UMEÅ UNIVERSITY

Separation and characterisation of Lol A, B and C proteins

Halaha Waduge Pasindu Nilanga Fernando

Master thesis: 30 hp
Supervisor: Karina Persson

Abstract

Prophyromonas gingivalis, as other Gram-negative bacteria, has two membranes, the outer and inner membrane. Both membranes contain lipoproteins which shape the membranes and protect against threats like antibiotics. Lipoprotein transport is vital for the survival of the bacteria and therefore promising as drug targets. To produce a successful antibiotic against those bacteria, we must understand how they transfer the lipoproteins over the periplasm, from the inner membrane to the outer membrane. The transportation system is known as Localization of lipoproteins (Lol) and there are two soluble proteins LolA and LolB, and one inner membrane complex LolCDE which facilitates this transportation.

This thesis project focuses on overexpressing and purifying recombinant forms of LolA, LolB and the periplasmic domain of LolC from *P. gingivalis* for structural and functional characterization.

Popular scientific summary

Lipoprotein trafficking is vital for all Gram-negative bacteria but has mainly been studied in *Escherichia coli* which is a γ-proteobacteria. For bacteria belonging to other phyla and classes much less is known. In this project I have studied *Porphyromonas gingivalis* which is an oral pathogen, that belongs to the phylum bacteroidota. *P. gingivalis* can cause tooth loss and chronic inflammation and is involved in many other diseases. As with many other bacterial pathogens it is desirable to eradicate it using directed antibacterial substances. This project describes three major proteins that are involved in the transport of lipoproteins in. *P. gingivalis*, LolA, LolB and LolC. The proteins that we are studying are recombinantly expressed in *E. coli*. The objects of this project were to use the purified proteins to analyze if they interact with each other, using different instrumental techniques and to solve the crystal structures of LolB and the periplasmic part of LolC.

Societal aspects

Antibiotic resistance is a global problem, hence novel antibiotics need to be designed. Therefore, it is important to discover novel drug targets. Here we are focusing on lipoprotein transport, which is a vital process for all Gram-negative bacteria, hence promising as drug targets. We are focusing on the phylum bacteroidota with the oral pathogen *P. gingivalis* as the model system.

Ethical aspects

There is no ethical effect on this project, since we work with bacteria and bacterial DNA, but in future steps ethical aspects may need to be considered, for example if drugs are developed that need to be tested in living animals and humans.

List of Abbreviations

LolA	Localisation lipoprotein A.
LolB	Localisation lipoprotein B.
LolC	Localisation lipoprotein C.
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophorosis.
BLI	Bio-Layer Interferometry.
PG	Porphyromonas gingivalis.
OM	Outer Membrane.
IM	Inner Membrane.
SOC	Super Optimal broth with Catabolite repression.
mM	Milli molar.
LB	Lysogeny broth.
IPTG	isopropylthio-β-galactoside.
MWCO	Molecular weight cut-off.
HIS	Histidine.
Da	Dalton.
XRD	X-Ray diffraction.
µl	Micro Liter.
RPM	Revolutions per minutes.
OM	Outer membrane.
OD_{600}	Optical density at 600 nanometres wavelength.
Ni-NTA	His60 Ni Super flow resin (Nitrilotriacetic acid with Ni).
TEV	Tobacco Etch Viru.

Author Contribution

In this thesis I have contributed my valuable time and skills, and the chemistry department of Umea University has provided Bio-Rad Gel filtration chromatography instrument, different kind of centrifugal instruments, different kinds of incubator instruments, heating blocks, Bio-Rad SDS-PAGE running instrument, heating blocks, BLI instrument, CB8 7SQ MosquitoR crystal robot, light microscope, micro pipets, several reagents, and chemicals. Mainly the methodological techniques and knowledge from Karina Persson (Associate professor chemistry department) and Deepika Jaiman (PhD Student at Biochemistry department) were the contributions for this thesis.

Table of contents

Abstract I
Popular scientific summary II
Societal aspects II
Ethical aspects II
List of Abbreviations III
Author Contribution III
1. Introduction 1
2. Experimental or Material and methods 2
  2.1 Transformation, overexpression, separation and purification of LolA 2
  2.2 Transformation, overexpression, separation and purification of LolB 4
  2.3 Transformation, overexpression, separation and purification of LolC 4
  2.4 Crystallization of LolA 4
  2.5 Crystallization and structure determination of LolB like. 5
  2.6 Crystallization of LolC 5
  2.7 BLI for Cleaved LolA, and His-tagged LolB like 5
  2.8 BLI for His-tagged LolA, and Cleaved LolC. 6
  2.9 BLI for His-tagged LolB Like, and Cleaved LolC 6
3. Results 7
  3.1 Results of SDS-PAGE, Gel filtration and protein concentration 7
  3.2 Results for crystalisation of LolA, LolB and LolC 15
  3.3 Results for BLI of LolA, LolB and LolC 19
4. Discussion 21
  4.1 Gel filtration, SDS-PAGE, and the concentrations 21
  4.2 Crystalisation 22
  4.3 BLI 23
5. Conclusions and Outlook 23
Acknowledgement 24
References 24
Appendix 26
  Appendix 1 26
  Appendix 2 28
  Appendix 3 29
  Appendix 4 29
  Appendix 5 30
  Appendix 6 31

1.Introduction

Gram-negative bacteria, such as *Porphyromonas gingivalis* (PG) and V*ibrio cholerae* (VC) has an outer membrane (OM) which provides a physical barrier for osmotic shocks, noxious compounds, and antibiotics (1,2). The OM also contains lipoproteins that are transported from the inner membrane (IM). In Gram-negative bacteria this trafficking process between IM and OM is performed by the membrane proteins and this function is crucial for the existence of the bacteria (3,4).

Roughly one-third of all proteins in the typical gram-negative bacterium cell are membrane proteins. The periplasm is home to soluble proteins that are involved in several processes such as nutrient import, disulfide bond formation, and peptidoglycan construction. Both the IM and OM contain integral membrane proteins, which usually take on a β-barrel shape in the OM and helical structures in the IM. Additionally, in both membranes there are lipoproteins, attached to the membrane by a lipid moiety in their N-terminus (5).

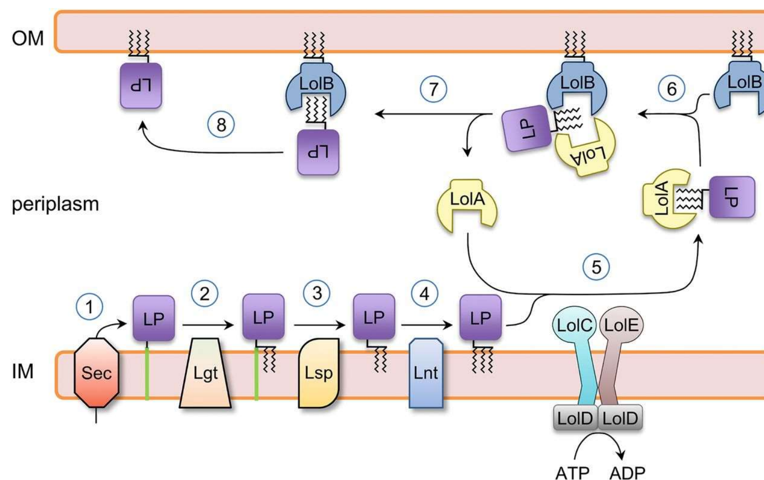

Figure 1

Figure 1.(8) An illustration of the stepwise conversion of prelipoprotein into triacylated lipoprotein with three inner membrane-bound Lgt, Lsp, and Lnt enzymes and lipoprotein trafficking from the inner membrane to the outer membrane, facilitated by the LolCDE complex, LolA, and LolB.

Prelipoproteins are produced in the bacterial cytoplasm and modified in the IM. Three membrane-bound enzymes, Prolipoprotein diacylglyceryl transferase (Lgt), Lipoprotein signal peptidase (Lsp), and Apolipoprotein N-acyltransferase (Lnt) sequentially modify prelipoproteins as they mature on the periplasmic face of the IM (6,7), resulting in proteins with an N-terminal lipid anchor. Lgt helps to add diacylglycerol to the side chain of a cysteine directly after the signal peptide and Lsp removes the N-terminal transmembrane anchor (signal peptide). Removal of the signal peptide generates a free amino group that can be acylated by Lnt resulting in a triacylated lipoprotein (8). The LolCDE complex facilitates the transfer of the triacylated lipoprotein to LolA. LolA then passes the lipoprotein to LolB via a mouth-to-mouth mechanism. Once this transfer is complete, LolA is released into the periplasm, leaving the lipoprotein bound to LolB. Subsequently, LolB delivers the lipoprotein to the outer membrane. In gram-negative bacteria some of the lipoproteins are transported to the outer membrane and a lipoprotein sorting motif in the N-

terminus determines its destination. The presence of Asp at +2 position (the acylated cysteine is +1) followed by certain residues at +3, the so-called Lol avoidance signal, signals that the protein is to stay in the IM(10,11). Otherwise the protein is transported to the inner leaflet of the OM (9). The five Lol proteins (LolA–E) make up the Lol machinery. Among these, lipoprotein sorting is started by LolCDE, an IM-embedded ATP-binding cassette transporter, which delivers OM-destined lipoproteins to LolA, a periplasmic chaperone, by selectively removing them from the outer leaflet of the IM (12,13,14). The LolA-lipoprotein complex is transported over the periplasm after which the lipoproteins are accepted by LolB, itself acylated and linked to the inner leaflet of the OM(15,16). A variety of lipoproteins that carry out a wide range of tasks have been identified as substrates of the Lol route(16).

The Lol system has mainly been studied in *E. coli* which is a γ-proteobacteria. Interestingly there are large variation in the acylation process and in the Lol system when comparing gram-negative bacteria of different classes and phyla. For instance, the Lnt enzyme has not been described for all bacteria and in the Lol system LolB is absent in many species. Here we are studying the Lol system in *P. gingivalis* which belongs to the phylum bacteroidota and is very distantly related to *E. coli*. Contrary to *E. coli*, has LolB not been described for *P. gingivalis*, however a thorough analysis of published protein sequences indicate that the bacterium encodes a lipoprotein that structurally resembles LolB. This protein is hereafter referred to as LolB-like. In this project I am studying the structure and function of the LolB-like protein and of LolA and the periplasmic domain of LolC. The long term goal is to understand the specificity and the differences among Lol systems of different phyla and classes in order to design a successful antibiotics. In this project I have overexpressed the *P. gingivalis* proteins in *E. coli* and purified them using affinity and size exclusion chromatography. Next, I have screened for crystallization conditions, collected X-ray diffraction data of the LolB-like protein and solved its structure. Further I have analyzed the interaction between the LolB-like protein and LolA using Bio-Layer Interferometry (BLI).

2. Experimental or Material and methods

2.1 Transformation, overexpression, separation, and purification of LolA.

LolA encoding plasmids were introduced to 100 µl of *E. coli* BL21 (DE3) competent cells in a 1.5 ml tube and a slight tap was done to mix them well before it was kept on the ice for 30 minutes. After 30 min cells were subjected to a 42°C heat shock for 30 sec and kept on ice for 2 min. 800 µl of antibiotic free SOC medium (Tryptone 2%, Yeast extract 0.5%, NaCl 10mM, KCl 2.5 mM) was added to the transformed cells and it was shaken at 200 RPM at 37°C for 1 hour. Then the cells were spread on a plate with antibiotics (kanamycin) and incubated at 37°C for overnight in an incubator.

400 ml of 5X LB medium (Tryptone 50g, Yeast extract 25g, NaCl 50g in 800ml distilled water) was diluted up to 2000 ml with water and 1.0ml of 100mg/ml kanamycin antibiotic. Colonies from the plates were used to inoculate 2×50 ml LB medium containing kanamycin in 100 ml conical flasks and incubated overnight in 200 RPM at 37°C. 50 ml of the cultures were transferred to two separate 2000 ml conical flasks containing 1000 ml LB + kanamycin and incubated at 200 RPM shaking at 37°C until their OD_{600} reached 0.6 absorbance. To induce the target protein

overexpression, 1ml of 100 mg/ml IPTG was added in to each culture and incubated at 18°C overnight. Both cultures were spun down at 4000 RPM and 20°C for 20 minutes and a 20µl of sample from pellet was taken for further SDS-PAGE run. The pellet was separated and stored at -80°C until the next step.

