*Strep*II-tagged MscL

Isolation and characterization

Rienk Sytze Dijkstra *Bachelor Research Project* *March-July 2009*

Aim

The aim of this bachelor research project is isolating functional *Strep*II-tagged G22C-MscL in high quantity and purity.

The picture on the cover shows the crystal structure of a StrepII-tag bound to one of the subunits of Strep-Tactin, which is an engineered version of streptavidin.

Abstract

Mechanosensitive channels are found in the membranes of cells from all sorts of living species and are critical in the response to touch, hearing and osmoregulation. The mechanosensitive channel of large conductance, MscL, functions as a safety valve to protect the prokaryotic cell against bursting because of hypo-osmotic downshifts. The pore of this channel is opened because it can sense membrane tension in the lipid bilayer. In this study, *Strep*II-tagged MscL from *Escherichea coli* (*Eco*-MscL) was isolated by means of *Strep*-Tactin affinity chromatography. This method yielded protein in high concentration (0.19 mg/ml) and high purity. The yield of the elution fractions could be improved more than tenfold by inducing the *E.coli* cells with 0.1% L-arabinose, instead of the recommended value of 0.001%. This study also confirmed that avidin was able to mask biotinylated host proteins, which led to a higher degree of purity. With the help of the G22C-mutation, which makes it possible to open the channel without applying tension, and the real-time fluorescence dequenching assay it was possible to measure the activity of the channel *in vitro*. The optimized reconstitution setup showed 100% activity for G22C-*Strep*II MscL by using 20 mg/ml azolectin for creating artificial liposomes and Triton X-100 from Fluka to titrate the lipids. Therefore, no loss of function was seen because of the introduction of the *Strep*II-tag into MscL. This research project thus has shown that the S*trep*II-tag is a practical recombinant tool for isolating MscL.

General information

Name of student:	Rienk Sytze Dijkstra
Student number:	S1531174
Email address:	R.S.Dijkstra@student.rug.nl

Credits:	20 ECTS
Course code:	CHBAPRO20E
Name of course:	14 Weeks Bachelor Onderzoeksproject
Period:	March - July 2009
Group:	Membrane Enzymology
Supervisors:	Dr. A. Koçer and Mr. J.P. Birkner
Examiner:	Prof. B. Poolman

Abbreviations

BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue
***Eco*-MscL**	MscL from *Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
G22C	*Eco*-MscL glycine to cysteine
His-tag	Hexahistidine tag
kDa	Kilo Dalton
MS	Mechanosensitive
MscL	Mechanosensitive channel of large conductance
MTSET	[2-(trimethylammonium)ethyl] methanethiosulfonate
OD	Optical density
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
***Tb*-MscL**	MscL from *Mycobacterium tuberculosis*
TM	Trans membrane
TY^+ broth	Trypton-yeast broth supplemented with 100 µg/ml Ampicillin and 10 µg/ml Chloramphenicol

Table of contents

1. Introduction

Mechanosensitive channels (MS) are channels that can be found in the membranes of prokaryotes and eukaryotes (1). These channels are capable of forming pores allowing ions and small molecules to flow in and out of the cell (2). Mechanosensitive channels have the ability to transduce mechanical tension into electrochemical signals (3) which make cells able to respond to stimuli as gravity, sound, touch and pressure (4). The patch-clamp technique (5) allowed the measurement of single MS channel activity, making it easier to study the channels. One of the mechanosensitive channels in prokaryotes has been of particular interest the last twenty years, namely the mechanosensitive channel of large conductance (MscL) (6). This channel plays an important role in osmotic regulation of bacteria (7). A bacterium in a natural environment comes in contact with constantly changing osmolarity, through the effects of, for example, rain. When exposed to a low osmotic environment, the cells start to swell because of a massive influx of water. This results in the buildup of turgor pressure, generating tension in the membrane, which is sensed by MscL. When the tension reaches a certain threshold value, the non-selective pore opens, allowing ions, small molecules and even small proteins to be released to the exterior of the cell (8) (9). MscL thus functions as an emergency release valve to protect the bacterial cell from bursting.

The mechanosensitive channel of large conductance from *E.coli, Eco*-MscL, is the best studied MS channel. The *mscL* gene, which fully encodes the protein forming of *Eco*-MscL, was first cloned and sequenced by Sukharev *et al.* (10), showing that the channel was formed out of proteins of 136 amino acids. Chang *et al.* resolved the structure of MscL in the closed state of the *Eco*-MscL homologue from *Mycobacterium tuberculosis* (*Tb*-MscL) by X-ray crystallography at 3.5 Å resolution (4). This showed that MscL is a homopentameric protein with each subunit having two α-helical transmembrane (TM) domains, TM1 and TM2, joined by a periplasmic loop and both the N- and C-terminus are found on the cytoplasm (**Figure 1**).

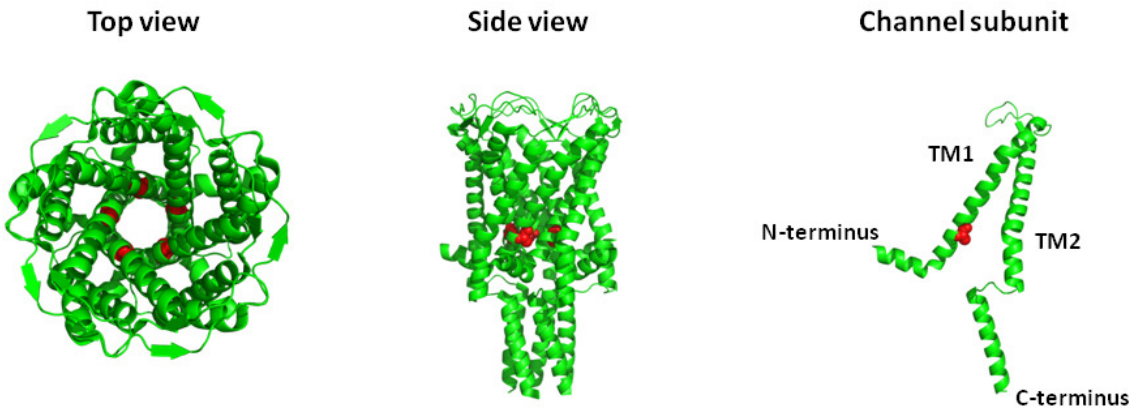

Figure 1: 3D crystal structure of the MscL homopentamer (left and middle) and the channel monomer (right) from *M. tuberculosis* (4). The alanine residues at position 20 in the constricted part of the pore are shown in red.

The five tightly packed TM1 helices form the constricted pore of the complex, whereas the TM2 helices surround the TM1 helices and interact with the lipids. Under normal conditions, MscL is closed with a constriction pore of about 2 Å wide. When the channel opens in response to tension in the membrane, it makes a large conformational change (11), resulting in a pore diameter of about 30 Å. The channel then 'flickers' between the opened and closed state (12) (13). In *Eco*-MscL, residue 22, by analogy to residue 20 in *Tb*-MscL, is buried within the constricted part of the channel and can be substituted to a cysteine, making charging of the amino-acid possible. The introduction of charged amino-acids to the constricted part of the channel by site-directed mutagenesis can lead to the spontaneous opening of the channel (14) (15).

The aim of study is to isolate *Strep*II-tagged G22C-MscL in high quantity and purity by means of *Strep*-Tactin affinity chromatography (**see 1.1**). In order to see if the *Strep*II-tag does not interfere with the function of MscL, the properties of the charged cysteine-mutation (**see 1.2**) will be used to determine the activity of the channel via a fluorescence dequenching assay (**see 1.3**).

1.1 The *Strep*II-tag as a recombinant tool to isolate MscL

After the cloning and sequencing of MscL, several methods have been used to isolate recombinant MscL from hosts like bacteria. One of the first used one-step purification methods to isolate MscL was the fusion of glutathione *S*-transferase (GST) to the N-terminus of MscL (16) (17). The GST-MscL fusion protein was purified by affinity chromatography of the cell lysate on glutathione-Sepharose beads. A thrombin cleavage site was introduced between the two proteins so that GST can be removed after purification. The advantage of GST is that the chromatography matrix is inexpensive and protein can be partially purified in high yields in a relatively short time (18). The drawback of this method is that ten additional amino acids are present at the N-terminal side of MscL, possibly interfering with the unidirectional passage of ions through the channel (17). Also, the affinity tag is a homodimer (19), making purification of oligomeric proteins, like for example MscL, difficult (20). Another disadvantage of GST is that the tag contains numerous exposed cysteines that can cause oxidative aggregation of the fusion proteins (19). It is also important to note that during overproduction the large sized GST-tag is a high metabolic burden for the cell. All the above lead to the choice of other affinity tags to purify MscL, like for example the hexahistidine tag (His-tag). The His-tag currently is the most commonly used tag for isolating MscL. The neighboring histidine-residues have a high affinity for metal ions (e.g. Ni^{2+}), and this feature is used in immobilized metal affinity chromatography (IMAC) (21) (22). The matrix mostly used for IMAC is Ni(II)-Nitrilotriacetic acid (Ni-NTA). The His-MscL fusion protein can be eluted from the column with imidazole, histidine or low pH. The advantages of the small His-tag compared to the GST-tag, is that it is relatively low in energy costs for the cell during overproduction. The Ni-NTA resin, just like the GST resin, is relatively inexpensive because it can be regenerated several times (20). The biggest drawback of the His-tag is the lower specificity of IMAC (20) compared to other affinity methods currently on the market, such as the *Strep*II-tag (23) which can bind more specifically to its affinity matrix. This lower specificity of the His-tag leads to a lower degree of purification from *E.coli* extracts (18). The *Strep*II-tag may therefore be an alternative choice as a recombinant tool to isolate MscL. The *Strep*-tag was originally selected from a genetic peptide library (24) as an oligopeptide (Trp-Arg-His-Pro-Gln-Phe-Gly-Gly) with high affinity for streptavidin. The *Strep*-tag specifically and reversibly binds to the position where the original substrate D-

biotin normally would bind in streptavidin (25). Since the *Strep*-tag was restricted to the carboxy-terminus of proteins, the optimized *Strep*II-tag (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) was developed, which gives more flexibility in the choice of the attachment site (26). With the engineering of an improved streptavidin-resin, called *Strep*-Tactin, a higher binding capacity was achieved making it more suitable for affinity chromatography (27). Furthermore, the *Strep*II-tag, just like the His-tag, does not hinder protein folding and it does not interfere with the function of most proteins (18, 23). However, a disadvantage of the *Strep*II-tag compared to the His-tag is the higher cost of the column material (18), although the price has been lowered lately. The *Strep*-Tactin affinity chromatography method is schematically illustrated in **Figure 2**. *Strep*II-MscL is eluted from the *Strep*-Tactin column using a low concentration of D-biotin.

