Recombinant expression of the cowpea chlorotic mottle virus

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Populärvetenskaplig sammanfattning

Virus är icke levande organismer som infekterar levande celler och förökar sig med hjälp av cellernas maskineri. De kan infektera alla typer av liv och de är totalt beroende av en värdcell. Alla virus innehåller genetiskt material samt en så kallad viruskapsid. Denna kapsid kan ha olika struktur och består av identiska proteinenheter. Kapsidens uppgift är att skydda det genetiska materialet. Detta projekt handlar om cowpea chlorotic mottle virus, även kallat CCMV, som är ett växtvirus med ikosaedrisk struktur vars associerings- och disassocieringsmekanismer fungerar som ett lämpligt modellsystem för att studera hur olika proteinenheter sätts ihop för att skapa en kapsid. Detta är ett passande modellsystem för att kapsiden kan disassociera och avlägsna det genetiska materialet för att sedan under specifika förhållanden associera tillbaka till den ursprungliga kapsiden.

Projektet har gått ut på att uttrycka CCMV genom rekombinant protein produktion. Proteinuttryck görs ofta i bakteriesystem eftersom bakterier både är lätta att odla och de växer snabbt. Dessutom producerar de ofta höga utbyten av rekombinant protein.

Escherichia coli, mer känd som *E.coli*, är en bakterieart och i detta projekt utnyttjas två olika slags *E.coli* celler.

Under projektet isolerades proteinenheter och det undersöktes under vilka förhållande som dessa proteinenheter associerar och skapar kapsider. Experimenten visade att med en associeringsbuffert och surt pH kunde troligen kapsider skapas.

Sammanfattningsvis är målet med projektet alltså att utveckla en metod för att rekombinant uttrycka CCMV följt av isolering av associeringskompententa dimerer.

Abstract

All viruses are composed of both genetic material and a capsid. The capsid is composed by identical protein subunits and the purpose is protection of the genetic material, in this case RNA. This project is about cowpea chlorotic mottle virus, also called CCMV, which is a plant virus and the disassembly and reassembly mechanisms of the capsid are investigated to understand how the capsid proteins associate to create capsids. These mechanisms are suitable to use as a model system to study protein self-assembly.
The capsid is able to disassembly and removes the genetic material. The capsid proteins can then assembly to an empty capsid without any genetic material.
The goal with this project is to develop a method to recombinant express CCMV and isolate dimers that are appropriate for assembly.
Previous research has tried to take advantages of a binding his-tag to facilitate the purification. These experiments have been unsuccessful. For that reason, this project doesn´t use a his-tag.
Plasmids containing the gene of interest were recombinant expressed in *E.coli* and purified with ammonium sulphate precipitation followed by gel filtration. Dynamic light scattering and mass spectrometry were performed on fractions from the gel filtration.
The solution conditions were changed to control assembly of capsid proteins. Dimers were isolated and experiments with a 50/50 mix of dimers and assembly buffer with different pH and incubation times were performed to create capsids. A 50/50 mix of dimers and assembly buffer with pH 4.5 and incubation for 30 minutes probably created capsids.
After the expression was the protein found in the soluble part of the clarified lysate. Previous research has found the protein in the inclusion bodies.
Further experiments should try to optimize the purification and also the solution conditions. The assembly-competent CCMV dimers can be recombinant expressed and isolated and thus useful in further biophysical experiments.

Introduction

Viruses are non-living organisms that infect cells and reproduce themselves by using the cells machinery. They can infect all types of life and they are totally depending on a host cell. All viruses are composed of both genetic material and a capsid that is a protein shell. Identical capsid proteins compose the capsid and the structure of virus capsids may be different. The function of the capsid is to enclose and protect the genetic material.
This project is about the cowpea chlorotic mottle virus, also called CCMV, which is a plant virus and the mechanisms of the disassembly and reassembly of the capsid are poorly understood and an interesting area to investigate. CCMV disassembly and reassembly mechanisms work as a suitable model system to explore capsid proteins reassembly to create a capsid. The protein is recombinant expressed as monomers that immediately creates dimers. The dimers are then associating to create capsids and these assembly mechanisms are the interesting question. Traditionally, CCMV is purified from plants. The plant is infected with the virus and then extracted. By using recombinant expression, it´s possible to study CCMV with mutations.
CCMV consists thus of a capsid that surrounds the central core of RNA. The capsid has a size of 28 nm and is composed of identical subunits, that is capsid proteins, with icosahedral arrangement. Icosahedral arrangement means a polyhedron with 20 equilateral triangles. The capsid proteins are creating a pattern where hexagons (six-sided polygons) and pentagons (five-sided polygons) interchange with each other as shown in *Figure 1*.[1]

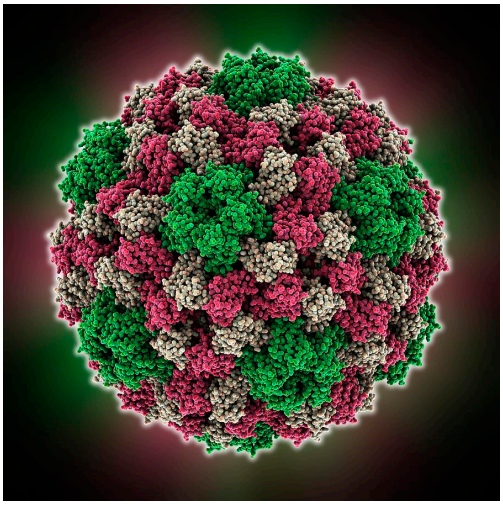

Figure 1. The structure of the CCMV capsid with T=3. Notice the interchange between the hexagons in white and pink and the pentagons in green. The illustration is taken from Fine Art America.[2]

[1] Speir A J.; Munshi S.; Wang G.; Baker S T.; Johnson E John. Structures of the native and swollen forms of cowpea chlorotic mottle virus determined by X-ray crystallography and cryo-electron microscopy. *Structure* **1995**, 27, pp 63-68
[2] https://fineartamerica.com/featured/cowpea-chlorotic-mottle-virus-capsid-science-photo-library.html

The icosahedral geometry of the capsids is known to be the structure of about half of the know virus families. Capsids with icosahedral arrangement are constructed by 60 T subunits, where T is an integer. In vivo does CCMV create capsids composed of 180 identical capsid proteins (T=3) where each capsid protein has a molecular weight of 20 kDa. The capsid with T=3 total molecular weight is consequently 3.6 MDa. In vitro, a capsid with T=2 also be formed by association by 12 pentamers of dimers as *Figure 2* shows. This capsid has a molecular weight of 2.4 MDa. The in vitro capsid with T=2 might be produced at high protein concentrations because of an overabundance of five-dimer nuclei.[3] Assembly reactions to create capsids don´t occur by a single high-order reaction. It rather requires a cascade of low-order steps. The relative simple geometric arrangement suggests that they just are a few types of mechanisms for icosahedral capsids.
Previous research suggests that the assembly of CCMV begins with formation of a pentamer of dimers, as the middle of *Figure 2* shows, and for T=3 it continue with cooperative addition of dimers.[4]

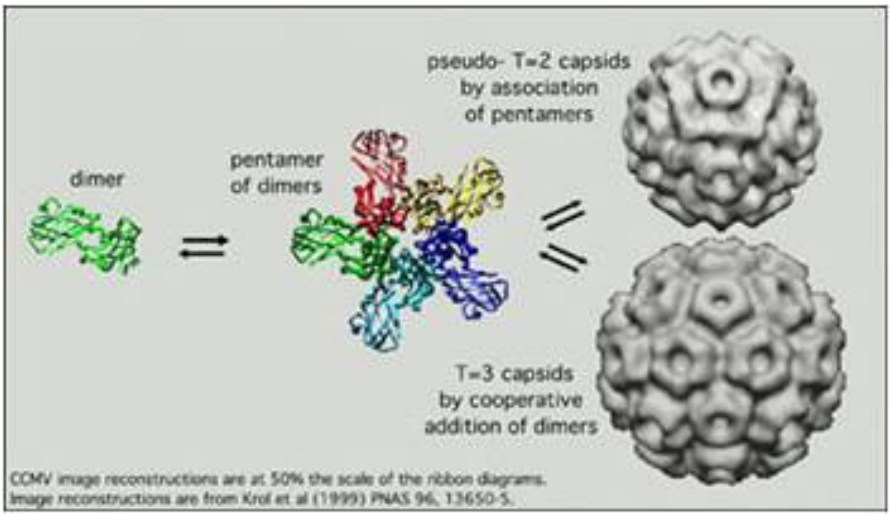

Figure 2. The assembly from dimers to a pentamer of dimers followed by capsid formation to either T=2 or T=3. The reactions have been examined by broad range of biophysical and biochemical techniques including fluorescence, light scattering, EM (including image reconstruction), and X-ray crystallography. The picture is taken from the Zlotnick Labatory.[4]

