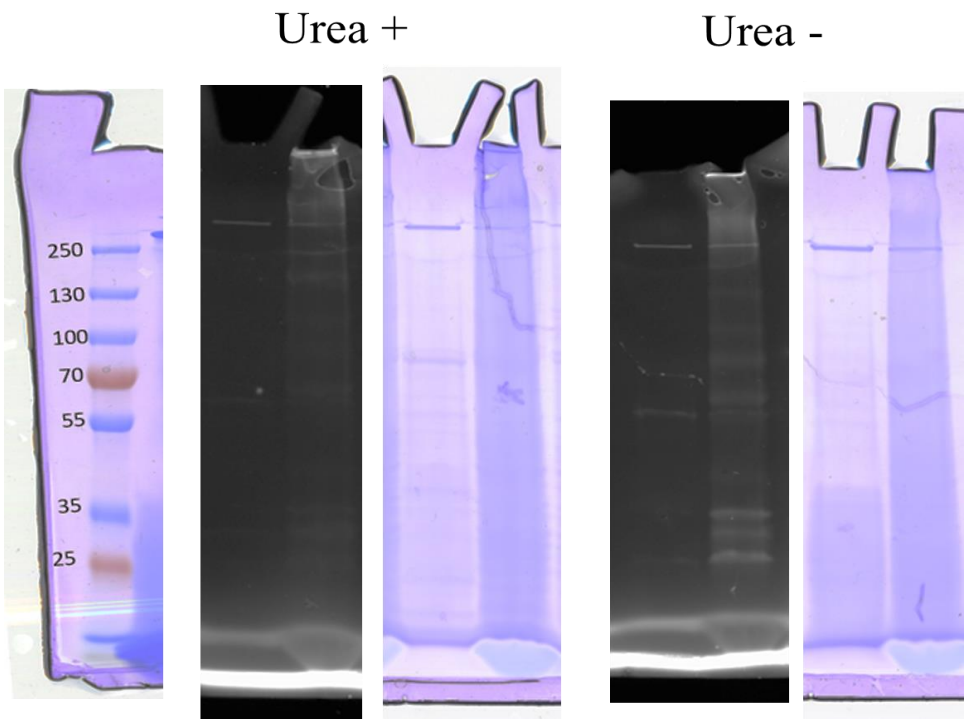


Figure 10: Fluorescent and Coomassie scans showing the final product of the membrane prep with and without the urea wash implemented. Supernatant 3 is the lane on the left, and pellet 3 on the right. Both membrane preps were conducted from the same cell batch. See Appendix 8.2.6 for uncut gels.

In Figure 10, we can observe that the sample without urea shows more degradation compared to the sample where urea wash was implemented. Due to these results, it was decided that the urea wash should be implemented in all future membrane preps conducted.

4.4 Solubilization and Purification

Utilizing the cells from the 20-hour induction and urea-washed membrane, the protein was solubilized and purified.

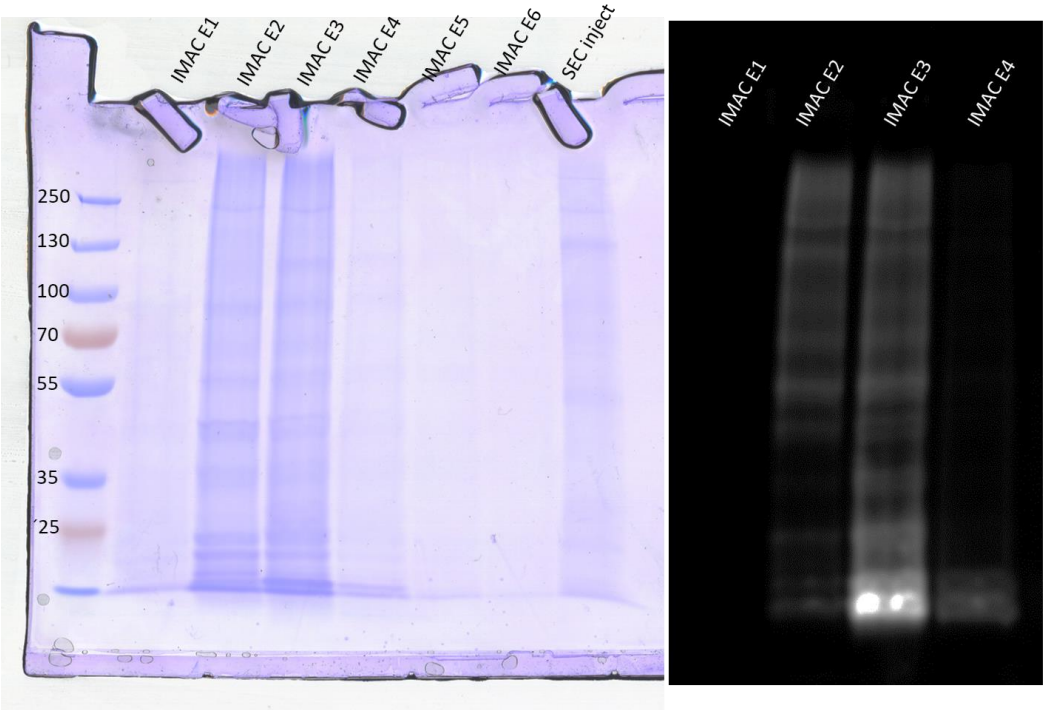


Figure 11: Fluorescent and Coomassie scans of an SDS-PAGE of IMAC elution fractions and the concentrated cleaved sample injected into the SEC. All samples are from a membrane prep done with urea-washed 20-hour induction samples. See Appendix 8.2.7 for uncut gels.