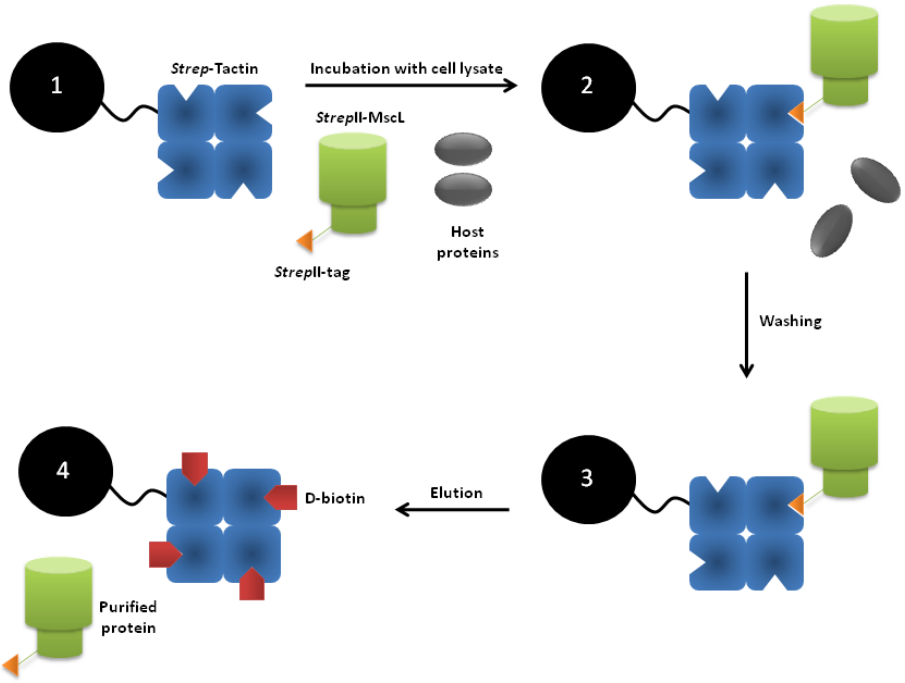

Figure 2: Schematic illustration of the *Strep*-Tactin affinity purification of *Strep*II-MscL. The *E.coli* lysate containing host proteins and the *Strep*II-tagged MscL fusion protein is incubated with immobilized *Strep*-Tactin column material (Steps 1 and 2). The host proteins are removed after a quick wash with a physiological buffer (Step 3). The protein is eluted via competition with D-biotin, which irreversibly binds to the *Strep*-Tactin resin. For clarity, only one of the five *Strep*II-tags of MscL is shown. This illustration is based on figure 1 of (23).

The effect of avidin on the purification of the *Strep*-Tactin isolation (23) will also be checked in this project. Avidin is known for binding to biotinylated proteins, just like the *Strep*-Tactin column material, but it has no affinity for the *Strep*II-tag (28). Therefore avidin 'masks' biotinylated proteins that normally would bind to the *Strep*-Tactin column, which can lead to a higher degree of purity.

1.2 MscL opening without applying tension

In order to characterize the functioning of *Strep*II-tagged MscL, a Gly-22 $\rightarrow$ Cys MscL mutant will be used in this project. This mild substitution has no strong effect on the growth of the host cell, although channel opening is a bit harder (14). Since wild type MscL contains no other cysteine residues, it is possible to chemically modify the cysteines of the five identical subunits within the channel. Charged methanethiosulfonate (MTS) reagents can be used to covalently bind to the cysteines of the protein via a disulfide bond. By charging MscL residues that are located within the hydrophobic pore constriction, the channel was shown to open without any applied tension on the membrane (15). In this project [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET) will be used to add positive charges to the *Strep*II-tagged MscL (**Figure 3**).

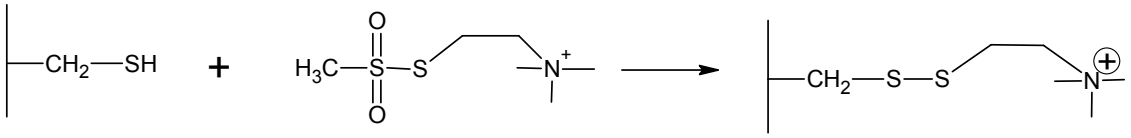

Figure 3: The reaction of cysteine with [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET), resulting in the formation of a disulfide bond. This reaction positively charges G22C-MscL, allowing the channel to be opened without applying tension on the membrane.

1.3 Real-time fluorescence dequenching assay

The channel activity of purified *Strep*II-G22C MscL will be followed by a real-time fluorescence dequenching assay (29). MscL now functions as a channel between the interior of the liposomes and the outside. Because G22C-MscL can be easily opened because of the cysteine mutation (as described above), the calcein is released from the proteoliposomes by addition of MTSET. The release leads to a decrease in effective concentration of calcein, and the dequenching associated to it, and so to an increase in fluorescence. This increase in fluorescence gives a good indication of the opening behavior of the channel. The fluorescence can be monitored at 515 nm (excitation at 495 nm) with a spectrofluorometer. In order to determine the maximal calcein release, all the liposomes are burst by adding an excess of detergent. To calculate the percentage released calcein the following formula is used:

$$\% \text{ Calcein release } = \frac{I_t - I_0}{I_{100} - I_0} \times 100$$

where:

I_0 = the initial fluorescence intensity because of background fluorescence of free calcein

I_t = the fluorescence intensity measured at time *t*

I_{100} = the fluorescence intensity measured after bursting all liposomes with detergent.

An illustration of a real-time fluorescence dequenching assay is reported in **Figure 4**.

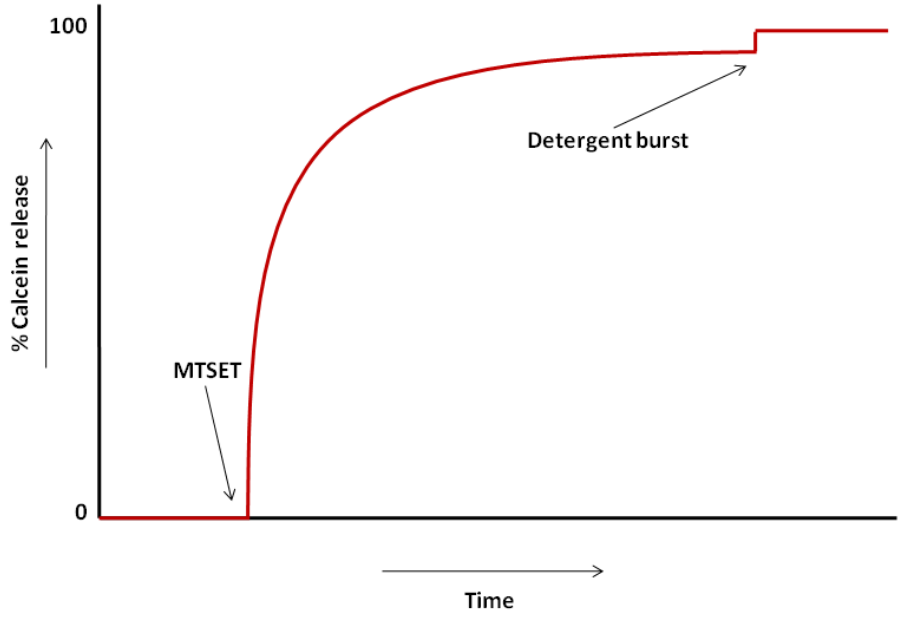

Figure 4: Illustration of a real-time fluorescence dequenching assay. A small amount of calcein-filled proteoliposomes are added into a cuvette with efflux buffer. After the background fluorescence is recorded (0%), the calcein release from inside the liposomes is activated by addition of MTSET into the buffer. After the release levels off, the liposomes are burst so that all the calcein is released (100%).

With the help of the G22C-mutation and the real-time fluorescence dequenching assay, it is possible to overcome the usage of time consuming techniques like patch clamp in order to get a general and quick idea of the activity of the channel *in vitro*.

2. Material

2.1 Reagents

- **TY^{+} broth** 16 g/l Bacto-Tryptone, 10 g/l Yeast-Extract and 5 g/l NaCl. The medium was supplemented with antibiotics for plasmid selection (100 µg/ml Ampicillin) and maintenance of chromosomal gene-disruption (ΔmscL::Cm^{res}; 10 µg/ml Chloramphenicol).
- **Solubilization buffer** 50 mM Na_2HPO_4 /NaH_2PO_4, pH 8.0, 300 mM NaCl and 1% (vol/vol) Triton X-100.
- **Wash buffer** solubilization buffer but with only 0.2% (vol/vol) Triton X-100.
- **Elution buffer** wash buffer containing 2.50 mg/ml D-biotin.
- **Coomassie Brilliant Blue solution** 0.25% Coomassie Brilliant Blue (Serva Blue R), 40% ethanol, 10% acetic acid.
- **Lipid buffer** 10 mM Na_2HPO_4/NaH_2PO_4, pH 8.0, 150 mM NaCl.
- **Calcein solution** 200 mM calcein in 10 mM Na_2HPO_4/NaH_2PO_4, pH 8.0.
- **Efflux buffer** 10 mM Na_2HPO_4/NaH_2PO_4, pH 8.0, 150 mM NaCl, 1 mM EDTA.
- **Blot buffer** 48 mM Tris, 39 mM glycine and 20% methanol (vol/vol).
- **Bradford reagent** 50 mg Coomassie Brilliant Blue G-250 in 5% (vol/vol) ethanol, 10% (vol/vol) phosphoric acid. Filter through Whatman #1 paper.
- Triton X-100 (10%, Fluka, lot. 13444815, filling. 44807191)
- *Strep*-Tactin Superflow (50%)
- *E.coli* strain PB104 having G22C-MscL with C-terminal *Strep*II-tag.

2.2 Equipment

- Constant Cell cell disrupter *(www.constantsystems.com)*
- FastPrep® instrument *(www.mpbio.com)*
- 10 L fermentor (www.applikon-bio.com)

3. Methods

3.1 Over-expression of G22C-*Strep*II MscL in *Escherichia coli*

3.1.1 L-arabinose induction determination

In order to optimize the over-expression of G22C-*Strep*II MscL, the best L-arabinose induction concentration was determined for the *Escherichia coli* strain PB104 carrying the plasmid p2BADb WT-*Strep*II[2]. To do so several small-scale cultures were grown and induced with different L-arabinose concentrations, the cells were disrupted and the cleared lysate was used for detection of the expression level of MscL in a Western blot.

The cultivation was done in 10 ml TY^+ broth overnight at 37 °C. The overnight culture was diluted 1000x in 3 ml TY^+ broth and cultivated in five tubes at 37 °C. To monitor the growth, the optical density at 600 nm (OD_{600}) was measured every hour. The expression of WT-*Strep*II MscL was induced at OD_{600} = ±0.7 (3 hr) with 0.001%, 0.002%, 0.01% and 0.1% L-arabinose and compared with a non-induced culture as a control. After 3-4 hours incubation, 2 ml of each culture was centrifuged at 14.000 x g for 2 min. The pellets were stored overnight at -20 °C.