One unique aspect of the CCMV in vitro disassembly and assembly system is the sensitivity of the chemical environment. Changes in ionic strength and pH-values can affect both the protein-protein and protein-RNA interactions. As *Figure 3* shows, a stable 40 kDa homodimer of CCMV capsid protein can be isolated around neutral pH. Empty capsids will be formed while lowering the pH. An important thing to notice is that formation of empty capsids is only an in vitro effect.[3]

[3] Zlotnick A.; Aldrich Ryan.; M.Johnson J.; Ceres P.; J.Young M. Mechanism of Capsid Assembly for an Icosahedral Plant Virus. *Virology* **2002**, 277, pp 450-456.
[4] http://www.indiana.edu/~zlotlab/projects.html

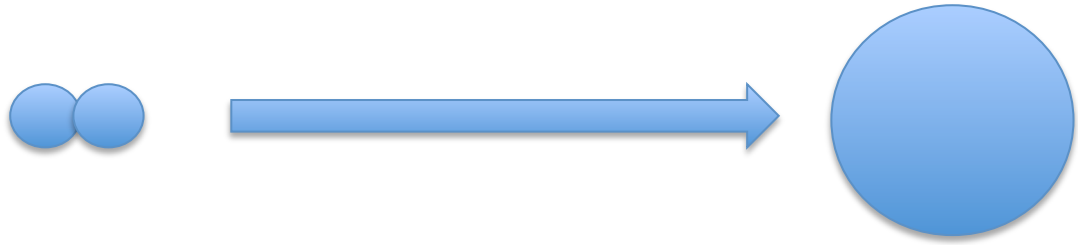

Figure 3. A simplified schematic picture from dimers to capsids by changing the pH-value and ionic strength

CCMV capsid protein has previously been recombinant expressed in *E.coli*. The in vitro disassembly and reassembly of CCMV CP to virus-like particles, that is empty capsids, were achieved by first adding disassembly buffer and perform ultra-sonication. The protein was found in the inclusion bodies. Capsids were created by dialysis against first dialysis buffer and then reassembly buffer.[5]

Ingemar Andre´s group has previously studied the disassembly and reassembly conditions for CCMV. The previous work tried to take advantages of a His-tag binding to the N-terminal to facilitate the purification. His-tag purification use immobilized metal affinity chromatography, IMAC, because the His-tag has a high affinity for metal ions and thus binds strongly to the IMAC column. The His-tag turned out to disrupt the disassembly into capsid protein dimers. Only after addition of chaotropes could dimers be observed. Because the protein was found in the inclusion bodies in all previous studies, refolding of the protein was necessary.
The goal with this project is to develop a method to recombinant express CCMV without a his-tag and isolate dimers that are appropriate for assembly.
The CCMV plasmid DNA without the His-tag was expressed in Rosetta cells followed by lysis of the cells. Ammonium sulphate precipitation was performed as crude purification. Ammonium sulphate was stepwise added to increase the ionic strength until the protein precipitate because it´s no longer stable. Different proteins precipitate at different saturation because of varied exposed charged residues. This was followed by fast protein liquid chromatography to separate proteins by their size. The project ends with attempts to create capsids from dimers and assembly buffer by changing pH and the incubation time. DLS and MS were performed on the fractions from FPLC.

[5] Hassani-Mehraban A.; Creutzburg S.; van Heereveld L.; Kormelink R. Feasibility of Cowpea chlorotic mottle virus-like particles as scaffold for epitope presentations. *BMC Biotechnology* **2015**

Methods

Sequencing

The plasmid containing the expression vector for the CCMV protein was sent away for Sanger sequencing (Eurofins, Sverige)[6] to confirm that the cloning experiment that removed the His-tag from the N-terminal was successful. 5 uL of plasmids were mixed with 1 uL reverse primer designed for the plasmid and 4 uL water.

Plasmid purification

Plasmid purification was performed to increase the amount of starting material available. It started with a bacterial transformation with XL1- Blue cells. The XL1- Blue cells were thawed on ice for 30 min and 1 uL of plasmid DNA was added to 1 aliquot of XL1- Blue cells. The solution was left on ice for 30 min to allow plasmid uptake followed by heat shock at 42 °C for 45 seconds. The solution rested on ice for 2 min and was then placed in a shaking incubator for 45 min after adding 500 uL SOC (without antibiotic). 50 uL respective 200 uL of the mixture was spread out on agar plates. The agar plates were incubated at 37 °C over night.[7]
The cells that contained the plasmid with gene of interest survived and formed colonies. A liquid bacterial culture with a single colony and 5 ml of LB media containing antibiotic ampicillin was inoculated over night at 37 °C. The plasmid DNA was purified with centrifugation by the GeneJET spin column kit. The purified plasmid DNA was stored at -20 °C.
One more transformation, with the same method as above, was made but this time into Rosetta cells. A glycerol stock was made from the petri plate with 200 uL that was incubated over night. The tube was stored in the -80 °C freezer.

Expression in Rosetta cells

Two starter cultures (one from the frozen stock solution and one from the 50 uL agar plate) with 5 ml LB media, 5 uL ampicillin and a small amount of the stock solution respective from the agar plate were grown over night in a shaking incubator at 37 °C. 2 ml of each starter culture was mixed with 100 ml media each containing ampicillin. The cells in this new growth cultures were incubated at 37 °C until they reached an OD600 of 0.6. IPTG with a final concentration of 1 mM was added to start the protein expression. The expression solutions were left over night in a shaking incubator at 20 °C.

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[6] https://www.eurofinsgenomics.eu/en/custom-dna-sequencing/eurofins-services/?fbclid=IwAR2-ZTJbdFczNT5sV29TQbx51Dh9La2ch73S0nUt4X0U2brlBchhABeNT4E

[7] https://www.addgene.org/protocols/bacterial-transformation/?fbclid=IwAR2EB1WBozak7kGemXWCvuZrFUYwlqfku49SUEZfVz7P_lPzc2ipyZagNE8

Four new bigger starter cultures with a small amount from the stock solution, 10 ml LB and 10 uL ampicillin were made after the first SDS-PAGE result and grown over night in a shaking incubator at 37 °C. 8 ml of each of the four starter cultures was mixed 450 ml LB with 450 uL ampicillin. The cells were incubated at 37 °C until they reach an OD600 of 0.6. IPTG with a final concentration of 1 mM was added again to start the protein expression. The expression solutions were left over night in a shaking incubator at 20 °C.

Purification

The growth cultures were centrifuged at 3220g for 20 min and each pellet was resuspended in 5 ml of disassembly buffer (0.020 M Tris-HCl, 0.9 M NaCl, 0.001 M DTT pH 7.4). The solutions were sonicated around 10 times, 30 seconds every time. The sonicated solutions were centrifuged for 15 min at 4000g. Each pellet was resuspended in 10 ml disassembly buffer containing 8 M urea. The solutions were sonicated again but this time with 10 seconds pulses until the pellet was entirely dissolved. Another 15 ml disassembly buffer was added and the samples were centrifuged at 15.000g for 15 min.
The four new bigger growth cultures were centrifuged at 9000g for 20 min and one of the pellets was resuspended in 25 ml of disassembly buffer (0.020 M Tris-HCl, 0.9 M NaCl, 0.001 M DTT pH 7.4). The other three were stored in the freezer.
The solution was sonicated 10 rounds of 1 min followed by centrifugation at 4000g for 15 min. The supernatant, that is the clarified lysate, was saved for ammonium sulphate precipitation.

SDS-PAGE

SDS-PAGE was performed to determine if the protein is in the soluble part of clarified lysate, the soluble part of inclusions bodies or the pellet part of inclusion bodies. The uninduced and induced cells were mixed with 5 uL of BugBuster and 45 uL disassembly buffer. 5 uL from each sample was mixed with 4 uL sample buffer and 11 uL water. The reference sample was PageRuler Prestained Protein Ladder Plus. The gel was loaded with 13 samples in total (reference, 2 uninduced cells, 2 induced cells, 2 soluble-clarified lysate, 2 pellet- clarified lysate, 2 soluble-inclusion bodies, 2 pellet-inclusion bodies).