In Figure 11, we can see that there is quite a lot of degradation present in the IMAC elutions, but also a band that corresponds to the correct size of the uncut protein (160.8 kDa). A band corresponding to the cleaved protein size (133.9 kDa) is also present in the sample used for the SEC; however, it is quite faint.

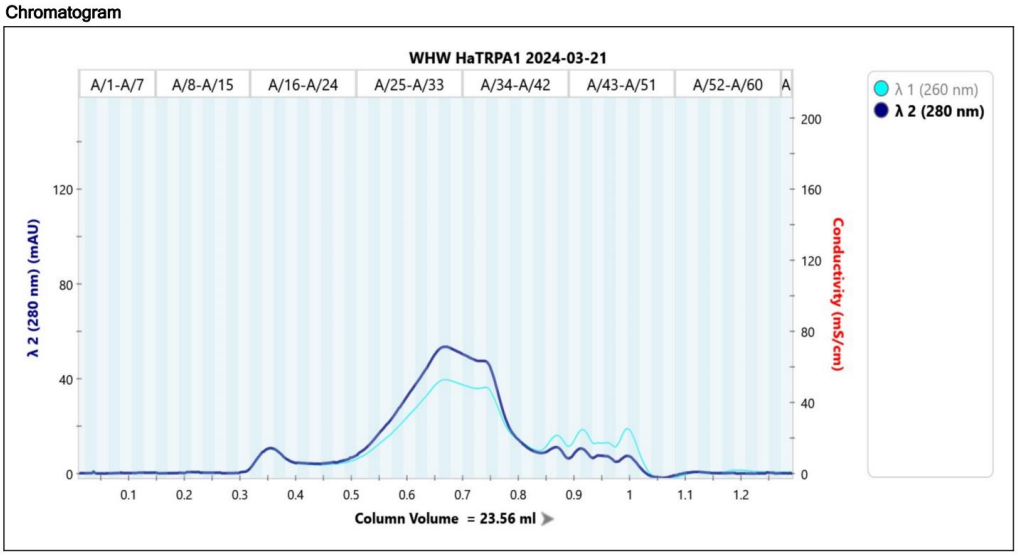


Figure 12: SEC chromatogram for cleaved HaTRPA1 from a membrane prep done with urea-washed 20-hour induction samples.

Figure 12 shows that the amount of protein in the sample used was insufficient to provide a notable peak, or it fell out of the column. Therefore, improving the solubilization and purification protocol is of great interest.

Table 1: Mass amount of the full-length construct and the truncated construct as well as the mass mount for the full-length construct with and without urea wash. Yield is also presented to view how much protein was lost in the concentration step.

	FL 45-hour (Urea-)	C2 45-hour (Urea-)	FL 45-hour (Urea+)
IMAC Elutions	2,56 mg	3,83 mg	3,34 mg
Concentrated Sample	1,54 mg	2,19 mg	1,94 mg
Yield	60%	57%	58%

Table 2: Mass amount of protein throughout the different steps of purification for the 20-hour and 45-hour induction samples for the full-length construct. The stepwise yield is presented, comparing the amount of protein in the previous step to the current step. The total yield is also presented, comparing the amount of protein in the current step to the total amount of protein observed in the first step.

	FL 20-hour (Urea+), Total mg	FL 45-hour (Urea+), Total mg	Yield Stepwise (FL, 20h, Urea+)	Yield Stepwise (FL, 45h, Urea+)	Yield Total (FL, 20h, Urea+)	Yield Total (FL, 45h, Urea+)

IMAC	3,05 mg	3,02 mg				
TEV	2,53 mg	2,71 mg	83%	90%	83%	90%
TEV Removal	2,43 mg	2,56 mg	96%	94%	80%	85%
Reverse IMAC	2,39 mg	2,49 mg	98%	98%	78%	83%
Concentrated Rev IMAC FT	1,32 mg	0,69 mg	55%	28%	43%	23%

From these results, the main issue currently is preventing the loss of protein throughout the purification. For every step in the purification, protein loss is inevitable. In table 1 and 2 we see that a lot of protein seems to be lost in the concentration step. However, due to the problems of degradation it is difficult to tell how much of the loss is intact protein. Therefore, the problems of protein loss during concentration might be solved by further preventing degradation.

