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Crystallization and physiological characterization of human aquaporin 10

Master's thesis in the Biotechnology Master's Programme

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Gothenburg, Sweden, 2012

Acknowledgements

This master thesis project was supported by **The Sven and Lilly Lawski's Foundation for Natural Science Research**. Thank you!

From the Lundberg Laboratory:
I would like to thank Jennie Sjöhamn for being a great support in the lab and for patiently answering all of my questions. It has been great to work with you!

I would also like to thank my supervisor, Kristina Hedfalk for the opportunity to work with aquaporins. It has been a learning process which has taught me to have patience with integral membrane proteins. It feels a bit sad to hand over the hAQP10 project to the next brave student but it is a step in solving the structure of this tough target. I am convinced that we will reach our goal in the near future.

Furthermore I would like to thank Richard Neutze for being my examiner and for taking the time to read through my report.

From Aquaporin A/S Denmark:
I would like to thank Julie Bomholt at Aquaporin for making me feel very welcome in a new lab environment. It has been great to work together and achieve very good results. I would also like to thank Kent Høier Nielsen for the intellectual input regarding the aquaporin skin project. Furthermore I would like to thank Claus Hélix Nielsen for always being positive and supportive regarding the project and for guiding the group in the right direction. It has been great to work together and feel like a part of the team. I am looking forward to further exciting research projects in the future!

From the Bispebjerg Hospital Denmark:
I would like to thank Tove Agner and Jakob Jungersted for our collaboration exploring the role of aquaporins in the skin. Thank you for always providing us with skin samples on demand and for having me follow you for a day at the hospital. It was an interesting learning experience. I am looking forward to further developing our collaboration together with the Aquaporin team.

I would also like to thank my dear friend and opponent Emelie Szabo for our great laughs together in the lab and for always keeping up the good spirit. I will never forget this year!

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Abstract

Integral membrane proteins control important biological processes in the cell. Being anchored to the membrane, they serve as sensors for changes in the environment in and out of the cell and they are responsible for signal transduction. They also serve as channels for the transport of ions, water and other substances and are often targets for drug development. Aquaporins are passive channels that are crucial for the survival of the cell under osmotic stress by regulating water and glycerol transport. Human aquaporin 10 (hAQP10) is an aquaglyceroporin found in the small intestine where it may be involved in the absorption of water and small nutrients. Recent studies suggest that hAqp10 might be also expressed in the *stratum corneum* of the skin and malfunctions of the protein might cause eczema. In this project hAQP10 was over-produced in the yeast *Pichia pastoris* and purified with chromatography techniques. Highly pure and concentrated protein sample was used in different crystallization setups aiming to get the three dimensional structure through X-ray diffraction. In addition, skin samples from scalpel scrapings and tape stripping were provided from a collaboration partner and analyzed with immunoblotting and immunohistochemistry. In conclusion, pre-crystal structures, spheroids, were generated having the protein in a mix of the detergents n-Dodecyl-β-D-Maltoside (DDM) and n-Octyl-β-D-Glucopyranoside (β-OG). Based on the immunoblot and the immunohistochemistry, the protein could be localized in the stratum corneum of the skin. This project lays a foundation for future work on structural studies of hAQP10 and other aquaglyceroporins.

Keywords: membrane proteins, aquaporins, crystallization, structure, function, physiology, *stratum corneum*, skin scrapings, tape stripping

Contents

1 Background

The function of integral membrane proteins is crucial for many important biological processes in the cell. These proteins serve as sensors for changes in the extracellular milieu and are involved in signal transduction through the membrane. They also function as channels by controlling or facilitating the transport of ions, water and other substances in or out of the cell. Membrane proteins are often targets for drug development because of their central role in the cells and information about their three dimensional structure is a very important tool for understanding their function in different biological processes at a molecular level.

Aquaporins are passive channels that are found in biological membranes in all organisms. They have highly conserved domains and are important for the survival of the cell under osmotic stress by controlling water or glycerol transport. There are 13 identified aquaporin homologues in mammals. Among these, AQP1, 2, 4, 5 and 8 show water selectivity only while AQP3, 7,9 and 10 transport glycerol and other small molecules together with water. Genetic defects in the aquaporin genes are involved in diseases such as diabetes insipidus, obesity and cataracts among others [1].

Solving crystal structures of membrane proteins facilitates the design of inhibitors or drugs to target different conditions. For example the crystal structure of the aquaporin from Plasmodium falciparum sheds light on novel drug design against malaria. Inhibitors can be constructed against the PfAQP to hinder the parasite from replicating itsself in host cells [2], [3].

Several structures of aquaporins from X-ray diffraction patterns have been reported in the databases. From the mammalian aquaporins there are crystal structures for AQP0 [4], AQP1 [5], AQP4 [6] and AQP5 [7]. There have also been reported crystal structures for bacterial and plant aquaporins. These are the bacterial aquaporins GlpF [8], AqpM [9], AqpZ [10] and the plant aquaporin SoPIP2 [11].

hAQP10 is one of the most recently identified members of the human aquaporin family of proteins. This protein was first reported in the databases as a poorly spliced version of 264 amino acids that was not fully functional. The protein lacked the sixth transmembrane domain (TM6) and had a different C-terminus as compared to its aquaglyceroporin homologues. These changes resulted in an incorrect folded state of the protein due to the loss of TM6 and also it affected its ability to transport glycerol and urea due to the altered hydrophilic C-terminal domain [12], [13]. The protein had reduced water transport ability as well. An independent study identified a correctly spliced form of hAQP10 consisting of 301 amino acids and with conserved sixth transmembrane domain and an intact C-terminus [14]. This protein is defined as an aquaglyceroporin found in the small intestine where it may be involved in the absorption of water and

small nutrients [15]. Both studies identified the hAQP10 protein to be localized in the small intestine.

hAQP10 has six transmembrane domains connected by intracellular and extracellular loops in between. A snake plot of hAQP10 is shown in Figure 1. Four units are joined together in a tetramer to form the water and glycerol channel.

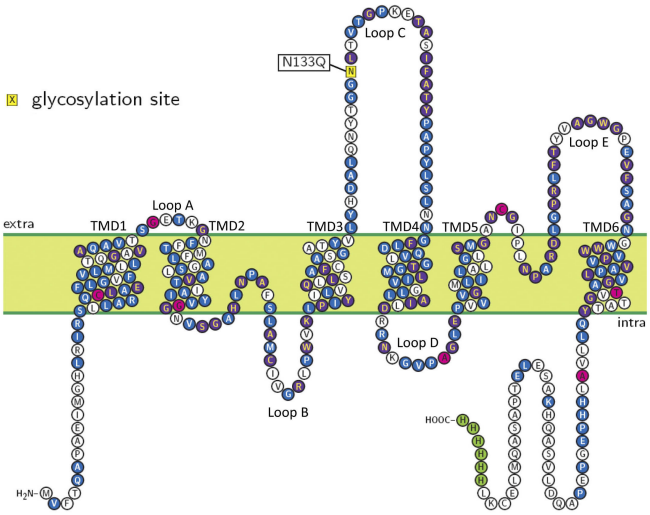

Figure 1: Snake plot showing the predicted arrangement in the membrane of the hAQP10 residues. The glycosylation site that was mutated (N133Q) is also shown in the picture. Figure modified from [16]

Recent studies indicate that hAqp10 is also expressed in the skin, where it might be involved in the hydration and elasticity of the skin or alternatively it might cause eczema [17]. As compared to many other aquaporins, hAQP10 is relatively uncharacterized and there is no known three-dimensional structure of this interesting target, neither of any other human aquaglyceroporin, in the databases. A reason why human aquaglyceroporins are underrepresented in the databases compared to the already solved crystal structures of homologues could be the low sequence identity between the aquaglyceroporins. Between hAQP10 and the GlpF homologue with a reported crystal structure there is only 36% sequence identity (Blastp). Similar numbers are seen between hAQP10 and another aquaglyceroporin with a solved crystal structure, PfAQP [2] where the sequence identity is 33% (Blastp).

Solving the crystal structure of hAQP10 leads the research closer to understanding the mechanisms of water and smaller nutrients uptake in the small intestine. Further, understanding how the hAQP10 subunits are assembled into a tetramer in a 3D structure gives insight in factors involved in ectopic eczema and how an eventually malfunctioning hAQP10 tetramer might cause the disease. Solving the structure of the protein further facilitates studying the lipid bilayer composition around the protein in the *stratum*

corneum. Novel, specific drugs can then be synthetized to address ectopic eczema. A crystal structure of a human aquaglyceroporin can then serve as a foundation for further crystallization studies on other human aquaglyceroporins and their respective disease causing mechanisms.

The protein used in this project is the N133Q mutant (FO216Δ) with a mutated glycosylation site [16]. A glycosylation is a co-translational or post-translational modification that consists in a carbohydrate being attached to a site in the protein. Glycosylations are the most common translational modifications that occur in a cell and they are very important for proper folding of proteins. For example, in hAQP2, glycosylation is important for the protein to exit the Golgi complex and be redirected to the plasma membrane [18]. For the hAQP10, glycosylations might be of importance to direct the protein to the apical region of the small intestine [19].

Glycosylation of proteins does not occur equally in all of the cells which might generate a non homogenous protein solution that contains both glycosylated and non-glycosylated proteins. This hinders the formation of crystals and therefore a recombinant protein is expressed instead which lacks glycosylation sites. The protein has two other additional glycosylation sites in the transmembrane domains which are sterically hindered and therefore do not get glycosylated.

The aim of this project is to determine the three dimensional structure of hAQP10 by X-ray diffraction. To achieve this goal, the protein is overproduced in *Pichia pastoris* and purified with chromatography techniques. Different buffer solutions are tested to find suitable conditions for crystal formation. The content of glycerol and NaCl in different detergents is varied to stabilize the protein and enable protein contact in the crystallization solution. Crystallization screens are run both manually and automated with a robot.

2 Theory

The hAQP10 project consists mainly of laboratory work but also some literature studies. The theory behind the methods that are used to characterize the protein is presented below.

2.1 Fermentor growth

P. pastoris is a methylotrophic yeast that has been shown to be useful in the overproduction of recombinant proteins. In *P. pastoris*, alcohol oxidase is an important enzyme involved in the utilization of methanol. When glucose is present the gene AOX1, encoding for the enzyme alcohol oxidase, is repressed but when methanol is used as a sole carbon source the AOX1 gene is strongly induced [20]. *P. pastoris* is used for the overproduction of hAQP10 by incorporating the hAqp10 gene under the control of the AOX1 promotor.

During the fermentation, parameters like pH, dissolved oxygen tension (DOT), stirring, temperature and feed are controlled to ensure optimal cultivation conditions. The fermentation is started as a batch cultivation where the cells consume the glycerol available in the start media. Glycerol consumption is seen as a decrease in the DOT curve until there isn't any glycerol available. When the glycerol has been entirely consumed, the DOT curve increases until reaching a 100% oxygen saturation in the media. The glycerol fed-batch phase is initiated then where glycerol is fed to the cells drop-wise to accustom the cells to a limiting carbon source and also to further produce biomass. The DOT curve shows fluctuations then due to oxygen consumption related to the glycerol consumption and re-saturation of the media with fresh oxygen. The cells are cultivated in a glycerol fed-batch mode for $\sim$24h and then the methanol fed-batch mode is initiated. Methanol is carefully fed to avoid accumulation which is toxic for the cells. Methanol is fed in a fed-batch mode together with sorbitol because it has been shown to shorten the response time to methanol and, also, it might enhance the production of heterologous proteins and reduce cell mortality compared to growth on methanol only [21].

2.2 Membrane preparation

Being an integral membrane protein, hAQP10 is integrated in the cell membranes. When expressed in *P. pastoris* the yeast cells need to be broken to obtain the cell membranes. *P. pastoris* cells are treated with breaking buffer and broken through passages in

X-press bombs or in a continous flow French-press cell. The cells are exposed to high pressure by passing through a hollow channel in the X-press bombs or in the French-press cell and thus they break. The cell material is centrifuged to get rid of the large cell debris pellet and then ultracentrifuged to obtain membranes with integral membrane proteins and proteins that interact with membranes. Finally the membrane pellet is washed with urea buffer and further ultracentrifuged to generate stripped *P. pastoris* membranes.

2.3 Protein purification

Stripped *P. pastoris* membranes are solubilized in solubization buffer containing 2% DM detergent and ultracentrifuged to get rid of non-solubilized material. Solubilized membranes are purified by using immobilized metal ion affinity chromatography (IMAC) and the protein is eluted with imidazole. Further, the purification is continued with size exclusion chromatography (SEC) where fractions of highly pure protein are collected.

2.3.1 IMAC

Immobilized metal ion affinity chromatography is used to bind proteins to a column that contains immobilized metal ions. Among these are Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} etc. These ions bind proteins that contain imidazole groups from histidine residues, thiole groups and indole groups. The N133Q mutant contains a His-tag that has affinity for Ni^{2+} which selectively will bind to the Ni^{2+} ions on the column. Other proteins and contaminants that don't bind to the column are washed away. The protein is eluted from the column by using elution buffer that contains high concentrations of imidazole [22].

2.3.2 SEC

Size exclusion chromatography is a technique used to separate particles based on their size and shape. This is done by using the molecular sieve properties of some materials. SEC is performed in a column containing cross-linked copolymers that are equilibrated with a mobile phase that is suitable for the particles that are being separated. Small particles interact with the hollow pores formed by the copolymers and appear late in the eluate. Large particles, in contrast, pass through the matrix easily and appear first in the eluate [22].

2.4 Crystallization

Crystal formation is enabled from contacts between residues in proteins. The protein slowly precipitates from a solution in a crystal conformation. For this to occur it is necessary to have a homogeneous and highly pure protein solution. Crystallization setups are done by varying different physical parameters that are involved in the solubility of the protein. Variations in pH, temperature, precipitants, ionic strength etc. produce a supersaturation in the solution that in turn leads to the formation of small aggregates formed by the collision of the molecules in the solution. Aggregates serve as nucleation sites for crystal growth. Membrane proteins need to be solubilized by detergents in order to have them in a solution. Detergents shield protein residues and might hinder crystal contacts.

In this project, highly pure protein will be crystallized following different crystallization conditions to find the optimal ones. The hanging drop crystallization method and the sitting drop crystallization method will be applied. These methods rely on vapor diffusion to produce supersaturation in the sample.

In the hanging drop method one drop of reservoir solution is added to one drop of concentrated protein on a cover glass. The glass is flipped and put on the well containing the same reservoir solution and thus generating a hanging drop over the well. The reservoir solution contains a pH buffer, a precipitant and additional additives. The precipitant concentration of the droplet is lower than the precipitant concentration in the reservoir solution in the well so vapour diffusion will occur. Vapour diffusion leads to the gradual increase in the concentration of the protein in the droplet and thus producing a supersaturated state followed by crystal formation. Crystals are then exposed to X-ray beams to obtain a diffraction pattern. The sitting drop method works in a similar way, only the drop is placed “sitting” above the reservoir solution.

2.5 Stratum corneum

The *stratum corneum* is the outermost layer of the eidermis that controls the intake of substances to the body or the excretion of these. This skin layer consists mostly of corneocytes which is dead skin cells that lack nuclei and organelles. These are generated from proliferating keratinocytes from lower skin layers. The *stratum corneum* contains also a lamellar lipid layer that is a result of the secretion of lamellar bodies [23]. There has been some indication that the *stratum corneum* might contain aquaporins to control the diffusion of water through the skin barrier but the evidence of hAQP10 has only been detected in mRNA extracts and not the actual translated protein [17].