35 ml lysis buffer (appendix 3) supplemented with 300 µl of Triton X-100 was added to a falcon tube containing the pellet. The pellet was resuspended. The solution was sonicated for 5 min while controlling the temperature in an ice bath and the sonication setting was, 10 sec sonication after every 20 sec rest to reduce heat accumulation. The sonicated suspension was separated in to two centrifuge tubes and centrifuged for 25 min at 10°C at 23000 RPM in a JA25.50 rotor. The supernatant was transferred into a falcon tube and 20 µl was kept for analysis on SDS-PAGE. 3 ml of His60 Ni Super flow resin (Takara Bio) was transferred into a 20 ml filter column and a full column volume of lysis buffer was used to wash the Ni Beads.

The washed Ni Beads were added to the supernatant in a falcon tube, and it was mixed and kept at 4°C for about 20 min allowing the protein to bind the Ni-beads. The supernatant and Ni bead mix were centrifuged for 8 min at 2000 RPM at 4°C and the supernatant (Flow through) was poured into a separate container. The Ni beads were washed with wash buffer and again centrifuged for 8 minutes at 2000 RPM and 4°C and drain off the wash buffer. Ni beads were washed two times and transferred into the filter column. One column volume of wash buffer was added into the filter column for further wash, and it was left to drain off. 10 ml of elution buffer was added on to the washed Ni Beads and the flow through was collected. 20 µl of 0.5M EDTA and 20µl of 1M DTT was added to the eluted protein and kept on ice.

The Ni beads were washed with one column volume of water and one column volume of wash buffer and Ni beads were transferred to the flow through from the first purification step and the purification was repeated.

Both elutes were pooled into a falcon tube and 50 µl of 1M DTT was added. This elute was separated into two aliquots. To change the buffer medium, one aliquot was concentrated using a 10,000 MWCO centrifuge filter (Millipore) at 4,000 RPM at 4°C. The centrifuge column was then filled with HEPES buffer (20 mM HEPES, pH 7.5, 0.3 M NaCl) twice to perform the buffer exchange. After the final spin, the buffer-exchanged protein was carefully collected and transferred to a Falcon tube. 500 µl of 2 mg/ml TEV protease was added into buffer-exchanged aliquot and it was kept in room temperature for 2 hours and then at 4°C overnight. Aliquot two was kept on ice.

The TEV cleaved protein was incubated with cleaned Ni beads for 2×20 minutes and it was poured into the filter column to collect the flowthrough, which contained the target protein without histag. The flowthrough was concentrated using 10000 MWCO centrifuge filter at 4000 RPM at 4°C. To change the buffer the centrifuge column was filled with HEPES (20 mM HEPES pH 7.5, 0.3M NaCl) buffer in the last run. The protein was concentrated to 1000 µl and transferred into to a 1.5ml tube. 20 µl of cleaved protein was separated and kept for SDS-PAGE.

The cleaved protein was purified on a gel filtration column (Superdex 200 16/60; Cytiva) connected to a Bio-Rad chromatography instrument. 4 ml fractions were collected separately with relevance of nice single peak in the chromatogram. 20 µl of each fraction was kept for SDS-PAGE analyis. A 12% SDS-PAGE was run to analyze pellet, supernatant, cleaved protein and fractions of the cleaved protein that was purified with gel filtration.

Peak fractions 3, 4, 5, 6 (Figure 4) were pooled and concentrated. The concentration of the protein was measured with a nanodrop instrument and was

aliquoted into 50 µl PCR tubes frozen in a N_2 bath and stored in -80°C. The concentration of cleaved LolA was 100 mg/ml (20 mM HEPES pH 7.5, 0.3M NaCl)

The protein aliquot two (non-cleaved LolA) was concentrated with a 10000MWCO concentrator at 4000 RPM at 4°C and the HEPES buffer (20 mM HEPES pH 7.5, 0.3M NaCl) was added at the last centrifuge to change the buffer medium. 20 µl of His-tagged LolA protein was separated and kept for SDS-PAGE.

The His-tagged protein was purified using size excusion chromatography as described for the cleaved protein. 20 µl of samples were separated and kept for each fraction for 12% SDS-PAGE. An SDS-PAGE was run for pellet, supernatant, His-tagged and fractions of His-tagged LolA 5,6,7 and 8. Peak fractions were pooled into the 10000 MWCO concentrator, and the pure His-tagged LolA was concentrated. The concentration of the protein was measured with a nanodrop instrument and was aliquoted into 50 µl PCR tubes, frozen in a N_2 bath and stored at -80 °C. The concentration of LolA His-tagged was 13.6 mg/ml (in 20 mM HEPES pH 7.5, 0.3M NaCl).

2.2 Transformation, overexpression, separation, and purification of the LolB-like protein.

The transformation, overexpression, separation, and purification techniques for the LolB-like protein was the same as in LolA.
The concentration of cleaved LolB-like protein was measeured to 26.7 mg/ml (20mM HEPES pH 7.5, 0.3M NaCl) and the concentration of LolB His-tagged to 24.2 mg/ml (In HEPES pH 7.5, 0.3M NaCl) using a nanodrop instrument.

2.3 Transformation, overexpression, separation, and purification of the periplasmic domain of LolC.

Unlike LolA and LolB-like, for LolC purification, 5% glycerol was used in every buffer (appendix 4) and for transformation we used C43 (DE3) competent cells. Overexpression, separation, and purification techniques were the same as in LolA , and LolB-like. While cleaving LolC, 0.5ml of 2.0mg/ml TEV protease was used. The concentration of LolC Cleaved was measured to 15.7 mg/ml (20 mM HEPES pH 7.5, 0.3M NaCl, 5% Glycerol) and the concentration of LolC His-tagged to 7.3 mg/ml (In 20 mM HEPES pH 7.5, 0.3M NaCl, 5% Glycerol) using a Nanodrop instrument.

2.4 Crystallization of LolA.

100 mg/ml (in 20 mM HEPES pH 7.5, 0.3M NaCl) LolA w/o histag was thawed in an ice bath and the concentration was adjusted to 5 mg/ml with 20mM HEPES pH 7.5 and 0.3 M NaCl.

One crystallisation 96 well plate (Swissci) was prepared with the Morpheus crystallization kit (Molecular Dimension). Each well was filled with 80 µl crystallization solution.

The crystallization plate was placed on the CB8 7SQ MosquitoR crystal robot and a 8 wells 5 µl reservoir strip was filled with 5 mg/ml protein. The settings of the CB8 7SQ MosquitoR crystal robot was set to mix 200 nl protein and 100 nl crystallization solution

The plate wascovered with sticky sealing tapes and carefully kept in a temperature-controlled room to facilitate crystal growth.

Every 24 hours for four days the plate was observed through light microscope and each drop was recorded.

2.5 Crystallization and structure determination of LolB.

The cleaved LolB-like protein at 27 mg/ml (In HEPES pH 7.5, 0.3M NaCl) was thawed on ice and the concentration was adjusted to 18 mg/ml with 20 mM HEPES pH 7.5 and 0.3 M NaCl.

Two crystallisation plates were prepared with MEMGOLD 1 and MEMGOLD 2 crystallization screens (Molecular Dimension). Each well was filled with 80 µl of crystallization solution.

Crystallisation plates were prepared as described above and needle like Crystals were observed in several conditions after 24 hours. In well G6 (MEMGOLD 1 crystallisation plate) we could observe proper, single crystals after 72 hours.

After flash freezing in liquid nitrogen the crystals were sent to the European Synchrotron Radiation Facility (ESRF) in Grenoble where X-ray diffraction data was collected. The data was automatically processed at the synchrotron. The structure was determined with the method molecular replacement using the program Phaser in the Phenix software. The initial search model was obtained from Alphafold. The structure was refined using phenix.refine and rebuilt by several rounds in WinCoot.

2.6 Crystallization of LolC.

Cleaved LolC protein at 16 mg/ml (In HEPES 7.5 pH, 0.3 M NaCl, 5% Glycerol) was thawed on ice and the concentration was adjusted to 5.3 mg/ml with the buffer of 20mM HEPES pH 7.5and 0.3 M NaCl.

One crystallisation 96-well plate was prepared with PGA MD 1-50 crystallization screen (Molecular Dimension). Each well was filled with 80 µl of buffer mix.

Crystalisation plates were prepared as described above but, Crystals were not observed.

2.7 BLI for cleaved LolA and His-tagged LolB Like.

In order to analyze the interaction between LolA and LolB, three PCR vials of His-tagged LolB and cleaved LolA protein were thawed on ice. The concentration of His-tagged LolB was adjusted to 1 µM with BLI buffer (20 mM HEPES pH 7.4) and cleaved LolA was diluted with the same buffer to make a concentration series of 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.125 µM, 1.562 µM.

The sample rack was prepared as below before introducing to the instrument.

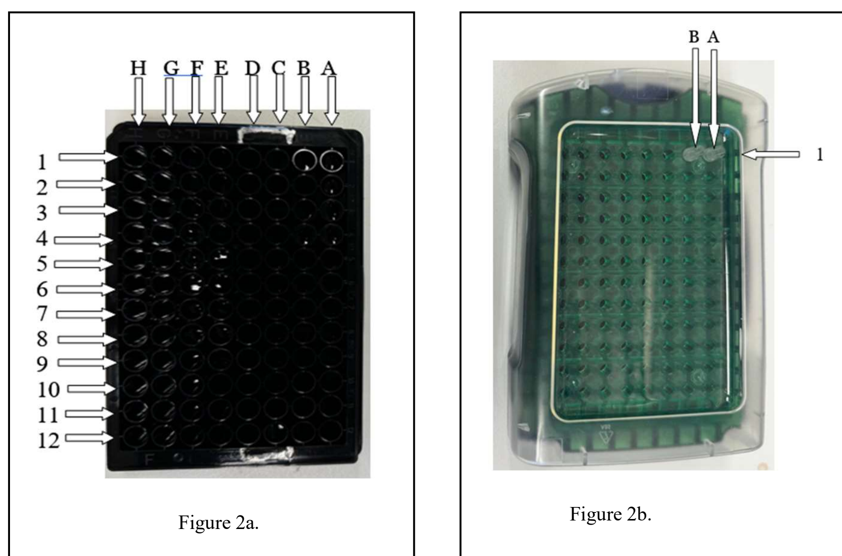

Figure 2a.

Figure 2b.

Figure 2a. BLI sample tray. In A and B rows, we use solutions to clean the sensors and, in E and F rows we use solutions to run the analysis. In each row, there are 12 wells.

Figure 2b. BLI sensor tray. The sensors were placed In row A and B number 1 positions. On 1A position sample running sensor and on 1B position control running sensor.

As per the figure 2a, E and F rows, wells 1 to 12 were filled with 200 µl of relevant solution. E1, E3, E12, F1, F2, F3 and F12 were filled with BLI buffer. 4E and 4F filled with 1M NaCl. 2E was filled with 1 µM His tagged LolB protein. 5 to 11 wells of both E and F rows were filled with the cleaved LolA protein with the ascending order of concentration. 1A, 4A, 1B and 4B are filled with BLI buffer 2A and 2B with 1 M imidazole and 3A and 3B with 1 M NaCl.

The sensors were cleaned with sensor cleaning program to remove any His-tagged protein on the sensor then the prepared sample tray was run with the desired sample running programme.

2.8 BLI for His tagged LolA and cleaved LolC.

In order to analyze the interaction between LolA and LolC, three PCR vials of His-tagged LolA and cleaved LolC protein were thawed on ice and the concentration of His tagged LolA was adjusted to 1 µM with BLI buffer (20 mM HEPES pH 7.4) and cleaved LolC was diluted with the same buffer to make a concentration series of 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.125 µM, 1.562 µM and follow the same procedure as in 2.7.

2.9 BLI for His tagged LolB and cleaved LolC.

To analyze the interaction between LolB and LolC, three PCR vials of His-tagged LolB and cleaved LolC protein were thawed in an ice bath and the concentration of

His tagged LolB was adjusted to 1 µM with BLI buffer (20 mM HEPES pH 7.4) and cleaved LolC was diluted with the same buffer (to make a concentration series of 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.125 µM, 1.562 µM and follow the same procedure as in 2.7.