To ensure that there is enough protein after the SEC, purification optimization needs to be done to decrease the loss for each step, or a method of pure force, purifying a large amount of membrane, needs to be used. While the pure force method may work to provide an adequate sample under a time constraint, in the long term, optimization in the purification protocol is needed to provide a sample through less wasteful methods.

5 Conclusions

The main conclusion to draw from this study is that despite the improvements made in the protocol, degradation and yield are still major obstacles in providing a sample adequate for Cryo-EM. The study showed that there is little difference in the amount of degradation between the full-length construct and the truncated construct of HaTRPA1. It is clear that a shorter induction time is beneficial in providing an undegraded protein sample, and implementing a urea wash in the membrane prep prevents further degradation. Some solubilization and purification protocol issues, such as degradation and protein loss, remain. Unfortunately, due to time constraints, we were not able to further optimize the solubilization and purification protocol or purify the 13-hour induction samples.

6 Future Aspects

The results of this study point towards a shorter induction, producing a less degraded protein sample. Therefore, future studies will likely focus on purifying the protein using shorter induction. The focus will, therefore, shift to improving the yield and optimizing the purification protocol in the hopes of providing a sample adequate for cryo-EM. In the short term, a large batch of membranes may be solubilized and purified to provide a sample for Cryo-EM. It is also a possibility that the sample sent away for Cryo-EM will have to be an uncleaved sample, as removing the TEV cleavage and the buffer exchanges would likely provide a sample with enough intact protein. The stability shown from the short induction samples also questions whether the C2 construct should be revisited. Since C2 provided a much higher molar concentration sample, it could produce a less degraded, high-yield sample if expressed with a shorter induction. Therefore, attempting another purification using C2 might be interesting to investigate the potential differences between the two complexes further.

While the shorter induction provides a less degraded sample, there is also some interest in attempting a long induction using a lower temperature. While the temperature used in this study was 30℃, Zhang et al. [2] successfully expressed human TRPs using a 48-hour induction at 15℃ in *Saccharomyces cerevisiae*. Even though Zhang et al. did not express TRPA1 and used a different host, evaluating a similar protocol for our host might be interesting. However, due to the apparent success of the 13-hour induction, this is unlikely to be explored currently unless a problem arises with the 13-hour induction protocol.

7 References

1. Kadir, L. A., Stacey, M., & Barrett-Jolley, R. (2018). Emerging Roles of the Membrane Potential: Action Beyond the Action Potential. *Frontiers in Physiology*, *9*. https://doi.org/10.3389/fphys.2018.01661
2. Zhang, L., Wang, K., Klaerke, D. A., Calloe, K., Lowrey, L., Pedersen, P. A., Gourdon, P., & Gotfryd, K. (2019). Purification of Functional Human TRP Channels Recombinantly Produced in Yeast. *Cells*, *8*(2), 148. https://doi-org.ludwig.lub.lu.se/10.3390/cells8020148
3. COSENS, D. J., & MANNING, A. (1969). Abnormal Electroretinogram from a Drosophila Mutant. *Nature*, *224*(5216), 285-287. https://doi.org/10.1038/224285a0
4. Koivisto, AP., Belvisi, M.G., Gaudet, R. *et al.* Advances in TRP channel drug discovery: from target validation to clinical studies. *Nat Rev Drug Discov* 21, 41–59 (2022). https://doi.org/10.1038/s41573-021-00268-4
5. Zhang, H., Wang, C., Zhang, K., Kamau, P. M., Luo, A., Tian, L., & Lai, R. (2022). The role of TRPA1 channels in thermosensation. *Cell insight*, *1*(6), 100059. https://doi-org.ludwig.lub.lu.se/10.1016/j.cellin.2022.100059
6. Sinica, V., & Vlachová, V. (2021). Transient receptor potential ankyrin 1 channel: An evolutionarily tuned thermosensor. *Physiological research*, *70*(3), 363–381. https://doi.org/10.33549/physiolres.934697
7. Macpherson, L., Dubin, A., Evans, M. *et al.* Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* 445, 541–545 (2007). https://doi.org/10.1038/nature05544
8. Suo, Y., Wang, Z., Zubcevic, L., Hsu, A. L., He, Q., Borgnia, M. J., Ji, R., & Lee, S. (2020). Structural Insights into Electrophile Irritant Sensing by the Human TRPA1 Channel. *Neuron*, *105*(5), 882-894.e5. https://doi.org/10.1016/j.neuron.2019.11.023
9. Yao, K., Dou, B., Zhang, Y., Chen, Z., Li, Y., Fan, Z., Ma, Y., Du, S., Wang, J., Xu, Z., Liu, Y., Lin, X., Wang, S., & Guo, Y. (2023). Inflammation-the role of TRPA1 channel. *Frontiers in physiology*, *14*, 1093925. https://doi.org/10.3389/fphys.2023.1093925