A FastPrep® instrument was used to mechanically disrupt the cells. Briefly, the pellets were resuspended in 400 µl of 25 mM Tris-HCl pH 8.0 and one scoop of glass beads (≤ 106 µm) was added. The suspended cells were disrupted for 2 x 20s at 6 while cooling the samples on ice in between for 5 min. The disrupted cells were centrifuged at 4.000 x g for 20 min at 4 °C to remove any inclusion bodies. The supernatant, cleared lysate, was used for further experiments.

20 µl of cleared lysate with 5 µl of 5x sample buffer was loaded onto a 12.5% SDS-Polyacrylamide gel (**see 3.2.3**). The gel was also loaded with 6 µl of PageRuler™ Plus Prestained Protein marker. The samples were stacked for 15 min at 100 V and then run for 30 min at 200 V. The gel was analyzed by a Western blot with streptavidin-AP conjugate antibody. A Bio-Rad Trans-Blot SD electrophoretic transfer cell was loaded from top to bottom as follows: filter paper (soaked in blot buffer), PVDF membrane (soaked in methanol

and washed with water), SDS-Polyacrylamide gel and another filter paper (soaked in blot buffer). To exclude excess moisture and air bubbles trapped in the filter papers and membrane, a glass rod was rolled over the surface. The transfer was done in 30 min at 0.08 A.

3.1.2 Fermentation

High quantities of membrane vesicles containing G22C-*Strep*II MscL were obtained from a 10 L fermentor culture of *Escherichia coli* strain PB104 carrying the plasmid p2BADb G22C-*Strep*II[2] cultivated in TY^+ broth. The fermentor was set to 37 °C, 500 rpm stirring, pH 7.5 (set with 4 M KOH) and minimum oxygen saturation of 30%. To monitor the growth, the optical density at 600 nm (OD_{600}) was measured every 30 min during the fermentation. The protein expression was induced in the mid-logarithmic phase (OD_{600} = 3.74, t = 220 min) with 0.1% L-arabinose. To prevent carbon source limitations an additional 0.2% glycerol was added upon induction. The stirring speed was increased to 800 rpm to increase the oxygen concentration. The cells were harvested when an OD_{600} of 6.94 was reached.

3.1.3 Harvesting of cells

The *E.coli* cells, grown as described above, were harvested by centrifugation at 6.891 x g for 15 min at 4 °C in a Beckman centrifuge with JLA 8.1000 rotor. The pellets were washed once with ice-cold 25 mM Tris-HCl pH 8 and the suspension was centrifuged again (6.891 x g, 15 min, 4 °C). The pellet was resuspended in ice-cold 25 mM Tris-HCl pH 8 to an OD_{600} of 116 (total volume of 600 ml). 10 aliquots of 40 ml samples were stored at -80 °C. The rest was directly used for preparation of the membrane vesicles.

3.1.4 Membrane vesicle preparation

For the preparation of membrane vesicles a total of 200 ml harvest was used. DNAse and RNAse were added to a final concentration of 0.5 mg/ml and $MgSO_4$ was added to a final concentration of 5 mM. The cells were homogenized for 15 min at 4 °C by using a magnetic stirrer. The cells were disrupted once with a Constant Cell cell disrupter (continuous setting) at 25 kpsi. The color of the cell suspension changed from yellow to red/brown after disruption. EDTA (pH 7) was added to a final concentration of 5 mM. The suspension was centrifuged low speed at 18.459 x g for 30 min at 4 °C with a Beckman centrifuge JA 25.50

rotor. The supernatant was ultra-centrifuged at 145.421 x g for 90 min at 4 °C with a Beckman Type 50.2 Ti rotor. The pellet (a red slurry) was resuspended with 25 mM Tris-HCl pH 8 by using a homogenizer. A total of 15 ml membrane vesicles with a concentration of 0.48 g/ml were collected and stored at -80 °C.

3.2 Isolation of G22C-*Strep*II MscL

3.2.1 *Strep*-Tactin affinity chromatography isolation

4 ml of membrane vesicles (0.48 g/ml) were added into 20 ml solubilization buffer and were incubated at 4 °C in a rotary mixer for 45 min. The suspension was ultra-centrifuged at 184.048x g for 30 min at 4 °C in a Beckman Type 50.2 Ti rotor. The supernatant with solubilized G22C-*Strep*II MscL was used for *Strep*-Tactin affinity chromatography. The isolation was performed in a cold room (4 °C). 2 ml 50% *Strep*-Tactin Superflow was washed with 4x 3 ml solubilization buffer. The washed column material was added to the supernatant of the ultra-centrifugation and was incubated for 15 min in a rotary mixer. The suspension was put into a 10 ml column holder from Bio-Rad and the flow through was collected. The column was then washed with 5x 1 ml and 1x 5 ml wash buffer. The protein was eluted in 16x 250 µl fractions.

3.2.2 Bradford Assay

To determine the protein concentration of the *Strep*-Tactin elution fractions a Bradford assay was done using the standard Bradford protocol. For making the calibration curve a BSA concentration gradient was used of 0, 0.079, 0.158, 0.237, 0.316 and 0.395 mg/ml. 10 µl of each BSA concentration sample together with 10 µl of the elution fractions were loaded on the 96 well plate. All samples were performed in duplicates and the bubbles formed in the wells were removed by using a pipette tip. 200 µl of Bradford solution was added to each well and the absorbance at 595 nm was measured in the Power Wave X from Bio-Tek instruments.

3.2.3 SDS-polyacrylamide gel electrophoresis

The purity of the *Strep*-Tactin elution fractions was determined with SDS-PAGE. The following pipette scheme was used for making two SDS-polyacrylamide gels:

12.5% running gel		stacking gel	
30% Acrylamide	4.17 ml	30% Acrylamide	0.67 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	0.5 M Tris-HCl pH 6.8	1.25 ml
10% SDS	0.1 ml	10% SDS	0.05 ml
H_2O	3.2 ml	H_2O	3.00 ml
10% APS	50 µl	10% APS	50 µl
TEMED	10 µl	TEMED	5 µl
Total volume:	10 ml	Total volume:	5 ml

For loading the SDS-polyacrylamide gels 20 µl samples were made of the *Strep*-Tactin column flow through-, wash- and elution fractions and the controls. The flow-through fractions were diluted 10x with elution buffer. 5 µl of 5x sample buffer was added to each sample before loading it onto the gel. The gels were also loaded with 10 µl of Low Molecular Weight marker or 6 µl of PageRuler™ Plus Prestained Protein marker. The samples were stacked for 30 min at 100 V and then run for 30 min at 200 V. After washing with water the gels were stained with Coomassie Brilliant Blue solution for 1 hour under gentle shaking. The gels were distained overnight with 20% ethanol, 10% acetic acid solution.

3.3 Determining activity of G22C-*Strep*II MscL

3.3.1 Reconstitution into artificial liposomes

In order to measure the activity of the G22C-*Strep*II MscL, it first has to be reconstituted into liposomes. 10 ml of 20 mg/ml azolectin (dissolved in chloroform) was evaporated under vacuum in a rotary evaporator in a 50 °C water bath until a thin film was formed. The lipids were rehydrated in 10 ml of lipid buffer. The rehydrated lipids were frozen and thawed 5 times in liquid nitrogen and a 50 °C water bath. After that the lipids were stored at -20 °C. 500 µL of 10 mg/ml *E.coli* lipids was extruded 11 times with a polycarbonate filter with pore diameter of 400 nm. 200 µL of the extruded lipids were added to an eppendorf cup and titrated with 15 µL Triton X-100. 196 µL of protein was added into titrated liposomes. The lipid, detergent, protein and buffer mixture was incubated for 30 min at 50 °C. After that, 200 µL calcein solution and 160 mg (wet weight) biobeads were added into the mixture and

the eppendorf cup was covered with aluminum foil and kept overnight in a rotary mixer at 4°C.

3.3.2 Fluorescence dequenching experiment

In order to separate the proteoliposomes from free calcein, the overnight calcein and proteoliposomes mixture was applied onto a Sephadex G50 size-exclusion column, which was equilibrated with the efflux buffer and run by gravity. The proteoliposomes proceed as a dark orange band on the elution front. 4 µL of collected proteoliposomes was added into 4 ml efflux buffer and divided over two cuvettes. The fluorescence was monitored at 515 nm (excitation at 495 nm) with a spectrofluorometer. After 1 min recording, 25 µL of freshly made 160 mM MTSET was added into one of the cuvettes and the fluorescence was recorded for ±5 min. The liposomes were burst by adding 100 µL Triton X-100 into each cuvette.

4. Results

4.1 Overexpression of G22C-*Strep*II MscL in Escherichia coli

4.1.1 L-arabinose induction determination

In order to optimize the overexpression of G22C-*Strep*II MscL, the best L-arabinose induction concentration was determined for *Escherichia coli* strain PB104 carrying the plasmid p2BADb WT-*Strep*II[2]. The Western blot of the L-arabinose induction determination is shown in **Figure 5**.

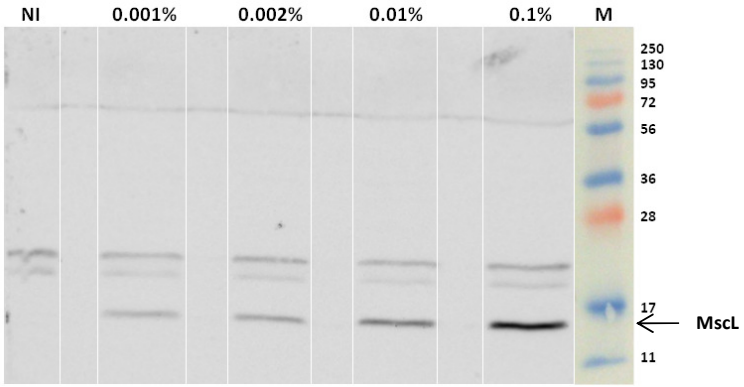

Figure 5: The Western blot of the L-arabinose induction determination. The blot shows the cleared lysate samples of the non-induced cells (NI), the L-arabinose induced cells (0.001% - 0.1%) and the PageRuler™ Plus Prestained Protein marker (M). The numbers on the right indicate the molecular weight values of the marker in kDa. The arrow indicates WT-*Strep*II MscL, this protein is not visible with the non-induced cells. The highest overexpression is found at an induction of 0.1% L-arabinose.

The non-induced cells sample shows two bands at ±20 kDa, which probably are biotinylated proteins normally produced by the *E.coli* cells. The WT-*Strep*II MscL bands of the induced samples (±15 kDa) show an increasing intensity with increasing concentrations of L-arabinose. Although 0.001% L-arabinose induction is recommended for the pBAD system (30), it shows the lowest intensity on the Western blot. The cells induced with 0.1% L-arabinose show the highest intensity and therefore give the highest amount of MscL protein.