3. Results

3.1 Results of SDS-PAGE, Gel filtration and protein concentrations.

To identify our target proteins, with or without histags, we conducted SDS-PAGE analysis. Following each gel filtration step, several fractions were pooled individually based on the gel filtration chromatogram to purify the target protein. The purified proteins were then concentrated, and their concentrations were quantified. All SDS-PAGE results, gel filtration chromatograms, and quantified purified protein concentrations are presented in the subsequent sections of the results below.

3.1.1 LolA

The practical results of the gel filtration runs and SDS-PAGE gel analyses related to cleaved and his-tagged LolA are detailed below. In each gel filtration run, an aggregated protein peak is observed prior to the pure protein peak. Due to their larger size, aggregated proteins elute faster than the pure protein fraction.

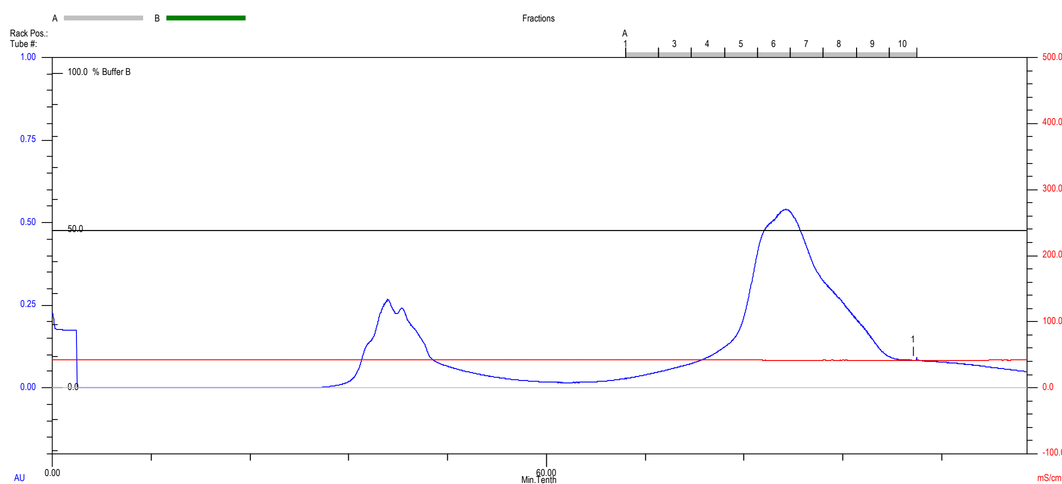

Figure 3.

Figure 3. Gelfiltration chromatogram of histagged LolA. This represents the gel filtration chromatogram of His-tagged LolA protein. The “Y” axis represents the absorbance at 280nm wavelength and “X” axis represent the time in minutes. Retention of LolA His-tagged is 90 minutes and fractions 2-10 were collected. The flow rate was 1 ml/min. The first peak at 40 minutes represents aggregated protein. The red line represent the gel filtration system pressure and black line shows the conductivity deviation of the matrix.

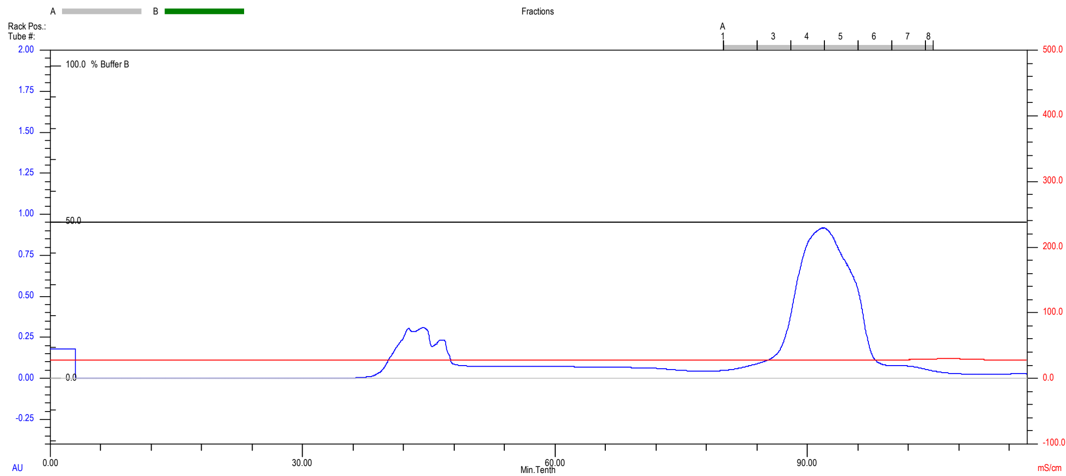

Figure 4.

Figure 4. Gel filtration chromatogram of cleaved LolA. This represents the gel filtration chromatogram of cleaved LolA protein. The "Y" axis represents the absorbance at 280nm wavelength and "X" axis represent the time in minutes. Retention of LolA cleaved is 93 minutes and fractions were collected from vial 2 to vial 8. The flowrate is 1 ml/min. The first peak at 42 minutes represents aggregated protein. The red line represent the gel filtration system pressure and black line shows the conductivity deviation of the matrix.

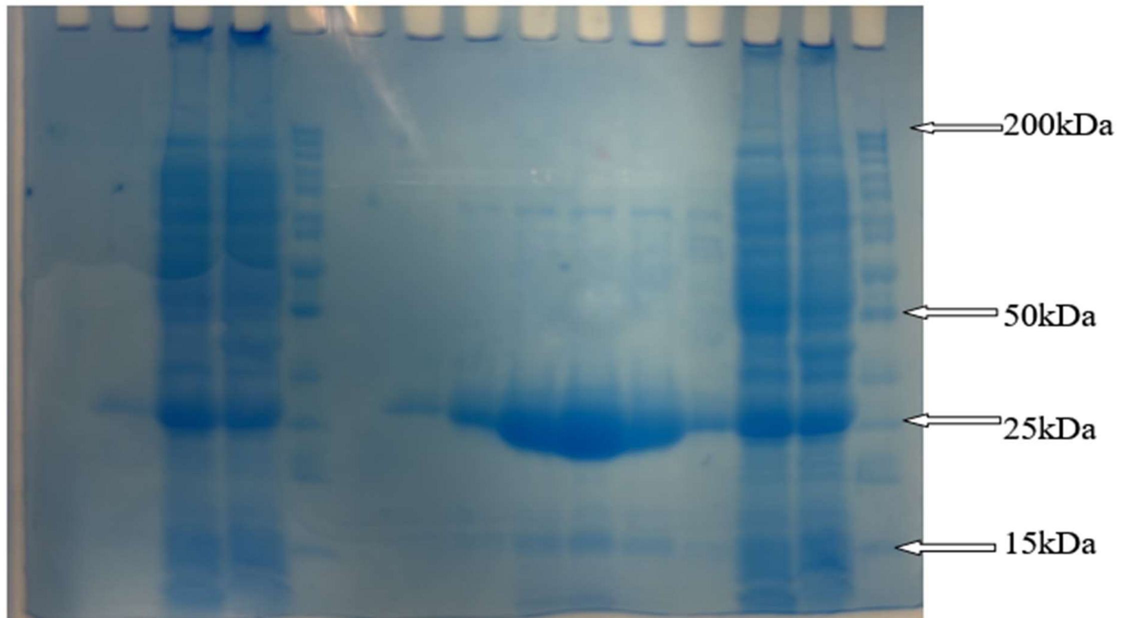

Figure 5.

Figure 5. SDS-PAGE analysis of HIS tagged LolA. Each well contains 4 μl of protein which denatured with 5X dye under 95°C on a heat block for 10 min. Well designations as below.

1. Empty, 2. Pellet, 3. Supernatant, 4. Supernatant, 5. Protein ladder, 6. Empty, 7. Fraction 2 of HIS tagged LolA gelfiltration, 8. Fraction 3 of HIS tagged LolA gelfiltration, 9. Fraction 4 of HIS tagged LolA gelfiltration, 10. Fraction 5 of HIS tagged LolA gelfiltration, 11. Fraction 6 of HIS tagged LolA gelfiltration, 12. Fraction 7 of HIS tagged LolA gelfiltration, 13. Supernatant, 14. Supernatant, 15. Protein ladder.

The protein ladder with 14 markers as 200 kDa, 150 kDa, 120 kDa, 100 kDa, 85 kDa, 70 kDa, 60 kDa, 50 kDa, 40 kDa, 30 kDa, 25 kDa, 20 kDa, 15 kDa, 10 kDa. 200 kD, 50 kD, 25 kD and 15 kD are labelled in the figure.

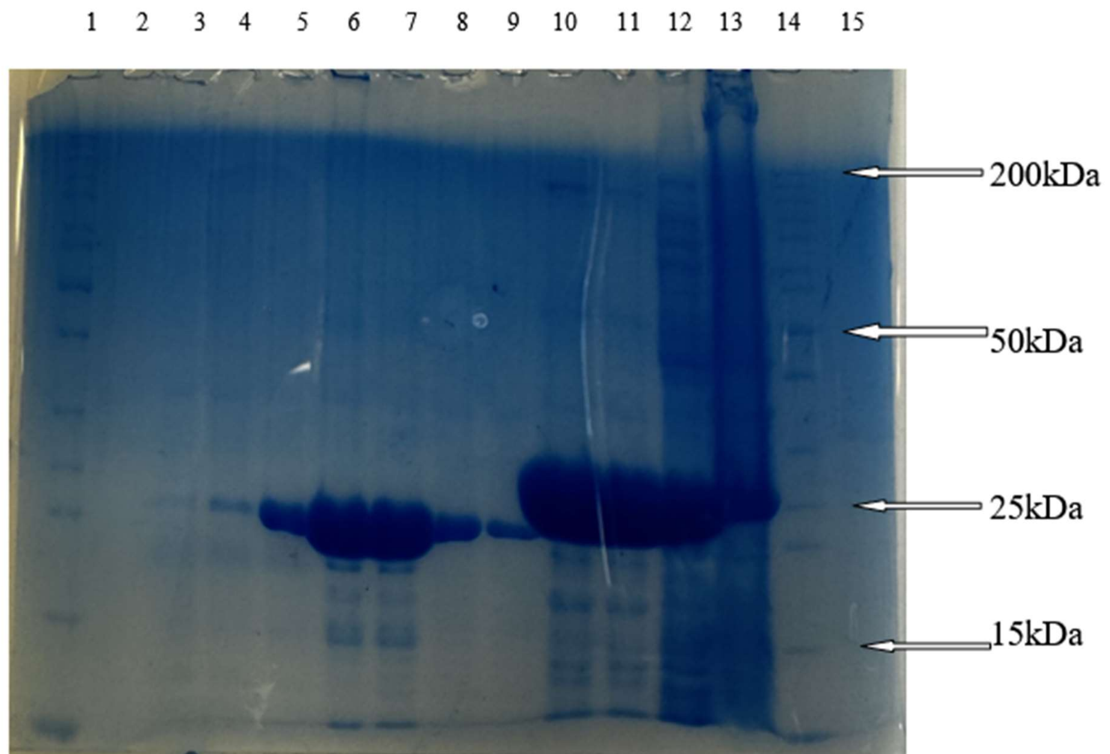

Figure 6.

Figure 6. SDS-PAGE run of cleaved LolA. Each well contains 4 μl of proteins which denatured with 5X dye under 95°C on a hot block for 10 min. Well are described below:

1.Protein ladder, 2. Empty, 3. Empty, 4. Fraction 3 of cleaved LolA gelfiltration run, 5. Fraction 4 of cleaved LolA gelfiltration run , 6. Fraction 5 of cleaved LolA gelfiltration run , 7. Fraction 6 of cleaved LolA gelfiltration run , 8. Fraction 7 of cleaved LolA gelfiltration run , 9. Fraction 8 of cleaved LolA gelfiltration run, 10. His tagged Lol A before gel filtration, 11. His tagged Lol A before gelfiltration, 12. Supernatant of LolA , 13. Pellet LolA, 14. Protein ladder, 15. Empty.

The protein ladder with 14 markers as 200 kDa, 150 kDa, 120 kDa, 100 kDa, 85 kDa, 70 kDa, 60 kDa, 50 kDa, 40 kDa, 30 kDa, 25 kDa, 20 kDa, 15 kDa, 10 kDa. 200 kD, 50 kD, 25 kD and 15 kD are labelled in the figure.

3.1.2 LolB like

The detailed results of gel filtration runs and SDS-PAGE gel analyses related to cleaved and his-tagged LolB are provided below. In the gel filtration run for His-tagged LolB, a very small aggregated protein peak is observed.