10. Hu, F., Song, X., & Long, D. (2021). Transient receptor potential ankyrin 1 and calcium: Interactions and association with disease (Review). *Experimental and therapeutic medicine*, *22*(6), 1462. https://doi.org/10.3892/etm.2021.10897
11. Clapham, D. Pain-sensing TRPA1 channel resolved. *Nature* 520, 439–441 (2015). https://doi.org/10.1038/nature14383
12. Williams, C.D., Dillon, A.B., Ennis, D. et al. Differential susceptibility of pine weevil, Hylobius abietis (Coleoptera: Curculionidae), larvae and pupae to entomopathogenic nematodes and death of adults infected as pupae. BioControl 60, 537–546 (2015). https://doi.org/10.1007/s10526-015-9658-3
13. *Advances in pine weevil resistance research may result in new weapons to battle the bugs | slu.se*. (n.d.). SLU.SE. https://www.slu.se/en/research/knowledge-bank/a2023/advances-in-pine-weevil-resistance-research-may-result-in-new-weapons-to-battle-the-bugs/#related-information-header
14. *pPICZA-EGFP vector map and sequence*. (n.d.). https://www.novoprolabs.com/vector/Vgeztmmrw
15. Moparthi, L., Sinica, V., Moparthi, V.K. *et al.* The human TRPA1 intrinsic cold and heat sensitivity involves separate channel structures beyond the N-ARD domain. *Nat Commun* 13, 6113 (2022). https://doi.org/10.1038/s41467-022-33876-8
16. Shimomura, O., Johnson, F.H. and Saiga, Y. (1962), Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from the Luminous Hydromedusan, *Aequorea*†. J. Cell. Comp. Physiol., 59: 223-239. https://doi.org/10.1002/jcp.1030590302
17. Stepanenko, O. V., Verkhusha, V. V., Kuznetsova, I. M., Uversky, V. N., & Turoverov, K. K. (2008). Fluorescent proteins as biomarkers and biosensors: throwing color lights on molecular and cellular processes. *Current protein & peptide science*, *9*(4), 338–369. https://doi.org/10.2174/138920308785132668
18. Cinelli, R.A.G., Ferrari, A., Pellegrini, V., Tyagi, M., Giacca, M. and Beltram, F. (2000), The Enhanced Green Fluorescent Protein as a Tool for the Analysis of Protein Dynamics and Localization: Local Fluorescence Study at the Single-molecule Level.

Photochemistry and Photobiology, 71: 771-776. https://doi.org/10.1562/0031-8655(2000)0710771TEGFPA2.0.CO2

19. Charlton, A., Zachariou, M. (2008). Immobilized Metal Ion Affinity Chromatography of Native Proteins. In: Zachariou, M. (eds) Affinity Chromatography. Methods in Molecular Biology™, vol 421. Humana Press. https://doi.org/10.1007/978-1-59745-582-4_2
20. Wang, K., Zhao, L., Li, T., Wang, Q., Ding, Z., & Dong, W. (2023). Selective Immobilization of His-Tagged Enzyme on Ni-Chelated Ion Exchange Resin and Its Application in Protein Purification. *International journal of molecular sciences*, *24*(4), 3864. https://doi.org/10.3390/ijms24043864
21. Nam, H., Hwang, B. J., Choi, D. Y., Shin, S., & Choi, M. (2020). Tobacco etch virus (TEV) protease with multiple mutations to improve solubility and reduce self-cleavage exhibits enhanced enzymatic activity. *FEBS open bio*, *10*(4), 619–626. https://doi-org.ludwig.lub.lu.se/10.1002/2211-5463.12828
22. Al-Tubuly, A.A. (2000). SDS-PAGE and Western Blotting. In: George, A.J.T., Urch, C.E. (eds) Diagnostic and Therapeutic Antibodies. Methods in Molecular Medicine, vol 40. Humana, Totowa, NJ. https://doi-org.ludwig.lub.lu.se/10.1385/1-59259-076-4:391
23. Ó'Fágáin, C., Cummins, P. M., & O'Connor, B. F. (2017). Gel-Filtration Chromatography. *Methods in molecular biology (Clifton, N.J.)*, *1485*, 15–25. https://doi.org/10.1007/978-1-4939-6412-3_2

8 Appendix

8.1 Amino Acid Sequences

The amino acid sequence of constructs FL and C2 with colours indicating different regions. The Ankyrin repeats in green S1-S4 in teal, S5-S6 and His-Tag in pink, TRP-domain in grey, coiled coil, and pore helix in red, TEV-site in blue, GFP in yellow.