4.1.2 Fermentation

Two 10L fermentations were done with *Escherichia coli* strain PB104 carrying the p2BAD b G22C-*Strep*II[2] MscL plasmid: one induced with 0.001% L-arabinose and one induced with 0.1% L-arabinose. *E. coli* strain PB104 does not produce wild-type MscL because the native *mscL* gene is disrupted by the insertion of the chloramphenicol resistance gene.

Figure 6 shows the measured OD_{600} plotted against time of the fermentation induced with 0.001% L-arabinose.

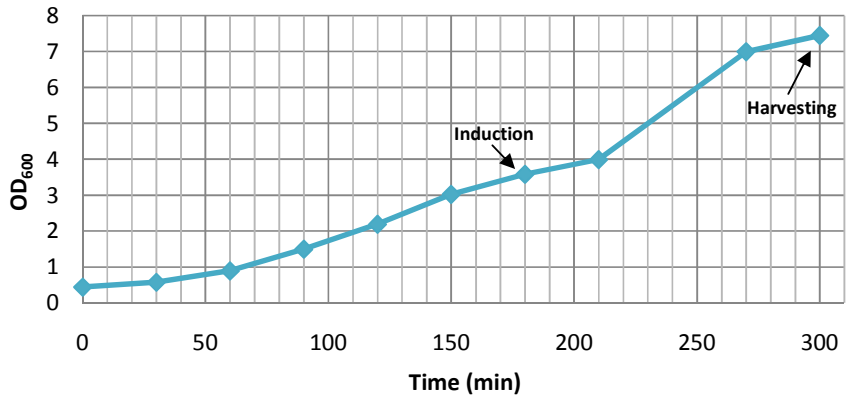

Figure 6: OD_{600} measurement of the fermentation of *E. coli* carrying the p2BAD b G22C-*Strep*II[2] MscL plasmid. The plot first shows a lag phase for 50 min followed by a logarithmic phase until an OD_{600} of 3.5 was reached. The induction was started with 0.001% L-arabinose at 185 min (OD_{600} = 3.58) with an additional 0.2% glycerol to prevent carbon source limitations. After a short lag phase the cells grew further in a logarithmic manner and were harvested at an OD_{600} of 7.45.

The *E.coli* cells mainly grow on amino acids and traces of glucose in the TY^{+} broth. Since glucose is a repressor of the transcription initiation of the L-arabinose operon in the pBAD system (31), growth media needed to be depleted of glucose before induction. This is achieved by waiting until the growth speed starts decreasing. The induction was started with 0.001% L-arabinose at an OD_{600} = 3.58 (185 min). After a short lag phase the cells grew further in a logarithmic manner and were harvested at an OD_{600} of 7.45. The short lag phase indicates that the cells had almost run out of carbon source and changed their metabolism for the additionally added glycerol.

The 10l fermentation induced with 0.1% L-arabinose was done in the exact same manner as described above. No important differences were found in growth behavior. The cells were induced at OD_{600} = 3.74 and were harvested at an OD_{600} of 6.94.

4.1.3 Harvesting of cells

A total of 600 ml cells (OD_{600} = 124) were harvested from the fermentation with 0.001% L-arabinose induction. The fermentation induced with 0.1% L-arabinose yielded 600 ml cells with an OD_{600} of 116.

4.1.4 Membrane vesicle preparation

Several membrane vesicles were prepared from the aliquots of two fermentation cultures. The fermentation culture induced with 0.001% L-arabinose yielded the following membrane vesicles:

- 15 ml of 0.816 g/ml membrane vesicles from 200 ml harvest. These vesicles were used for the standard protocol *Strep*-Tactin isolation (**See 4.2.1**) and for the isolations to check to effect of avidin on the purification (**See 4.2.5**).
- 19 ml of 0.695 g/ml membrane vesicles from 240 ml harvest. These vesicles were used for the double scale-up *Strep*-Tactin isolation (**See 4.2.2**).
- 14 ml of 0.445 g/ml membrane vesicles from 160 ml harvest. These vesicles were used for the quadruple scale-up *Strep*-Tactin isolation (**see 4.2.3**).

The fermentation culture induced with 0.1% L-arabinose yielded 15 ml of 0.481 g/ml membrane vesicles from 200 ml harvest. These vesicles were used for the final *Strep*-Tactin isolation **(see 4.2.4).**

4.2 Isolation of G22C-*Strep*II MscL

The first *Strep*-Tactin affinity chromatography isolation of G22C-*Strep*II MscL was performed with membrane vesicles from the 0.001% L-arabinose induced cells by following the standard protocol (23). In order to optimize the yield of the isolation, several scale-up isolations were done with higher amounts of membrane vesicles and column matrix volume. The optimized isolation conditions were then used for the 100 fold higher induced fermentation. The purification of the *Strep*-Tactin isolation was also optimized by checking the effect of the addition of avidin in the solubilization buffer.

4.2.1 Standard protocol *Strep*-Tactin isolation

The first *Strep*-Tactin isolation of G22C-*Strep*II MscL was done with 1.63 g membrane vesicles, 1 ml *Strep*-Tactin Superflow (50%) column matrix and 250 µl elution fractions based on the standard protocol. Also 0.067 mg/ml avidin was added to the solubilization buffer. The Coomassie Brilliant Blue (CBB)-stained SDS-polyacrylamide gel is shown in **Figure 7**.

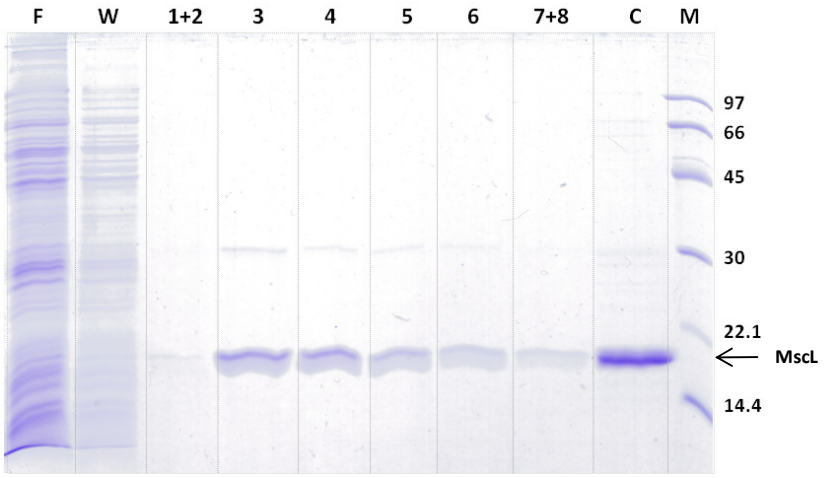

Figure 7: CBB-stained SDS-PAGE of standard protocol isolation of G22C-*Strep*II MscL. The gel shows the *Strep*-Tactin column flow-through and wash fractions (F, W), the *Strep*-Tactin elution fractions containing purified mono- and dimeric G22C-*Strep*II MscL (1-8), the heteromonomeric MscL control (C) and the low molecular weight marker (M). The numbers on the right indicate the molecular weight values of the marker in kDa.

The SDS-PAGE clearly shows that G22C-*Strep*II MscL was isolated with high purity (see arrow). Elution fractions show no contaminants compared to the flow-through and wash fractions. The band at ±30 kDa is probably a not fully reduced (dimeric) form of MscL.

The protein concentrations of all the elution fractions were determined by Bradford assay (**Figure 8**).

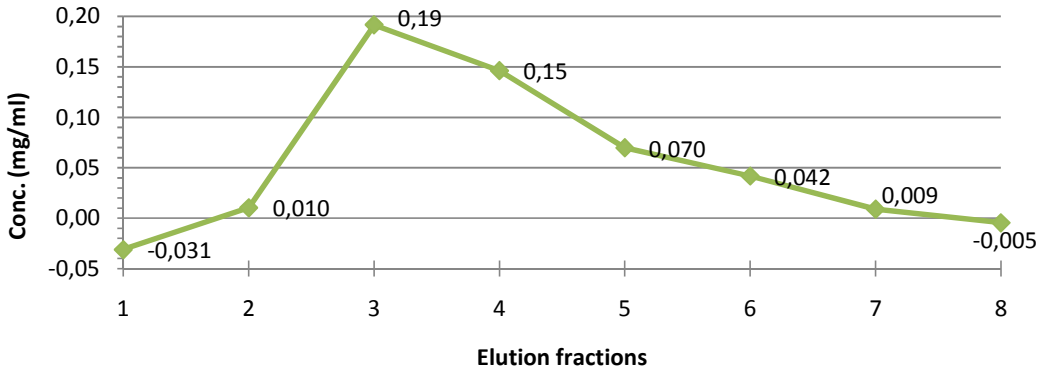

Figure 8: Protein elution profile of the standard protocol isolation of G22C-*Strep*II MscL. The numbers in the graph indicate the protein concentrations of the corresponding elution fractions in mg/ml.

The plot shows an expected protein elution profile. The first two elution fractions contain no protein. The protein concentration then suddenly increases to a maximum of 0.19 mg/ml at elution fraction 3, followed by fraction 4 with 0.15 mg/ml protein. After this the

concentration decreases to zero. Fractions 3 and 4 were combined yielding 0.5 ml of ±0.17 mg/ml purified protein.

4.2.2 Double scale-up *Strep*-Tactin isolation

In order to improve the yield of the *Strep*-Tactin isolation, a scale-up isolation was done with a higher amount of membrane vesicles and twice the column matrix volume. The isolation was done with 2.78 g membrane vesicles, 2.0 ml *Strep*-Tactin Superflow (50%) column matrix and 250 µl elution fractions. A lower value of 0.026 mg/ml avidin was added to the solubilization buffer because the purity of the previous isolation was already very high. The CBB-stained SDS-polyacrylamide gel is shown in **Figure 9**.

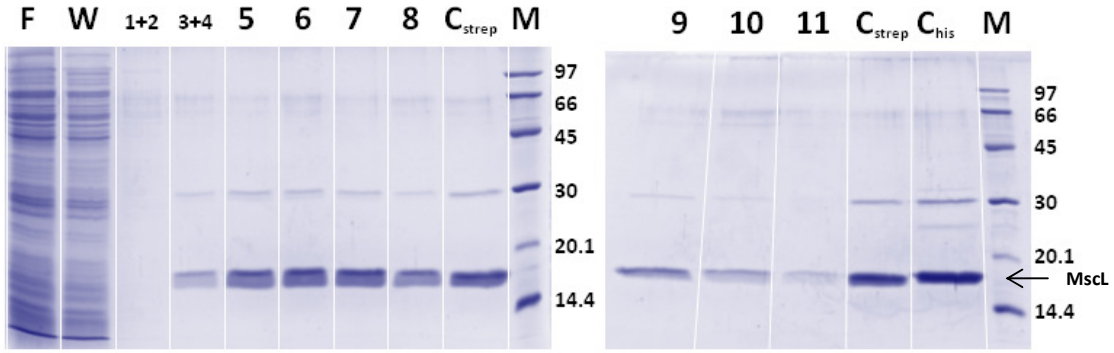

Figure 9: CBB-stained SDS-PAGE of the double scale-up isolation of G22C-*Strep*II MscL. The gel shows the *Strep*-Tactin column flow-through and wash fractions impurities (F, W), the *Strep*-Tactin elution fractions containing purified mono- and dimeric G22C-*Strep*II MscL (1-11), the *Strep*II-tagged MscL control (C_{strep}), the His-tagged MscL control (C_{his}), and the low molecular weight marker (M). The numbers on the right indicate the molecular weight values of the marker in kDa.