2.5.1 Tape stripping method

Tape stripping is a non-invasive method used to characterize dermatological formulations [24]. Here the same procedure is used to asses cells of the *stratum corneum* layer of the skin to detect human aquaporin 10 and human aquaporin 3. Tape strips containing adhesive films are placed on a marked area of the skin, pressure is applied and the tape strip is removed. The first tape strip is discarded and ten following samples are taken with different tape strips. The strips containing skin cells are immediately placed in eppendorf tubes and stored in a -80°C freezer.

2.5.2 Skin scraping method

A marked skin area is scraped with a scalpel and skin flakes containing cells from the *stratum corneum* are collected in a vial. The vial is immediately frozen and stored in a -80°C freezer.

3 Method

For a detailed description of the materials and buffers used in this project see the Appendix section.

3.1 Fermentor growth

The fermentors (Belach Bioteknik AB), some flasks and BMGY media were autoclaved before the fermentation to ensure a sterile environment. Six precultures were prepared by mixing 5 ml BMGY media together with hAQP10-N133Q mutant colonies in falcon tubes. Two falcon tubes contained a small amount of cells, two contained a medium amount of cells and two contained a large amount of cells. The falcon tubes were put in the incubator at 29°C, 225rpm over night. The following day OD_{600} was measured and the two cultures with OD_{600} closest to 10 were diluted to 50ml BMGY in E-flasks. The E-flasks were incubated at 29°C, 225rpm for 4h until reaching OD_{600} between 2 and 6. Fermentation was initiated by innoculation with the 50ml cultures to the 1.5L starting media. A trace salts solution was added (4.35mL/L) to the starting media and also to the additional feeding flasks of glycerol methanol and sorbitol (12mL/L) during the fermentor cultivation. When the initial glycerol was entirely consumed, the DOT curve dropped and started to rise towards 100% again. The glycerol fed batch phase was initiated after 26h and the methanol fed-batch phase was initiated after additional 7h. *P. pastoris* cells were fed with sorbitol (60%) and methanol (100%) in a mixed feed mode, ratio 1:1. The cells were harvested after 50h of cultivation on mixed feed by centrifugation in a JLA-8.1000 rotor at 6000rpm, 45min, 4°C. The cell pellets from the fermentor cultivation were weighted and stored in a -20°C freezer.

3.2 Membrane preparation

To generate *P. pastoris* membranes containing the hAQP10 protein the yeast cells need to be broken. Breakage of the cells was done using either an X-press or a French-press. The following methods are presented below.

3.2.1 X-press

Fermentor pellet was thawn and resuspended in Breaking Buffer following the ratio 1g cells to 1ml buffer. The cell liquid was poured into six prefrozen X-press bombs and stored in the -20°C freezer over night. The cells were broken the following day by three

passages through the X-press bombs due to high pressure. The bombs were dismantled and the collected pellet with broken cells was thawn and resuspended in an appropriate amount of Breaking Buffer for centrifugation in a JA10 tube. The resuspended pellet was homogenized with a syringe or a homogenizer followed by centrifugation in a JA10 rotor.

3.2.2 French-press

Fermentor pellet was thawn and resuspended in Breaking Buffer following the ratio 1g cells to 3ml buffer. The cells were subjected to high pressure and broken by three passages through a continuous flow French-press cell.

Homogenized pellet from the X-press or pressed cell liquid from the french press was poured into JA10 tubes and spun twice in a JA10 rotor (Beckman Coulter, 10 000g, 20min, 4° and 15 000g, 30min, 4°C). Spinning in low velocities is done to discard the large cell debris and unbroken cells. The membranes together with the integral membrane proteins are in the supernatant.

The supernatant was poured in Ti45 tubes and after spun at 42 000rpm, 90min, 4°C. Spinning in higher velocities results in the formation of a pellet that contains membranes, integral membrane proteins and proteins that interact with membranes.

The pellets from the previous spin were pooled together and homogenized with a homogenizer in an appropriate amount of Urea Wash Buffer for centrifugation an a Ti45 tube. The homogenized material is poured in a Ti45 tube and spun at 42 000rpm, 90min, 4°C. Urea wash is done to dispose of proteins that interact with membranes generating stripped *P. pastoris* membranes that contain the hAQP10 protein.

Washing the membranes with Urea Wash Buffer generates a loose pellet. The supernatant after the urea wash spin was carefully removed with a syringe and the pellet was homogenized in Breaking Buffer. The homogenized material was poured into a preweighted Ti45 tube and spun at 42 000rpm, 60min, 4°C to generate a firm pellet. The supernatant was discarded and the pellet was weighted. The pellet was homogenized in Membrane Resuspension Buffer by using a homogenizer to assume a final total protein concentration of 4mg/ml. The assumption is that 4mg total protein per milliliter is equal to 7ml buffer per gram of membrane. The homogenized pellet was frozen in liquid nitrogen and stored at -80°C.

3.3 Protein purification

P. pastoris membranes at a total protein concentration of 4 mg/ml were mixed with an equal volume of solubilisation buffer to generate a detergent concentration of 1%. The mixture was left stirring in a 8°C room for 1h. Non-solubilized material was discarded by centrifugation in a Ti70.1 rotor at 42 000rpm, 30min, 4°C.

IMAC was prepared during the centrifugation time.

3.3.1 IMAC

For the IMAC purification Ni-NTA slurry (Qiagen) was used.

2ml of 50% Ni-NTA Slurry was transferred to a falcon tube and washed twice with 25ml mQ and centrifugation at 3 000g, 10min, 4°C. The resin was equilibrated with buffer EQ for 5min and then pelleted by spinning again at 3 000g, 10min, 4°C.

Solubilized material from the Ti70.1 centrifugation was transferred to the falcon tube containing the equilibrated Ni-NTA slurry together with 30mM imidazole. The mixture was incubated on a rolling table in the 8°C room for 2h.

Material that was not bound to the slurry was disposed of by centrifugation at 4 000g, 10min, 4°C. The pelleted slurry with bound hAQP10 protein to it was transferred to a gravity flow column. The column containing the slurry was washed with Wash Buffer and the protein was eluted using Elution Buffer300 and Elution Buffer500 each containing 300mM and 500mM imidazole respectively. 1ml fractions were collected manually and left on ice over night.

The fractions from IMAC were evaluated on a Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Fractions showing a band between 26kDa and 34kDa that looked pure on the gel were pooled together and concentrated by centrifugation (3 000g, 4°C) to a volume of approximately 250μl in a 20ml Vivaspin concentration tube (Sartorius Stedim Biotech), 100 000 MWCO. The concentrated protein sample was filtered through a 0.2μm filter (Anapore) before being used in a SEC assay.

3.3.2 SEC

The Superdex200 gelfiltration column (24ml, GE Healthcare) was equilibrated with Gelfiltration Buffer and the concentrated, filtered protein sample was loaded. Fractions of 400μl were collected in a deep-well block and kept on ice. Fractions under the protein peak according to the chromatogram were evaluated on an SDS-PAGE. Pure fractions

were pooled together and concentrated by centrifugation (4 000g, 4°C) in a 2ml Vivaspin concentration tube (Sartorius Stedim Biotech), 100 000 MWCO. The sample was concentrated to a concentration of 8mg/ml before being used for crystallization setups. The concentration of the protein sample was measured on a NanoDrop Spectrophotometer (SWAB) at 280nm following the Lambert-Beer law: $A = \epsilon lc$, where ϵ = 41160 (Protparam). All experiments except the screen with the detergent NG were run on a 24ml Superdex200 column. The NG screen was run with a 3ml Superdex200 column.

3.4 Cystallization

The hanging drop method and the sitting drop method were used to screen for different crystallization conditions. Crystallization conditions with the hanging drop method were tested manually by adding a 1μl drop of concentrated protein sample ($\sim$8 mg/ml) to a 1μl drop of reservoir solution on a cover glass. Crystallization conditions with the sitting drop method were tested with the HoneyBee Robot (Cartesian Technology).

3.5 Immunohistochemistry

Tape strips containing cells of the *stratum corneum* were permeabilized by adding 1 ml 20$\%$ ethanol/mQ or 20$\%$ ethanol/PBS and incubated at 50°C for 24h. 1 ml PBT solution was added and the tapes were washed twice for 5 minutes by centrifugation on a small centrifuge. 250μl of primary antibody dilution (anti-hAQP10, Sigma-Aldrich) was added to each eppendorf tube and the tapes were incubated with the antibody overnight on a rolling table. The tapes were washed 4x5min by adding 1ml PBS to the eppendorf tubes for each washing step. 100μl secondary antibody dilution was added and the tapes were incubated with the antibody for 3h. The tapes were washed again 4x5min in 1ml PBS. hAQP10 was visualized on a fluorescence microscope following the emission wavelength of 546nm of the label on the secondary antibody.

3.6 Immunoblot on skin cells

Vials containing skin scrapings were taken out of the freezer and thawn on ice. Sample buffer (2x) stock was prepared by adding a protease inhibitor cocktail (1:1000) and pmsf (1:1000). 400μl from the stock was added to each vial and the vial was left vortexing up and down in room temperature for 3h. 20 μl from each vial was loaded in a 4-12$\%$ Bis-Tris SDS gel and the gel was run at 140V until the dye ran off the gel. The gel was blotted onto a PVDF membrane and transfer to the membrane was run overnight

at 30V and 4°C. The WesternBreeze Chromogenic Western Blot Immunodetection Kit (Life Technologies) was used to detect the proteins on the membrane. The immunoblot protocol was followed with slight changes. The membrane was incubated with the primary antibody (anti-hAQP10, Sigma-Aldrich) for 3h, with the secondary antibody for 1h and overnight with the chromogenic substrate. The detected protein was viewed as visible purple bands on the membrane.

4 Results

The results from the different experiments are presented below.

4.1 Fermentor growth

A fermentor cultivation with the N133Q mutant was done to generate material for the project. The fermentation curves are shown in Figure 2 and Figure 3.

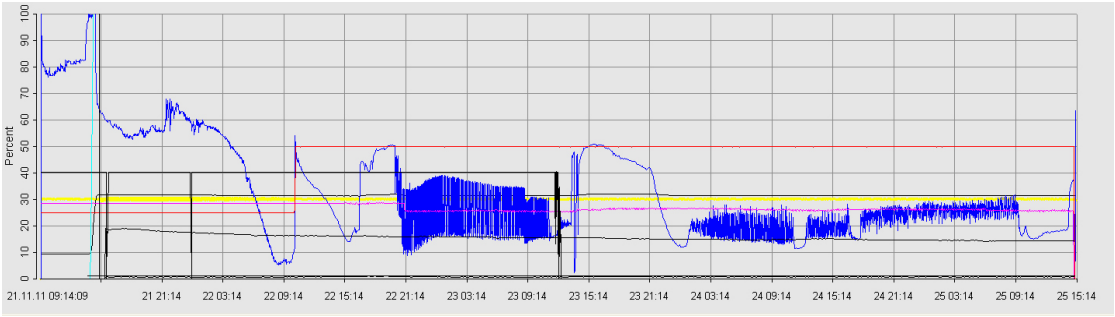

Figure 2: The fermentation curves showing the change in the controllable parameters DOT, pH, biomass etc in Fermentor 1.

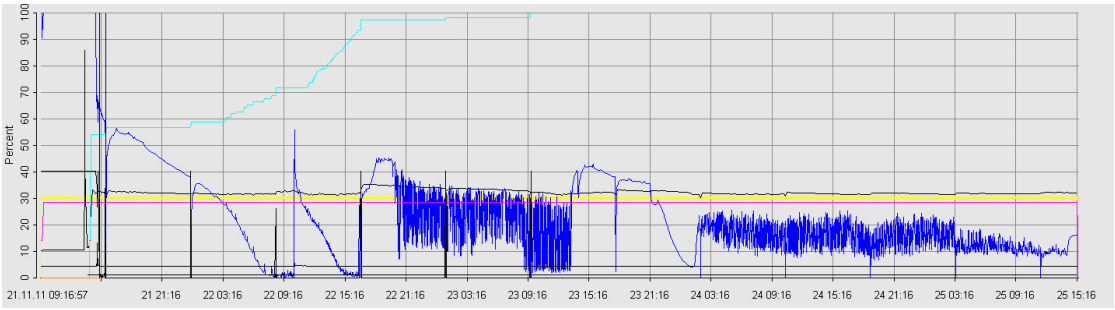

Figure 3: The fermentation curves showing the change in the controllable parameters DOT, pH, biomass etc in Fermentor 3.

The fermentation log-file is shown in Figure 4.

FO216Delta

11/21/2011

Fermentor3	FO216Delta
OD in precultures	1.08
11/21/2011 14:30	inocculering
11/21/2011 15:47	nolla biomassan
11/22/2011 10:20	1 ml AF added
11/22/2011 20:37	Glycerol feed batch started
11/23/2011 13:31	Methanol feed batch started
11/24/2011 2:00	respond to meoh
11/25/2011	Harvest

Culture volume:	1550 ml
Gram cells:	
Used Glycerol:	100 ml
Used Methanol:	50 ml
Used NH3:	100 ml
Response to MeOH:	12h
Used Sorbitol (60%):	500 ml

Culture volume:	
Gram cells:	
Used Glycerol:	
Used Methanol:	
Used NH3:	
Response to MeOH	

Fermentor1	FO216Delta
OD in precultures	1.55
11/21/2011 14:30	inocculering
11/21/2011 15:47	nolla biomassan
11/22/2011 10:20	1 ml AF added
11/22/2011 16:12	1 ml AF added
11/22/2011 20:37	Glycerol feed batch started
11/23/2011 13:31	Methanol feed batch started
11/23/2011 21:14	1 ml AF added
11/24/2011 1:30	respond to MeOH
11/24/2011 9:21	1 ml AF added
11/24/2011 17:52	decreased Feed to setp1
11/25/2011 9:53	2 ml AF added
11/25/2011 15:11	Harvest

Culture volume:	1950 ml
Gram cells:	
Used Glycerol:	200 ml
Used Methanol (100%):	100 ml
Used Sorbitol (60%):	250 ml
Used NH3:	100 ml
Response to MeOH:	12h
Note:	

Figure 4: The fermentation log-file showing the different time-points during the fermentation and also the consumed glycerol, methanol, sorbitol and NH_3

The fermentor cultivation showed a slightly odd behaviour of the cells. Very little glycerol, methanol and sorbitol from the feed was consumed. The result was a low amount of pellet after harvesting the cells.

Start OD_{600} was around 1 when the fermentors were inoculated compared to the recommended OD_{600} between 4 and 6. Also the inoculated amount of cells was half the recommended amount (50 ml instead of 100 ml per fermentor). These are factors that might have influenced the fermentor cultivation.

4.2 Optimization of the protocols

Samples were taken during solubilization of the membranes at 1h, 2h and 3h. The samples were run on an SDS-PAGE and after blotted in a immunoblot. The immunoblot is shown in Figure 5.