C2 construct (Δ1-708): 712 residues, 81.8 kDa, cut by TEV 461 aa, 53.5 kDa

>C2_HaTRPA1_TEV_GFP_8xHis

MSAHGRVELLAHPLSQKYLQMKWNSYGKYFHLTNLLFYCIFLGSITCFSSQLMEHEKALNFS
YIKYSNMTTEQKYAERKSEILDVQINYSMYICAMAILVFIVLNGAREIAQMIQQKCMYFFNP
INLVTWCLYGATIVMVLPIFGGEIYEIQFSFASLVVFLSWFNLLLLLQRFDQVGIYVVMFLE
ILQTLIKVLMVFSILIIAFGLAFYILLSRGDHLSFKTIPMALIRTFSMMLGEIDFLGTYVKP
YYLSNKEENTFLPFPIPAFLILGLFMVLMPILLMNLLIGLAVGDIESVRRNAQLKRLAMQVV
LHTELERKLPRRWLERVDKAEITEYPNESKCKKGILDFILKKWFGSPFSEDSDIVMENSEDY
VVSELAKTKNKLRQISQALETQNQFLRLIVQKMEIKTEADDIDEGVPLRNTSHGHSSKWTSP
KIRKKIKSVLSFSNRANSTLEENLYFQSAGGSVSKGEELFTGVVPILVELDGDVNGHKFSVS
GEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGY
VQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMA
DKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRD
HMVLLEFVTAAGITLGMDELYKHHHHHHHH

FL construct: 1420 residues 160 kDa, cleaved by TEV 1169 aa 131.8 kDa

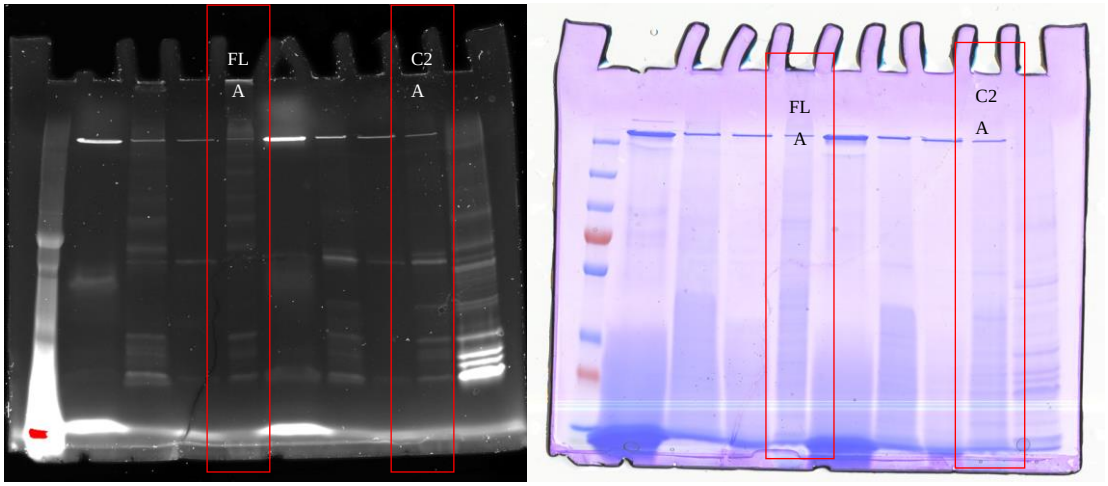
>FL_HaTRPA1_TEV_GFP_8xHis

MSNSFRSLVSAYGTESELQNMLPSDVENGQDKTSNGEAVCSIASSPYRILRAAESGNLDMFQ
RLYIQDPSRLSIQDPRGRTTAHQAASRNKINILTFINQQGGDLNAQDNVGNTPLHVAVESEA
LDAVDYLLTVGVKTDILNEKKQAPVHLATELSKISVLERMAKYKDKIDIEQGGEHGRTALHI
AAIYDHDACARVLISDFGASPRKPCNNGYYPIHEAAKNASSKTMEVFLAWGESLGCTRDEMI
SFYDAEGNVPLHSAVHGGDIRAVELCIRSGAKISTQQHDLSTPVHLACAQGAIEIVKIMFQM
QPEEKMACLASCDVQKMTPLHCAAMFDHPEIVEYLISEGADCNPIDKERRSPLLLAALRGGW