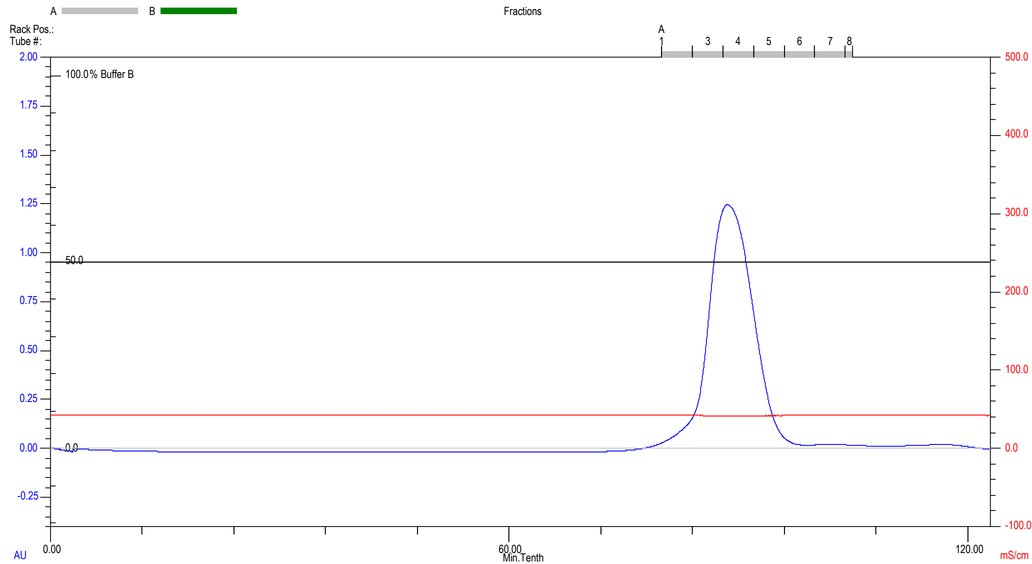

Figure 7.

Figure 7. Gel filtration chromatogram of His-tagged LolB-like protein. The "Y" axis represents the absorbance at 280 nm wavelength and "X" axis represent the time in minutes. Retention of His-tagged LolB protein is 86 minutes after the injection and fractions 2-8 were collected. The flow rate is 1 ml/min.The red line represent the gel filtration system pressure and black line shows the conductivity deviation of the matrix.

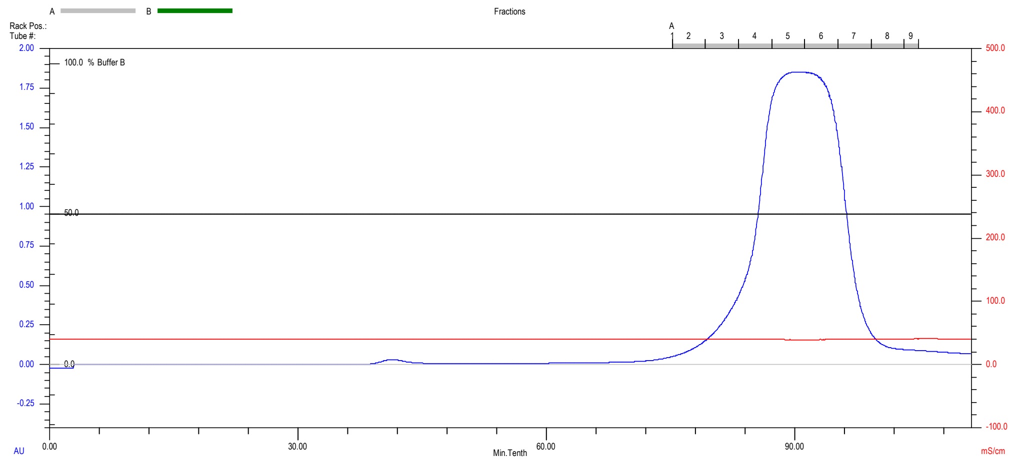

Figure 8.

Figure 8. Gel filtration chromatogram of cleaved LolB-like protein. The "Y" axis represents the absorbance at 280 nm wavelength and "X" axis represent the time in minutes. Retention of cleaved LolB protein is 90 minutes after the injection and fractions were collected from vial 2 to vial 9. The flowrate is 1 ml/min.The red line represent the gel filtration system pressure and black line shows the conductivity deviation of the matrix.

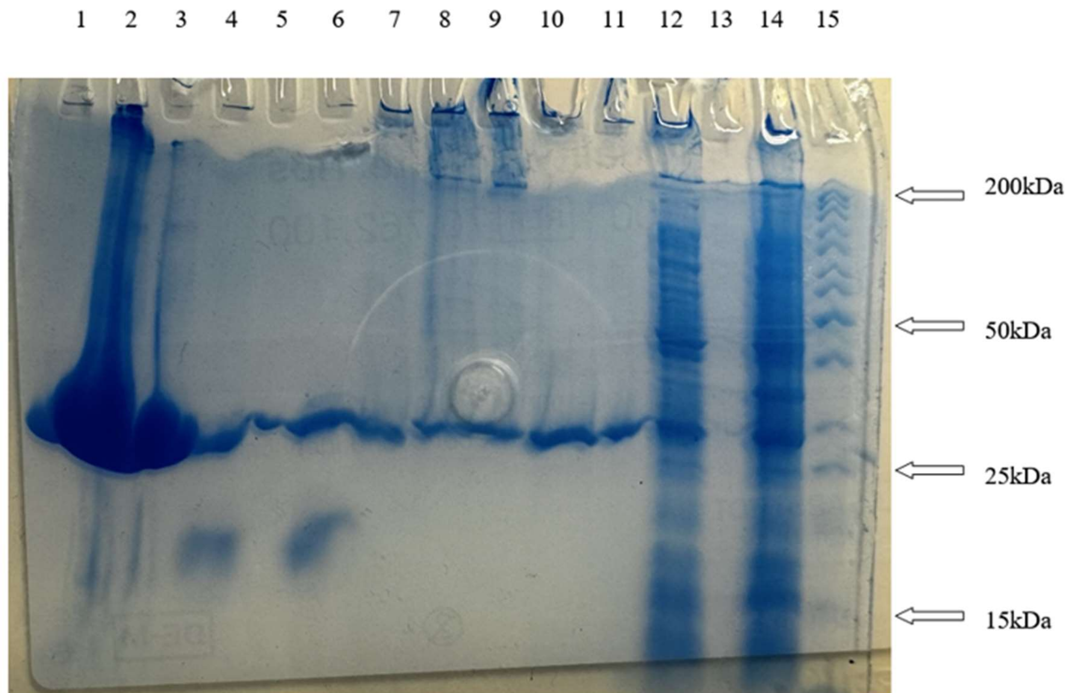

Figure 9.

Figure 9. SDS-PAGE run of HIS tagged and cleaved LolB. Each well contains 4 µl of proteins which denatured with 5X die under 95°C on a heat block for 10 min**.** well designations as below.

1. Empty, 2. Cleave LolB, 3. His tag LolB, 4. Fraction 3 of cleaved LolB gelfiltratioin run, 5. Fraction 4 of cleaved LolB gelfiltratioin run, 6. Fraction 5 of cleaved LolB gelfiltratioin run, 7. Fraction 6 of cleaved LolB gelfiltratioin run, 8. Fraction 7 of cleaved LolB gelfiltratioin run, 9. Fraction 3 of His tagged LolB gelfiltratioin run, 10. Fraction 4 of His tagged LolB gelfiltratioin run, 11. Fraction 5 of His tagged LolB gelfiltratioin run, 12. Supernatant, 13. Empty, 14. Pellet, 15. Protein ladder.

The protein ladder with 14 markers as 200 kDa, 150 kDa, 120 kDa, 100 kDa, 85 kDa, 70 kDa, 60 kDa, 50 kDa, 40 kDa, 30 kDa, 25 kDa, 20 kDa, 15 kDa, 10 kDa 200 kD, 50 kD, 25 kD and 15 kD are labelled in the figure.

3.1.3 LolC

The detailed results of gel filtration runs and SDS-PAGE gel analyses related to cleaved and his-tagged LolC are presented below. In each gel filtration run, an aggregated protein peak is observed.

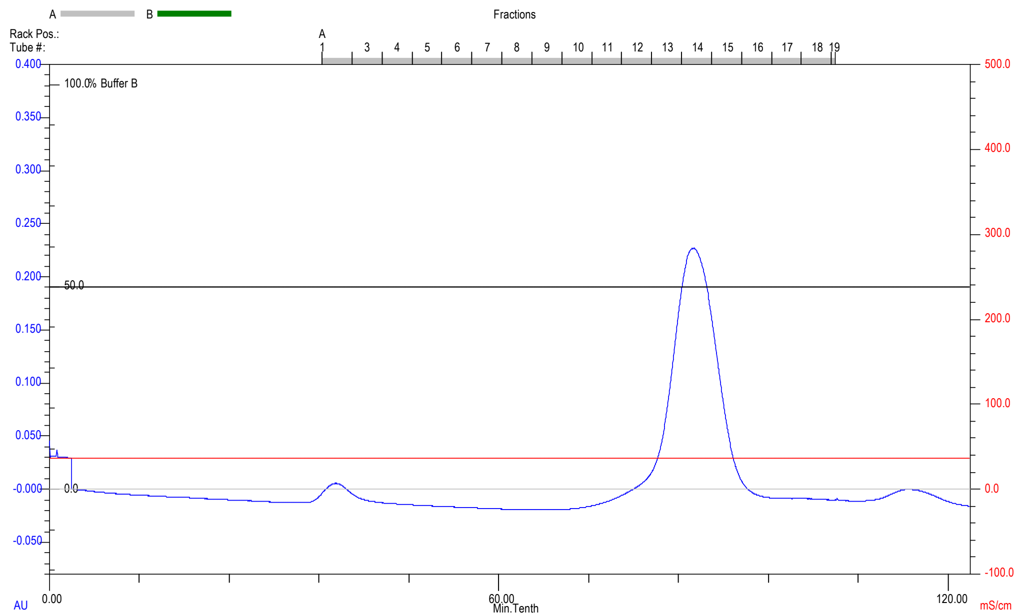

Figure 10.

Figure 10. Gel filtration chromatogram of HIS tagged LolC protein. The "Y" axis represents the absorbance at 280nm wavelength and "X" axis represent the time in minutes. Retention of His-tagged LolC protein is 85 minutes after the injection and fractions were collected from vial 2 to vial 19. The flowrate is 1 ml/min. The first peak at 36 minutes represents aggragated protein. The red line represent the gel filtration system pressure and black line shows the conductivity deviation of the matrix.

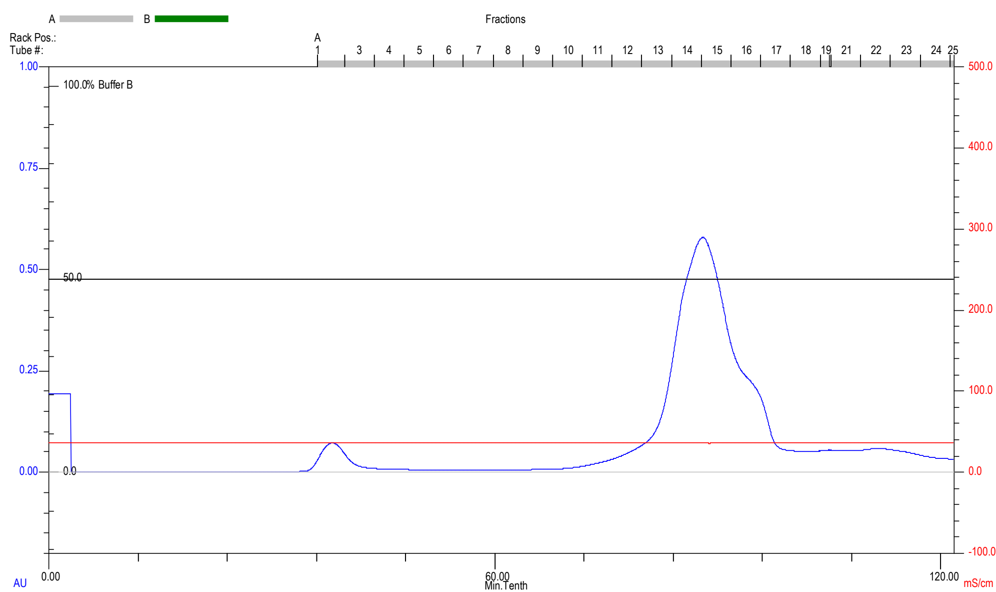

Figure 11.