The bands at the arrow of the elution fractions show that G22C-*Strep*II MscL was isolated with very high purity.

The protein concentrations of all the elution fractions were determined by Bradford assay (**Figure 10**).

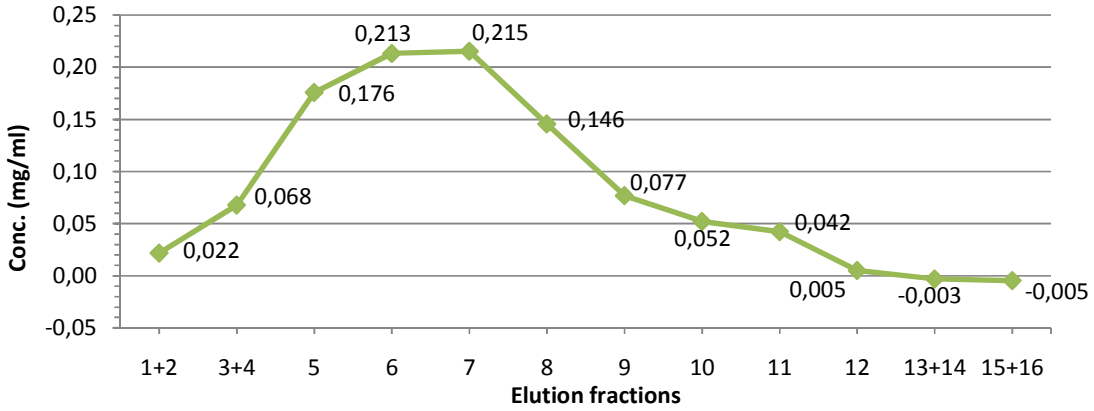

Figure 10: Protein elution profile of the double scale-up isolation of G22C-*Strep*II MscL. The numbers in the graph indicate the protein concentrations of the corresponding elution fractions in mg/ml.

The same elution profile was found as the first isolation but with more high-concentrated fractions. Fraction 5-8 were combined yielding 1.0 ml of ±0.19 mg/ml purified G22C-*Strep*II MscL.

4.2.3 Quadruple scale-up *Strep*-Tactin isolation

In order to improve the yield of the *Strep*-Tactin isolation even more, a quadruple scale-up isolation was done with 4.0 ml *Strep*-Tactin Superflow (50%) column matrix and 500 µl elution fractions. Because of a mistake in the calculation of the membrane vesicle concentration a too low amount of 3.56 g of membrane vesicles was used (the aim was to use ±6.4 g vesicles). No avidin was added to the solubilization buffer. The protein concentrations of all the elution fractions were determined by Bradford assay (**Figure 11**).

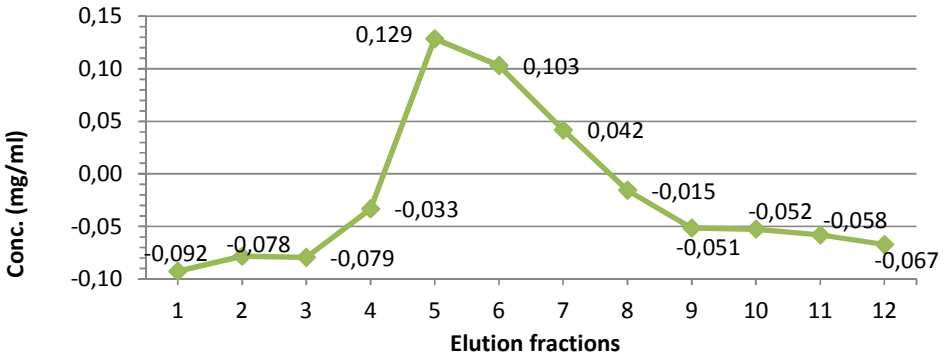

Figure 11: Protein elution profile of the quadruple scale-up isolation of G22C-*Strep*II MscL. The numbers in the graph indicate the protein concentrations of the corresponding elution fractions in mg/ml.

A similar elution profile was found as the other two isolations but with much less protein. Fraction 5 and 6 were combined yielding 1.0 ml of ±0.12 mg/ml purified G22C-*Strep*II MscL. Because of the low yield and low concentration of protein no SDS-polyacrylamide gel electrophoresis was done for this isolation.

4.2.4 *Strep*-Tactin isolation of 0.1% L-arabinose fermentation culture

G22C-*Strep*II MscL was also purified from the membrane vesicles of the 0.1% L-arabinose induced fermentation culture. The double scale-up *Strep*-Tactin isolation protocol (**see 4.2.2**) was used for this isolation because this yielded the best results for the 0.001% L-arabinose membrane vesicles. The isolation was done with 1.9 g membrane vesicles (the aim was to use 2.8 g, but the concentration of the membrane vesicles was lower than expected because of a miscalculation), 2.0 ml *Strep*-Tactin Superflow (50%) column matrix and 250 µl elution fractions.

The SDS-PAGE showed that the higher induction of 0.1% L-arabinose gave much better results (**Figure 12**).

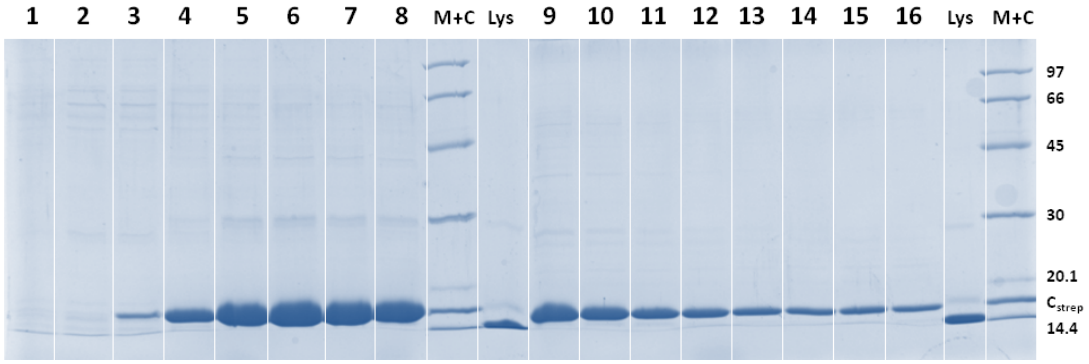

Figure 12: CBB stained SDS-PAGE of the isolation of G22C-*Strep*II MscL from 0.1% L-arabinose induced *E.coli* cells. The gel shows the *Strep*-Tactin elution fractions containing purified monomeric G22C-*Strep*II MscL (1-16), the *Strep*-tagged MscL control (0.1 mg/ml) combined with the low molecular weight marker (M+C) and 0.5 mg/ml lysozyme (Lys). The numbers on the right indicate the molecular weight values of the marker in kDa.

G22C-*Strep*II MscL was isolated with high purity but this time with much higher yield. 0.5 mg/ml lysozyme (Lys) was loaded on the gel to determine the protein concentrations of the elution fractions. The amount of protein is at least ten times higher than the previous isolations from the 0.001% L-arabinose induced cells.

The Bradford assay showed the same elution pattern as in the previous isolations but with more than tenfold higher protein concentrations (**Figure 13**).

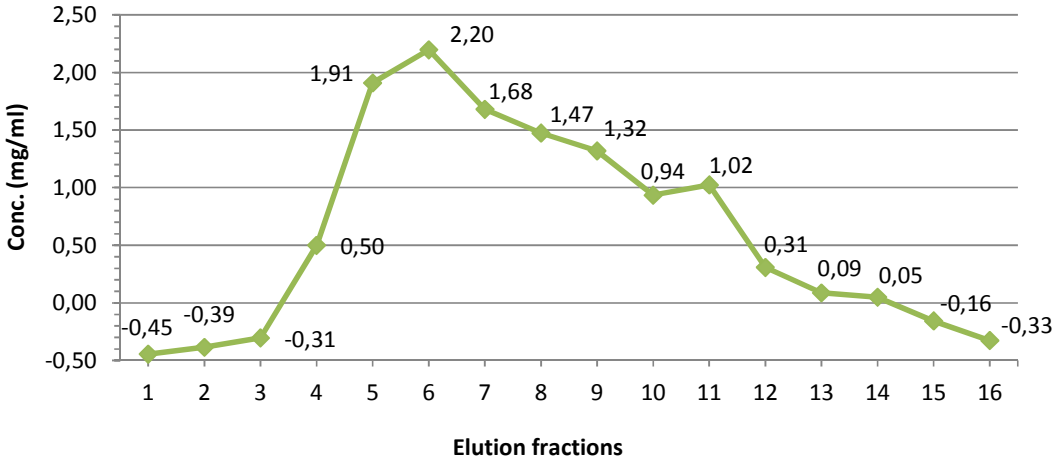

Figure 13: Protein elution profile of the isolation of G22C-*Strep*II MscL from 0.1% L-arabinose induced *E.coli* cells. Fractions 5-11 contained the highest protein concentration with an average of ~1.5 mg/ml. The numbers in the graph indicate the protein concentrations of the corresponding elution fractions in mg/ml.

Elution fractions 5-11 were combined yielding 1.75 ml of ±1.5 mg/ml purified G22C-*Strep*II MscL.

4.2.5 Effect of avidin on purification

To confirm the possible positive effect of avidin on the purification of G22C-*Strep*II MscL, two additional isolations were done; one with and one without the addition of avidin. The standard protocol was followed (**see 4.2.1**). 0.026 mg/ml avidin was added into the solubilization buffer. Elution fractions were analyzed by SDS-PAGE and Western blotting. The results are shown in **Figure 14**.