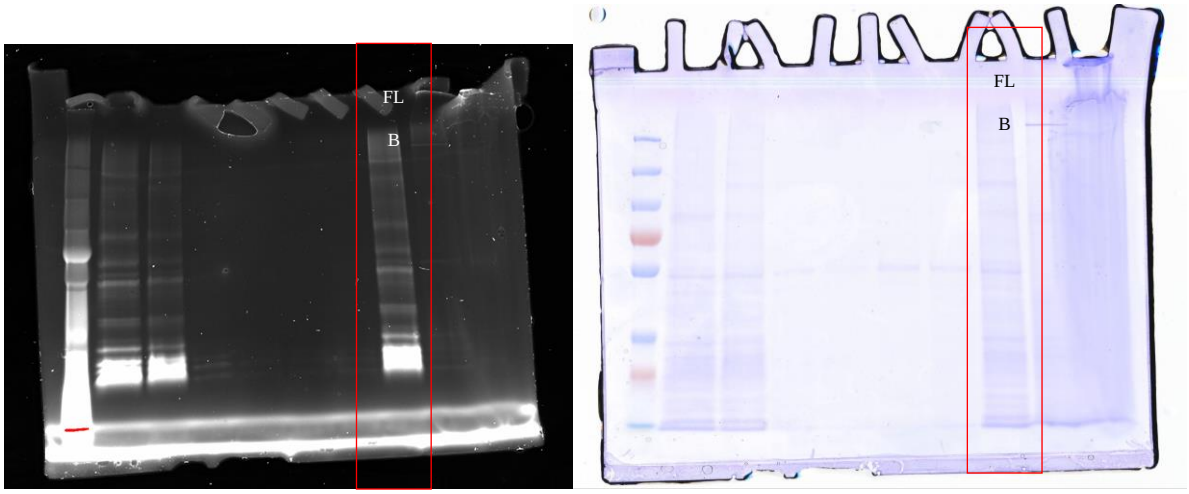
KTVHSLIRLGADINIKDINKRNVLHLVVMNGGQLEQFAAEAKSKESLLQLLNEKDLTGCSPL
HYASREGHIRSLENLIRLGACINLKNNNNESPLHFAARYGRYNTVKQLLDSEKGNFIINESD
GEGMTPLHIASQQGHTRVVQLLLNRGALLHRDHNGRNPLHLAAMNGYTQTIELLLSVHSHLL
DQLDKDGNTALHLATMENRPNVIAQLLSMKCKLLYNQTEMSAIDYAIYYKYLEAALAMVTHE
DRAEEIMVLKSPKNPCVSLALIASMPRVFEAVQDKCIIKANCKKDSKLFSIKYSFCCLQCNT
VTEEIDEKTGDKIVEKEPTPLPALNAMVAHGRVELLAHPLSQKYLQMKWNSYGKYFHLTNLL
FYCIFLGSITCFSSQLMEHEKALNFSYIKYSNMTTEQKYAERKSEILDVQINYSMYICAMAI
LVFIVLNGAREIAQMIQQKCMYFFNPINLVTWCLYGATIVMVLPIFGGEIYEIQFSFASLVV
FLSWFNLLLLLQRFDQVGIYVVMFLEILQTLIKVLMVFSILIIAFGLAFYILLSRGDHLSFK
TIPMALIRTFSMMLGEIDFLGTYVKPYYLSNKEENTFLPFPIPAFLILGLFMVLMPILLMNL
LIGLAVGDIESVRRNAQLKRLAMQVVLHTELERKLPRRWLERVDKAEITEYPNESKCKKGIL
DFILKKWFGSPFSEDSDIVMENSEDYVVSELAKTKNKLRQISQALETQNQFLRLIVQKMEIK
TEADDIDEGVPLRNTSHGHSSKWTSPKIRKKIKSVLSFSNRANSTLEENLYFQSAGGSVSKG
EELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTY
GVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGI
DFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGD
GPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKHHHHHHHH

8.2 SDS PAGE

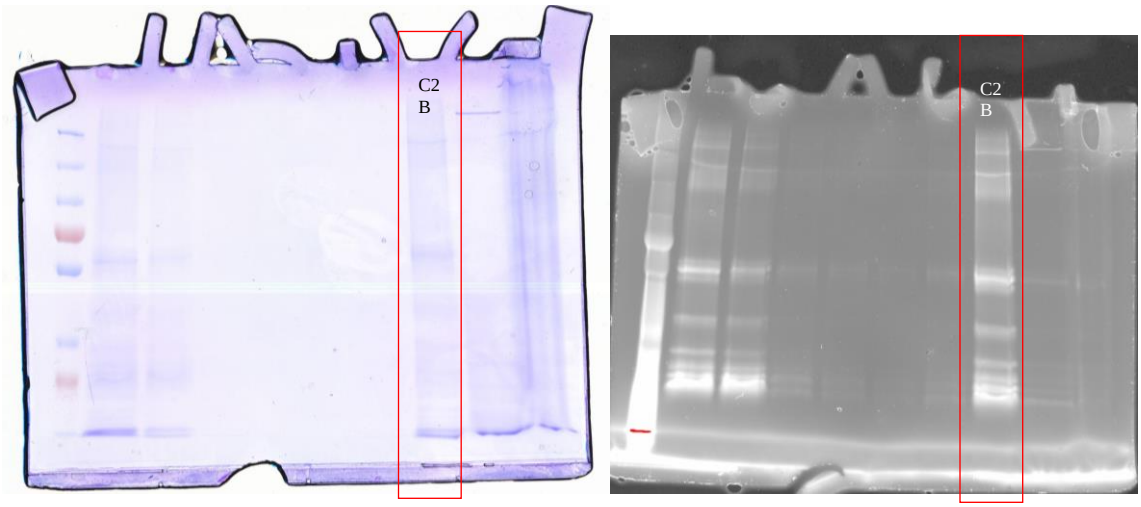
8.2.1 Membrane prep for FL and C2, 45-hour