Figure 11. Gel filtration chromatogram of Cleaved LolC protein. The "Y" axis represents the absorbance at 280 nm wavelength and "X" axis represent the time in minutes. Retention of cleaved LolC protein is 88 minutes after the injection and fractions were collected from vial 2 to vial 25. The flowrate is 1 ml/min. The first peak at 38 minutes represents aggragated protein. The red line represent the gel filtration system pressure and black line shows the conductivity deviation of the matrix.

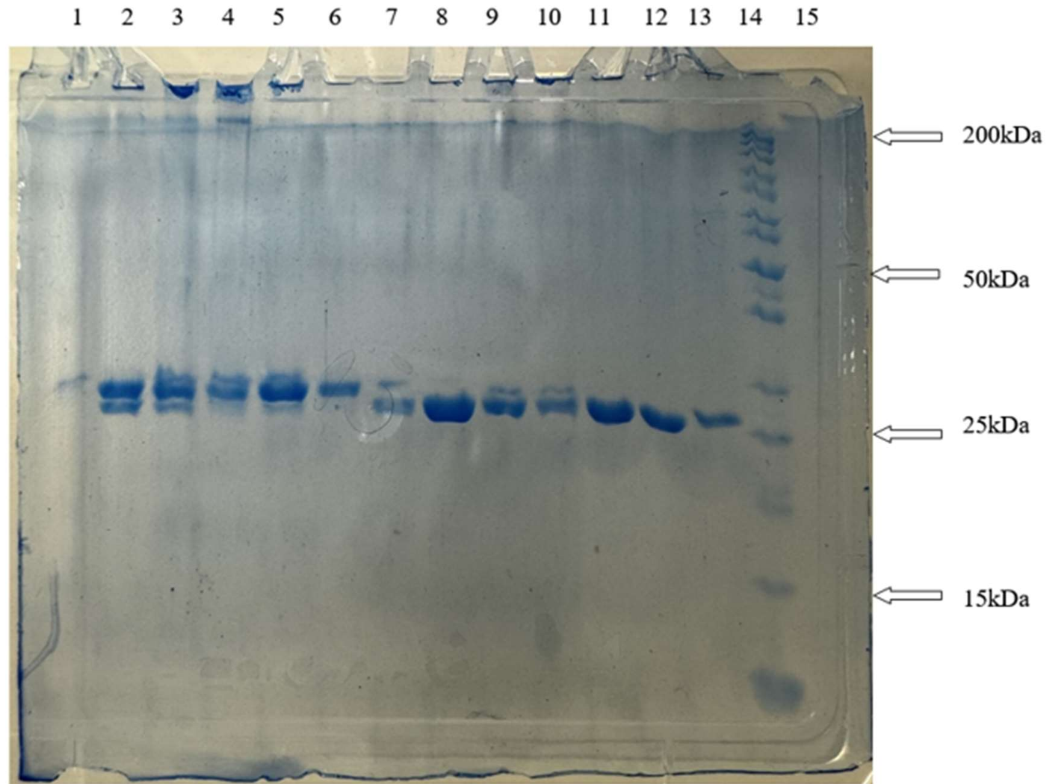

Figure 12.

Figure 12. SDS-PAGE run of His tagged and cleaved LolC. The protein fractions from fraction collector and each well contains 4 µl of proteins which denatured with 5X die under 95°C on a heat block for 10 min**.** well designations as below.

1. Empty, 2. Fraction 11 of His tagged LolC gelfiltration run , 3. Fraction 12 of HIS tagged LolC gelfiltration run , 4. Fraction 13 of His tagged LolC gelfiltration run , 5. Fraction 14 of Histagged LolC gelfiltration run , 6. Fraction 15 of His tagged LolC gelfiltration run , 7. Fraction 11 of cleaved LolC gelfiltration run , 8. Fraction 12 of cleaved LolC gelfiltration run , 9. Fraction 13 of cleaved LolC gelfiltration run , 10. Fraction 14 of cleaved LolC gelfiltration run , 11. Fraction 15 of cleaved LolC gelfiltration run , 12. Fraction 16 of cleaved LolC gelfiltration run , 13. Fraction 17 of cleaved LolC gelfiltration run , 14. Protein ladder, 15. Empty.

The protein ladder with 14 markers as 200 kDa, 150 kDa, 120 kDa, 100 kDa, 85 kDa, 70 kDa, 60 kDa, 50 kDa, 40 kDa, 30 kDa, 25 kDa, 20 kDa, 15 kDa, 10 kDa. 200 kD, 50 kD, 25 kD and 15 kD are labelled in the figure.

The concentrations of all Lol proteins can be found in Table 1. The concentrations were determined using a nanodrop instrument, with the absorbance of 1A corresponding to 1 mg/ml. To obtain the actual protein concentration, the nanodrop-measured concentration must be divided by the theoretically calculated extinction coefficient of each protein. The extinction coefficient, along with the calculated molecular weight and theoretical pI, was derived using the Expasy software based on the sequence data provided in Appendix 1.

All purified proteins except LolC cleaved and LolC His-tagged are in 20 mM HEPES pH 7.5, 0.3M NaCl. LolC cleaved and LolC His-tagged proteins are in 20 mM HEPES pH 7.5, 0.3M NaCl, 5% glycerol.

Table 1. Practical and theoretical results of purified proteins. This represents the measured concentrations, retention times, theoretically determined molecular weights and pI.

Purified Protein name	Final concentration (mg/ml)	Gel filtration main protein peak retention (Minutes)	Calculated molecular weight (Da)	Calculated theoretical pI values
LolA His tagged	13.6	90	23548.83	8.73
LolA Cleaved	100	93	22464.62	8.41
LolB His tagged	24.2	86	31074.34	7.21
LolB Cleaved	26.7	90	29992.13	6.64
LolC His tagged	7.3	85	27723.51	5.09
LolC Cleaved	15.7	88	26641.30	4.59

3.2 Results for crystallisation of LolA, LolB and LolC

Protein crystallisation is unpredictable, and we can expect the crystals sometimes with unexpected conditions. In this thesis we have obtained results in 5 mg/ml LolA concentration with Morpheus screen, 18 mg/ml LolB with MEMGOLD1 and MEMGOLD2 screens and 5.3 mg/ml LolC with PGA MD 1-50. We have received lots of precipitation and few needle like crystals but in MEMGOLD1 screen with 18 mg/ml LolB, we got nice crystals in well number G6 (0.05M Zinc acetate di-hydrate, 0.05M MES 6.1pH 11% W/V PEG 8000). We tried to repeat the crystallization with LolB under same conditions as mentioned above but it was not successful.

Crystals of the LolB-like protein were analyzed with XRD at the ESRF. X-ray data was collected and the structure was solved with Phaser and refined to 2.1 Å resolution (Appendix 6) with Phenix.refine and WinCoot.

3.2.1 Crystallisation of LolA

Every 24 hours, the LolA crystallization plate was examined under a light microscope. Needle-like crystals were observed in a few wells. However, none of these needle-like crystals developed into well-formed, proper crystals. The needle-like primary crystals are shown in Figure 13.

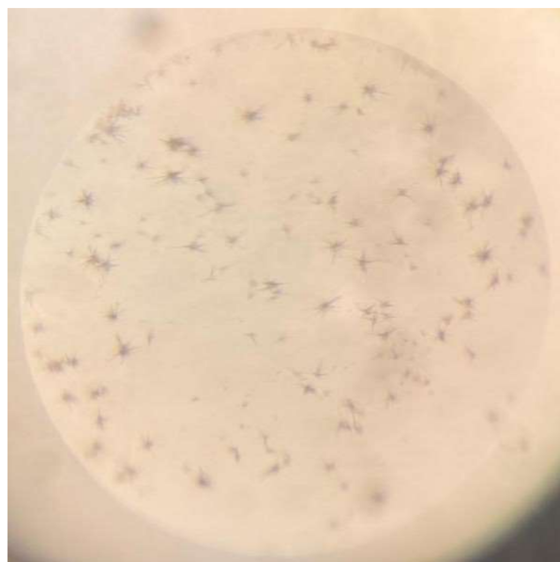

Figure: 13.

Figure 13. Needle-like primary crystals of LolA. This image under the light microscope. The buffer screen was Morpheus, and the well designation was D5. The concentration of the LolA was 5 mg/ml in 20 mM HEPES pH 7.4.

3.2.2 Crystallisation of LolB

The LolB crystallization plate was examined under a light microscope every 24 hours. Needle-like crystals were observed in several wells. However, well-formed, proper crystals were successfully identified in well G6 after 72 hours. This was the only well where fully developed single crystals were observed. The crystals found in well G6 are shown in Figure 14.

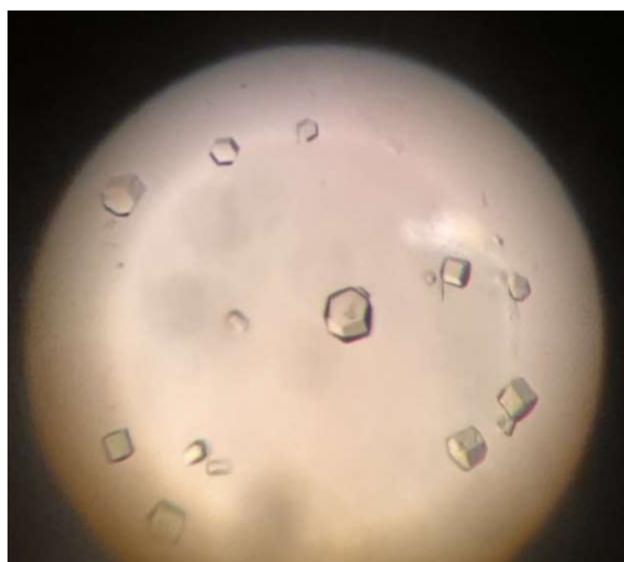

Figure: 14.

Figure 14. LolB crystals under the light microscope. The crystallization screen was MEMGOLD1, and the well was G6. The concentration of the LolB-like protein was 18mg/ml in 20 mM HEPES pH 7.4.

Diffraction data that were collected and processed and the ESRF were downloaded and used to solve the structure. The structure was determined by molecular replacement using the programme Phaser in the Phenix software. During refinement, using Phenix refine, the R-work and R-free values were monitored for each refinement step. These values represent the quality of atomic model obtain from the crystallographic data. R-work represents 90% of the data that is used for refinement and measures the how well the calculated model match the experimentally obtained diffraction patterns (17). R-free is used as a quality control and shows how well the model fits data (10%) that has not been used for refinement. Typically, the R-free value is higher than R-work value in each refinement step and both values should decrease with each refinement step.

In my refinement procedure I obtained 0.5454 and 0.5440 R-work and R-free values respectively after the first refinement but after 12 rounds of refinements the Rwork and Rfree had decreased to 0.2480 and 0.2909 respectively. The space group was $P6_522$ and there were two moleules in the asymmetric unit (see Appendix 6).The Figure 15b comprises 14 β-strands that form an open barrel, that together with the N-terminal helix constitute a binding cleft. The cleft is filled with two short helices and a loop. This structure has similarities with both LolA and LolB proteins that have been solved previously. The resulting structure looks like a cross-over with features of both LolA and LolB.

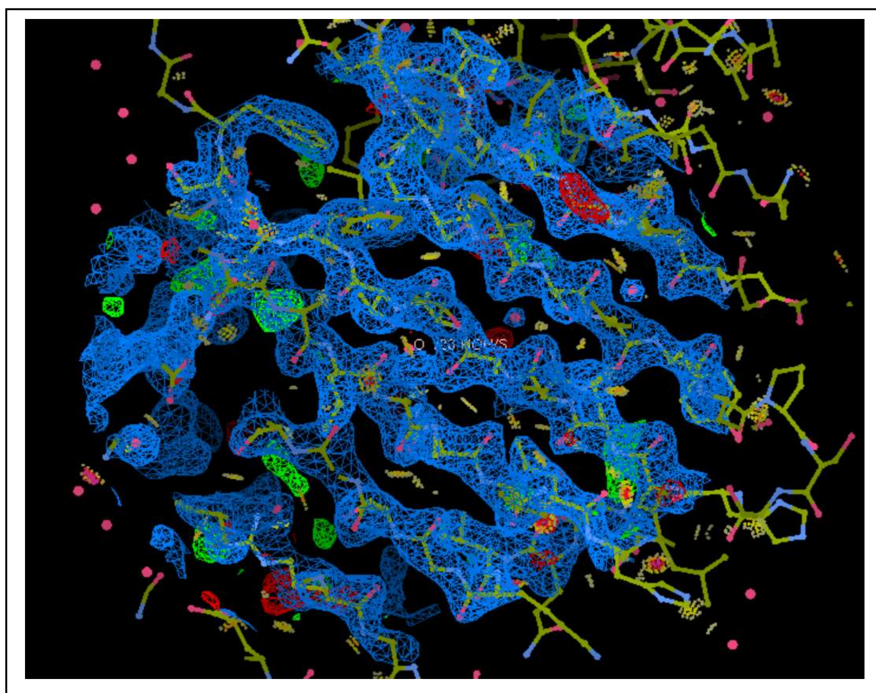

Figure:15a.