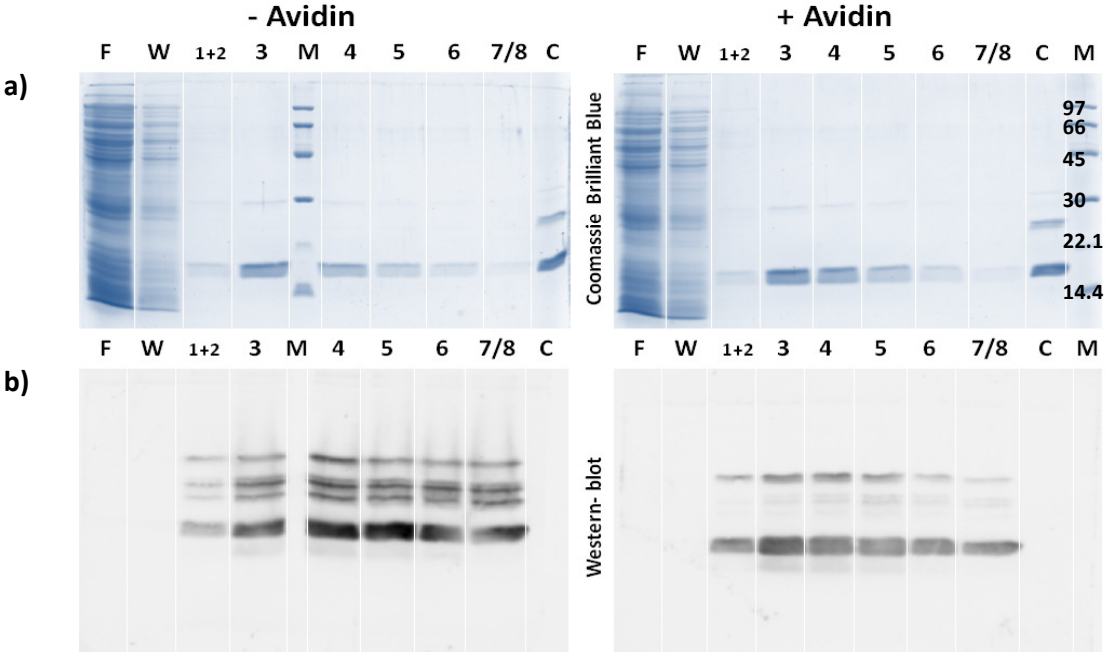

Figure 14: SDS-polyacrylamide gel results of the G22C-*Strep*II MscL isolations in the absence (left) and presence (right) of avidin. The gels and blots illustrate the *Strep*-Tactin column flow-through and wash fractions (F, W), the *Strep*-Tactin elution fractions (1-8), the G22C-His MscL control (C) and the low molecular weight marker (M). The numbers on the right indicate the molecular weight values of the marker in kDa. **A)** CBB-stained SDS-PAGEs. The elution fractions contain purified mono- and dimeric G22C-*Strep*II MscL. No significant differences can be seen between the two different isolation methods. **B)** Western blots of the SDS-PAGEs. It is clear that the flow-through and wash fractions do not contain any *Strep*-tagged protein. The elution fractions contain purified mono- and dimeric G22C-*Strep*II MscL. The isolation done without avidin has more contamination than the isolation done with avidin, proving the positive effect of the reagent on the purification of G22C-*Strep*II MscL.

No differences in purity between both isolation methods can be seen from the SDS-PAGE shown above. However, the Western blots show a clear difference; the isolation with the addition of avidin has less contamination than the isolation without avidin. The two additional bands above the G22C-*Strep*II MscL band are probably the same biotinylated proteins found with the L-arabinose induction determination (**see 4.1.1**).

The Bradford assay for both isolations showed roughly the same elution profile as in the other isolation, the results are shown in **Figure 15**.

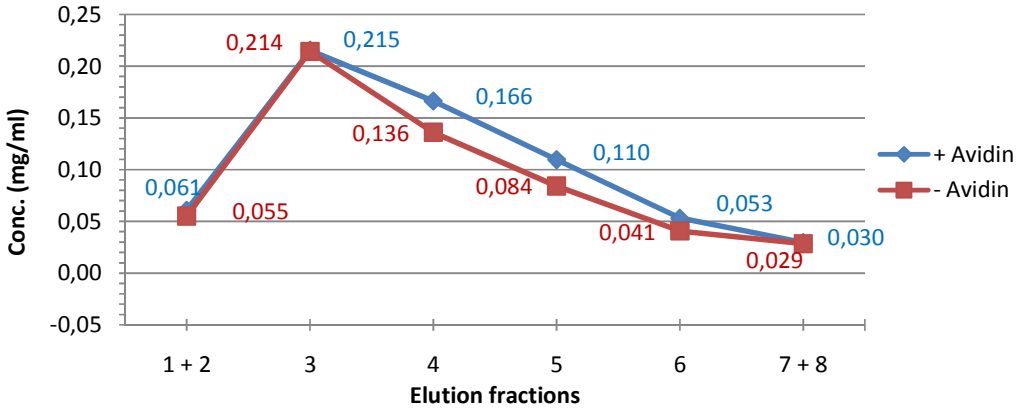

Figure 15: Protein elution profile of the G22C-*Strep*II MscL isolations to see the effect of avidin on the purification. Two graphs are shown; the isolation with presence of avidin is colored in blue and the isolation with absence of avidin is colored in red. The numbers in the graph indicate the protein concentrations of the corresponding elution fractions in mg/ml.

Fraction 3 contained the highest protein concentration for both isolation methods (~0.21 mg/ml). The results are therefore more or less the same as in the standard protocol isolation of G22C-*Strep*II MscL (**see 4.2.1**).

4.3 Determining activity of G22C-*Strep*II MscL

The activity of G22C-*Strep*II MscL was determined by means of fluorescence dequenching. Since the protein is in a detergent-lipid solution after purification it first needs to be reconstituted into artificial liposomes. Several reconstitutions were done with different lipid compositions and detergents to get the best activity of the channel. G22C-His MscL was used as a positive control to make good comparison possible.

The first reconstitution of G22C-*Strep*II MscL was done in 20 mg/ml azolectin with protein to lipid ratio of ±1:100 (wt:wt). The lipids were titrated with Anapoe-X-100. The fluorescence dequenching assay did not show any significant activity for both the His-tagged and the *Strep*II-tagged G22C-MscL, although in early studies more than 80% activity was achieved for G22C-His MscL with azolectin. The reason for this can be that the detergent was not good anymore, the lipids batch was not good anymore or that the *Strep*-Tactin elution buffer has a negative effect on the channel.

In order to see if other lipids work better, azolectin was replaced by a fixed lipid composition of 10 mg/ml DOPE/DOPC (7:3, wt:wt), while keeping the same protein to lipid ratio of

±1:100. This reconstitution setup did not show any significant activity, so for future reconstitutions azolectin will be used.

In order to determine if the low activities were caused by the detergent, Triton X-100 (Sigma, 100% diluted to 10%) was used instead of Anapoe-X-100. This yielded an activity of ~32% for G22C-His Mscl but the G22C-*Strep*II channel still showed no significant activity. Based on these results it was decided not to use Anapoe-X-100 anymore for future reconstitutions, and to continue using Triton X-100.

To see if the *Strep*-Tactin elution buffer was causing the lower activity for the *Strep*II-tagged MscL, G22C-His MscL was diluted with *Strep*-Tactin buffer and also with the Ni-NTA elution buffer. The reconstitution was done with 20 mg/ml azolectin and a protein to lipid ratio of 1:83 (wt:wt). The fluorescence dequenching experiment showed that the protein activity was equal in both buffers (~18%), concluding that the elution buffer was not causing the lower activity.

In order to find activity for the *Strep*II-tagged MscL, azolectin was replaced by 10 mg/ml *Escherichia coli* lipids. G22C-*Strep*II MscL was used from the double scale-up isolation (**see 4.2.2**) with a protein to lipid ratio of 1:53 (wt:wt). The following amounts of reagents were used:

Reconstitution:	***E.coli* lipids (10 mg/ml)**	**Triton X-100 (10%, Sigma)**	**Calcein buffer (200 mM)**	**Biobeads (wet weight)**	**G22C-*Strep*II MscL (0.19 mg/ml)**	**His-MscL (0.19 mg/ml)**
G22C-*Strep*II (1:53, wt:wt)	200 µL	14 µL	200 µl	160 mg	200 µL	-
G22C-His (1:53, wt:wt)	200 µL	14 µL	200 µl	160 mg	-	200 µL

The results of the fluorescence dequenching experiment is shown in **Figure 16**.

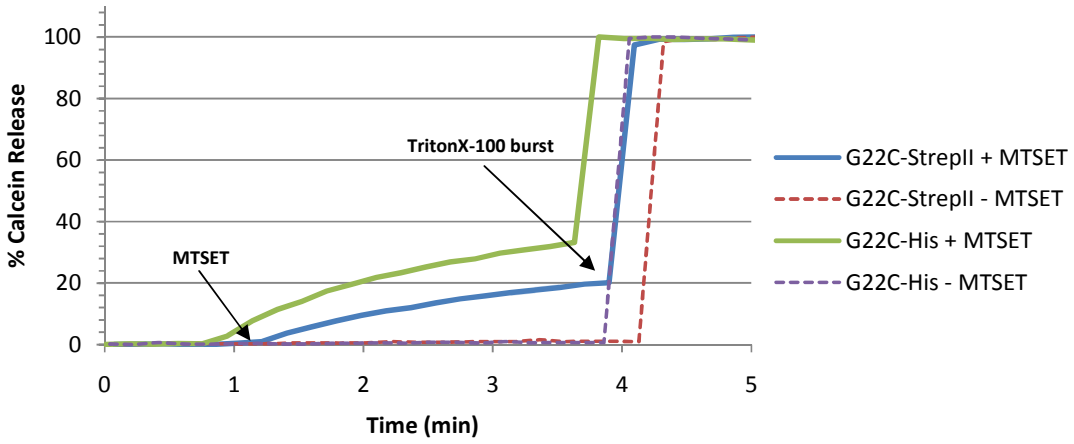

Figure 16: fluorescence dequenching assay results of G22C-*Strep*II and G22C-His MscL reconstituted in 10 mg/ml *E.coli* lipids. This figure shows the percentage release of calcein from the proteoliposomes into the buffer measured by fluorescence at 515 nm (excitation 495 nm).

Although G22C-His MscL shows the same activity as before (~33%), G22C-*Strep*II MscL now shows some activity (~19%).

The reconstitution setup needed to be improved further as both channels showed low activity. Based on results from our own lab (N. Mukherjee *et al*, unpublished work), which gave more than 80% activity for G22C-His MscL, a new reconstitution was done with a fresh batch of 20 mg/ml azolectin. Also another detergent switch was done; Triton X-100 (100% diluted to 10%) from Sigma was replaced by Triton X-100 (10%) from Fluka. G22C-*Strep*II MscL was used from the *Strep*-Tactin isolation of the 0.1% L-arabinose induced cells (**see 4.2.4**) in a protein to lipid ratio of about 1:50. The real ratio is unknown because of the uncertainty in the protein concentration. The following amounts of reagents were used:

Reconstitution:	**azolectin (fresh batch, 20 mg/ml)**	**Triton X-100 (10%, Fluka)**	**Calcein buffer (200 mM)**	**Biobeads (wet weight)**	**G22C-*Strep*II MscL (~0.2 mg/ml)**	**G22C-His MscL (0.22 mg/ml)**
G22C-*Strep*II (~1:50, wt:wt)	100 µL	15 µL	200 µl	160 mg	196 µL	-
G22C-His (1:50, wt:wt)	100 µL	15 µL	200 µl	160 mg	-	196 µL

The results of the fluorescence dequenching assay are shown in **Figure 17**.