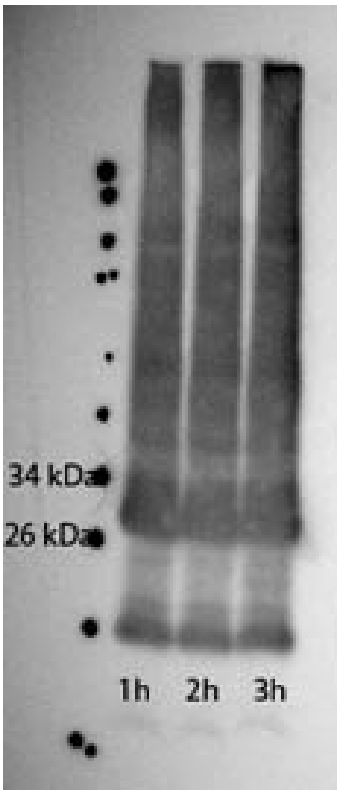

Figure 5: The immunoblot showing the three different solubilization samples at 1h, 2h and 3h. The ladder is shown to the left.

The immunoblot in Figure 5 shows that the amount of solubilized protein does not increase between the three samples so one can decrease the solubilization time to 1h instead of 3h.

Three other samples were taken during incubation with imidazole. The immunoblot is shown below.

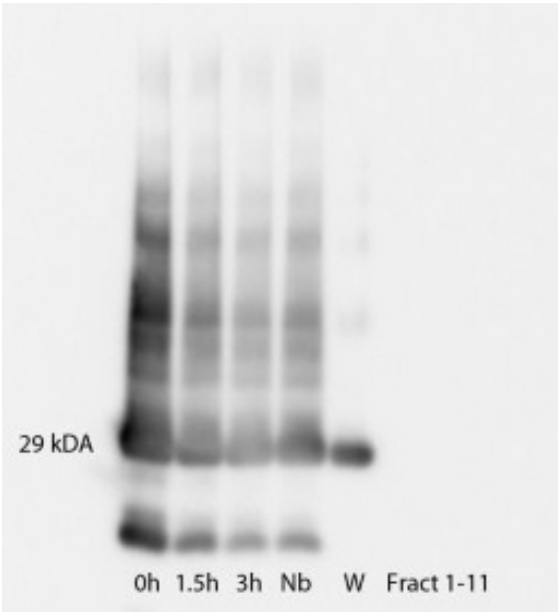

Figure 6: The immunoblot showing the three different samples under incubation with imidazole at 0h, 1.5h and 3h. The ladder is shown to the left.

The immunoblot in Figure 6 shows that there is less protein on the sample the more it is incubated with imidazole. Samples after 1.5h and 3h do not differ that much from each other so the incubation time with imidazole can be optimized to 1.5h instead of 3h incubation.

4.3 Purification in DM

Stripped *P. pastoris* membranes were solubilized in solubilisation buffer containing the detergent n-Decyl-β-D-Maltopyranoside (DM). IMAC purification was done manually by eluting fractions from a gravity flow column. The fractions were run on an SDS-PAGE which is shown in Figure 7.

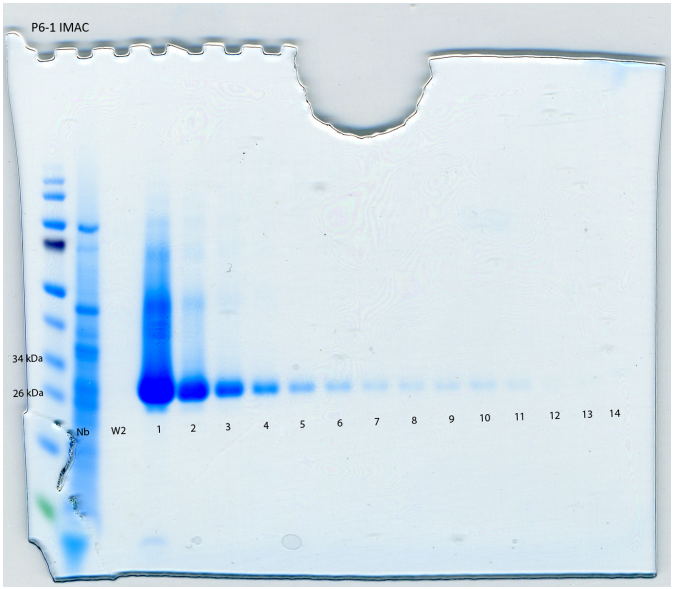

Figure 7: The SDS-PAGE showing the different fractions after elution with 300mM and 500mM imidazole. In the figure Nb and W2 correspond to samples taken to determine the amount of non-bound protein to the Ni-NTA slurry respectively amount of protein that is released from the gel during the wash step. The ladder is shown to the left.

The SDS-PAGE shows that there is a little amount of protein in the not-bound (Nb) fraction, no protein in the wash fraction and that there is protein in fractions 1-14. The amount of protein in the not-bound fraction is much less than the protein amount in fractions 1-14 and no protein can be observed in the wash so one can conclude that the IMAC purification was successful.

Fractions 1-14 are further concentrated to $\sim$250μl and further purified on a Superdex200 column. The protein is purified in gel filtration buffer containing the detergent DM. The chromatogram is shown in Figure 8.

GF buffer
20mM Tris-HCl pH 7.5
100mM NaCl
0.3% DM

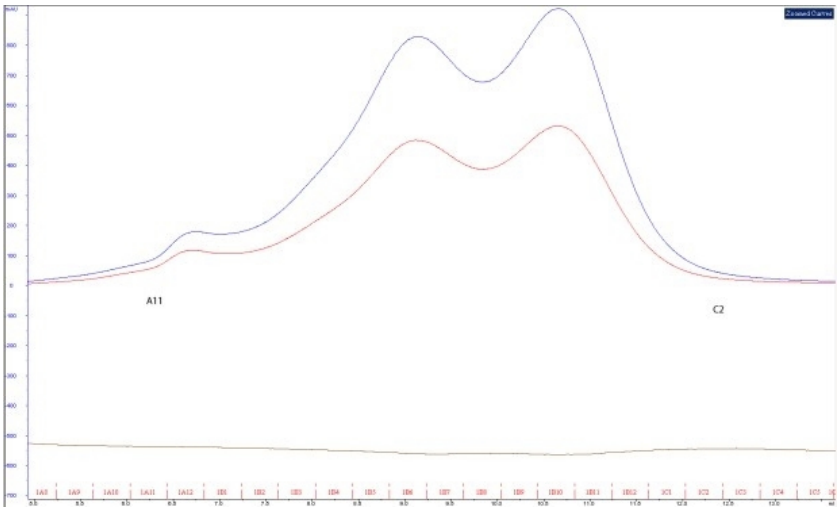

Figure 8: The chromatogram showing the fractions under the protein peak after gel filtration. Fractions A11-C2 are under the protein peak.

Fractions A11-C2 are run on an SDS-PAGE to see the amount of protein in the different fractions and also if the fractions look pure and free from any degradation product. The SDS-PAGE is shown in Figure 9.

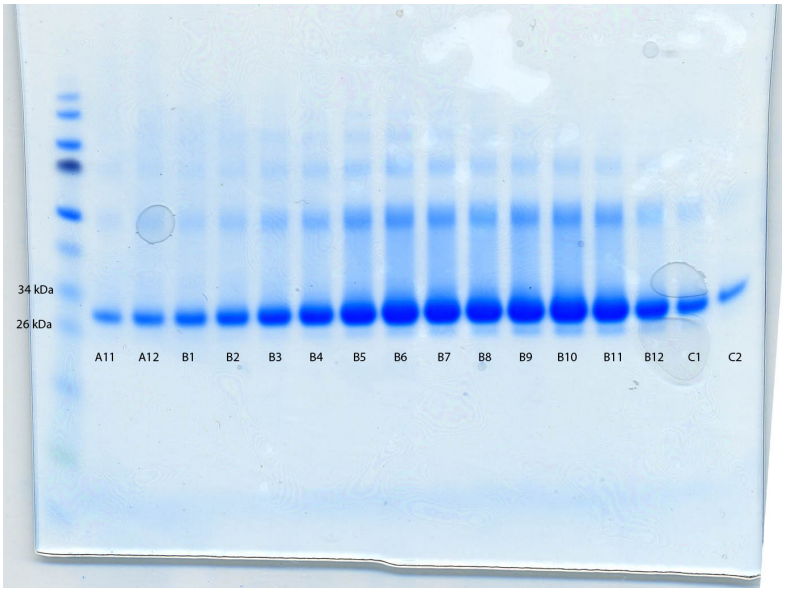

Figure 9: The SDS-PAGE showing the fractions under the protein peak after gel filtration. Fractions A11-C2 are under the protein peak. The ladder is shown to the left.

The SDS-PAGE on fractions A11-C2 shows that these fractions contain the hAQP10 protein and that the samples look relatively pure. The gel shows also a laddering effect of the bands which has also been observed in previous work.

All of the fractions are concentrated to a concentration of 8,57mg/ml and the concentrated protein sample is used for crystallization setups following the schedule in Table 1.

Table 1: Table showing the crystallization setups tested for the protein in DM

			PEG400	120	130	140	150	160	170
			mQ	280	270	260	250	240	230
100mM				1	2	3	4	5	6
50	pH 6.3		A	24% PEG, pH=6.3	26% PEG, pH=6.3	28% PEG, pH=6.3	30% PEG, pH=6.3	32% PEG, pH=6.3	34% PEG, pH=6.3
50	pH 7.8		B	24% PEG, pH=7.8	26% PEG, pH=7.8	28% PEG, pH=7.8	30% PEG, pH=7.8	32% PEG, pH=7.8	34% PEG, pH=7.8
50	pH 9.3		C	24% PEG, pH=9.3	26% PEG, pH=9.3	28% PEG, pH=9.3	30% PEG, pH=9.3	32% PEG, pH=9.3	34% PEG, pH=9.3
50	pH 10.8		D	24% PEG, pH=10.8	26% PEG, pH=10.8	28% PEG, pH=10.8	30% PEG, pH=10.8	32% PEG, pH=10.8	34% PEG, pH=10.8

There was a formation of round crystals which unfortunately didn't diffract. DM in combination with hAQP10 has been tested previously at the Lundberg laboratory and it doesn't seem promising as a detergent for crystallization assays. An Experimental design was performed instead by trying different buffer solutions with the detergent n-Nonyl-β-D-Glucopyranoside (NG). The goal was to find buffer conditions that generate a stable protein.

4.4 Experimental Design

The experimental design was performed on a small Superdex200 column (column volume:3 ml). Two factors are investigated at two different levels. The NaCl concentration is varied between a low value (100mM) and a high value (300mM) and the amount of glycerol is varied between 0% (no glycerol) and 10% glycerol. The different gel filtration buffers are shown below.

GF1
20mM Tris-HCl pH 7.5
100mM NaCl
0.4% NG

GF2
20mM Tris-HCl pH 7.5
100mM NaCl
10% Glycerol
0.4% NG

GF3
20mM Tris-HCl pH 7.5
300mM NaCl
0.4% NG

GF4
20mM Tris-HCl pH 7.5
300mM NaCl
10% Glycerol
0.4% NG

For a comparison, the assay is also run with GF buffer containing DM.

GF
20mM Tris-HCl pH 7.5
100mM NaCl
0.3% DM

The four gel filtration assays generated four different chromatograms. These are shown below.

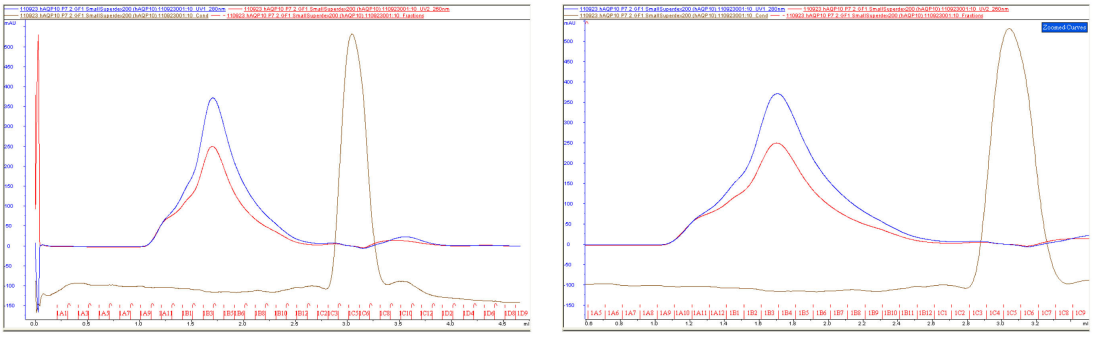

Figure 10: The chromatogram when the protein is purified in NG using GF1. The standard chromatogram is shown to the left and the zoomed area under the protein peak is shown to the right.

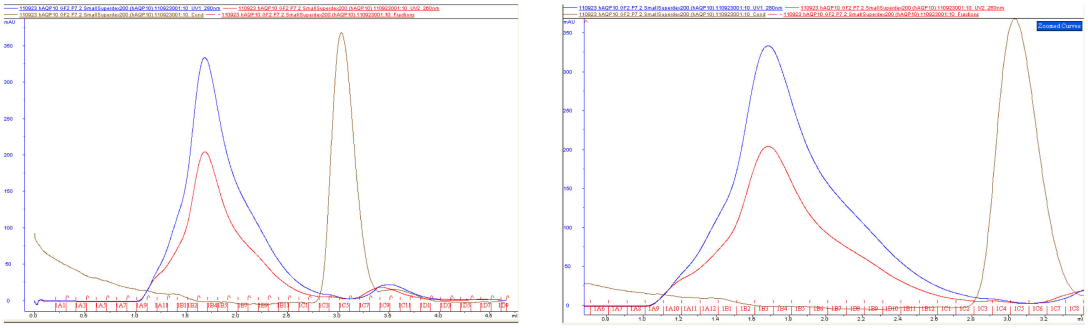

Figure 11: The chromatogram when the protein is purified in NG using GF2. The standard chromatogram is shown to the left and the zoomed area under the protein peak is shown to the right.

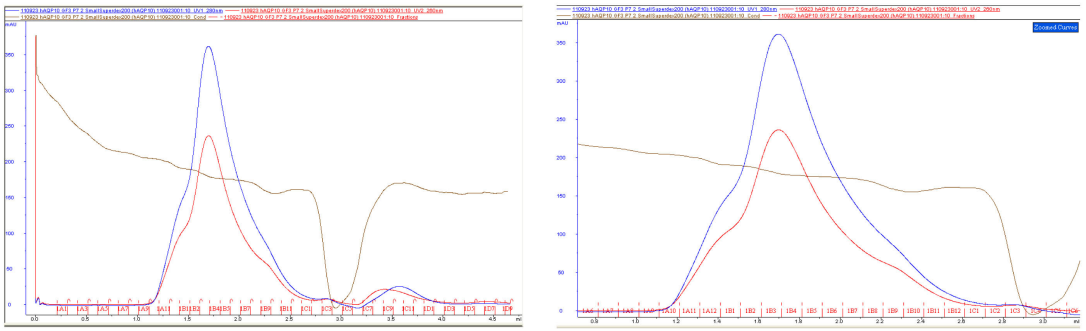

Figure 12: The chromatogram when the protein is purified in NG using GF3. The standard chromatogram is shown to the left and the zoomed area under the protein peak is shown to the right.