Figure15a. Electron density map for the LolB structure. The data which collected after analyzing the XRD of Crystals from figure 6 was refined with Pheonix and wincoot software tools. The model is depicted in its electron density.

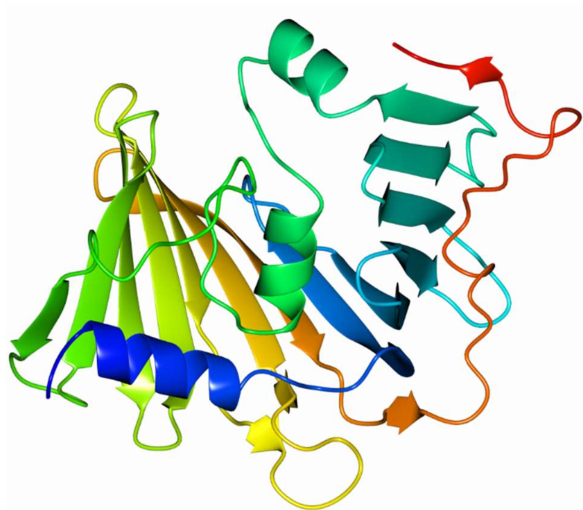

Figure:15b.

Figure15b. The ribbon diagram (Richardson diagram) of the LolB protein. provides the idea of its structural features, including α-helices, β-sheets, and binding clefts.

3.2.3 Crystallisation of LolC

The LolC crystallization plate was examined under a light microscope at 24-hour intervals. Apart from the presence of precipitates and clear drops, no crystals were observed. A clear drop is shown in Figure 16.

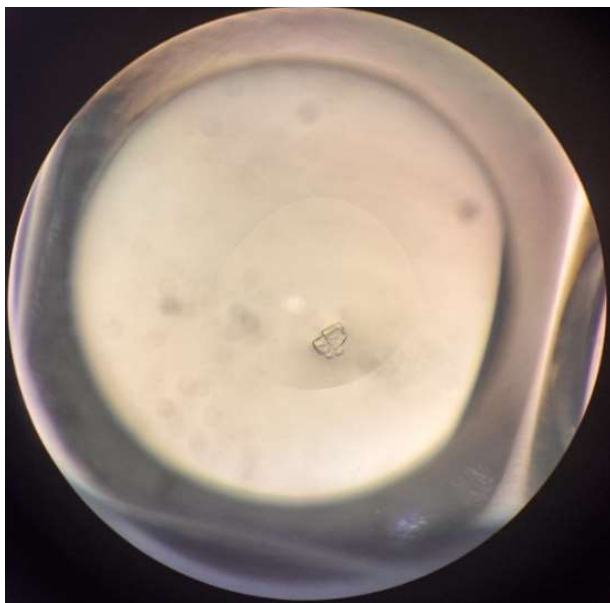

Figure: 16.

Figure:16. A clear drop under the light microscope. The buffer screen was PGA MD 1-50, and the well designation was E5. The concentration of the LolC was 5.3mg/ml.

3.3 Results for BLI of LolA, LolB and LolC

In the BLI experiment, the protein was bound to the Ni-sensor through its His-tag. Different concentrations of the cleaved protein, placed in different wells of the sample tray, interacted with the His-tagged protein, producing a measurable response. After each sample sequence on the BLI instrument, data was obtained as curves which shows the blank run, total response, sample response, and a graph plotting the response equiblirium against the cleaved protein concentration. Figure 18 shows the blank response over time, while Figure 18 shows the sample and blank responses over time in blue, and the sample-only response over time in red. Figure 19 shows the relationship between the response equiblirium and the cleaved protein concentration, gives data to determine the interaction rate constant.

3.3.1 Results of BLI of Cleaved LolA and HIS tagged LolB.

Under BLI analysis Cleaved LolA and HIS tagged LolB was the only combination became success.

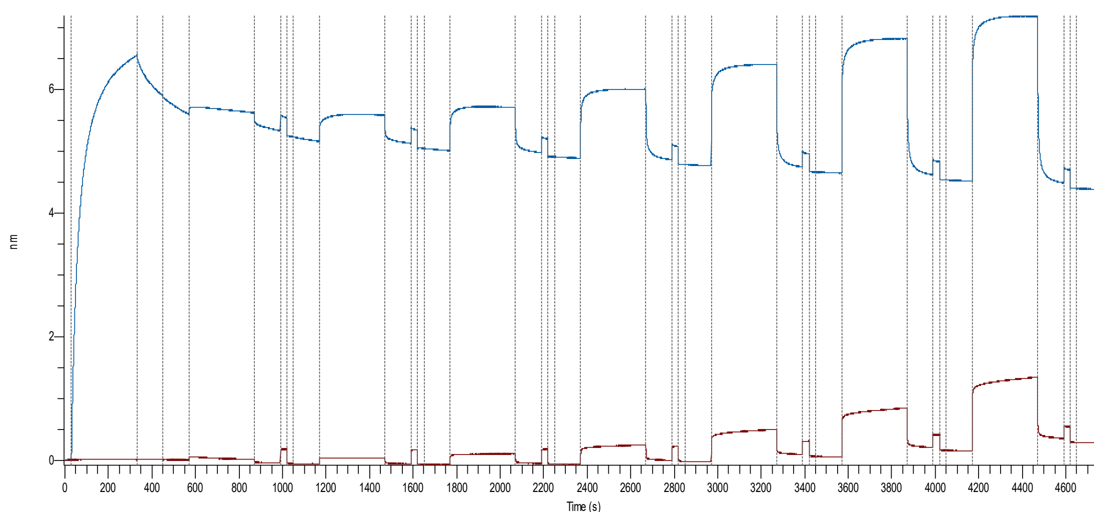

Figure 17.

Figure 17. BLI response of His tag LolB with cleaved LolA. X axis represent the time and the Y axis represent the response of binding. The blue colour line represent the binding responses of HIS tagged LolB with cleaved LolA and Ni-NTA sensor with all other background (blank). The red colour line indicates the responses of HIS tagged LolB with cleaved LolA (response with out the background). Each bump of responses along the X axis indicates the change of cleaved LolA concentration.

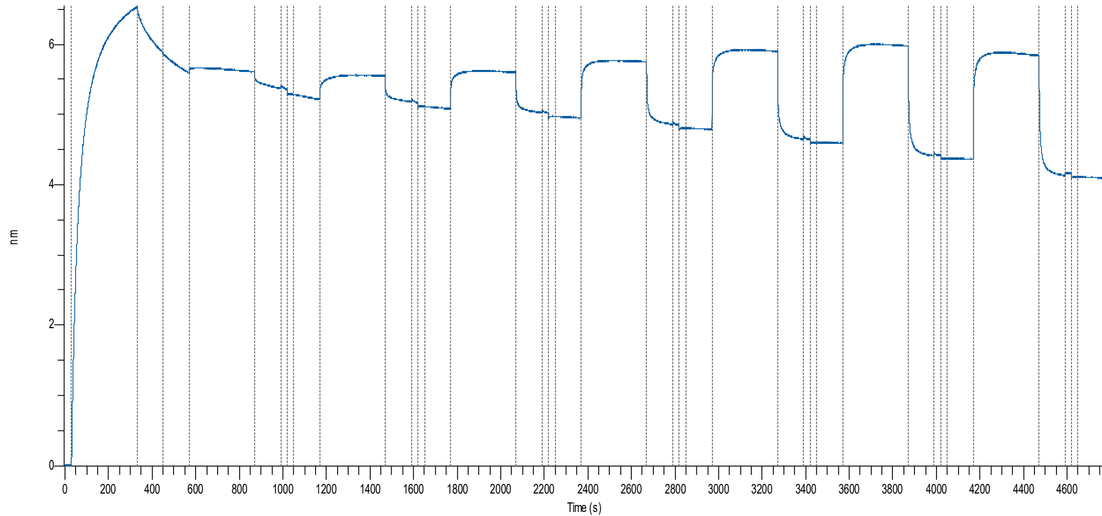

Figure 18.

Figure 18. The BLI response of Ni-NTA sensor with cleaved Lol A.. X axis represent the time and the Y axis represent the change of response on Ni-NTA sensor with the change of cleaved LolA concentration. Each bump of responses along the X axis indicates the change of cleaved LolA concentration.

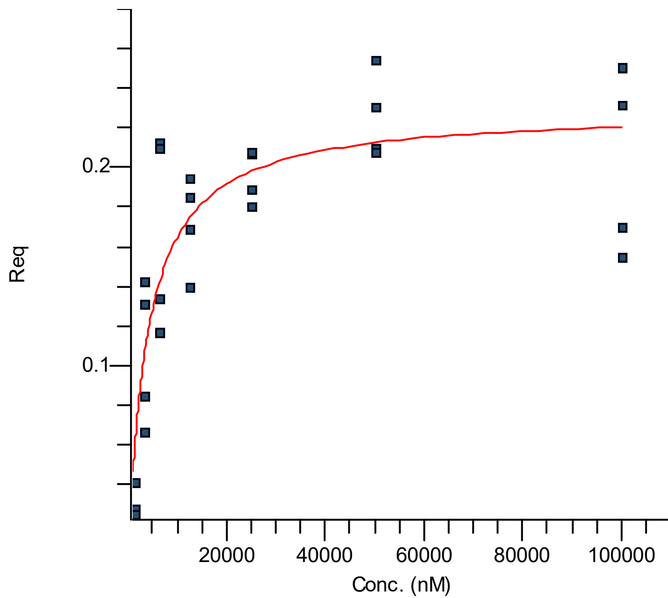

Figure 19.

$$Response = \frac{Rmax \times Conc.}{(KD + Conc.)}$$

Equation 1.

Equation 1. The equation enables the calculation of the dissociation constant (KD), which is a key parameter indicating the interaction rate constant.

Response= Response at relevant LolA cleaved concentration.
Rmax = Maximum response at relevant LolA cleaved concentration.
KD = Rate constant of interaction.
Conc. = LolA cleaved concentration.

Figure 19. the graph of the response equilibrium against the concentration of LolA cleaved interact with his tagged LolB in nM. The X axis represent the concentration of LolA cleaved which bind with LolB his tagged on the sensor and Y axis represent Response which calculated as per the equation 1. For this curve we have used four data sets.

The rate constant (KD) as per the Figure: 19 is $3.9\times10^{-6} \pm 0.91\times10^{-6}$ molar The uncertinity of the rate constant is so high due to random errors bot the value is still greater than the uncertinity so we can accept this value and this means that LolA cleaved interacts with LolB his tagged.

3.3.2 Results of BLI of Cleaved LolC with HIS tagged LolB and HIS tagged LolA.

For some reason LolC did not give any valuable responses with LolA so, there are no results that can be reported. Probably because there is no interaction or because LolC is not stable. In theory there should be no interaction between the periplasmic domain of LolC and LolB.

4. Discussion

4.1 Gel filtration, SDS-PAGE, and the concentrations.

When we consider the gel filtration chromatograms of LolA His-tagged and LolA cleaved we can see the elution times as 90 minutes and 93 minutes respectively. The calculated molecular weight of LolA His tagged and LolA cleaved are 23548.83 Da and 22464.62 Da, and the small difference in the elution time confirm that cleaving of the LolA had properly happened. As per the theory of elution in gel filtration the bigger partical elutes faster than smaller so we can have a hint of our theoretical values of proteins are match with the practical details. The spectrum shape in both chromatograms were not nicely spread because the proteins concentrations in column are high so column separation get disturb but, both have single peak and it can be considered as more pure fractions.