Ammonium sulphate precipitation

An online calculator[8] was used to estimate the amount of ammonium sulphate that should be added to get 15 %, 30 %, 45 %, 60 % and 90 % saturation of the clarified lysate. The start volume was 22 ml and the start temperature was 20 °C. Ammonium sulphate was added slowly while stirring. The samples were centrifuged at 20 000g. Each pellet was resuspended in 3 ml disassembly buffer and more ammonium sulphate was added to the supernatant. SDS-PAGE was performed again, with the same method as before, to determine where the protein precipitates.

[8] http://www.encorbio.com/protocols/AM-SO4.htm

Another pellet was resuspended in disassembly buffer and this time were lysozyme and benzonase also added. Ammonium sulphate precipitation was performed again according to the result for SDS-PAGE. The pellet was resuspended in disassembly buffer and lithium chloride with a final concentration of 500 mM was added to half of the solution. Lithium chloride was added to precipitate RNA.

Fast protein liquid chromatography

The first column that was used was Superdex 75 HiLoad 26/60 (320 ml). 6 ml of the solution with LiCl from ammonium sulphate precipitation was injected into a 5 ml loop. The speed was 2.6 ml/min. This was repeated two more times with the solution with LiCl and one time with the solution without LiCl.
SDS-PAGE was performed again, with the same method as before, to confirm that one of the peaks in the chromatograms actually was the CCMV protein. The gel was loaded with 13 samples.
Fraction 16, 17 and 18 (corresponding to the second peak) of the three rounds with LiCl were pooled together. Fraction 14 and 15 respective 11, 12 and 13 (corresponding to the first peak) were also pooled together. The same for fraction 11 to 18 for the round without LiCl.
A smaller column was changed to, Superose 6 10/300 GL (24 ml) with a 0.5 ml loop. The speed for all rounds with Superose 6 was 0.3 ml/min.
Two rounds were carried out where 0.5 ml of the pooled fractions 16, 17, and 18 with LiCl was injected.
300 uL of assembly buffer (0.1 M Sodium citrate, 1 M NaCl, pH 5.25) and 300 uL of the pooled fractions 16, 17, and 18 with LiCl were mixed and left for 30 min. The pH of the mixture was 5.5. 0.5 ml of the mixture was injected. This was repeated two more times. The running buffer for the column was assembly buffer plus 1 mM DTT.
A mixture of the same volumes and pH was left over night and 0.5 ml was injected.
400 mL of assembly buffer and 400 uL of the pooled fractions 16, 17 and 18 with LiCl were mixed and the pH was lowered to 4.5. 0.5 ml of the solution was injected after 30 min.

Dynamic light scattering

1 ml of each of the six pooled tubes (3 with LiCl and 3 without LiCl) from FPLC with Superdex 75 were filtered and then centrifuged for 5 min at 20 000 g to remove dust. DLS was measured for each one.

Mass spectrometry

Mass spectrometry was performed on two bands in the SDS-PAGE gel from the FPLC Superdex 75 fractions. The two bands in the same fraction around 20 kDa were cut out and measured. MALDI-TOF was used.

Results

This project was trying to take advantages that the protein was found in the soluble part of the clarified lysate. The purification methods were based on purification of dimers. When dimers were reached, experiments with different pH values were performed to create capsids.

Sequencing

The project started with a solution of plasmids that should contain the CCMV gene without the His-tag. Since no one actually analysed if the removal of the His-tag was successful, the plasmid was sent away for sequencing and the result is shown below.

This is one part of the sequence of the plasmid that was sent away:

3'5' Frame 2
P P **Stop** I D F L P G A I M P Y R E R F C A I R W C P G S R R S P L C D S C I R K Q P S S R L R P L S T A A A R N G
A C K E M A P N S P P A T G P A T I P T P K Q A L MS P K W R A R S S P S V M S A I **Stop** A P A T A P V A P
V M P A T M R P A **Stop** R I E I S I P R N **Stop** Y D S L **Stop** G N C E R I T I P L **Stop** K **Stop** F C L T L R R R Y TM S
T V G T G K L T R A Q R R A A A R K N K R N T R V V Q P V I V E P I A S G Q G K A I K A W T G Y S V S K W T
A S C A A A E A K V T S A I T I S L P N E L S S E R N K Q L K V G R V L L W L G L L P S V S G T V K S C V T E T
Q T T A A A S F Q V A L A V A D N S K D V V A A M Y P E A F K G I T L E Q L T A D L T I Y L Y S S A A L T E G
D V I V H L E V E H V V

This is the sequence for CCMV capsid protein:

>sp|P03601|CAPSD_CCMV Capsid protein OS=Cowpea chlorotic mottle virus OX=12303 GN=ORF3b PE=1 SV=4
MSTVGTGKLTRAQRRAAARKNKRNTRVVQPVIVEPIASGQGKAIKAWTGYSVSKWTASCAAAEAKVTSAITISLPN
ELSSERNKQLKVGRVLLWLGLLPSVSGTVKSCVTETQTTAAASFQVALAVADNSKDVVAAMYPEAFKGITLEQLT
ADLTIYLYSSAALTEGDVIVHLEVEHVRPTFDDSFTPVY

This is the sequence for His-tagged CCMV capsid protein:

TMGHHHHHHHHHLCSGHIDDDDNHTSLEVLFQGPHMSTVGTGKLTRAQRRAAARKNKRNTRVVQPVIVEPIASGQ
GKAIKAWTGYSVSKWTASCAAAEAKVTSAITISLPNELSSERNKQLKVGRVLLWLGLLPSVSGTVKSCVTETQTTA
AASFQVALAVADNSKDVVAAMYPEAFKGITLEQLTADLTIYLYSSAALTEGDVIVHLEVEHVRPTFDDSFTPVY

Notices that the three grey highlighted parts are the same except from the final few amino acids.
More important, while comparing the first sequence with the His-tagged sequence, it´s proven that the N-terminal and cleavage site have been removed. The green highlighted
part in the last sequence is gone in the first one.

SDS-PAGE 1

A first round of SDS-PAGE was carried out to determine where the protein was located after protein expression and lysis of the cells. Before protein expression, two cultures, one from the frozen stock solution and one from the agar plate, were cultured and after separation of everything all the way to inclusion bodies, one sample for each one was loaded on the gel.