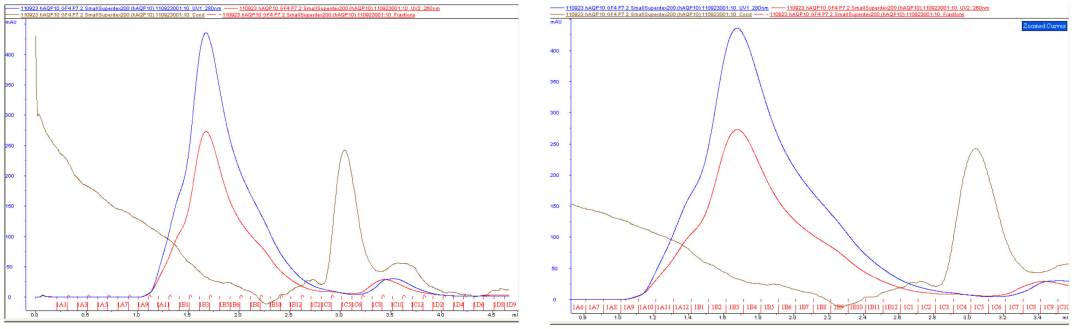

Figure 13: The chromatogram when the protein is purified in NG using GF4. The standard chromatogram is shown to the left and the zoomed area under the protein peak is shown to the right.

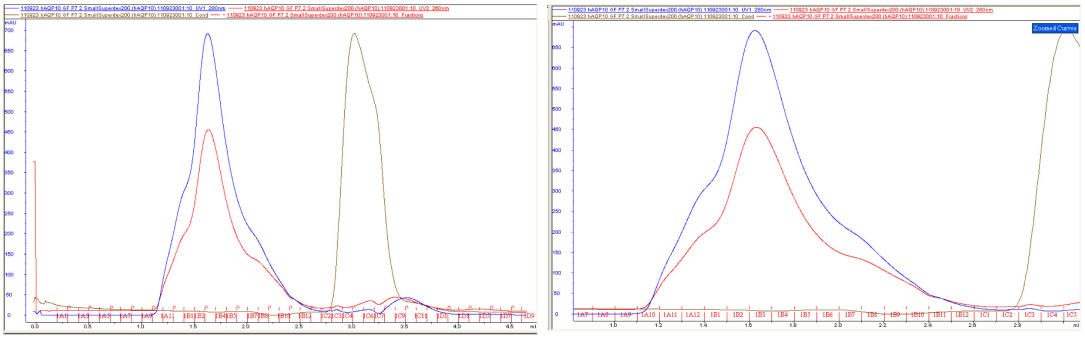

Figure 14: The chromatogram when the protein is purified in DM. The standard chromatogram is shown to the left and the zoomed area under the protein peak is shown to the right.

In all of the chromatograms the protein peak comes at 1.7ml and the curves look smooth. Fractions A10-B12 lie under the protein peak and these are run on SDS-PAGEs to determine the amount and purity of the fractions. The SDS-PAGEs are shown in Figure 15 and Figure 16. The reference SDS-PAGE for the purification in DM is shown in Figure 17.

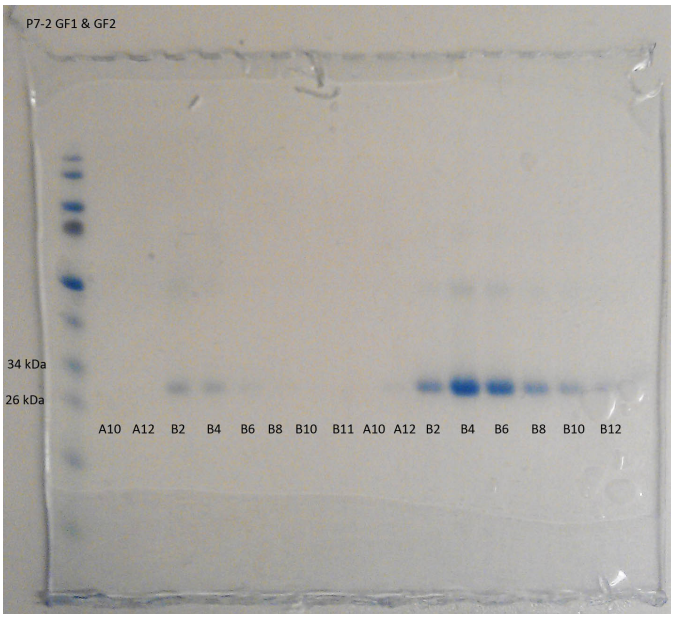

Figure 15: The SDS-PAGE showing the fractions under the protein peak after gel filtration. Fractions A10-B12 lie under the protein peak. Fractions from both GF1 (to the left of the gel) and GF2 (to the right of the gel) are shown in the figure. The ladder is shown to the left.

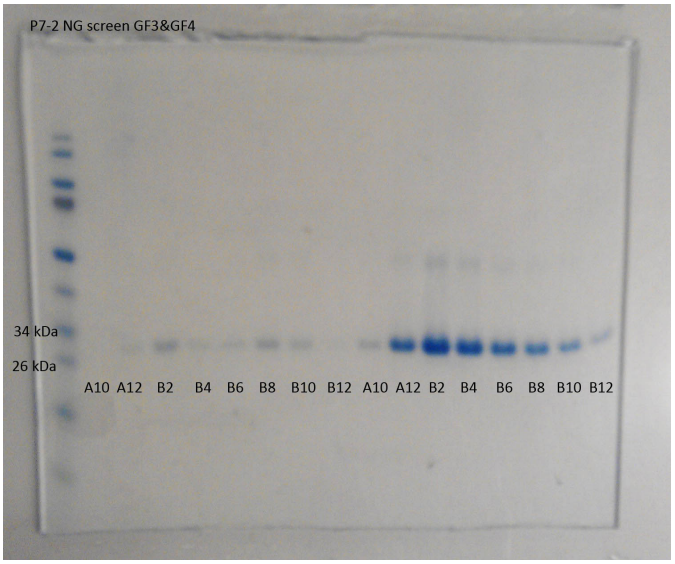

Figure 16: The SDS-PAGE showing the fractions under the protein peak after gel filtration. Fractions A10-B12 lie under the protein peak. Fractions from both GF3 (to the left of the gel) and GF4 (to the right of the gel) are shown in the figure. The ladder is shown to the left.

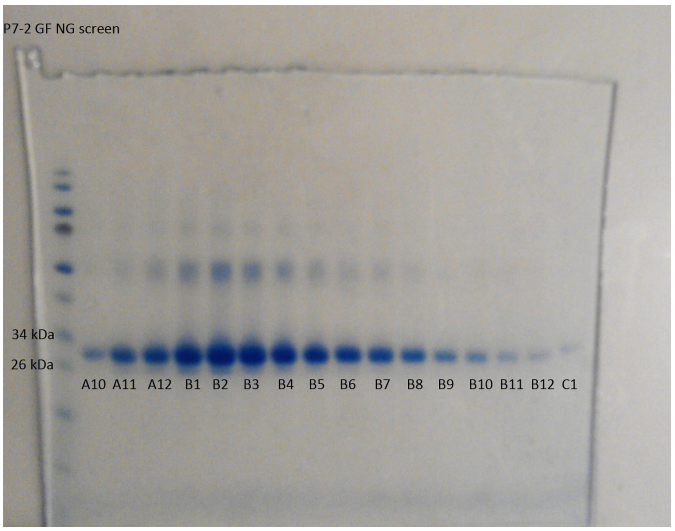

Figure 17: The SDS-PAGE showing the fractions under the protein peak after gel filtration in the detergent DM. Fractions A10-C1 lie under the protein peak. The ladder is shown to the left.

From the SDS-PAGE one can conclude that glycerol in the gel filtration buffer leads to the elution of more protein compared to not having glycerol in the buffer.

The fractions under the protein peak were concentrated further. The final protein concentration is shown below.

GF1: 0.18 mg/ml
GF2: 1.68 mg/ml
GF3: 0.50 mg/ml
GF4: 2.57 mg/ml
GF: 3.04 mg/ml

The protein was concentrated the most in the buffers where glycerol was present. However GF2 and GF4 contain 300mM NaCl and 100mM NaCl respectively. To investigate if the NaCl concentration affects the results, two assays are set up; one containing 300mM NaCl and one containing 100mM NaCl in the gel filtration buffer. The following SEC experiments are all run in a 24ml Superdex200 column.

4.5 300mM NaCl, 10% Glycerol

Stripped *P. pastoris* membranes were solubilized in solubilization buffer containing the detergent DM. IMAC purification was done manually by eluting fractions from a gravity flow column. The fractions were run on an SDS-PAGE which is shown in Figure 18.

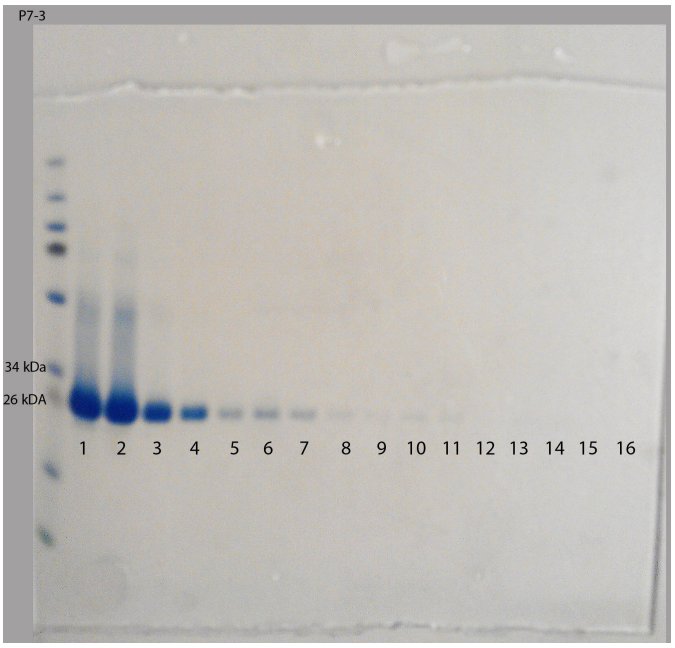

Figure 18: The SDS-PAGE showing the different fractions after elution with 300mM and 500mM imidazole. The ladder is shown to the left.

The SDS-PAGE shows that there is protein in fractions 1-16 and that the fractions look pure. Fractions 1-16 are further concentrated to $\sim$250μl and further purified on a Superdex200 column. The chromatogram is shown in Figure 19.

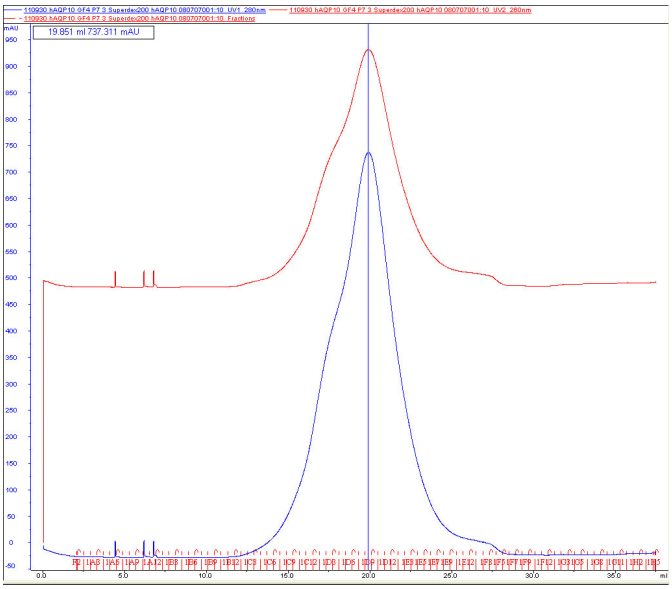

Figure 19: The chromatogram showing the fractions under the protein peak after gel filtration. Fractions C9-D12 are under the protein peak.

GF buffer
20mM Tris-HCl pH 7.5
300mM NaCl
0.4% NG

Fractions C9-D12 are run on an SDS-PAGE to see the amount of protein in the different fractions and also if the fractions look pure and free from any degradation product. The SDS-PAGE is shown in Figure 20.

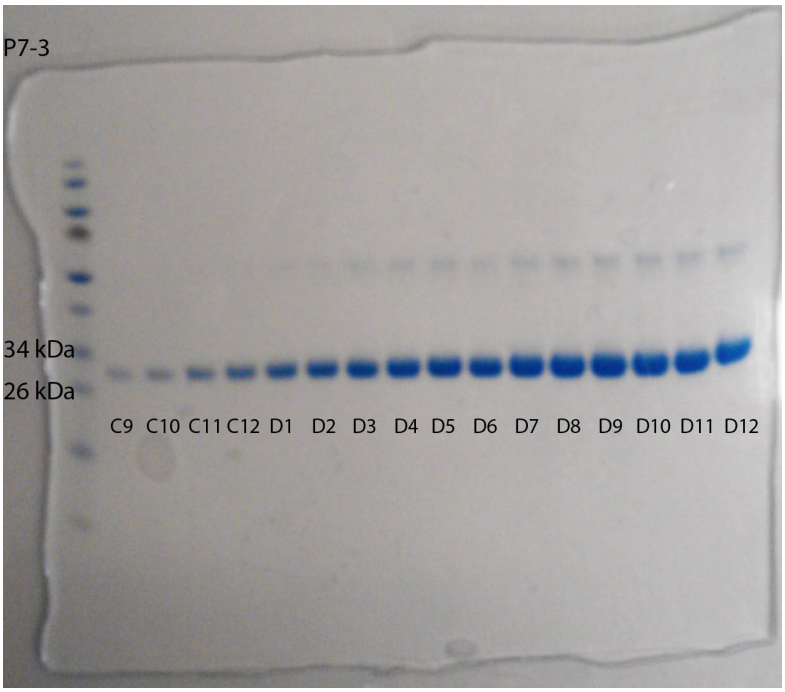

Figure 20: The SDS-PAGE showing the fractions under the protein peak after gel filtration. Fractions C9-D12 are under the protein peak. The ladder is shown to the left.

The SDS-PAGE on fractions C9-D12 shows that these fractions contain the hAQP10 protein and that the samples look relatively pure. The gel doesn't show a laddering effect of the bands but instead distinct dimer bands for each fraction.

All of the fractions are concentrated to a concentration of 9,02mg/ml and the concentrated protein sample is used for crystallization setups following the schedule in Table 2.

Table 2: Table showing the crystallization setups tested for the protein in NG

			PEG400	120	130	140	150	160	170
			mQ	280	270	260	250	240	230
100mM				1	2	3	4	5	6
50	pH 6.3		A	24% PEG, pH=6.3	26% PEG, pH=6.3	28% PEG, pH=6.3	30% PEG, pH=6.3	32% PEG, pH=6.3	34% PEG, pH=6.3
50	pH 7.8		B	24% PEG, pH=7.8	26% PEG, pH=7.8	28% PEG, pH=7.8	30% PEG, pH=7.8	32% PEG, pH=7.8	34% PEG, pH=7.8
50	pH 9.3		C	24% PEG, pH=9.3	26% PEG, pH=9.3	28% PEG, pH=9.3	30% PEG, pH=9.3	32% PEG, pH=9.3	34% PEG, pH=9.3
50	pH 10.8		D	24% PEG, pH=10.8	26% PEG, pH=10.8	28% PEG, pH=10.8	30% PEG, pH=10.8	32% PEG, pH=10.8	34% PEG, pH=10.8
			Li_2SO_4 50 ul in all of the wells						

Increasing the NaCl concentration seems to shift the elution of the proteins at 20 ml in NG instead of 12 ml in DM.