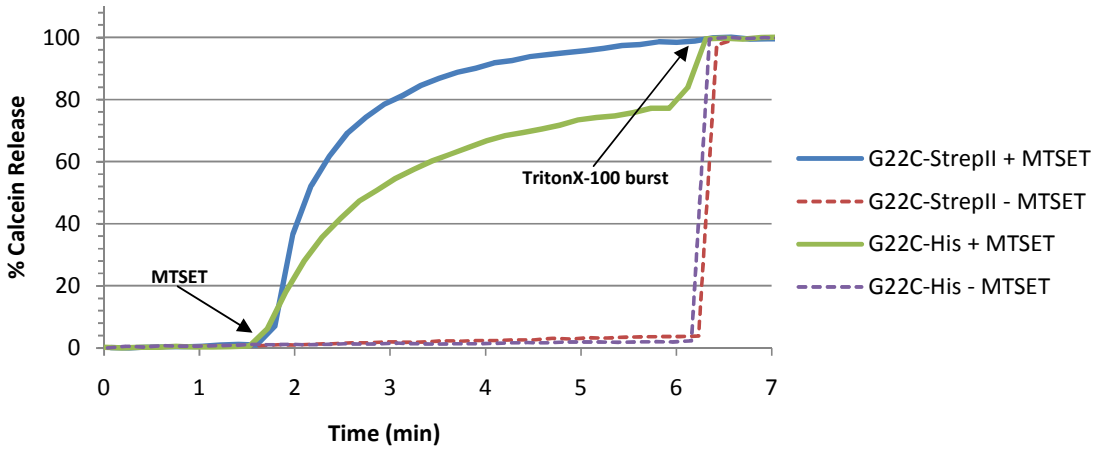

Figure 17: Results of the fluorescence dequenching assay of G22C-*Strep*II and G22C-His MscL reconstituted in a fresh batch of azolectin (20 mg/ml) with protein to lipid ratio of ±1:50 (wt:wt). This figure shows the percentage release of calcein from the proteoliposomes into the buffer measured by fluorescence at 515 nm (excitation 495 nm).

The *Strep*II-tagged MscL shows ~100% calcein release and the His-tagged MscL shows 77% calcein release.

5. Discussion and conclusions

5.1 Overexpression of G22C-*Strep*II MscL in *Escherichia coli*

The L-arabinose induction determination showed that it is always good to verify recommended values for a slightly different system. The suggested value of 0.001% L-arabinose was determined for *E.coli* strain MC1061, while strain PB104, which is a derivative of AW405 (32) (33), was used in this project. The fermentation yielded much less protein than the fermentation with 100-fold higher inducer concentration. It should be noted that strain PB104, unlike MC1061, can use arabinose as an energy source (30). Therefore, the actual amount of L-arabinose that can initiate transcription in the pBAD system is much lower than expected. However, the increase in expression levels with higher inducer concentration is unmistakable. The growth behavior of the 10L fermentations induced with 0.001% and 0.1% L-arabinose did not show any significant differences. Both cultures were growing in the same manner and were induced and harvested at the same time point. From the final OD_{600} of ≥7.0 can be concluded that a high amount of *E.coli* cells were cultivated.

5.2 Isolation of G22C-*Strep*II MscL

The SDS-polyacrylamide gel electrophoresis showed that the first *Strep*-Tactin isolation of G22C-*Strep*II MscL yielded highly pure protein. Nearly all proteins that were present in the flow-through and the wash fractions were removed in the elution fractions. This result confirms the high affinity of the *Strep*II-tag for the *Strep*-Tactin Superflow affinity column, while it is still possible to remove the protein with D-biotin during elution. The Bradford assay showed that high concentrated fractions (0.17 mg/ml) were collected but with very low yield (0.5 ml). Based on these results it was decided that the purity and concentration of the protein was good enough for activity measurements. However, the isolation method required optimization as new isolations had to be done repeatedly.

In order to get more protein per *Strep*-Tactin isolation, a double scale-up isolation was performed. The aim was to use double the amount of membrane vesicles and double the column matrix volume. Though, because of a mistake in the calculation of the membrane vesicle concentration, a too low amount of vesicles was added. By using only 70% more membrane vesicles and double the amount of *Strep*-Tactin Superflow, the yield could be

doubled (1.0 ml), while remaining the same purity and concentration (0.19 mg/ml). The yield would probably have been better if the correct amount of vesicles was added into the solubilization buffer. The elution fractions were compared with a His-tagged MscL control on a SDS-PAGE, showing a slightly higher degree of purity for the *Strep*II-tag.

A quadruple scale-up isolation was done in order to improve the protein yield even more. The aim was to use four times the amount of membrane vesicles and column volume compared to the standard protocol. Again, by a calculation mistake a too low amount of vesicles was added (only 2.2 times). This isolation method yielded no more than 1 ml of 0.12 mg/ml G22C-*Strep*II MscL, which is lower than the double scale-up isolation. A possible reason for this is that too much column matrix volume was used per amount of membrane vesicles. The concentration of *Strep*II-tagged protein per fraction is then lower as it is more diluted by the higher elution volume.

The yield of the *Strep*-Tactin affinity chromatography isolation could be improved greatly by changing to membrane vesicles from the 0.1% L-arabinose induced cells (**Figure 18**).

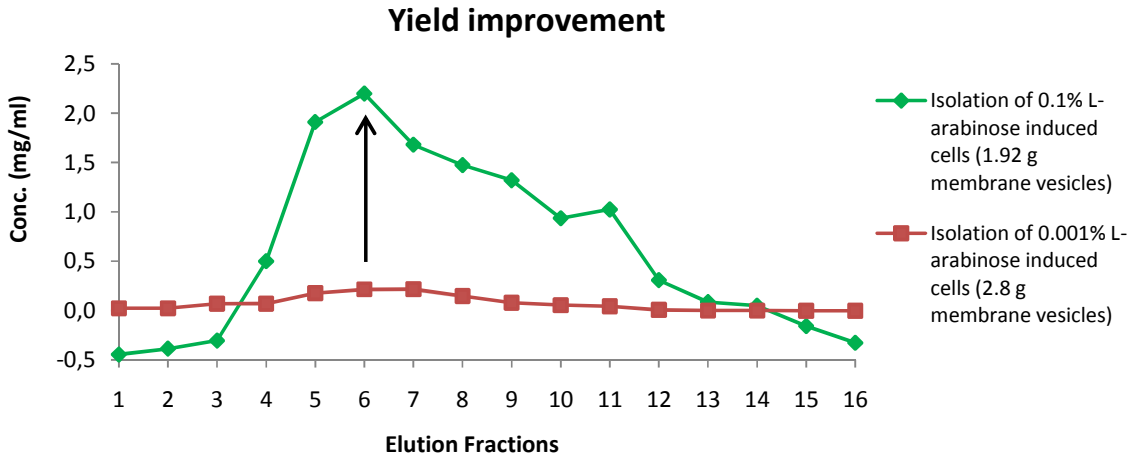

Figure 18: Bradford assays of the double-scale-up isolation of 0.001% L-arabinose induced cells (red, see 4.2.2) and the isolation done with the 0.1% L-arabinose induced cells (green, see 4.2.4). This comparison shows the improvement achieved by using a 100-fold higher L-arabinose concentration during induction. It should be noted that the isolation with the higher induction concentration was performed with ~30% less amount of vesicles, making the differences even bigger.

The same conditions were used as the double-scale up isolation because this yielded the best results so far. The isolation was done with 1.92 g membrane vesicles and 2.0 mL *Strep*-Tactin Superflow (50%) column matrix. The aim was to use 2.8 g vesicles, but the

concentration of the membrane vesicles was lower than expected because of a miscalculation. The SDS-PAGE showed that G22C-*Strep*II MscL was isolated with the same high purity as in the other isolation but in much greater quantity. By comparing the lysozyme bands with the bands of the elution fractions it is clear that a lot of fractions contain more than 0.5 mg/ml purified protein. The Bradford assay confirmed these results, showing that some fractions contained more than 2 mg/ml *Strep*II-tagged MscL. The real concentrations of all the fractions were difficult to determine because the BSA standard of the Bradford assay was not correct. Elution fractions that according to the Bradford assay contained no protein had a clear thick band on the SDS-PAGE, even though both methods use the same dye for staining. Nonetheless, it is clear that a lot of protein is produced with the 100-fold higher induction concentration. The preliminary results of the L-arabinose induction determination are therefore confirmed with this isolation. It is possible that even better results can be obtained by optimizing the amount of vesicles added per isolation. This needs to be tested in future studies.

Not only the yield, but also the purification was optimized for the *Strep*-Tactin isolation by adding avidin into the solubilization buffer. The effect of avidin on the purification of G22C-*Strep*II MscL was analyzed by doing two isolations; one with and one without the addition of avidin. The SDS-PAGEs showed that there is no clear difference in purity between both isolation methods. However, the Western blots did show a clear difference; the isolation with the addition of avidin had less contamination than the isolation without avidin. Avidin thus has a positive effect on the purification of *Strep*II-tagged MscL, although in a small extent. These results confirmed the findings that were addressed by T.G Schmidt and A. Skerra (23). Because of the small effect on the purification and no effect on the activity of the channel it is safe to say that avidin is not crucial for the *Strep*-Tactin isolation.

5.3 Determining activity of G22C-*Strep*II MscL

The reconstitutions and fluorescence dequenching assays made it possible to study the function of the *Strep*II-tagged MscL channels. The reconstitution setup needed a lot of optimization in order to find any activity for the His- and *Strep*II-tagged G22C-MscL mutants. By using 10 mg/ml *E.coli* lipids and Triton X-100 from Sigma, G22C-*Strep*II MscL showed the first significant activity of ~19%. Although the activity is somewhat lower than G22C-His

MscL (~33%), it is clear that the *Strep*II-tag does not interfere with the functionality of the protein in a drastic way. Even though the channels show low activity, one would expect all calcein to be released after a certain period of time. This is not the case for both reconstitutions because the curves plateau at 20-40%. No clear reason for this phenomenon can be deduced. It is possible that because of the not fully optimized reconstitution setup only a small percentage of all the proteoliposomes have active MscL. The liposomes that contain no MscL, or do have MscL but in the opposite orientation, may not be opened by addition of MTSET. When Triton X-100 is added all the proteoliposomes will burst, even the liposomes that contain no (functional) protein, leading to lower activity percentages. Therefore, the low activity for both channels is mainly because of artifacts in the reconstitution setup and are not caused by intrinsic properties of the channels.

The reconstitution setup could be improved greatly by using a fresh batch of 20 mg/ml azolectin and Triton X-100 (10%) from Fluka (**Figure 19**).