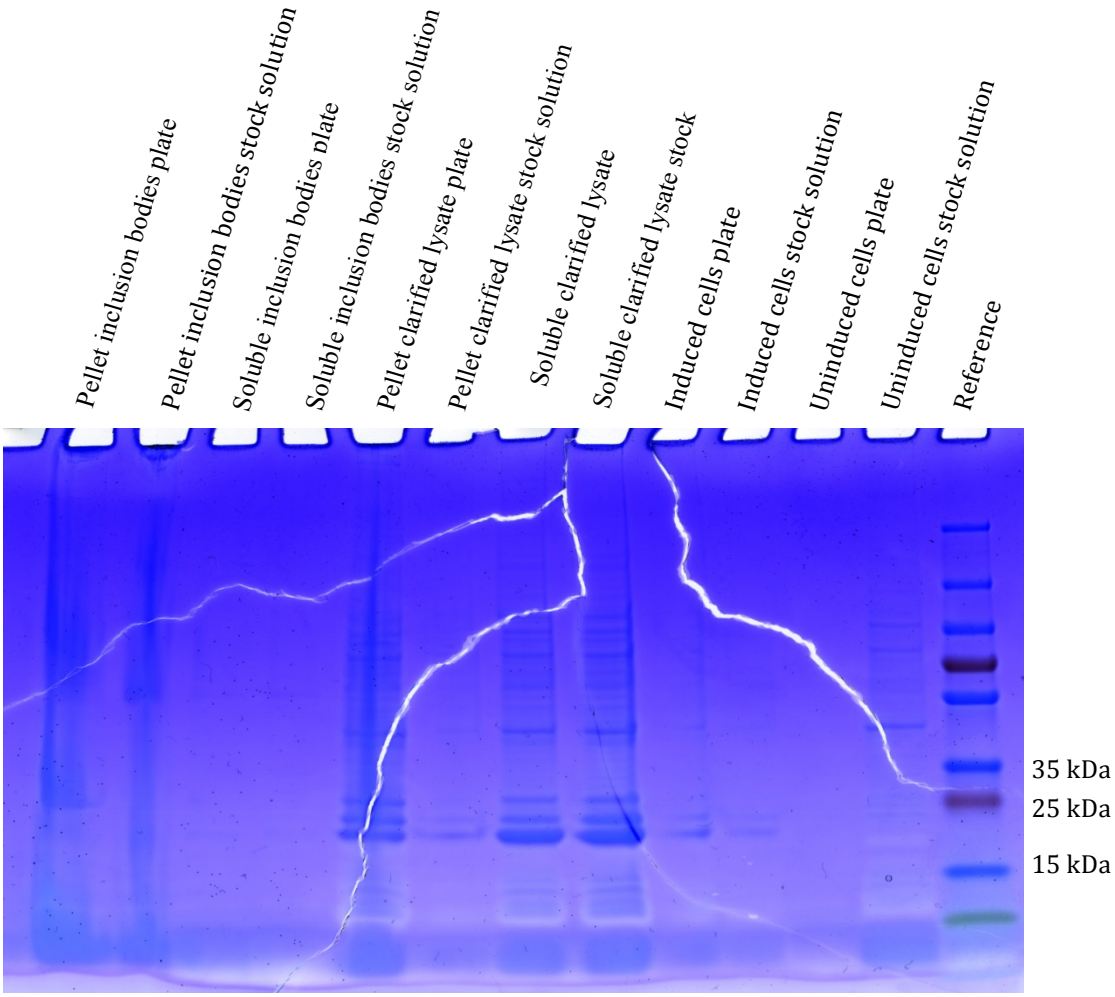


Figure 4. The SDS-PAGE gel that identified where the protein was after lysis

Figure 4 shows the gel with samples all the way from uninduced cells to inclusion bodies. The gel shows two significantly strong bands around 20 kDa for both the soluble parts of the clarified lysate. This indicates that the protein is found there and that the soluble part of the clarified lysate was moved on to the next step.

SDS-PAGE 2

A second round of SDS-PAGE was carried out on the fractions from ammonium sulphate precipitation to determine at which saturation of the clarified lysate the protein precipitated.

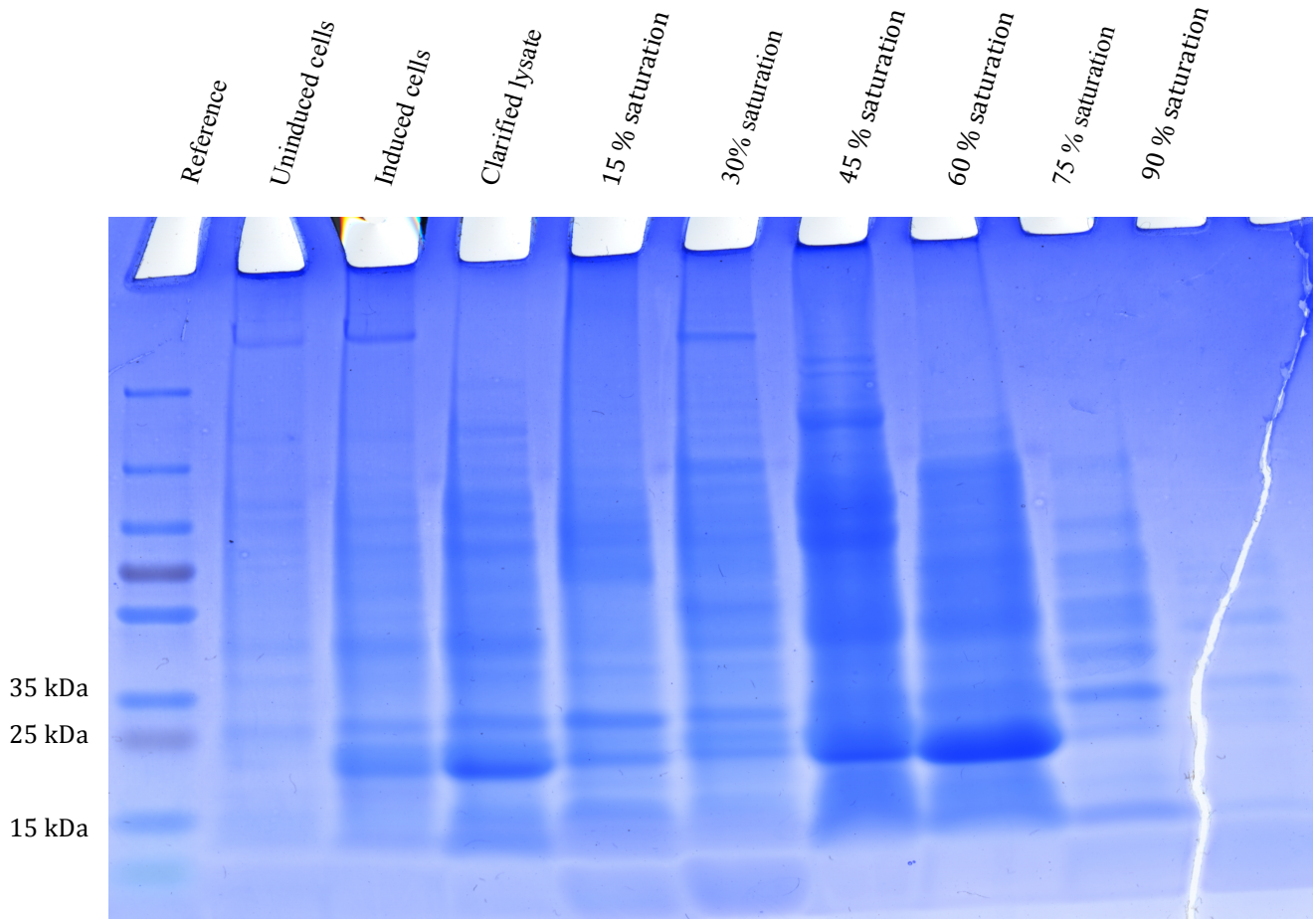


Figure 5. The SDS-PAGE gel that identified where the protein precipitated

Figure 5 shows the gel after stepwise increased saturation of ammonium sulphate of the clarified lysate. Two remarkably bands especially around 20 kDa are shown for 45 % respective 60 % saturation. This suggests that the protein precipitate from 45-60 % saturation. LiCl was added to half of the solution of the resuspended pellet from 45-60 % saturation to precipitate RNA.

FPLC Superdex 75

Fast protein liquid chromatography with Superdex 75 as column was performed to further purify and isolate dimers based on size-exclusion after ammonium sulphate precipitation. Four rounds were carried out in total. Three rounds of the resuspended pellet from 45-60 % saturation with LiCl and one round without LiCl.

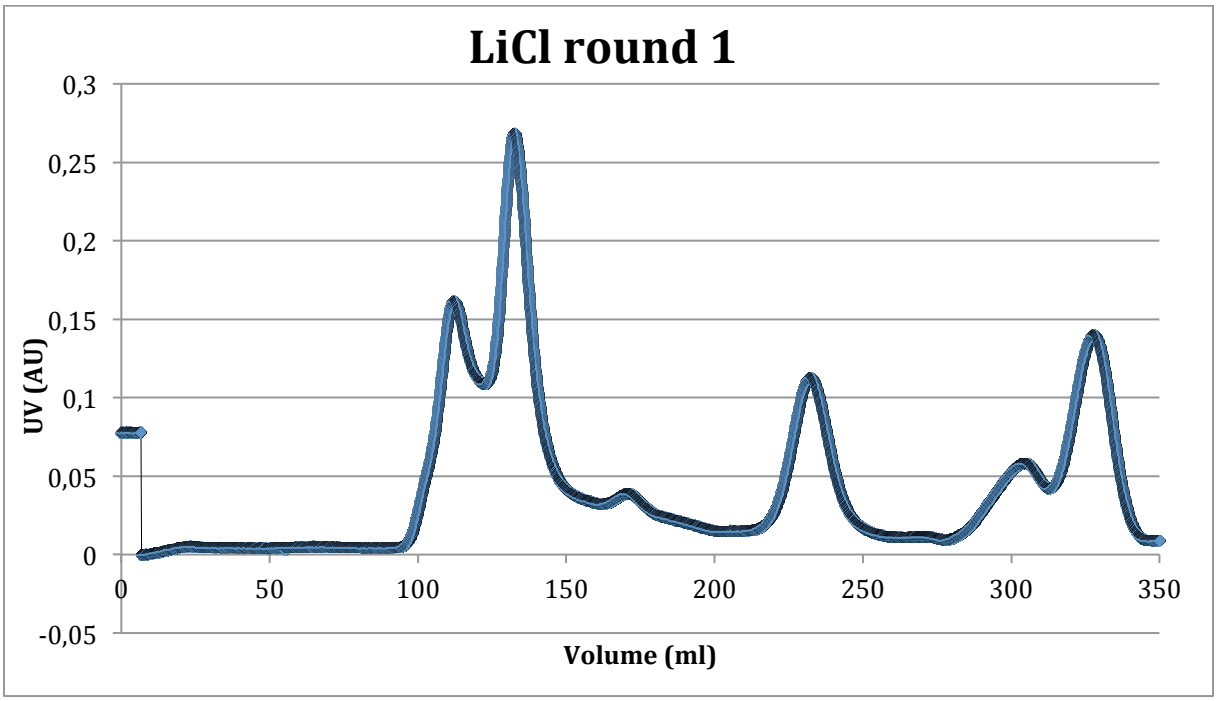


Figure 6. The UV as a function of the volume in ml

Figure 6 shows the chromatogram for the first round of 5 ml sample with LiCl. The chromatogram shows significantly peaks around 110 ml, 130 ml, 230 ml and 330 ml. The peak around 130 ml corresponds to something with a weight around 50 kDa comparing to the calibration curve for this column (see appendix *Figure 15*).