4.6 100mM NaCl, 10% Glycerol

Stripped *P. pastoris* membranes were solubilized in solubilisation buffer containing the detergent DM. IMAC purification was done manually by eluting fractions from a gravity flow column. The fractions were run on an SDS-PAGE which is shown in Figure 21.

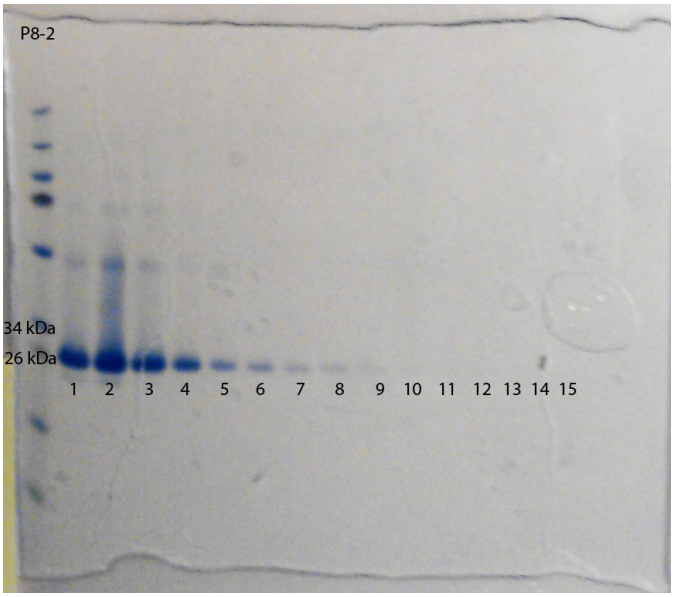

Figure 21: The SDS-PAGE showing the different fractions after elution with 300mM and 500mM imidazole. The ladder is shown to the left.

The SDS-PAGE shows that there is protein in fractions 1-15 and that the fractions look pure. Fractions 1-15 are further concentrated to $\sim$250μl and further purified on a Superdex200 column. The chromatogram is shown in Figure 22 and the zoomed curves are shown in Figure 23.

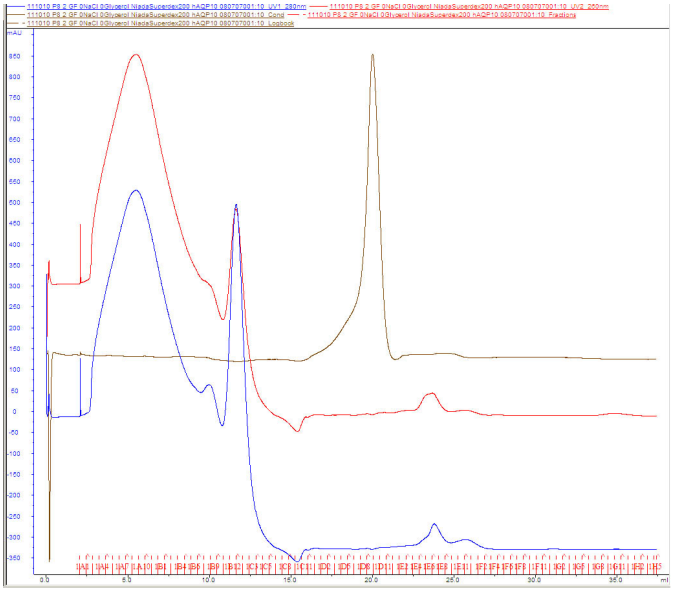

Figure 22: The chromatogram showing the fractions under the protein peak after gel filtration. Fractions B8-C9 are under the protein peak.

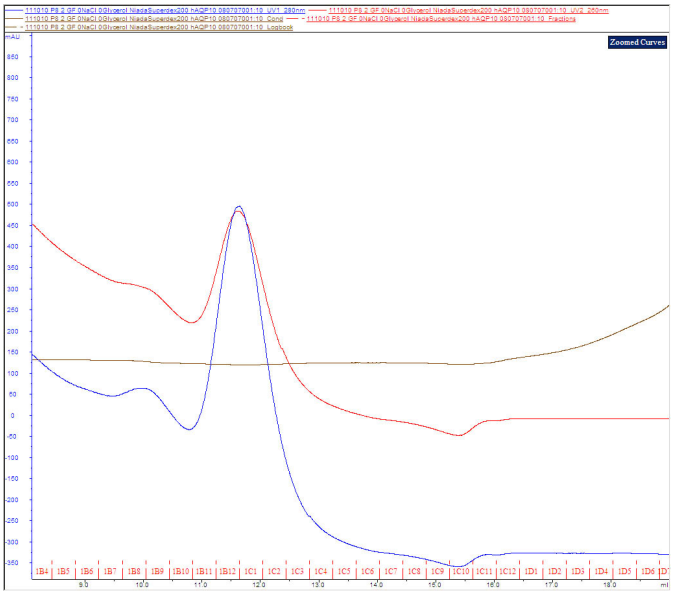

Figure 23: The chromatogram showing the fractions under the protein peak after gel filtration. Fractions B8-C9 are under the protein peak.

GF buffer
20mM Tris-HCl pH 7.5
100mM NaCl
10% Glycerol
0.4% NG

Fractions A3-B6 (first peak) and B7-C9 (second peak, protein peak) are run on two SDS-PAGEs to see the amount of protein in the different fractions and also if the fractions look pure and free from any degradation product. The SDS-PAGEs are shown in Figure 24 and Figure 25.

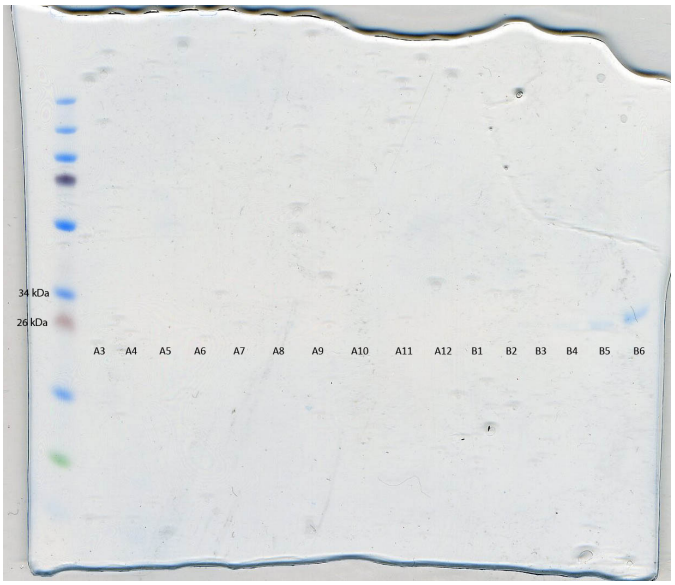

Figure 24: The SDS-PAGE showing the fractions under the first peak after gel filtration. Fractions A3-B6 are under the first peak. The ladder is shown to the left.

The SDS-PAGEs in Figure 24 and Figure 25 shows that there are bands on fractions B1-C8 but according to the chromatogram, fractions A3-B7 might be aggregates of the protein since they are eluted so early so these fractions are not concentrated further. Instead fractions B8-C9 under the protein peak at 12 ml are concentrated to 7.49mg/ml in a total volume of $\sim 49 \mu$l. The concentrated protein sample is used for crystallization setups following the schedule in Table 3.

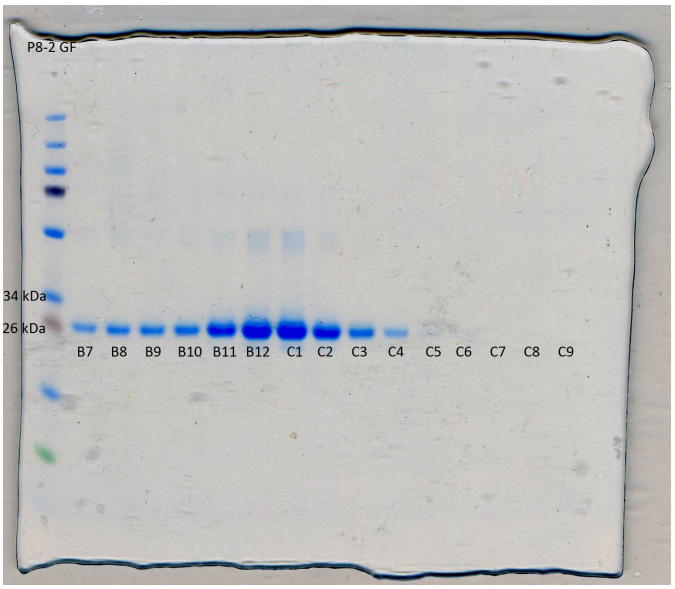

Figure 25: The SDS-PAGE showing the fractions under the protein peak after gel filtration. Fractions B7-C9 are under the protein peak. The ladder is shown to the left.

Table 3: Table showing the crystallization setups tested for the protein in NG

			PEG400	120	130	140	150	160	170
			mQ	280	270	260	250	240	230
100mM				**1**	**2**	**3**	**4**	**5**	**6**
50	**pH 6.3**		**A**	24% PEG, pH=6.3	26% PEG, pH=6.3	28% PEG, pH=6.3	30% PEG, pH=6.3	32% PEG, pH=6.3	34% PEG, pH=6.3
50	**pH 7.8**		**B**	24% PEG, pH=7.8	26% PEG, pH=7.8	28% PEG, pH=7.8	30% PEG, pH=7.8	32% PEG, pH=7.8	34% PEG, pH=7.8
50	**pH 9.3**		**C**	24% PEG, pH=9.3	26% PEG, pH=9.3	28% PEG, pH=9.3	30% PEG, pH=9.3	32% PEG, pH=9.3	34% PEG, pH=9.3
50	**pH 10.8**		**D**	24% PEG, pH=10.8	26% PEG, pH=10.8	28% PEG, pH=10.8	30% PEG, pH=10.8	32% PEG, pH=10.8	34% PEG, pH=10.8
			Li_2SO_4 50 ul in all of the wells						

A lower NaCl contration (100mM) seems to stabilize the protein more and generate larger amounts of concentrated protein compared to the case with 300mM NaCl. However the amount of concentrated protein is still relatively low.

The detergent n-Octyl-β-D-Maltopyranoside (OM) was tested in the search of finding a detergent that stabilizes the protein and also is suitable for crystal formation.

4.7 OM, 10% Glycerol

Stripped *P. pastoris* membranes were solubilized in solubilisation buffer containing the detergent DM. IMAC purification was done manually by eluting fractions from a gravity flow column. The fractions were run on an SDS-PAGE which is shown in Figure 26.

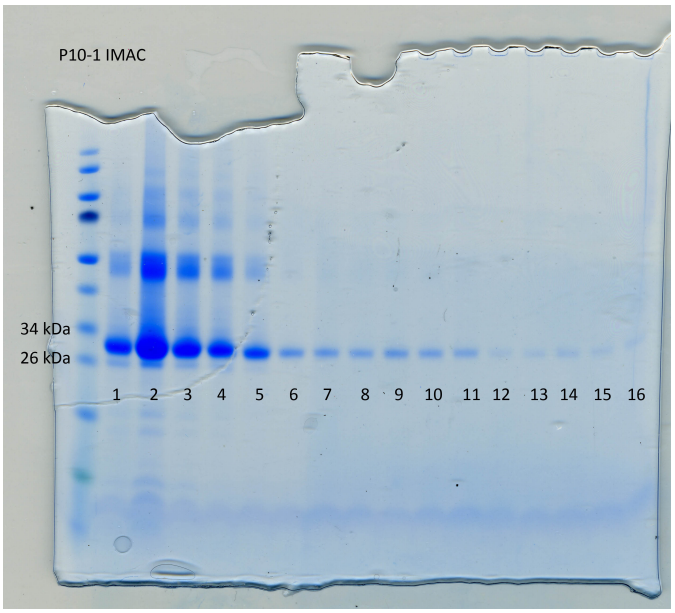

Figure 26: The SDS-PAGE showing the different fractions after elution with 300mM and 500mM imidazole. The ladder is shown to the left.

The SDS-PAGE shows that there is protein in fractions 1-16 and that the fractions look pure. Fractions 1-16 are further concentrated to $\sim$250μl and further purified on a Superdex200 column. The chromatogram is shown in Figure 27 and the zoomed curves are shown in Figure 28.

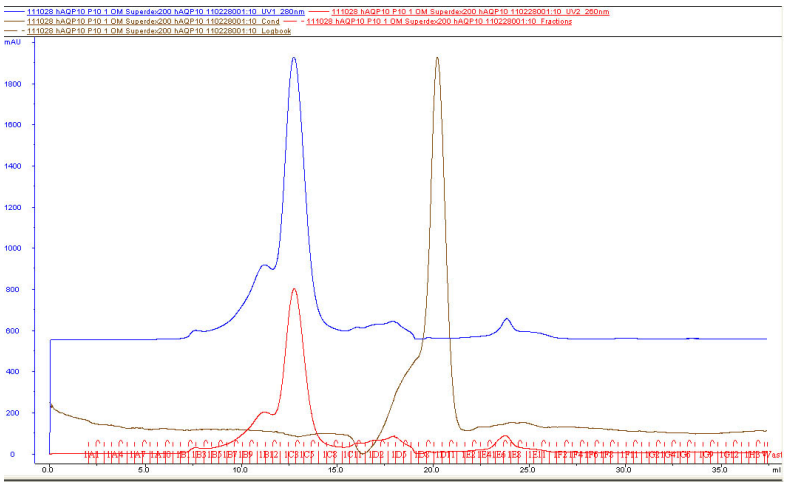

Figure 27: The chromatogram showing the fractions under the protein peak after gel filtration. Fractions B4-C7 are under the protein peak.

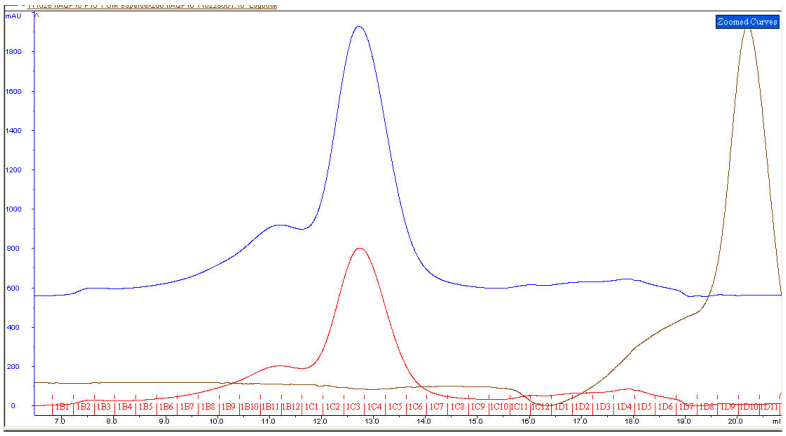

Figure 28: The chromatogram showing the zoomed curves at the protein peak after gel filtration. Fractions B4-C7 are under the protein peak.