As per the SDS-PAGE runs, practically the gel filtration fractions and pure LolA proteins are lighter than 25000 Da with the protein marker ladder, and both SDS-PAGE runs clearly shows that LolA His tagged is larger than Lol A cleaved, and this also confirms that the cleaving of His tagged Lol A was successful.

When we consider the gel filtration chromatograms of LolB His tagged and LolB cleaved we can see the elution times as 86 minutes for LolB His tagged and 90 minutes for LolB cleaved. The calculated molecular weight of LolB His tagged and LolB cleaved are 31074.34 Da and 29992.13 Da respectively as per the theoretical calculations and the elution times represents that cleaving of the LolB had properly happened. The spectrum shape in both chromatograms were nicely spread and, since they both have single peak, it can be considered as more pure fractions than the

sample what we injected to the gelfiltration. In LolB cleaved the spectrum looks saturated.

As per the SDS-PAGE runs, practically the gel filtration fractions and pure LolB proteins are heavier than 25000 Da with the protein marker ladder, and SDS-PAGE runs clearly shows that LolB His tagged is heavier than LolB cleaved, and this also confirms that the cleaving of His-tag LolB was successful.

When we consider the gel filtration chromatograms of LolC His tagged and LolC cleaved we can see the elution times as 85 minutes for LolA His tagged and 88 minutes for LolC cleaved. The molecular weight of LolC His tagged and LolC cleaved are 27723.51 Da and 26641.30 Da respectively as per the theoretical calculations and the elution times represents that cleaving of the LolC had properly happened. The spectrum shape in both chromatograms were nicely spread and, since they both have single peak, it can be considered as more pure fractions.

As per the SDS-PAGE runs, practically the gel filtration fractions are heavier than 25000 Da with the protein marker ladder, and SDS-PAGE runs clearly shows that Lol C His tagged fractions are heavier than Lol C cleaved, and this also confirms that the cleaving of His tagged Lol B was successful.

While purifying the LolC proteins, we had to use 5% of Glycerol in all buffer mediums because, in practically, separation of LolC is difficult due to its aggregation so, the glycerol can interact with large patches of hydrophobic on protein and create barrier between proteins to avoid aggrigation and further more glycerol helps to avoid unfoldin of the proteins (18).

Concentrations of all Lol proteins were measured by nanodrop instrument, and it was measured as 1A=1mg/ml and, to get real protein concentration we must divide concentration by nano drop from theoretically calculated extinction coefficient of Lol protein .

4.2 Crystallisation

While crystalising LolA we used the Morpheus kit and this mixture generated needle like crystals in some wells, but they were to small for X-ray data collection.

For crystallization of the LolB-like protein we used MEMGOLD1 and MEMGOLD2 kits. Several needles like crystals and some nice crystals were obtained in these screen. The best crystals were obtained in well number G6 in the MEMGOLD1 kit. (0.05M Zinc acetate di-hydrate, 0.05M MES 6.1pH 11% W/V PEG 8000). We tried to crystalize LolB with the same conditions manually but we were not successful. Hence, we extracted crystals from the well G6 of the screen and analysed for XRD, and the data was interpreted and refined with Phenix and wincoot software. Initially the electron densities were perfectly matched with expected structure of LolB but, there were five miss located amino acids and we had to delete them and re-introduce as per the available electron densities and they were Lysine (at 228), Methionine (at 229), Arginine (at 230), Phenylalanine (at 231), Threonine (at 232). We had to rearrange the angles of amino acids and add water molecules to the available electron densities to get the minimum R-work and the R-free values with several rounds of refinement. The final R-work and R-free values are 0.2480 and 0.2909 respectively.The final refined structure can be seen in Figure 15. In Figure 15 we can see blue electron density which shows the aligned electron densities with the model, green electron density indicate that something should be added to the model and red coloured electron densities shows atoms that are included in the model that do not correlate with the experimental data.

This is the first structure of a LolB from *Bacteroidota*, marking a significant breakthrough in our understanding of this protein family. The structural elucidation of LolB from *Bacteroidota* provides new insights into its role in lipoprotein trafficking. This discovery opens up avenues for exploring the unique features of lipoprotein transport in *Bacteroidota*, potentially aiding in the development of novel therapeutic strategies targeting this pathway in these bacteria.

While crystalising LolC we used PGA MD 1-50 kit and unfortunately no crystals were obtained.

4.3 BLI Experiment

The experiment of LolB his tagged with LolA cleaved gave very good results and the rate constant of interact (KD) is $3.9\times10^{-6} \pm 0.91\times10^{-6}$ molar for LolA and LolB. This is a value that is similar to what has been obtained for the LolA-LolB interaction in for instance *V. cholerae* (19).

LolC cleaved didn't give any response against LolB his tagged and LolA his tagged.

In these all experiments we had to use extra pure fractions of all proteins and we had to do extra purifications with Ni- beads but those steps decreased our original protein concentrations and while thawing the LolA cleaved it got precipitated so we had to add 20mM HEPES pH 7.5, 0.3 M NaCl buffer to the PCR tube with freezed LolA cleaved protein before thawing to avoid precipitation and, re analysed the protein concentration to perform final dilutions.

Before starting any experiment, we cleaned the sensors and sample trays to remove any protein remaining and we used 1M imidazole to clean and remove any his tagged proteins on sensors which can be interfere for the results.

In LolB his tagged and LolA cleaved analysis we maintained experiment with the control. In control we didn't used His tagged LolB to bind on sensor to check how the LolA concentrations (backgrounds) interact with sensor and this run was considered as blank run and it can be seen in Figure: 18 which shows high background effect on sensor depend on the increasing LolA cleaved protein.

In the LolB HIS-tagged and LolA-cleaved BLI analysis, we can observe a clear interaction between HIS-tagged LolB and cleaved LolA. This is the first time it has been practically proven that LolA interacts with LolB. As shown by the red line in Figure 17, the binding response increases with the increasing concentration of cleaved LolA. When we plot the curve (Figure 19) with the response at equilibrium against the concentration of bound cleaved LolA, we can determine the rate constant (KD) related to the binding rate between LolB and LolA, which demonstrates that our extracted LolA and LolB are actively interacting with each other.

5. Conclusions and Outlook

1. LolA and LolB interact but we could not detect any interaction between LolC and LolA or LolB. The periplasmic domain of LolC should theoretically interact with LolA, however it may be possible that the purified LolC was not stable enough for an interaction that could be detected.
2. LolC expression and purification is complicated, so we had to give special condition with 5% glycerol in its medium.
3. We could obtain perfect crystals of LolB with 0.05M Zinc acetate di-hydrate, 0.05M MES 6.1pH 11% W/V PEG 8000 buffer combination in MEMGOLD1

screen but when we tried to re crystalize the LolB with in house made solutions and different plates we did not obtain any crystals.

4. The chromatograms, retention times in gel filtration runs, the theoretical molecular weights and practically obtained SDS-Page results with protein ladder are exactly matching with each other so our extracted proteins are pure.
5. In BLI runs we got huge background so, it may happen because of our cleaved LolA has slight number of His-tagged proteins which can be increase with the concentration of cleaved LolA and different LolA concentrations has slightly different matrixes can give a response respect to the BLI buffer response.
6. Software tools have excellent capabilities on structure refinement, but we must adjust the angle of amino acids to get the best refinement, and despite high resolution flexible parts of the protein have no electron density and the structure can no be built.
7. In protein structure the water molecules and the buffer background affect the structure of protein.
8. While concentrating the protein, be careful about pore size of the MWCO filter which helps you to not to lose the protein with flowthrough.
9. While thawing highly concentrated freezed proteins, it can lead to precipitate and waste part of the valuable protein. So, it is good to maintain lower concentrations in pure proteins before freeze it to - 80°C.
10. Do not try to increase the Ni-beads volume more than what is recommended. It can reduce the final yield of the target protein.
11. Overall, we could crystallize, refine the structure, and prove the interaction capabilities of LolB like protein. We can understand, the LolB protein that we can find in E. coli is almost similar to the LolB like protein that we overexpressed in E. coli from Porphyromonas gingivalis plasmid because it could interact with LolA protein. Since LolB like protein was successfully interacted with LolA, we get a hint that Bacteroidota has a functional LolB protein and that is the main achievement of this project.

Acknowledgement

I would like to thanks to associate Professor Karina Persson who gave me this opportunity to do my thesis in biochemistry and the great support with the theoretical and practical knowledge of this thesis. And I would like to thank the PHD student Deepika Jaiman who helped me with the knowledge and her resources to accomplish my target. Finally, I would like to thank for everyone who works in Umea university Biochemistry department to letting me use their valuable time, expensive instruments, reagents and solutions to help me to make this dream come true.

References

1. Konovalova A, Kahne DE, Silhavy TJ. Outer membrane biogenesis. *Annu rev Microbial* 71:539-556 (2017)
2. Henderson JC, et al. The power of asymmetry: Architecture and assembly of the gram-negative outer membrane lipid bilayer. *Annu rev Microbial* 70:255-278 (2016)

3. Grabowicz M, Silhavy TJ. Redefining the essential trafficking pathway for outer membrane lipoproteins. *Proc Natl Acad Sci USA 114:4769-4774.* (2017)
4. Yakushi T, Tajima T, Matsuyama S, Tokuda H, Lethality of covalent linkage between mislocalized major outer membrane lipoprotein and the peptidoglycane of *Escherichia coli. J Bacteriol* 179:2857-2862 (1997)
5. Jessica El Rayes, Raquel Rodríguez-Alonso, Jean-François Collet, Lipoproteins in Gram-negative bacteria: new insights into their biogenesis, subcellular targeting and functional roles. J sciencedirect. 61, 25-34 (2021)
6. von Heijne G. The structure of signal peptides from bacterial lipoproteins. Protein Eng.;2:531–534. pmid:2664762 (1989)
7. Noland CL, Kattke MD, Diao J, Gloor SL, Pantua H, Reichelt M, et al. Structural insights into lipoprotein N-acylation by Escherichia coli apolipoprotein N-acyltransferase. Proc Natl Acad Sci.;114:E6044–E6053. pmid:28698362 (2017)
8. E. Kaplan, N.P. Greene, A. Crow, & V. Koronakis, Insights into bacterial lipoprotein trafficking from a structure of LolA bound to the LolC periplasmic domain, J Proc. Natl. Acad. Sci. U.S.A. 115 (31) E7389-E7397, (2018).
9. Narita S, Tokuda H. Sorting of bacterial lipoproteins to the outer membrane by the Lol system. Methods Mol Biol.;619:117–129. pmid:20419407 (2010)
10. Hara T, Matsuyama S-i, Tokuda H. Mechanism underlying the inner membrane retention of Escherichia coli lipoproteins caused by Lol avoidance signals. J Biol Chem.;278:40408–40414.(2003)
11. Yamaguchi K, Yu F, Inouye M. A single amino acid determinant of the membrane localization of lipoproteins in E. coli. Cell.;53:423–432. pmid:3284654 (1988)
12. Yakushi T, Masuda K, Narita S, Matsuyama S, Tokuda H. A new ABC transporter mediating the detachment of lipid-modified proteins from membranes. Nat Cell Biol.;2:212–218. pmid:10783239 (2000)
13. Tajima T, Yokota N, Matsuyama S, Tokuda H. Genetic analyses of the in vivo function of LolA, a periplasmic chaperone involved in the outer membrane localization of Escherichia coli lipoproteins. FEBS Lett.;439:51–54. pmid:9849875 (1998)
14. Kaplan E, Greene NP, Crow A, Koronakis V. Insights into bacterial lipoprotein trafficking from a structure of LolA bound to the LolC periplasmic domain. Proc Natl Acad Sci.;115:E7389–E7397. pmid:30012603 (2018)
15. Matsuyama S, Yokota N, Tokuda H. A novel outer membrane lipoprotein, LolB (HemM), involved in the LolA (p20)-dependent localization of lipoproteins to the outer membrane of Escherichia coli. EMBO J.;16:6947–6955. pmid:9384574 (1997)
16. Okuda S, Tokuda H. Model of mouth-to-mouth transfer of bacterial lipoproteins through inner membrane LolC, periplasmic LolA, and outer membrane LolB. Proc Natl Acad Sci U S A.;106:5877–5882. pmid:19307584 (2009)
17. Wang J. Estimation of the quality of refined protein crystal structures. Protein Sci. 2015 ;24(5):661-9. doi: 10.1002/pro.2639. Epub (2015)
18. Vagenende, Vincent, Yap, Miranda G. S., Trout, Bernhardt L. J. Mechanisms of Protein Stabilization and Prevention of Protein Aggregation by Glycerol. J. ACS. 48, 11084-11096 (2009)
19. Jaiman D, Nagampalli R, Persson K. A comparative analysis of lipoprotein transport proteins: LolA and LolB from Vibrio cholerae and LolA from Porphyromonas gingivalis. Sci Rep. 24;13(1):6605. (2023)

Appendix

Appendix 1

Appendix 1 represent the clone and DNA sequences of LolA, LolB, and LolC for all His-tagged and cleaved proteins.