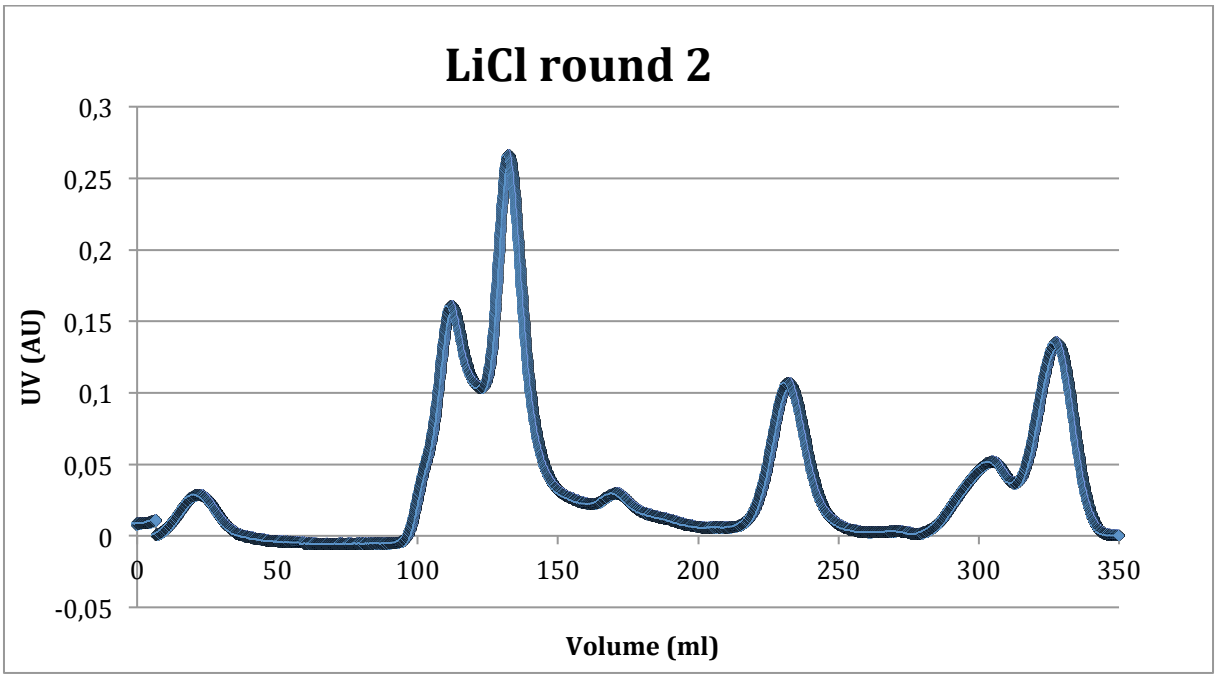


Figure 7. The UV as a function of the volume in ml

Figure 7 is the chromatogram for the second round of 5 ml sample with LiCl. The result is almost identical compared with the result for *Figure 6*.

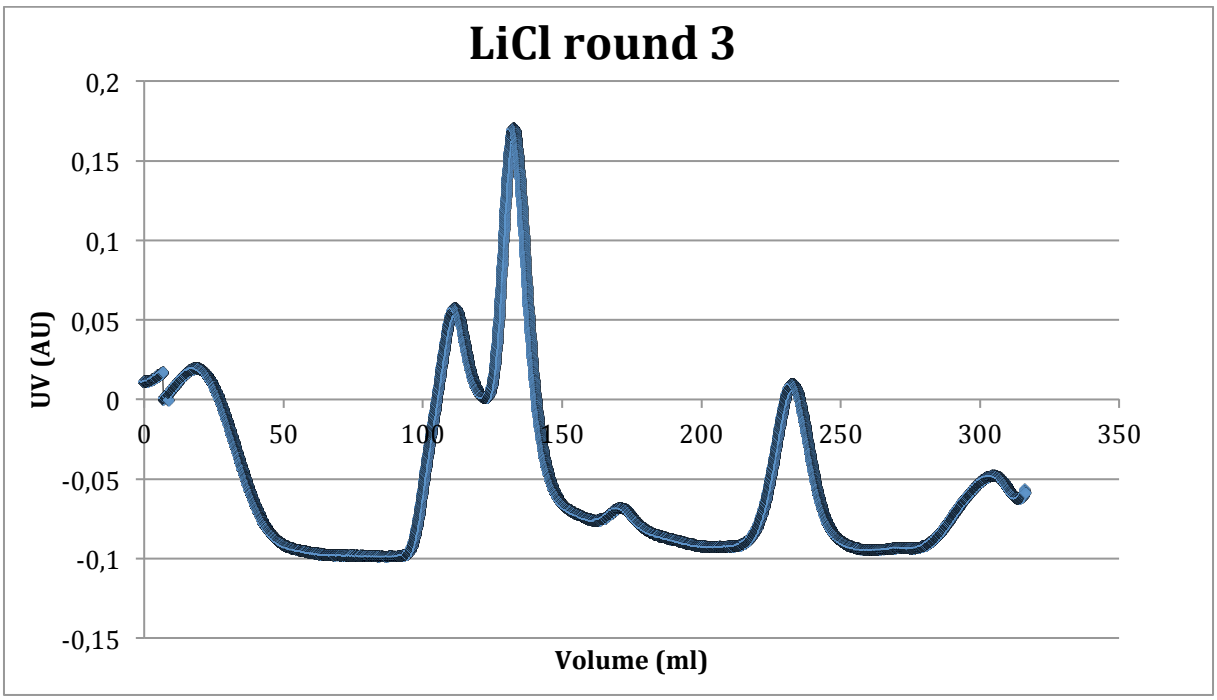
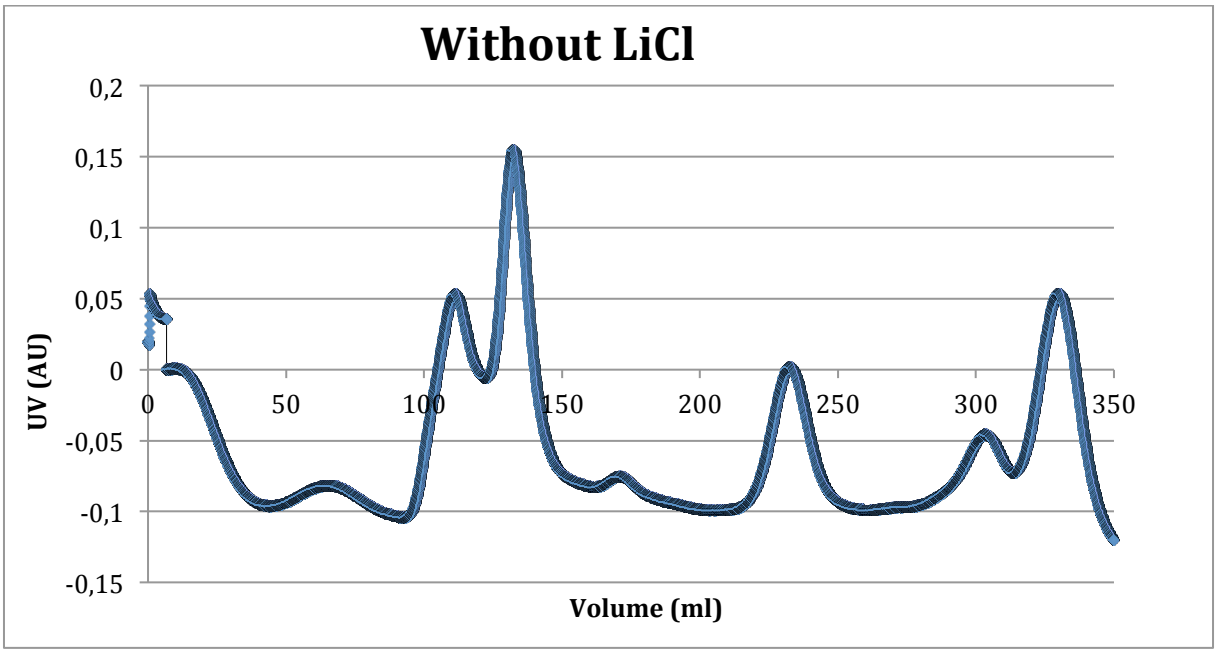


Figure 8. The UV as a function of the volume in ml

Figure 8 is the chromatogram for the third round of 5 ml sample with LiCl. The result is almost identical compared with the result for *Figure 6* and *Figure 7*. It just looks a little bit different because the baseline wasn´t set to zero.