GF buffer

20mM Tris-HCl pH 7.5
100mM NaCl
10% Glycerol
1.8% OM

Fractions B4-C7 lie under the protein peak and therefore these fractions are run on an SDS-PAGE to see the amount of protein in the different fractions and also if the fractions look pure and free from any degradation product. The SDS-PAGE is shown in Figure 29.

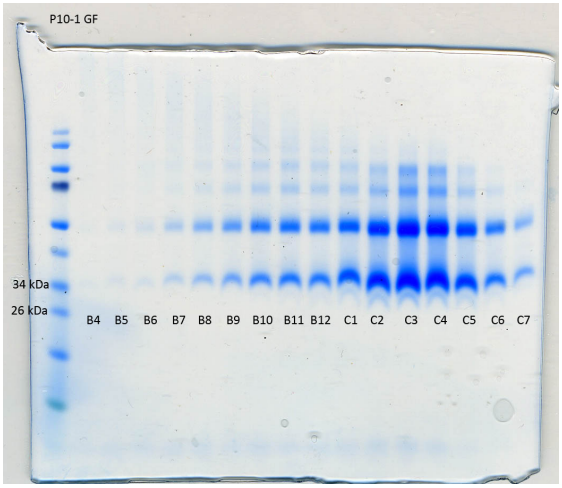

Figure 29: The SDS-PAGE showing the fractions under the protein peak after gel filtration. Fractions B4-C7 are under the protein peak. The ladder is shown to the left.

The SDS-PAGE on fractions B4-C7 shows that these fractions contain the hAQP10 protein and that the samples look relatively pure. The gel shows a laddering effect of the bands which correlates to previous work done on the hAQP10 protein and can be interpreted as a good sign. The bands look slightly bended which might be due to old 1xMES running buffer.

Fractions C1-C7 are concentrated to a concentration of 8.96mg/ml in a total volume of $\sim 95\mu$l and the concentrated protein sample is used for crystallization setups with the HoneyBee Robot. The MemGold crystallization conditions are tested.

Another parallel purification in the OM detergent was done where the fractions under the protein peak were concentrated to 10.56mg/ml in a total volume of $\sim 68\mu$l. Crystallization conditions from the MemGold screen were tested with the HoneyBee Robot simultaneously as the previous robot screen. This is done to determine if the concentration of the protein affects the crystallization.

No crystals were observed from the robot plate. The protein concentration doesn't seem to affect the formation of crystals with the MemGold screen.

4.8 DDM, OM

Stripped *pichia* membranes were solubilized in solubilisation buffer containing the detergent n-Dodecyl-β-D-Maltopyranoside (DDM). IMAC purification was done manually by eluting fractions from a gravity flow column. The fractions were run on an SDS-PAGE which is shown in Figure 30.

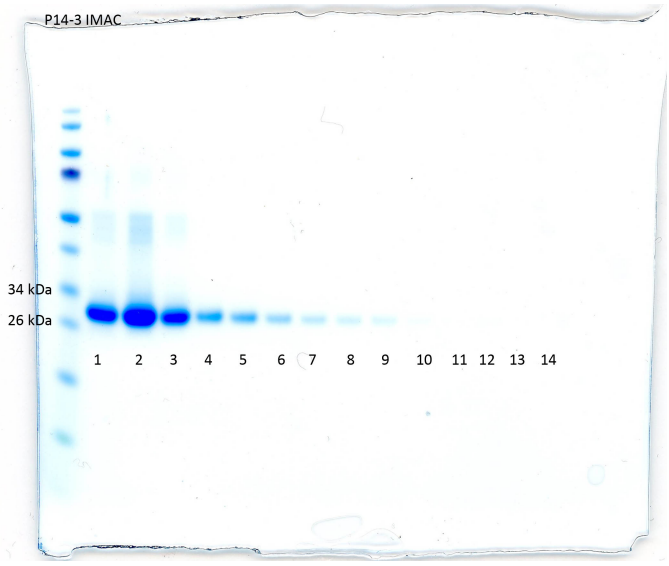

Figure 30: The SDS-PAGE showing the different fractions after elution with 300mM and 500mM imidazole. The ladder is shown to the left.

The SDS-PAGE shows that there is protein in fractions 1-14 and that the fractions look pure. Fractions 1-14 are further concentrated to $\sim$250μl and further purified on a Superdex200 column. The chromatogram is shown in Figure 31 and the zoomed curves are shown in Figure 32.

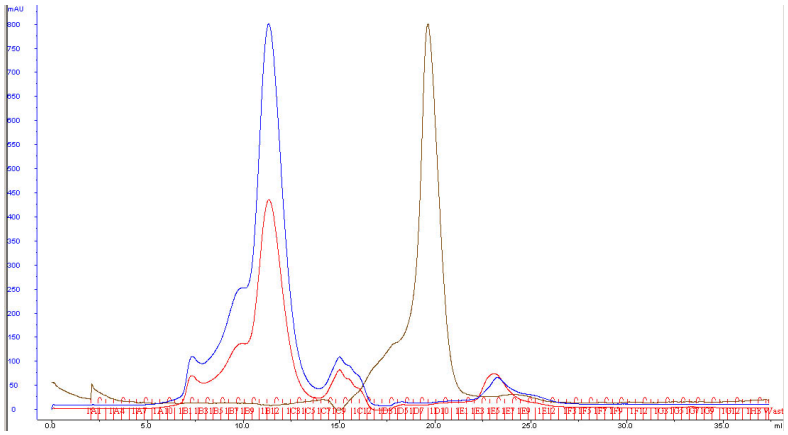

Figure 31: The chromatogram showing the fractions under the protein peak after gel filtration. Fractions B10-C5 are under the protein peak.

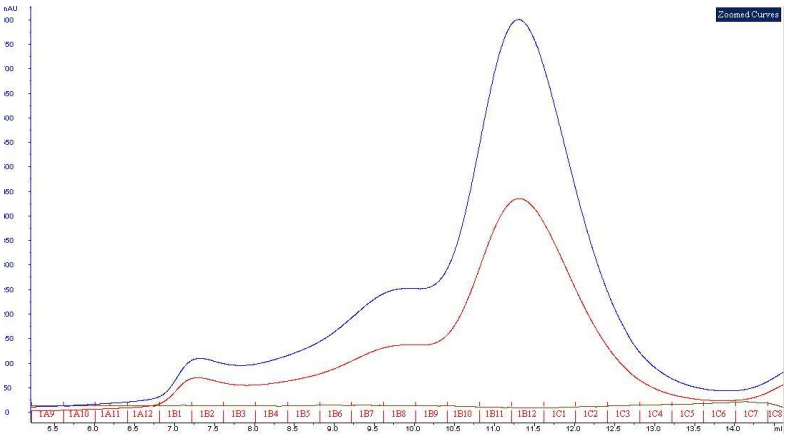

Figure 32: The chromatogram showing the zoomed curves at the protein peak after gel filtration. Fractions B10-C5 are under the protein peak.

GF buffer
20mM Tris-HCl pH 7.5
100mM NaCl
10% Glycerol
1.8% OM

Fractions B10-C5 lie under the protein peak and therefore fractions B9-C6 are run on an SDS-PAGE to see the amount of protein in the different fractions and also if the fractions look pure and free from any degradation product. The SDS-PAGE is shown in Figure 33.

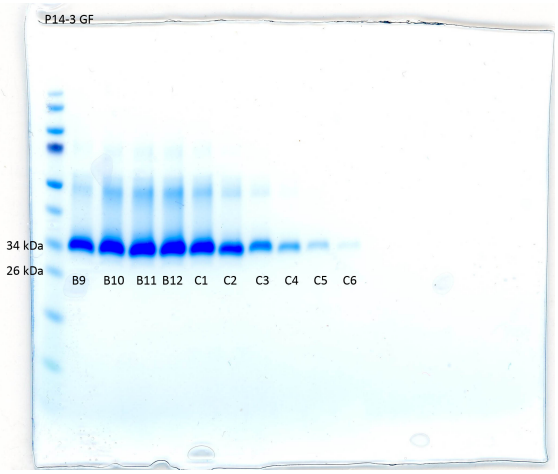

Figure 33: The SDS-PAGE showing fractions B9-C6 after gel filtration. Fractions B10-C5 are under the protein peak. The ladder is shown to the left.

Fractions B9-C6 contain the hAQP10 protein and the samples look pure. The gel shows a laddering effect of the bands.

Fractions B10-C5 are concentrated to a concentration of 12.78mg/ml in a total volume of $\sim$50μl and the concentrated protein sample is used for crystallization setups with the HoneyBee Robot. The MemGold crystallization conditions are tested.

No crystals were formed at the time of the screen but there was formation of precipitate and in some wells formation of a brown precipitate. OM doesn't seem promising as a detergent for crystallization. The focus was shifted back to the detergent NG and in trying to stabilize the protein with DDM.

4.9 DDM, NG

Stripped *pichia* membranes were solubilized in solubilisation buffer containing the detergent DDM. IMAC purification was done manually by eluting fractions from a gravity flow column. The fractions were run on an SDS-PAGE which is shown in Figure 34.

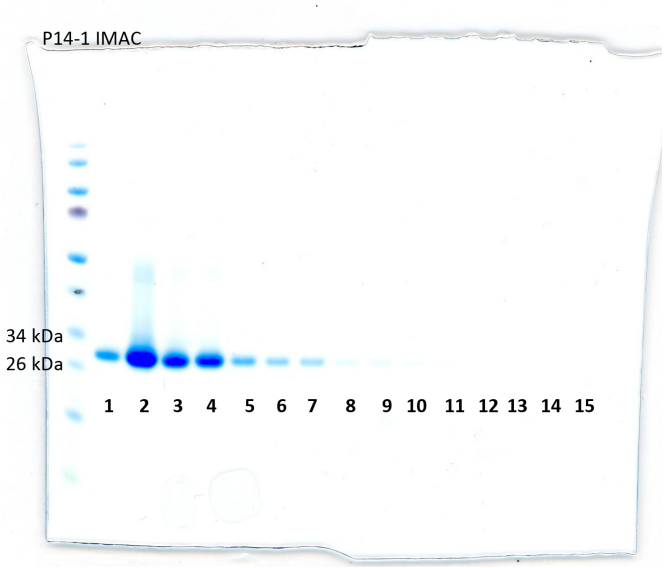

Figure 34: The SDS-PAGE showing the different fractions after elution with 300mM and 500mM imidazole. The ladder is shown to the left.

The SDS-PAGE shows that there is protein in fractions 1-15 and that the fractions look pure. Fractions 1-15 are further concentrated to $\sim$250μl and further purified on a Superdex200 column. The chromatogram is shown in Figure 35 and the zoomed curves are shown in Figure 36.

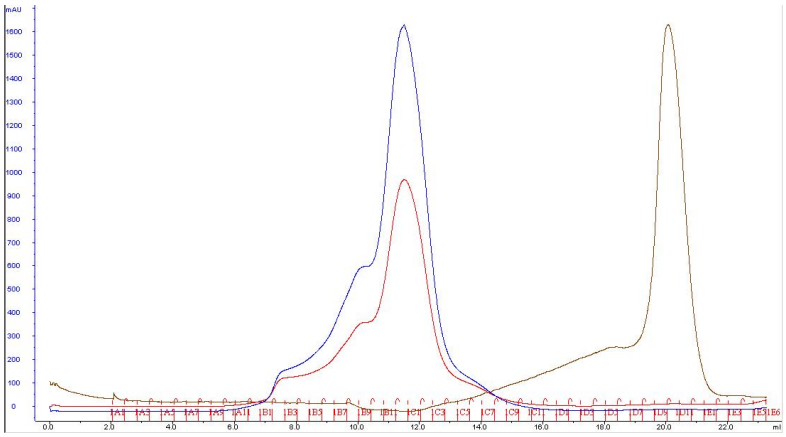

Figure 35: The chromatogram showing the fractions under the protein peak after gel filtration. Fractions B10-C5 are under the protein peak.

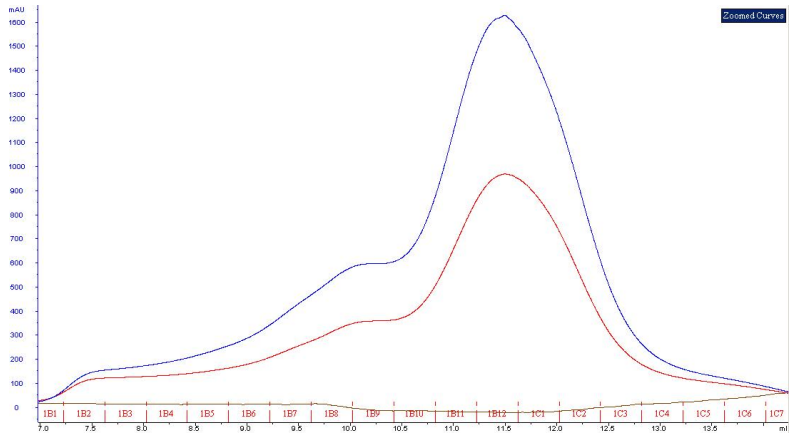

Figure 36: The chromatogram showing the zoomed curves at the protein peak after gel filtration. Fractions B10-C5 are under the protein peak.

GF buffer

20mM Tris-HCl pH 7.5
100mM NaCl
10% Glycerol
0.4% NG

Fractions B10-C5 lie under the protein peak and therefore these fractions are run on an SDS-PAGE to see the amount of protein in the different fractions and also if the fractions look pure and free from any degradation product. The SDS-PAGE is shown in Figure 37.

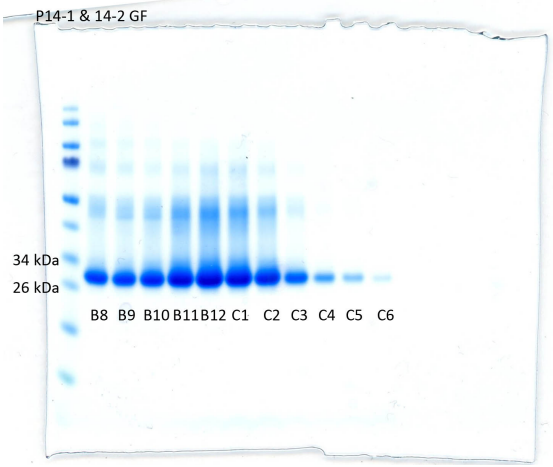

Figure 37: The SDS-PAGE showing the fractions under the protein peak after gel filtration. Fractions B10-C5 are under the protein peak. The ladder is shown to the left.

The SDS-PAGE on fractions B8-C6 shows that these fractions contain the hAQP10 protein and that the samples look pure. The gel shows a laddering effect of the bands.

Fractions B10-C5 are concentrated to a concentration of 7.52mg/ml in a total volume of $\sim$120μl and the concentrated protein sample is used for crystallization setups with the HoneyBee Robot. The MemGold, MemStart and MemSys crystallization conditions are tested. Mainly precipitate is seen in the crystallization wells. Another detergent is tested, n-Octyl-β-D-Glucopyranoside (β-OG) which has shorter side chains than NG.

4.10 DDM, β-OG

Stripped *P. pastoris* membranes were solubilized in solubilisation buffer containing the detergent DDM. IMAC purification was done manually by eluting fractions from a gravity flow column. The fractions were run on an SDS-PAGE which is shown in Figure 38.