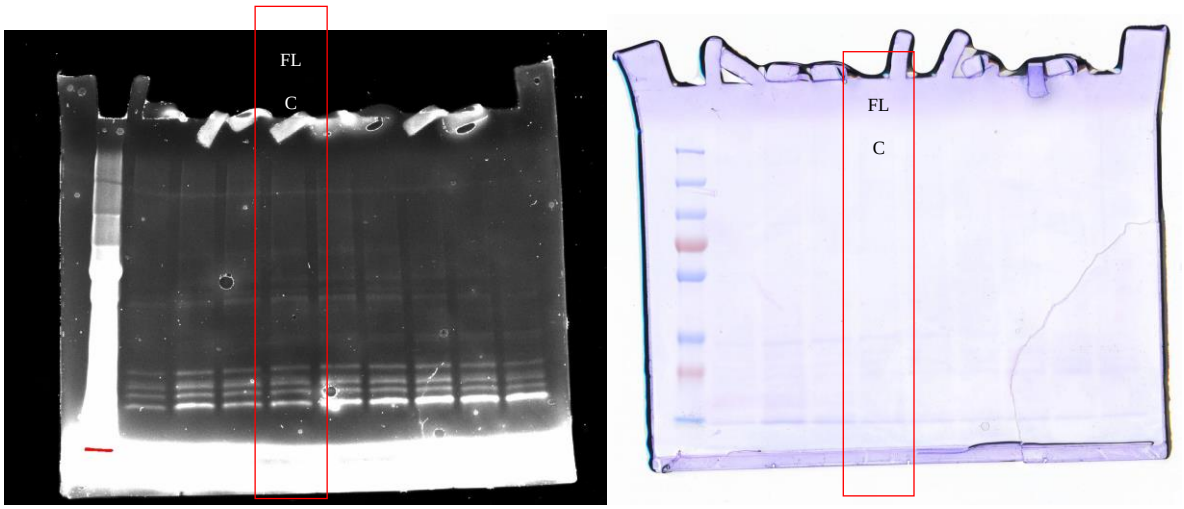
8.2.2 FL IMAC 45-hour



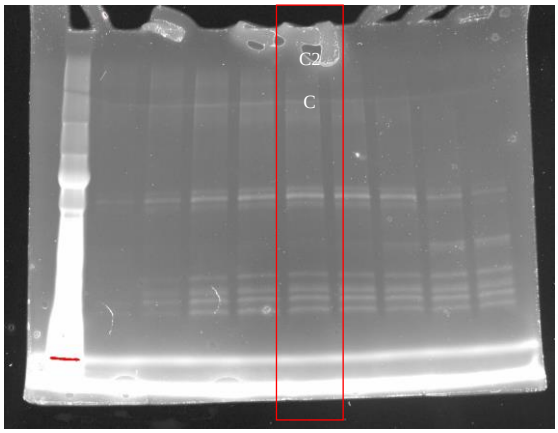
8.2.3 C2 IMAC 45 hour

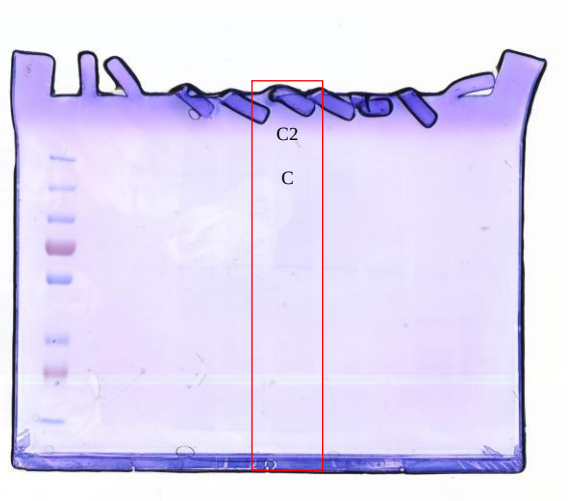


8.2.4 FL SEC 45-hour

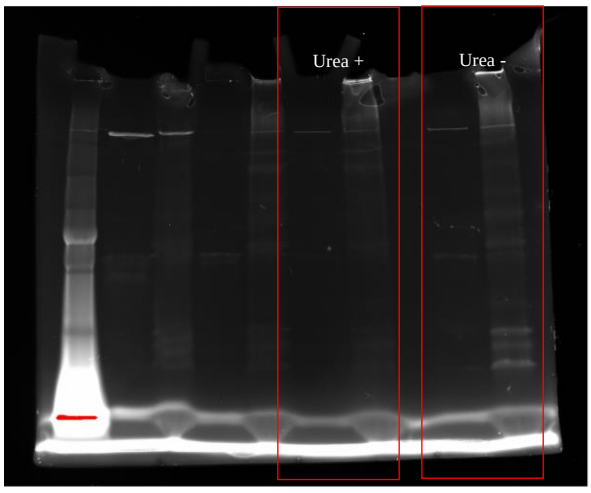


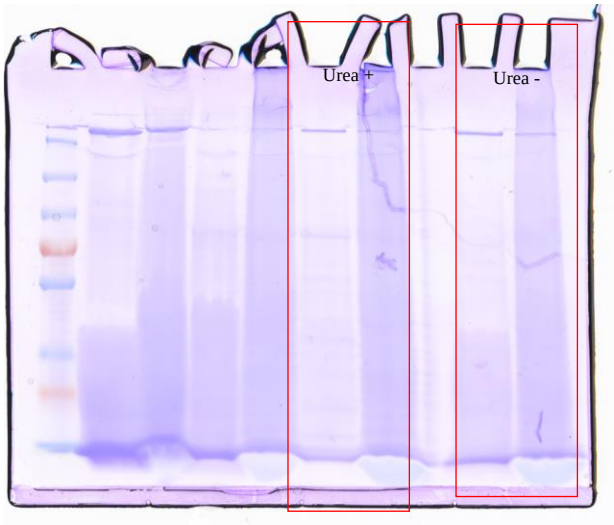