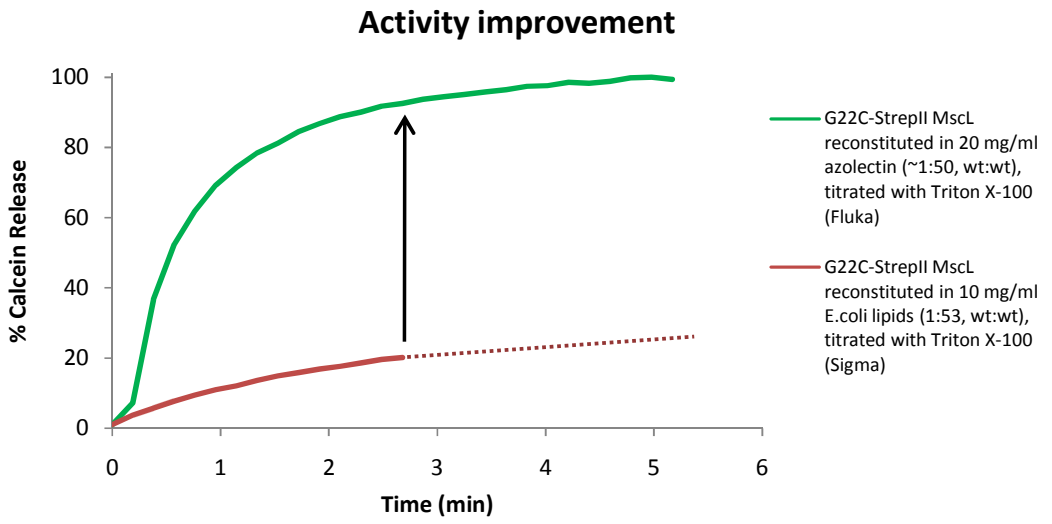

Figure 19: Fluorescence dequenching assay results of two different reconstitution setups (see 4.3). This comparison shows the improvement in activity achieved by using different batches of detergent, lipids and protein.

This time, G22C-*Strep*II MscL showed full activity since no clear difference in calcein release was observed after the Triton burst. This means that all the proteoliposomes contained functional protein. The His-tagged G22C mutant also showed high activity (~77%), although a bit lower than the *Strep*II-tagged mutant. This can be due to differences in the protein to lipid ratios between the two reconstitutions because the exact concentration of the G22C-

*Strep*II MscL is not known. It is also possible that the higher induction of 0.1% L-arabinose leads to more properly-folded protein than the recommended value of 0.001%. The reason why a different Triton X-100 batch works better is probably because detergents are known for aging in which oxidative reactions form other, unknown, compounds. This decrease in quality makes the actual detergent concentration lower, which effects the titration of the lipids. It is also possible that the newly formed compounds can interact with the cysteines of the G22C-mutant, making it impossible for MTSET to form a disulfide bond.

Drawing a conclusion about the differences of the *Strep*II-tag and the His-tag on the activity of MscL was difficult as the fluorescence dequenching assay only shows qualitative differences. The quality of the reconstitution is influenced by many variables, such as the detergent, the lipids, the temperature, the protein, etc. However, the fluorescence dequenching experiment unambiguously showed that the *Strep*II-tag does not inactivate the channel in any way. Nevertheless, the *Strep*II-tag can still influence the opening behavior of the channel, not visible with the fluorescence dequenching assay. In order to determine the actual differences between the two tags, single channel recordings need to be done with patch clamp or black lipid membrane.

In conclusion, this study has shown that the protein yield of the *Strep*-Tactin affinity chromatography could be improved more than tenfold by inducing the *E.coli* cells with 0.1% L-arabinose, instead of the recommended value of 0.001%. It is shown that avidin is able to mask biotinylated host proteins, leading to a higher degree of purity. The fluorescence dequenching experiments showed that G22C-*Strep*II MscL is an active channel by using 20 mg/ml azolectin and Triton X-100 from Fluka during the reconstitution. No loss of function could be found because of the *Strep*II-tag. This research project thus has proven that the S*trep*II-tag is a practical tool for isolating MscL.

6. References

1. *Molecular Basis of Mechanotransduction in Living Cells.* **Hamill, O.P. and Martinac, B.** 2001, Physiological Reviews, Vol. 81 (2), pp. 685-740.

2. *Gating-associated conformational changes in the mechanosensitive channel MscL.* **Yoshimura, K., Usukura, J. and Sokabe, M.** 2008, PNAS, Vol. 105 (10), pp. 4033-4038.

3. *Mechanosensitive channels.* **Sackin, H.** 1995, Annu. Rev. Physiol., Vol. 57, pp. 333-353.

4. *Structure of the MscL Homolog from Mycobacterium tuberculosis: A Gated Mechanosensitive Ion Channel.* **Chang, G., et al.** 1998, Vol. 282, pp. 2220-2226.

5. *Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches.* **Hamill, O.P., et al.** 1981, Pfügers Arch. Eur. J. Physiol., Vol. 391, pp. 85-100.

6. *Multiple mechanosensitive ion channels from Escherichia coli, activated at different thresholds of applied pressure.* **Berrier, C., et al.** 1996, J. Memb. Biol., Vol. 151, pp. 175-187.

7. *Protection of Escherichia coli cells against extreme turgor by activation of MscS and MscL mechanosensative channels: identification of genes required for MscS activity.* **Levina, N.** 18, 1999, EMBO J., pp. 1730-1737.

8. *Release of thioredoxin via the mechanosensitive channel MscL during osmotic downshock of Escherichia coli cells.* **Ajouz, B., et al.** 1998, The Journal of biological chemistry, Vol. 273 (41), pp. 26670-26674.

9. *Elongation Factor Tu and DnaK Are Transferred from the Cytoplasm to the Periplasm of Escherichia coli during Osmotic Downshock Presumably via the Mechanosensitive Channel MscL.* **Berrier, C., et al.** 2000, Jornal of Bacteriology, Vol. 182 (1), pp. 248-251.

10. *A large-conductance mechanosensitive channel in E. coli encoded by mscL alone.* **Sukharev, S.I., et al.** 1994, Nature, Vol. 368, pp. 265-268.

11. *Conformational changes involved in MscL channel gating measured using FRET spectroscopy.* **Corry, B., et al.** 2005, Biophysics J., Vol. 89 (No. 6), pp. L49-L51.

12. *Open channel structure of MscL and the gating mechanism of mechanosensitive channels.* **Perozo, E., et al.** 2002, Nature, Vol. 418, pp. 942-948.

13. *Bacterial mechanosensitive channels: Experiment and theory (Review).* **Corry, B. and Martinac, B.** 2008, Biochimica et Biophysica Acta, Vol. 1778, pp. 1859-1870.

14. *Hydrophilicity of a single residue within MscL correlates with increased channel mechanosensitivity.* **Yoshimura, K., Batiza, A. and Schroeder, M.** 1999, Biophys. J., Vol. 77, pp. 1960-1972.

15. *Chemically charging the pore constriction opens the mechanosensitive channel MscL.* **Yoshimura, K., Batiza, A. and Kung, C.** 5, 2001, Biophys. J., Vol. 80, pp. 2198-2206.

16. *Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase.* **Smith, D.B. and Johnson, K.S.** 1988, Gene, Vol. 67 (1), pp. 31-40.

17. *Purification and Functional Reconstitution of the Recombinant Large Mechanosensitive Ion Channel (MscL) of Escherichia coli.* **Häse, C.C., Le Dain, A.C. and Martinac, B.** 1995, The Journal of Biological Chemistry, Vol. 270 (31), pp. 18329-18334.

18. *Comparison of affinity tags for protein purification.* **Lichty, J.J., et al.** 2005, Protein Expression Purification, Vol. 41 (1), pp. 98-105.

19. *Conformational stability of pGEX-expressed Schistosoma japonicum glutathione S-transferase: a detoxification enzyme and fusion-protein affinity tag.* **Kaplan, W.** 1997, Protein Science, Vol. 6, pp. 399-406.
20. *Making the most of affinity tags.* **Waugh, D.S.** 2005, Trends Biotechnol., Vol. 23, pp. 316-320.
21. *Genetic Approach to Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate Adsorbent.* **Hochuli, E., Bannwarth, W. and Döbeli, H.** 1988, Nature Biotechnology, Vol. 6, pp. 1321 - 1325.
22. *Perspectives of immobilized metal affinity chromatography.* **Gaberc-Porekar, V. and Menart, V.** 2001, J. Biochem. Biophys. Methods, Vol. 49, pp. 335–360.
23. *The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins.* **Schmidt, T.G. and Skerra, A.** 2007, Nature Protocols, Vol. 2 (6), pp. 1528-1535.
24. *The random peptide library-assisted engineering of a C-terminal affinity peptide, useful for the detection and purification of a functional Ig Fv fragment.* **Schmidt, T.G. and Skerra, A.** 1993, Protein Engineering, Vol. 6 (1), pp. 109-122.
25. *Molecular interaction between the Strep-tag affinity peptide and its cognate target, streptavidin.* **Schmidt, T.G., et al.** 1996, J. Mol. Biol., Vol. 255, pp. 753-766.
26. *Improved affinity of engineered streptavidin for the Strep-tag II peptide is due to a fixed open conformation of the lid-like loop at the binding site.* **Korndörfer, I.P. and Skerra, A.** 2002, Protein Science, Vol. 11 (4), pp. 883-893.
27. *Mutagenesis of a flexible loop in streptavidin leads to a higher affinity for the Strep-tag II peptide and improved performance in recombinant protein purification.* **Voss, S. and Skerra, A.** 1997, Protein Engineering, Vol. 10, pp. 975-982.
28. *Use of the Strep-tag and streptavidin for detection and purification of recombinant proteins.* **Skerra, A. and Schmid, T.G.** 2000, Methods Enzymol, Vol. 326, pp. 271-304.
29. *Synthesis and utilization of reversible and irreversible light-activated nanovalves derived from the channel protein MscL.* **Koçer, A., Walko, M. and Feringa, B.L.** 2007, Nature Protocols, Vol. 2, pp. 1426-1437.
30. *Maximizing Functional Transport Protein Expression Controlled by the AraC/PBAD System in E. coli.* **Geertsma, E.R., van den Bogaard, G. and Poolman, B.** Chapter 2, 2005, Thesis E.R. Geertsma "What lies between: Functional interfaces in a dimeric transporter", pp. 13-22.
31. *The Escherichia coli L-arabinose operon: binding sites of the regulatory proteins and a mechanism of positive and negative regulation.* **Ogden, S.** June 1, 1980, PNAS, Vol. 77 (6), pp. 3346-3350.
32. *Nonchemotactic Mutants of Escherichia coli.* **Armstrong, J.B., Adler, J. and Dahl, M.M.** 1967, J. Bacteriol., Vol. 93 (No.1), pp. 390-398.
33. *Membrane topology and multimeric structure of a mechanosensitive channel protein of Escherichia coli.* **Blount, P., et al.** 1996, The EMBO Journal, Vol. 15 (no. 18), pp. 4798-4805.