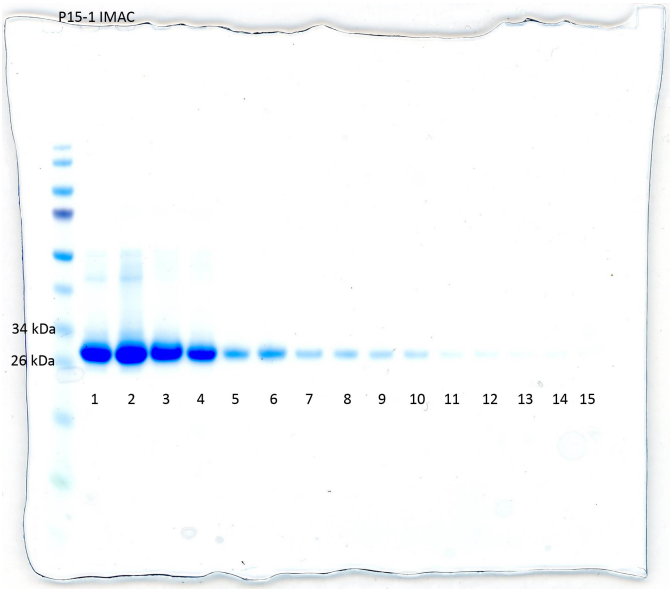

Figure 38: The SDS-PAGE showing the different fractions after elution with 300mM and 500mM imidazole. The ladder is shown to the left.

The SDS-PAGE shows that there is protein in fractions 1-15 and that the fractions look pure. Fractions 1-15 are further concentrated to $\sim$250μl and further purified on a Superdex200 column. The chromatogram is shown in Figure 39 and the zoomed curves are shown in Figure 40.

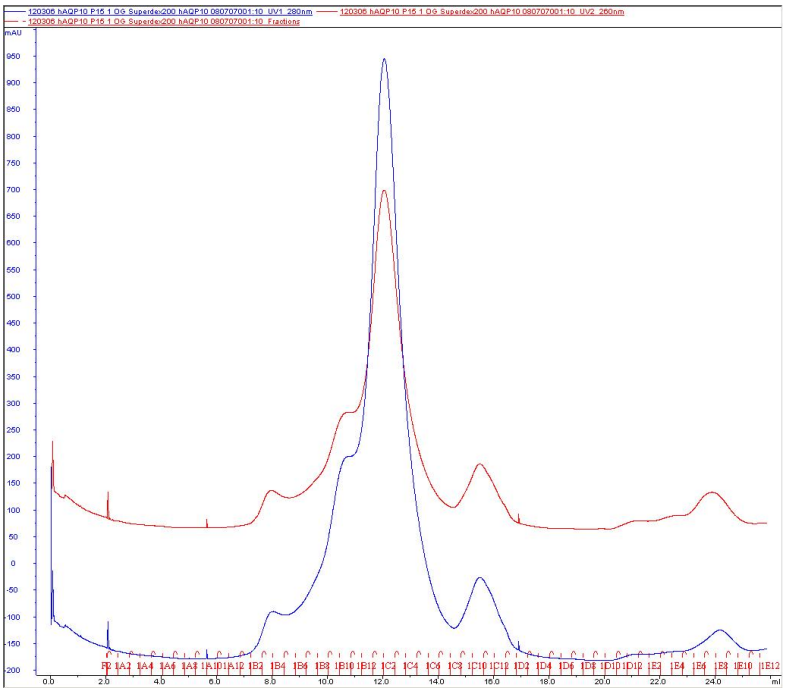

Figure 39: The chromatogram showing the fractions under the protein peak after gel filtration. Fractions B12-C7 are under the protein peak.

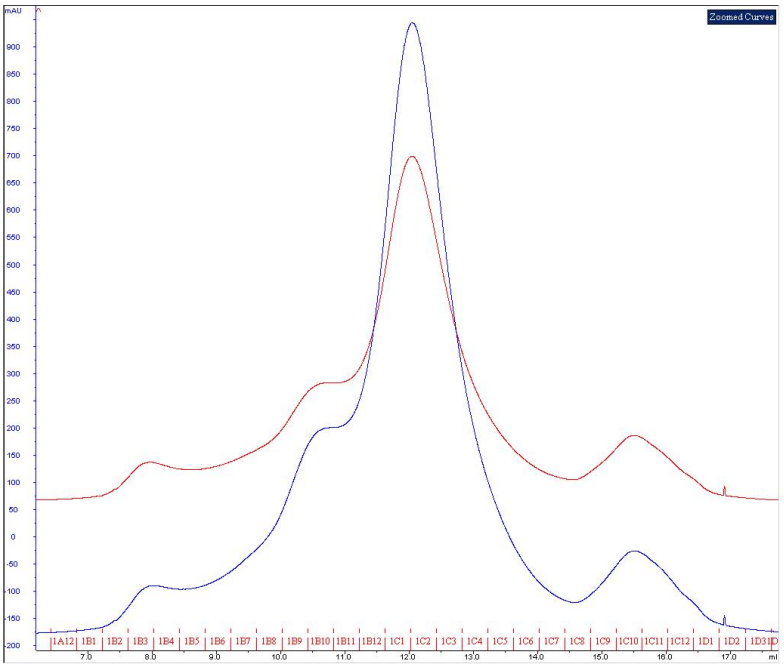

Figure 40: The chromatogram showing the zoomed curves at the protein peak after gel filtration. Fractions B12-C7 are under the protein peak.

GF buffer

20mM Tris-HCl pH 7.5
100mM NaCl
10% Glycerol
1% β-OG

Fractions B8-C8 are run on an SDS-PAGE to see the amount of protein in the different fractions and also if the fractions look pure and free from any degradation product. The SDS-PAGE is shown in Figure 41.

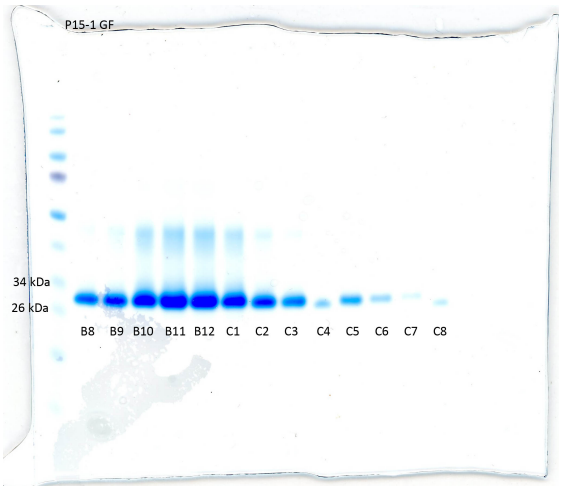

Figure 41: The SDS-PAGE showing the fractions under the protein peak after gel filtration. Fractions B8-C8 are are run in the SDS-PAGE. The ladder is shown to the left.

The SDS-PAGE shows that fractions B12-C7 contain the hAQP10 protein and that the samples look pure. The gel shows a laddering effect of the bands. These are further concentrated to a concentration of 16.67mg/ml in a total volume of $\sim$35μl and the concentrated protein sample is used for crystallization setups with the HoneyBee Robot. The MemGold crystallization conditions are tested.

Another purification was run in parallel where the fractions under the protein peak were concentrated to 10.54mg/ml in a total volume of $\sim$90μl. The concentrated protein sample was used for crystallization setups with the HoneyBee Robot. The conditions tested were those in the MemStart and MemSys both in room temperature and in the cold room.

In comparison to the other crystallization setups, β-OG seems to be the more promising detergent.Spheroids, which is a pre-crystallization phase were viewed on the robot plate in room temperature.

4.11 Immunohistochemistry of tape strips

The immunostaining of tape strips with fluorescent antibodies showed the presence of hAQP10 in *Stratum Corneum* skin cells. The results are shown in Figure 42.

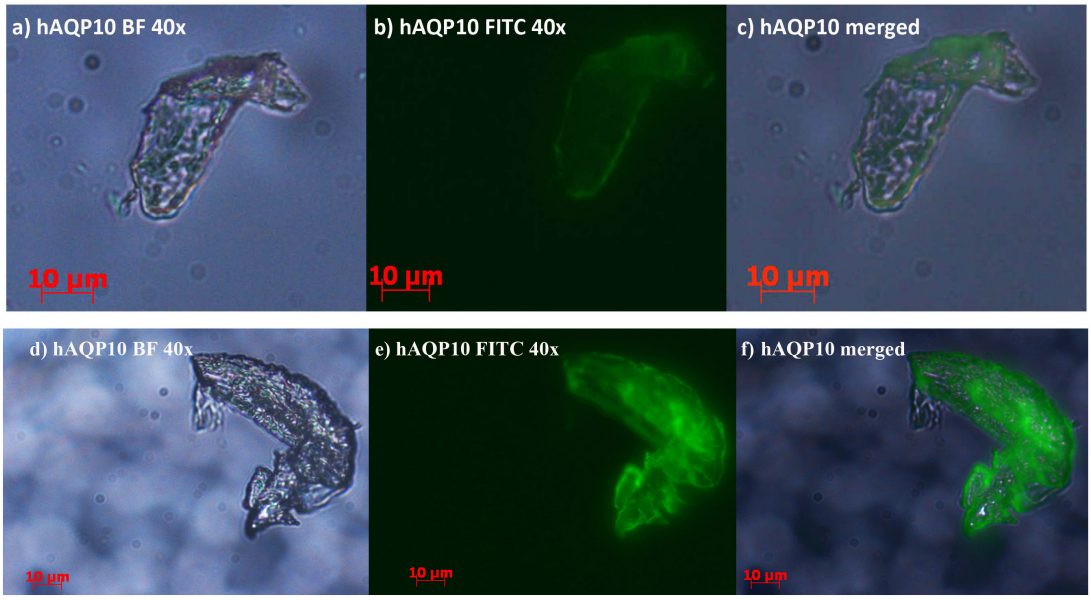

Figure 42: Microscope figures where the hAQP10 from skin samples on tape strips is detected with fluorescence. Two different corneocytes are shown in the figures with brightfield microscopy in a) and d), fluorescence in b) and e) and merged pictures in c) and f).

4.12 Immunoblot of skin scrapings

Skin samples from scalpel scrapings were incubated with 2x SDS sample buffer and loaded on an SDS-PAGE. The gels were then blotted on pvdf membranes and detected with antibodies. The immunoblot is shown in Figure 43.

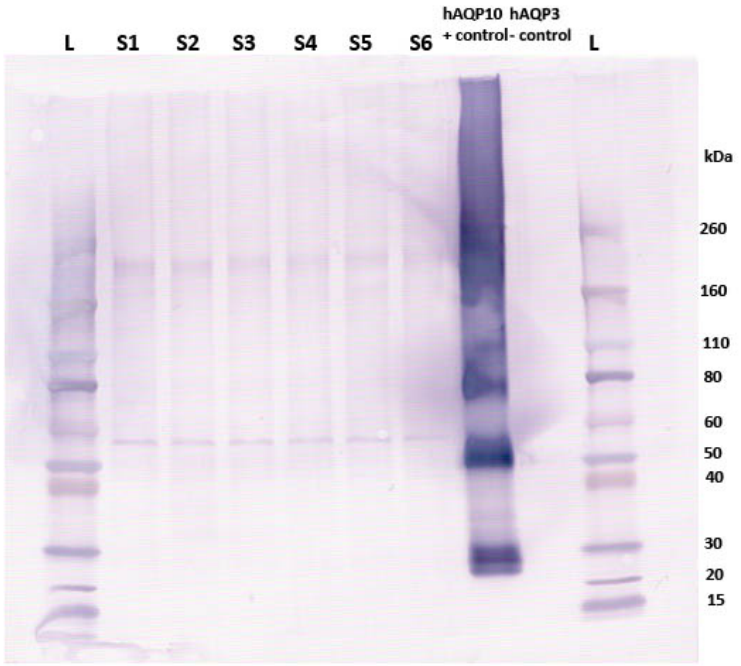

Figure 43: Immunoblot showing bands for the hAQP10 in five samples from the same person and scraping. The bands correlate with the positive control at the size of a dimer (∼60kDa) of hAQP10 overexpressed in *P. pastoris* and purified with IMAC and SEC. A negative control was also loaded in the gel for hAQP3 to ensure antibody specificity.

The immunoblot shows that there are bands about the size of a hAQP10 dimer (around ∼60kDa) that also correlate to a band in the positive control. Bands of multimer size (∼250kDa) can also be seen in all the wells.

4.13 Summary of purifications

A summary of all the performed purifications is shown in Figure 44. The most successful experiments are highlighted in the figure.

	Sol	Pur	Conc mg/ml	V µ	Cryst screen	Plates	Comments
P6-1	DM	DM	8.57		PEG/pH	2x24	Round "crystals", no diffraction
P6-2	DM	DM					Protein lost
P6-3	DM	DM	56.0				Not realistic conc?, frozen
P7-1	DM	NG	8.13	20.0	PEG/pH	1/2x24	Formation of precipitate, no crystals
P7-2	DM	NG screen					100mM NaCl + 10% Glycerol is good
P7-3	DM	NG	9.02	27.0	PEG/pH	2x24	Formation of precipitate, no crystals
P8-1	DM	NG 0%glc, 0mM NaCl					Aggregation, salt is important
P8-2	DM	NG	7.49	49.0	PEG/pH	2x24	Formation of precipitate, no crystals
P7-4+7-5	DM						Detergent dilution, 2 peaks in chromatogram
P9-1	DM	OM	11.3	70.0			
P9-2	DM	NG + Lipids	N/A	40.0			Not measurable concentration, frozen
P10-1	DM	OM	8.96	100.0	MemGold	Robot	Nothing
P10-2	DM	OM	10.56	68.0	MemGold	Robot	Nothing, concentration doesn't matter
P10-3	DM	OM	8.26	60.0			
P7-6	DM	OM	9.21	65.0			
P11-1	DM	OM					Frozen
P11-2	DM	OM					Frozen
P11-3	DM	OM					Aggregation due to storage in conc tube
P8-3+9-3	DM	OM					Aggregation due to storage in conc tube
P12-1	DM	DM	10.95	90.0			Denmark batch
P12-2	DM	DM	10.26	100.0			Denmark batch
P12-3	DM	DDM	8.26	120.0			Denmark batch
P12-4	DM	DDM	9.39	140.0			Denmark batch
P13-1	DDM						Protein lost, too low concentration of DDM
P13-2	DDM						Protein lost, too low concentration of DDM
P10-4+13-3	DDM						
P14-1	DDM	NG	7.52	120.0	MemGold	Robot	Formation of precipitate, no crystals
P14-2	DDM	NG	7.52	120.0	MemStart+Sys	Robot	Formation of precipitate, no crystals
P14-3	DDM	OM	12.78	50.0	MemGold	Robot	Formation of precipitate in high concentration
P15-1	DDM	β-OG	16.67	35.0	MemGold	Robot	Formation of precipitate, no crystals
P15-2	DDM	β-OG	10.54	90.0	MemStart+Sys	2xRobot	Spheroids in room temperature
P15-3	DDM	β-OG					

Figure 44: Summary of perfomed experiments. The most successful experiments are highlighted in the figure.