9. The UV as a function of the volume in ml

Figure 9 shows the chromatogram for the only round with 5 ml sample without LiCl. Still, the result is very similar to the chromatograms for the rounds with LiCl (*Figure 6,7* and *8*).

Dynamic light scattering

Dynamic light scattering is a method that is able to determine the size of particles by measuring the diffusion. DLS was measured on several samples but the only relevant result with good quality is shown below.

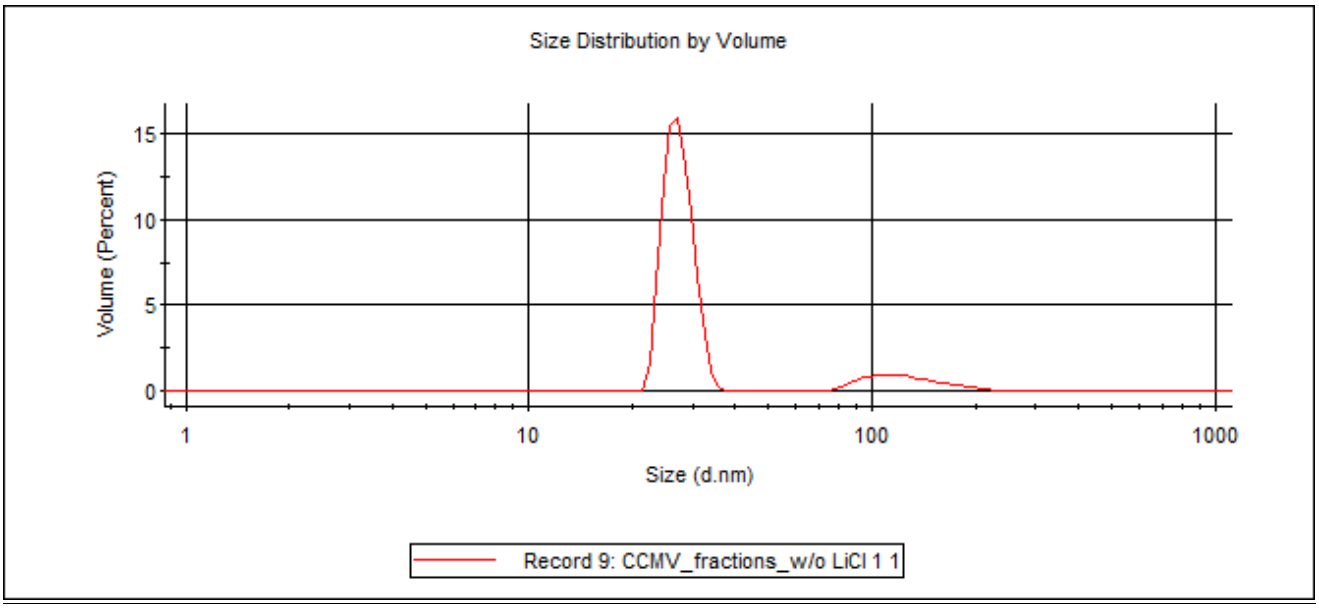


Figure 10. The volume in percent as a function of the diameter size in nanometers

Figure 10 is the DLS-result of the pooled fractions 11,12 and 13 without LiCl which corresponds to the first peak in chromatogram in *Figure 9*. It shows a significantly peak at 28 nm and a smaller and broader peak around 100 nm.

SDS-PAGE 3

A third and last round of SDS-PAGE was carried out with different fractions from FPLC with Superdex 75 corresponding to the two first peaks in the chromatograms.

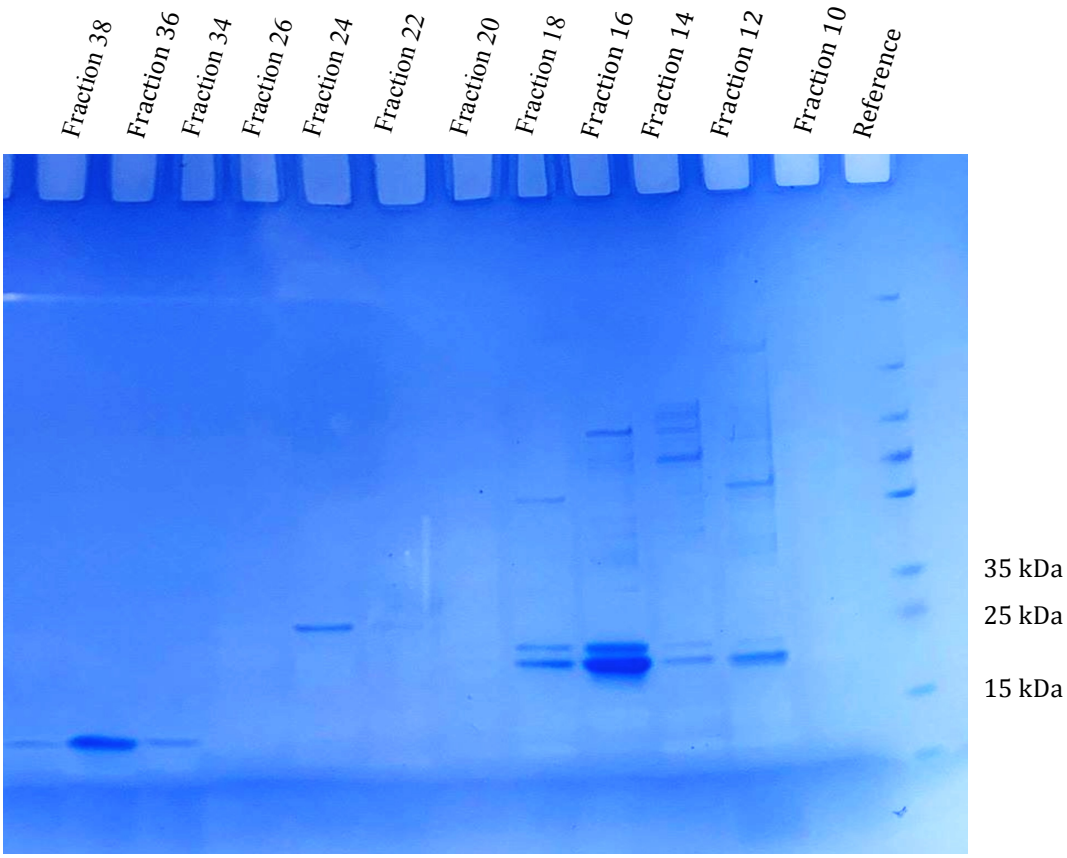


Figure 11. The SDS-PAGE gel that identified in which fractions the dimers were

Figure 11 shows the gel for 12 fractions from the first FPLC round for Superdex 75. Two remarkably bands, both around 20 kDa, is shown in fractions 16. The lower band is the strongest one.

Mass spectrometry

The first gel band, the top one, at approximately 20 kDa in *Figure 11* and fraction 16 is according to MS measurements chloramphenicol acetyltransferase. The protein sequence coverage was 20 %.
The second gel band, also at approximately 20 kDa in fraction 16, corresponds to the protein in interest, CCMV capsids protein, and the protein sequence coverage was 28 %.