8.2.5 C2 SEC 45-hour



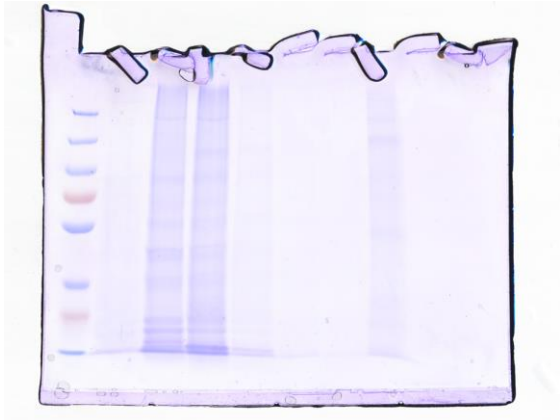


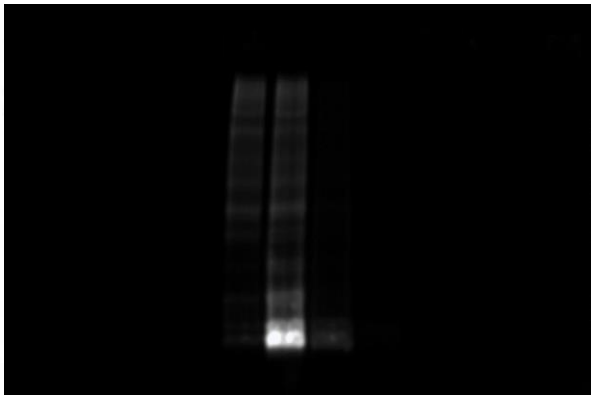
8.2.6 UREA wash



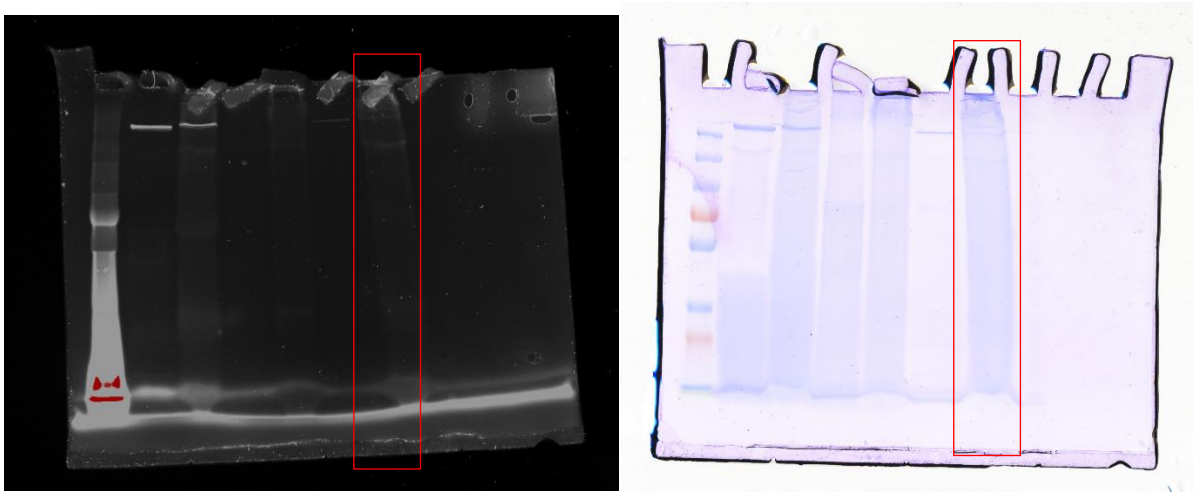


8.2.7 IMAC FL 20-hour





8.2.8 Membrane Preparation 20-hour



8.2.9 Membrane Preparation 13-hour