Clone of LolA (with the histag)

MKHHHHHHPMSDYDIPTTENLYFQGAMQRTAEGELQAAAKHLANPDGTRI
DFQAETIAPNDMGSSPLSSGSLILKGNQFRLSFGSITAVFDGKKTLSYYDASE
NTLNISHPTNAELAMINPLIILTRSEAGYRTAMLPPTKGGKVIGLTPKTANGIK
QIELQVDSRDSRPTGAMITLEDGTKIVTKITGISRTKASPGLFRLMKKDYPGV
EVVDLR

LolA (with the his-tag) DNA sequence

ATGAAACATCACCATCACCATCACCCCATGAGCGATTACGACATCCCCAC
TACTGAGAATCTTTATTTTCAGGGCGCCATGCAACGCACAGCAGAAGGC
GAACTGCAAGCAGCAGCCAAGCACCTTGCAAACCCCGATGGAACACGAA
TAGACTTTCAGGCAGAGACCATAGCTCCCAACGACATGGGCTCATCACC
CCTCTCTTCCGGTAGCCTCATCCTCAAAGGCAATCAGTTTCGGCTCAGCT
TCGGCTCTATCACTGCCGTATTCGATGGCAAGAAAACGCTCTCTTACTAC
GATGCTTCCGAGAACACGCTCAACATTTCCCACCCGACGAATGCGGAAT
TGGCTATGATCAACCCGCTGATCATTCTCACCCGATCCGAAGCCGGCTA
TCGCACAGCCATGCTGCCTCCGACGAAAGGAGGAAAGGTGATCGGTCT
CACGCCTAAAACGGCTAACGGCATCAAACAAATCGAGCTACAGGTTGAT
TCTCGGGACTCACGTCCGACCGGTGCCATGATTACTCTGGAAGATGGGA
CAAAGATCGTTACAAAAATCACCGGTATCAGTCGCACGAAAGCTTCTCCC
GGCCTGTTCCGACTCATGAAGAAAGACTATCCGGGCGTAGAAGTTGTCG
ATCTCCGATAACTCGAG

Clone of LolB (with the his-tag)

MKHHHHHHPMSDYDIPTTENLYFQGAMGNDMEVSSPAALFANIKEHYPRQ
DRFSATGKAILRFDQKEVNTRIELTLVRNRGIRLVAMPFPLVVAGRAWITPEG
MTVTDAINKRYVTASYSQLSELTGIELSYRAFESLFLAQLFKADGASIVASDLL
LSTGAQKGHLLSYQDNRKMEYISEIGSNRRPLSISIYDPSTHYRLATTYSSFR
KYGAEHNLPANLLLQVLHLGQVKGSLSLDLPKMRFTDIDETDVTPRVNTSTY
RRMTLEDLSELF

LolB (with the his-tag) DNA sequence

ATGAAACATCACCATCACCATCACCCCATGAGCGATTACGACATCCCCAC
TACTGAGAATCTTTATTTTCAGGGCGCCATGGGCAACGATATGGAAGTCA
GTAGCCCTGCTGCTCTTTTTGCCAATATCAAGGAGCACTACCCTCGACAG
GACAGATTCAGTGCAACGGGCAAAGCTATTCTCCGATTCGATCAAAAGG
AGGTGAATACACGTATCGAATTGACCCTCGTTCGCAATCGTGGTATCCG
CCTCGTTGCCATGCCTTTTCCTCTCGTAGTGGCAGGGCGTGCATGGATT
ACA
CCCGAAGGTATGACAGTAACTGATGCCATTAACAAACGCTACGTAACAG
CCTCTTATTCGCAGCTTAGCGAACTCACCGGTATCGAACTCTCCTATCGT
GCCTTCGAATCGCTTTTCCTCGCACAGCTATTCAAAGCTGATGGTGCTTC
C
ATTGTGGCATCGGATCTTTTACTTTCGACCGGTGCACAAAAGGGACATTT
GCTTTCCTATCAAGACAACCGAAAA
ATGGAATATATATCAGAAATCGGTAGCAATAGGCGACCTCTTTCCATCAG
TATATACGATCCCTCCACCCACTATAGGCTTGCTACTACCTATTCTTCTTT
CCGAAAGTATGGTGCCGAGCATAATTTGCCGGCAAACTTGCTTTTGCAA
GTACTGCATCTCGGACAGGTTAAAGGCTCTCTTTCCCTTGATCTGCCTAA
GATGAGATTTACAGACATTGATGAAACAGATGTAACCCCTCGCGTCAATA
CTTCCACCTATCGCAGAATGACGTTGGAAGATCTCTCCGAGCTTTTCTAA
GGTACC

LolC Clone (with the his-tag)

MKHHHHHHPMSDYDIPTTENLYFQGAMNGYEALIMTHSAVTDPPLMIRSAD
NSLIKADDKTLLTALEAEGIGSYSFILTGEGLVKTKYRQQAVSLMGVDDRYPR
TVKIDSIVFAGTFATDTLSGATALNVGAAIATEMQLGAGFVEAVEVIVPRRIGL
INPLVPAGAFKSLQGQVASVFASGLQPEDNSIILSIDSLRKLLDYSDHEAEAV
AIQLQTGSDAEQIALHLKETLRDSYQVLDLAGQHPEITHLVAMEK

LolC (with the histag) DNA sequence

ATGAAACATCACCATCACCATCACCCCATGAGCGATTACGACATCCCCAC
TACTGAGAATCTTTATTTTCAGGGC
GCCATGAATGGGTACGAAGCTCTGATCATGACGCATTCGGCAGTAACGA
TCCCCCTCTTATGATTCGTTCGGCAGACAATTCGCTGATAAAGGCAGATG
ATAAAACTCTGCTCACAGCTCTCGAAGCGGAAGGGATCGGTTCGTACAG
C
TTTATCCTCACCGGAGAAGGTCTTGTAAAGACGAAGTATCGACAGCAGG
CTGTTTCTCTCATGGGTGTGGATGATCGCTATCCCCGCACTGTGAAGATA
GACTCCATCGTCTTTGCAGGCACATTCGCTACGGATACGCTATCCGGAG
CT
ACAGCTCTGAATGTCGGTGCAGCCATCGCAACAGAAATGCAGCTCGGTG
CAGGCTTTGTAGAAGCTGTGGAGGTAATTGTTCCCCGTCGTATCGGGCT
GATCAATCCACTTGTTCCGGCCGGTGCATTCAAGTCGCTCCAAGGACAA
GTGGCCTCGGTATTTGCATCCGGCCTGCAACCCGAGGACAATTCCATCA
TCCTTTCCATAGACAGTCTGCGCAAACTGCTCGATTATTCGGATCATGAA
GCAGAAGCCGTGGCCATACAGCTACAAACGGGTAGCGATGCAGAGCAA
ATAGCTCTCCACTTAAAAGAGACTCTGAGGGACAGCTACCAAGTACTCG
ATTTGGCCGGACAGCATCCCGAAATCACTCACTTGGTAGCGATGGAGAA
GTAAGGTACC

Appendix 2

Appendix 2 represent the valuable experimental and calculated data of of LolA, LolB, and LolC for all His-tagged and cleaved proteins.

Protein name	Gel filtration retention (Minutes)	Calculated molecular weight (Da)	Final concentration (mg/ml)
LolA His tagged	90	23548.83	13.6
LolA Cleaved	93	22464.62	100
LolB His tagged	86	31074.34	24.17
LolB Cleaved	90	29992.13	26.68
LolC His tagged	85	27723.51	7.27
LolC Cleaved	88	26641.30	15.66

Appendix 3

Appendix 3 represent the buffer compositions for LolA and LolB transformations and separations.

Buffer name	Composition
Lysis buffer	50mM Tris pH 7.5, 0.3M NaCl, 10mM imidazole
Wash Buffer	50mM Tris pH 7.5, 0.3M NaCl, 30mM imidazole
Elution buffer	50mM Tris pH 7.5, 0.3M NaCl, 0.3M imidazole
Gel filtration buffer	20mM Tris pH 7.5, 0.3M NaCl
HEPES buffer	20mM HEPES pH 7.5, 0.3 NaCl

Appendix 4

Appendix 4 represent the buffer compositions for LolC transformations and separations.

Buffer name	Composition
Lysis buffer	50mM Tris pH 7.5, 0.3M NaCl, 10mM imidazole, 5% Glycerol.
Wash Buffer	50mM Tris pH 7.5, 0.3M NaCl, 30mM imidazole, 5% Glycerol.
Elution buffer	50mM Tris pH 7.5, 0.3M NaCl, 0.3M imidazole, 5% Glycerol.
Gel filtration buffer	20mM Tris pH 7.5, 0.3M NaCl, 5% Glycerol.
HEPES buffer	20mM HEPES pH 7.5, 0.3 NaCl, 5% Glycerol.

Appendix 5

Appendix 5 represent the relevant protein with the relevant well in all four SDS-PAGE gel runs.

Well number	Figure 5	Figure 6	Figure 9	Figure 12
1	Empty	Protein ladder	Empty	Empty
2	Pellet	Empty	Cleave LolB	Fraction 11 of Figure 10
3	Supernatant	Empty	HIS tag LolB	Fraction 12 of Figure 10
4	Supernatant	Fraction 3 of Figure 4	Fraction 3 of Figure 8	Fraction 13 of Figure 10
5	Protein ladder	Fraction 4 of Figure 4	Fraction 4 of Figure 8	Fraction 14 of Figure 10
6	Empty	Fraction 5 of Figure 4	Fraction 5 of Figure 8	Fraction 15 of Figure 10
7	Fraction 2 of Figure 3	Fraction 6 of Figure 4	Fraction 6 of Figure 8	Fraction 11 of Figure 11
8	Fraction 3 of Figure 3	Fraction 7 of Figure 4	Fraction 7 of Figure 8	Fraction 12 of Figure 11
9	Fraction 4 of Figure 3	Fraction 8 of Figure 4	Fraction 3 of Figure 7	Fraction 13 of Figure 11
10	Fraction 5 of Figure 3	His tagged LolA	Fraction 4 of Figure 7	Fraction 14 of Figure 11
11	Fraction 6 of Figure 3	His tagged LolA	Fraction 5	Fraction 15 of Figure 11
12	Fraction 7 of Figure 3	Supernatant	Supernatant	Fraction 16 of Figure 11
13	Supernatant	Pellet	Empty	Fraction 17 of Figure 11
14	Supernatant	Protein ladder	Pellet	Protein ladder
15	Protein ladder	Empty	Protein ladder	Empty

Appendix 6

Appendix 6 represent the processing and refinement statistics for the LolB-like protein. .

	lolB
Wavelength	
Resolution range	47.34 - 2.1 (2.12 - 2.1)
Space group	P 65 2 2
Unit cell	94.679 94.679 104.942 90 90 120
Total reflections	
Unique reflections	16765 (523)
Multiplicity	
Completeness (%)	99.93 (98.49)
Mean I/sigma(I)	
Wilson B-factor	42.54
R-merge	
R-meas	
R-pim	
CC1/2	
Reflections used in refinement	16765 (523)
Reflections used for R-free	0 (0)
R-work	0.2480 (0.3032)
R-free	0.2909 (0.3793)
Number of non-hydrogen atoms	1949
macromolecules	1878
ligands	1
solvent	70
Protein residues	237
RMS(bonds)	0.008
RMS(angles)	1.01
Ramachandran favored (%)	94.47
Ramachandran allowed (%)	3.83
Ramachandran outliers (%)	1.70
Rotamer outliers (%)	3.41
Clashscore	7.94
Average B-factor	49.45
macromolecules	49.51

UMEÅ UNIVERSITY