FPLC Superose 6

The previous column Superdex 75 was used to purify and separate mixture between different particles like dimers, caspsids etc.
Superose 6 is a smaller column and suitable if you only have small volumes.
A sample of fraction 16,17 and 18 with LiCl from Superdex 75 was injected and the result is shown below.

Figure 12. The UV as a function of the volume in ml

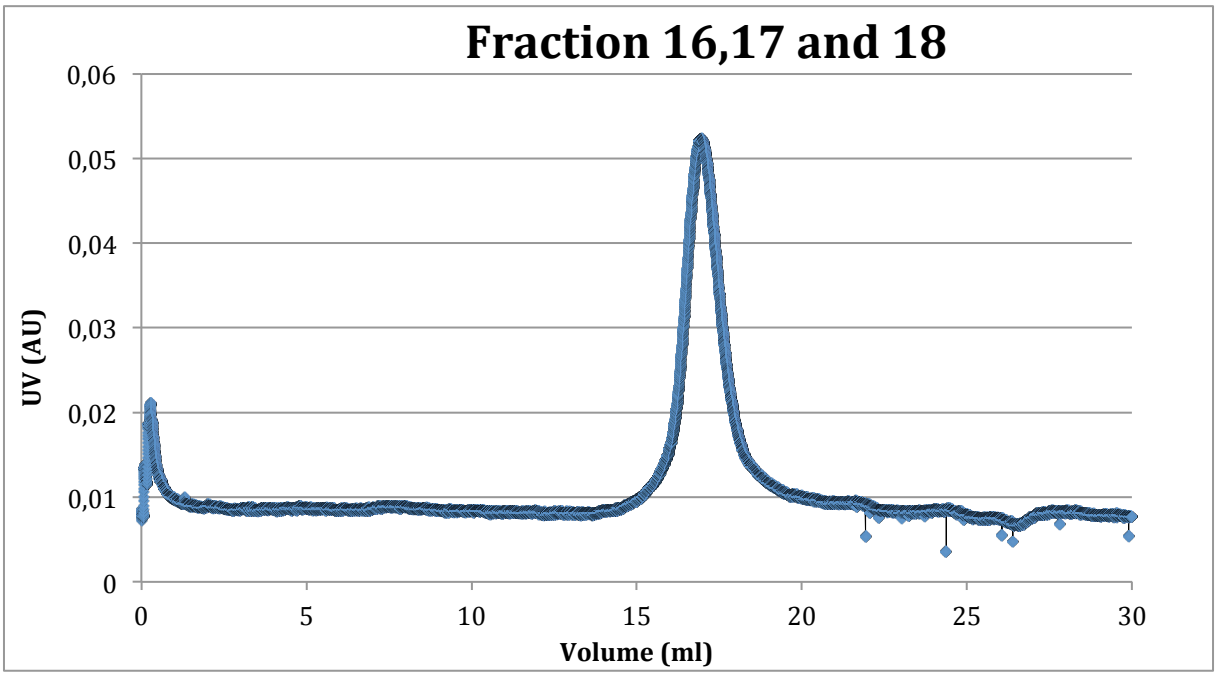


Figure 12 shows the chromatogram for the pooled fractions 16,17 and 18 with LiCl from Superdex 75. A strong peak is shown after 17 ml and that corresponds to molecules with a weight around 40 kDa according to the calibration curve for this column (see appendix *Figure 16*).

Mixtures between dimers and assembly buffer under different conditions were explored to create capsids. Below are the results for two of the experiments.

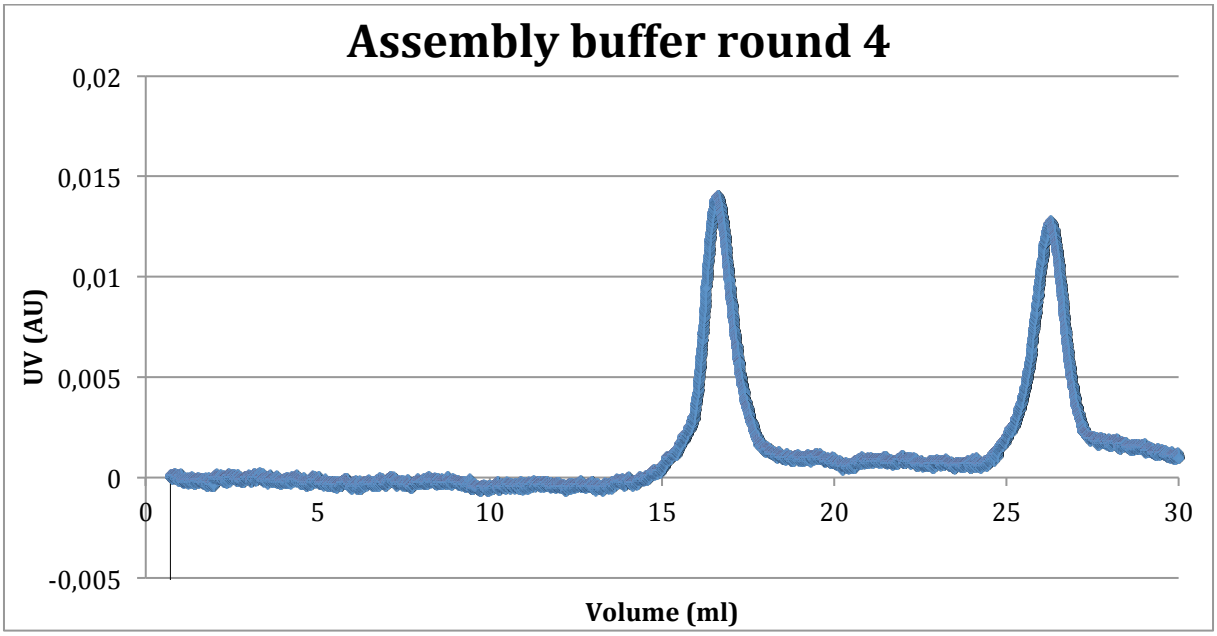


Figure 13. The UV as a function of the volume in ml

Figure 13 shows the chromatogram for a mix between 300 uL assembly buffer and 300 uL of the pooled fractions 16,17 and 18 with LiCl. The mixture was incubated for 30 min and the pH was 5.5. Two peaks are shown after 16 ml and 26 ml.

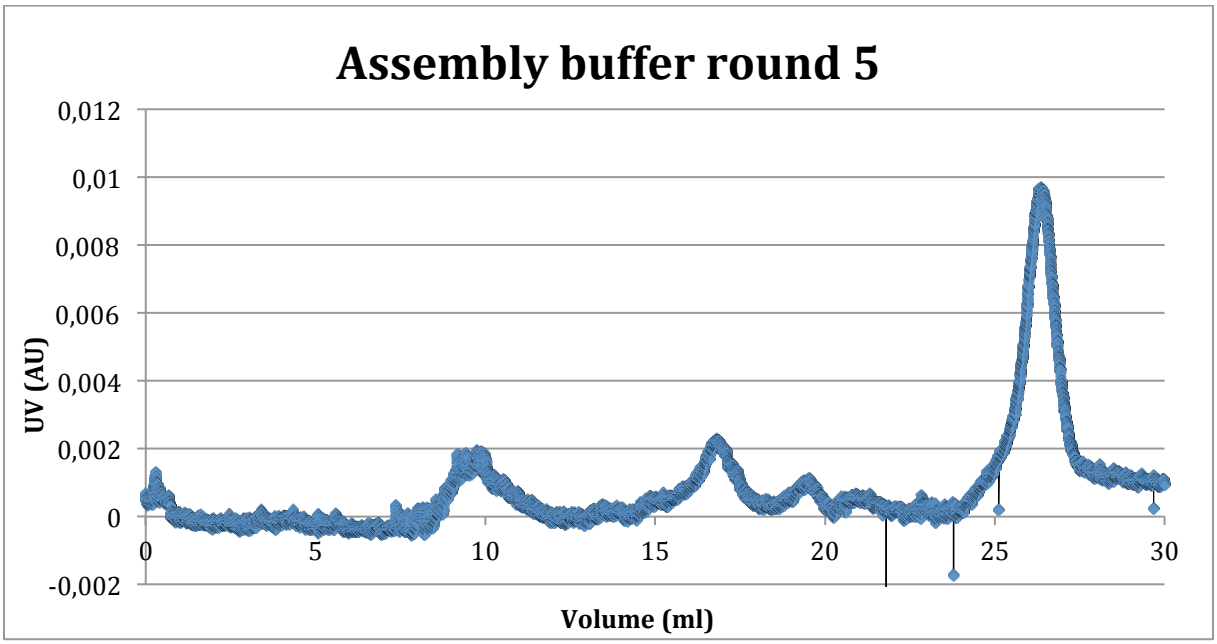


Figure 14. The UV as a function of the volume in ml

Figure 14 shows the chromatogram for a mix between 400 uL assembly buffer and 400 uL of the pooled fractions 16,17 and 18 with LiCl. The mixture was again incubated for 30 min but the pH was adjusted to 4.5. Two small peaks are shown around 10 ml and 17 ml. A strong peak is shown after 26 ml.