5 Discussion

Water channels are crucial to the survival of the cell under osmotic pressure. Human aquaporin 10 is both a water and glycerol transporter that hasn't been extensively studied yet. There is no three dimensional structure reported in the databases which could be due to a number of reasons. One possible factor that might influence the crystallization of hAQP10 is the large Loop C that is situated between transmembrane domain three (TMD3) and four (TMD4). The loop might hinder the proteins to get closer and form crystals. Loop C contains one of the three predicted glycosylation sites. A glycosylation forms a further branching on the aminoacid asparagine 133 further hindering the crystal formation. Therefore the hAqp10 gene was mutated on this glycosylation site by exchanging the asparagine 133 to a glutamine in order to increase the likelihood of crystal formation.

Purification in DM

Previous work on hAQP10 has shown that the protein is stable when solubilized and purified in DDM and DM. DDM binds strongly to the protein and generates a stable protein but it is not suitable for crystallization assays due to it's long chains (12 carbon groups) that might influence crystal formation. A detergent with shorter chains is preferred and therefore the protein was solubilized and purified in DM. The DM purification generated a concentrated protein sample that was used for crystallization assays. Round crystals were formed but no diffraction pattern could be obtained which suggests that DM is not suitable for crystallization assays and another detergent with shorter chains is needed. DM has shorter chains than DDM but in general it doesn't seem to be any major differences in the protein stability. It is believed that DM does not bind as strongly to the protein as DDM does, one reason being DDM's low critical micelle concentration (CMC). DM can therefore still be used to solubilize the protein and then it can be successfully exchanged to another detergent with shorter chains during the gel filtration step.

Experimental Design

The experimental design with the detergent NG showed that the presence of glycerol in the gel filtration buffer leads to the elution of more protein compared to the case with no glycerol in the buffer. Also the protein can be concentrated at higher concentration without aggregating with glycerol present. Glycerol might serve as an additional stabilizing agent together with detergent for the hydrophobic domains of the protein. It

is therefore advisable to include glycerol in the gel filtration buffer when handling the hAQP10 protein.

NaCl, 10% Glycerol

Another factor that is important for protein stability is the NaCl concentration. Two different assays were run, one with 300mM NaCl and one with 100mM NaCl, both in the presence of 10% glycerol. A high NaCl concentration seems to shift the elution of hAQP10 at 20 ml instead of normally being eluted at 12 ml from the Superdex200 column. This suggests that a high salt concentration affects the protein negatively when it comes to the stability of the hAQP10 tetramer. In a superdex200 column large molecules are eluted first and smaller molecules are eluted later. Because the protein is first eluted at 20 ml we have reason to believe that the tetramer has been split into smaller units and that is what is seen in the eluate. The aquaporin 10 water channel isn't preserved as a whole channel in higher NaCl concentrations. This is also seen in the SDS-PAGE after the gel filtration where only two states of the protein can be seen, a monomer and a dimer instead of a laddering effect with the different states of the protein that is usually seen after gel filtration. A lower protein concentration seems to stabilize the protein more and generate larger amounts of concentrated protein compared to the case with 300mM NaCl. However the amount of concentrated protein sample is still low (7.49mg/ml, 49μl) which is an indication that the protein might not be stable enough for crystallization assays.

OM, 10%Glycerol

OM is another detergent that was tested which generated larger amounts of concentrated protein sample compared to NG. OM has the same head group than DDM and DM but shorter side chains (8 carbon groups). DDM and DM have previously been shown to be preferred detergents for protein stability but not for crystallization assays due to long side chains. OM has shorter side chains which suggests that it might be more suitable for crystallization assays while still considering protein stability. However this detergent didn't generate any crystals either and there was barely any formation of precipitates. The protein concentration in OM might be too low at the tested standard concentration 8-10mg/ml and a higher concentration might be more favourable.

DDM, OM

Another trial was tested with the protein solubilized in DDM and purified in OM and later concentrated to a concentration of 15mg/ml. This generated brown precipitate which is an indication of protein degradation and a negative influence on the crystallization process. It is clearly an improvement compared to previous trials where nothing could be seen in the crystallization wells. One could try to find a more suitable concentration of the protein between 8mg/ml and 15mg/ml and see if it leads to protein crystals.

DDM, NG

Solubilization in DDM seems to stabilize the protein and generate more of the concentrated protein sample. The detergent NG seemed more exciting for crystallization trials but it didn't generate much of the concentrated protein sample. Another trial was tested with solubilization in DDM and purification in NG to see if there were any improvements in the protein stability and amount of concentrated protein sample. The protein could be concentrated at 7.5mg/ml at a volume of 120μl which is a clear improvement compared to previous trials where we could only get 20-30μl concentrated sample. No crystals could be seen in the crystallization plates but there was precipitation, which is a good sign.

DDM, β-OG

β-OG has even shorter chains than NG and has often been used in the three dimensional structures that have been reported in the databases. Considering the improvement in stability and concentrated protein sample in NG, another trial was set up by solubilizing the protein in DDM and purifying in β-OG. The sample could be concentrated up to 16.7mg/ml at a volume of 35μl which was enough for a robot screen. Another purification was done in parallel where the sample was concentrated to 10.5mg/ml at a volume of 90μl. The hAQP10 has never before been stable enough to concentrate in β-OG. As previously mentioned, a reason for the stability could be that DDM molecules bind strongly to the protein residues and do not get exchanged entirely for β-OG molecules in the gel filtration step. One possible theory is that we might get a mix of both DDM and β-OG molecules after the purification steps which seems to be stabilizing for the hAQP10 tetramer.

Further studies need to be carried to investigate the potential of DDM and β-OG detergent molecules for crystallization trials. Hopefully this will lead in the near future to

a three dimensional structure of the first human aquaglyceroporin to be reported in the databases.

Immunoblot of skin scrapings

The hAQP10 protein has recently been detected in the skin *stratum corneum* through mRNA studies. In this project the hAQP10 protein was detected in skin samples by an immunoblot of skin scrapings and immunostaining of tape strips. Both methods have been successful in detecting the protein. In the PVDF membranes from the immunoblot the protein can be seen as bands of a dimer size that correlate to a band in the positive control. The antibody is specific for the hAQP10 because no bands can be seen for the negative control (hAQP3). Because the samples are from the same person and from the same vial containing the skin scrapings sample, the bands are all similar and of the same size. Further trials were carried with samples from different persons but these results are not shown here due to secrecy. What can also be seen in the immunoblot is bands of a multimer size for all the samples. This could be a sign of aggregation due to the handling and preparation of the samples. Since it is real human tissues we are working with, we cannot expect all of the proteins in the tissue to be solved by the detergent. The clear bands that we see are proteins that have been solved by the detergent and can run through the gel but the multimer sized bands could be proteins and tissue rests that form aggregates. Overall the dimer bands are really clear and convincing that what we are seeing is the hAQP10.

Immunohistochemistry of tape strips

From the immunostaining of the tape strips we could see fluorescent pictures of corneocytes. This was later confirmed the collaboration partners from the Bispebjerg Hospital that what is seen in the pictures is a corneocyte with a typical fold. Trials were run with blank samples to outrule the possibility of artifacts showing as fluorescence. Barely anything could be seen in the blank samples stained with fluorescent antibodies which further strengthens the fact that what is detected in the pictures is the hAQP10 protein.

6 Conclusions

The most important conclusions from this project are presented below.

- The protein doesn't form crystals when it is solubilized and purified in DM.
- The protein is not so stable in NG when solubilized in DM. The amount of concentrated protein is relatively low.
- The protein is stable in NG when solubilized in DDM. This is probably due to DDM binding strongly to the protein residues and not being substituted entirely for NG detergent molecules during the purification steps.
- A lower NaCl contration (100mM) seems to stabilize the protein more and generate larger amounts of concentrated protein compared to the case with 300mM NaCl.
- Glycerol seems to increase the eluted amount of protein during gel filtration compared to not having glycerol at all. 10% glycerol gives good results in this project.
- Increasing the NaCl concentration from 100mM to 300mM in the presence of 10% glycerol in the gelfiltration buffer seems to affect the stability of the hAQP10-tetramer negatively. The protein is eluted at 20 ml instead of the expected 12 ml.
- No NaCl and no glycerol in the gel filtration buffer (NG) results in the formation of aggregates during the concentration of the protein.
- The protein is rather stable in OM. The protein doesn't form crystals when it is purified in OM at different concentrations and the tested conditions are those in MemGold, MemStart and MemSys.
- The protein is not stable when solubilized in DM and purified in NG or β-OG. The amount of concentrated protein is relatively low in the NG case.
- The protein is stable when solubilized in DDM and purified in NG or β-OG. The amounts of concentrated protein are much higher then when the protein is solubilized in DM.
- The DDM in solubilization and β-OG in purification trials have been the most successful in the crystallization assays generating spheroids which is a pre-crystallization phase.
- Solubilization in DM and purification in NG have also been very interesting in crystallization assays.

- Generally more is seen in the crystallization plate when the detergent used has a glycopyranoside head group.
- The hAQP10 protein is present in the *stratum corneum* of the skin where it most probably is involved in the hydration of the skin.

7 Future work

Currently there is no crystal formation for the hAQP10 protein in the different tested conditions. The closest to a crystal have been the pre-crystallization structures called spheroids which were viewed in the combo of DDM and β-OG. The next step would be to set up a manual crystallization screen by using the commercially available screens and concentrated protein in the DDM/β-OG mix. If the results are reproducible and there is still no crystal formation then one could try to optimize and screen for the conditions around the lead.

The advantage by using the crystallization robot is that the human factor is eliminated. The drops that are set up by the robot are generally homogenous and of the same size. The major disadvantage is that the drops are set up in room temperature and the crystal plate is then transferred to the 4°C room. This could influence the crystal formation by generating unstable crystals or no crystal formation at all. Also generally crystals that are generated by the robot are very small and hard to fish. Such issues could be eliminated by setting up a screen manually in the 4°C room.

Another track is to look into the detergent NG more closely and try to optimize the gel filtration buffer to generate a more stable protein. Large amounts of the protein are needed to do a screen with the commercially available crystallization screens (0.5-1μl drops in a 96 well plate).

One could always test other detergents but it seems that with aquaglyceroporins, trial and error assays are not productive. Another strategy should be pursued where focus is laid on protein stability by directed evolution. A stable protein is required for crystal formation. One could try to stabilize the hAQP10 tetramer with random mutagenesis and screen for mutants with functional protein in detergents that are suitable for crystallization. In this project the protein was solubilized in DDM and purified in β-OG due to previous trials resulting in aggregation of the protein when solubilized and purified in β-OG. The approach of random mutagenesis and screening for stability in β-OG will be pursued by a master thesis student in the near future.

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Appendices

A Membrane preparation

Breaking buffer

50mM KPi, pH 7.5
5% Glycerol

Urea wash buffer
4M Urea (mw 60.06 g/mol)
5mM Tris (mw 121.14 g/mol)
2mM EDTA
2mM EGTA
Change pH to 9.5 with HCl

Membrane resuspension buffer
20 mM Hepes (mw 238.3 g/mol) pH 7.8
50 mM NaCl (mw 58.44 g/mol) 10% Glycerol
2mM β-mercaptoethanol (14.9M)

1M KPi pH 7.5
The base will have pH 9.1 and the acid pH 4.2 A 50/50 mix will give pH 6.6. Since we want pH 7.5 more of the base is needed.

Base K_2HPO_4 (mw 174.18) 174g

Acid KH_2PO_4 (mw 136.09) 68g

To 100 ml base approximately 25 ml acid was added to get the correct pH

EDTA: Ethylene-dinitrilo-tetraacetic-acid, C6H16N2O8, mw 292.25 g/mol, Titriplex II

EGTA: Ethylene-glycol-tetraacetic-acid C14H24N2O10, mw 380.35 g/mol, Titriplex IV

0.1M EGTA EGTA 1.9g Change pH to 8.0 with NaOH

B Protein purification

Solubilisation buffer
Membrane resuspension buffer
2 % n-Decyl-β-D-Maltopyranoside (DM)
0.02 % n-Dodecyl-β-D-Maltopyranoside (DDM)
1.8 % n-Octyl-β-D-Maltopyranoside (OM)
0.4 % n-Nonyl-β-D-Glucopyranoside (NG)
1 % n-Octyl-β-D-Glucopyranoside (β-OG)

2xNi-buffer
40 mM HEPES pH7.8
600mM NaCl (Mw 58.44 g/mol)
20% Glycerol
4mM β-mercaptoethanol
0.6% n-Decyl-β-D-Maltopyranoside (DM)

Buffer EQ
2xNi-buffer
mQ
10mM imidazole

Wash buffer
2xNi-buffer
mQ
50mM imidazole

Elution buffer300

2xNi-buffer
mQ
300mM imidazole

Elution buffer500
2xNi-buffer
500mM imidazole

GF buffer
20mM Tris-HCl pH 7.5
100mM NaCl
0.3% DM or another detergent

C Fermentor growth

Start media	**1.5 L**
Phosphoric acid 85%	40mL
Calcium sulphate	1.4 g
Potassium sulphate	27.3 g
Magnesium sulphate heptahydrate	22.35 g
Potassium hydroxide	6.20 g
Glycerol 87%	69 g
MQ water	to 1.5 L
PTM_1 trace salts	**1.0 L**
Cupric sulphate pentahydrate	6.0 g
Sodium iodine	0.08 g
Manganese sulphate monohydrate	3.0 g
Sodium molybdate dihydrate	0.2 g
Boric acid	0.02 g
Cobolt chloride	0.5 g
Zinc chloride	20.0 g
Ferrous sulphate	65.0 g
Biotin	0.2 g
Sulphuric acid	5 mL
MQ water	to 1.0 L

BMGY media for pre-cultures	**500 mL**
Yeast Extract	5 g
Pepton aus fleisch	10 g
mQ	350 mL
1M KPi, pH 6	50 mL
10x YNB	50 mL
500x B	1 mL
10x GY	50 mL
Glycerol feed	**500 mL**
Glycerol 87%	287 mL (353 g)
PTM_1 trace salts	6 mL
mQ	to 500 mL
Methanol feed	**250 mL**
Methanol 100%	250 mL
PTM_1 trace salts	3 mL

D Immunohistochemistry

Antibodies stock solutions:

Anti-AQP3 Stock: 0.4 mg/ml
Anti-AQP3 is supplied as lyophilized powder from PBS, 1% BSA and 0.025% sodium azide. To make a 0.4 mg/ml solution, the content of the vial is dissolved in 50μl mQ. The solution is aliquoted in 5μl per eppendorf tube and stored at minus 20°C until usage.

Anti-AQP10 Stock: 0.5mg/ml
Anti-AQP10 is supplied as 50μg lyophilized powder from PBS buffer with 2% sucrose. To make a 0.5mg/ml solution, the concent of the vial is dissolved in 100μl mQ. The solution is aliquoted in 10μl per eppendorf tube and stored at minus 20°C until usage.

Alexa Fluor 546 goat anti-rabbit IgG
The antibody is supplied as 50μl liquid with 2mg/ml concentration. The solution is aliquoted in 10μl and stored at minus 20°C until usage.

PBS: 200ml
1 PBS tablet is dissolved in 200ml mQ.

PBT (PBS + BSA + Triton X-100) solution: 50ml
49.5ml PBS

0.05ml Triton X-100
0.1g BSA
Bring into solution by vortexing 5min.

Blocking solution (PBT + goat serum): 10ml
9.5ml PBT
0.5ml goat serum
Mix together