Discussion

The project started with a successful sequencing result. The N-terminal and cleavage site were removed and the sequence of the plasmid that was sent away almost matched the entire sequence for the CCMV capsid protein.
The first SDS-PAGE round identified where the protein was after lysis. The protein was found in the soluble part of the clarified lysate. The capsid protein weight is 20 kDa. The bands corresponding to the protein in the gel were as expected found around 20 kDa. Comparing this to previous research results, it´s surprising that the protein was found in the soluble part of the clarified lysate. As described in the introduction, the protein was found in the soluble part of the inclusions bodies before. That corresponds to the pellet part of the clarified lysate. The low temperature over night expression probably led to that the protein was found in the soluble part of the clarified lysate. This is usefully result because it´s easier to purify the protein from the soluble fraction and no refolding from inclusion bodies is needed.
The second SDS-PAGE round identified where the protein precipitated. The protein, again around 20 kDa as expected, was found to precipitate from 45-60 % saturation of ammonium sulphate.
The first chromatogram shown in *Figure 6* is having a peak around 130 ml and it corresponds to something with a weight around 50 kDa. A dimer has a weight of 40 kDa. The peak around 130 ml might be dimers. The same applies to *Figure 7,8* and *9.* The result for the injection without lithium chloride (*Figure 9)* looks the same as the injection with LiCl (*Figure 6,7* and *8*). No remarkable difference appears.
DLS was measured on several pooled fractions from FPLC but only one graph gave a useful result with acceptable quality. This one graph is shown in *Figure 10* and indicates that a big part of the volume in the pooled fractions of 11,12 and 13 without LiCl, which corresponds to the first peak in the chromatogram in *Figure 8,* is capsids with a diameter of 28 nm. These capsids are capsids expressed from *E.coli*, not capsids that have been formed by adding assembly buffer and consequently changing the pH value. Capsids are probably the only relevant thing that can be shown with good quality in DLS because smaller particles like dimers are very hard to see because of their sensitivity.
The third round of SDS-PAGE was to confirm that the aggregate around 50 kDa in fraction 16 actually was dimers. Two bands around 20 kDa were shown in the gel for fraction 16. One of the bands is probably the capsid protein.
Mass spectrometry proved that one of the bands, the strongest band, actually was the capsid protein. The other band corresponds to chloramphenicol acetyltransferase and it´s unclear why there is a band corresponding to this protein.
The chromatogram in *Figure 12* shows a peak around 17 ml and it´s corresponds to molecules with a weight around 40 kDa. It should be dimers. Superose 6 is better to separate big aggregates than Superdex 75 and should also prove that it actually is dimers in the peak around 130 ml in *Figure 6*. The chromatogram in *Figure 13* shows two significantly peaks but compared with the calibration curve, it´s low weight particles like dimers and no capsids.
The chromatogram in *Figure 14* shows one additional peak around 9 ml and it corresponds to something with the same weight as capsids. It´s probably capsids but further experiments are needed to confirm that.

Conclusions and future aspects

When reached dimers after expression and purification, experiments with a 50/50 mix of dimers and assembly buffer with different pH and incubation times were performed to create capsids. A 50/50 mix of dimers and assembly buffer with pH 4.5 and incubation for 30 min before injection probably created capsids. Still, the solution mostly consisted of dimers and just a little percent of capsids.

The protein was found in the soluble part of the clarified lysate and this was probably due to the low temperature over night expression. This result facilitates the purification methods for further experiments.

Further experiment should examine if the particles that were created under the conditions described above actually is capsids. One could also try to optimize the formation of capsids. Try several pH values and many different incubation times to maximize the capsid formation.

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I would like to thank my supervisor Ingemar André for letting me do my project in his research group and for helping me along the project. I am grateful for this opportunity and for everything I learned the last couple of weeks.
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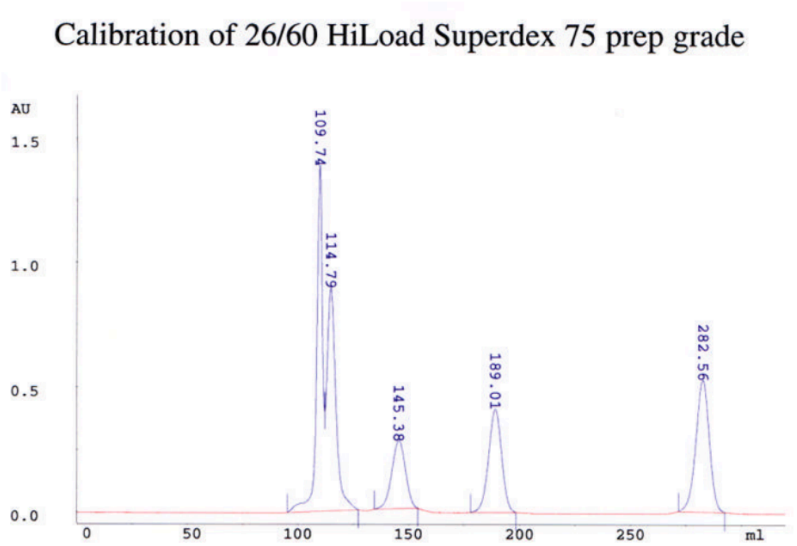
Appendix



Protein/compound	M_r (10^3)	log(M_r)	V_{el} (ml)
Thyroglobulin	670	5.826	109.7
gamma-Globulin	158	5.199	114.9
Aldolase	158	5.199	117.4
BSA	67	4.826	128.6
Ovalbumin	44	4.643	145.4
Chymotrypsinogen A	25	4.398	175.1
Myoblobulin	17	4.230	189.0
Blue Dextran	>2000		109.5
Vitamin B-12	1.35		282.6

Figure 15. The calibration curve for Superdex 75 column[9]

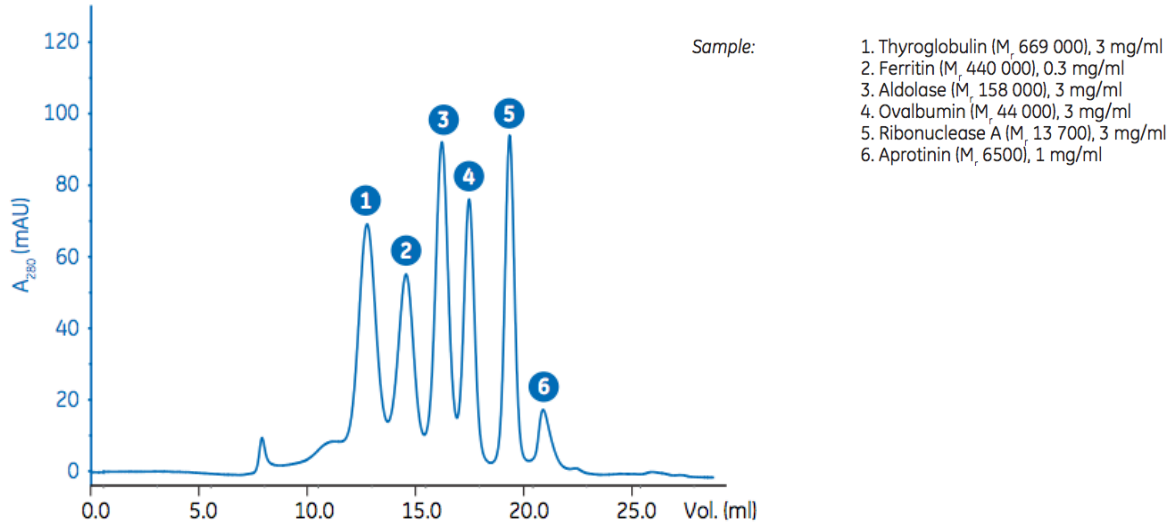


Figure 16. The calibration curve for Superose 6 column[10]

[9]https://www.embl.de/pepcore/pepcore_services/protein_purification/chromatography/hiload26-60_superdex75/

[10] https://www.gelifesciences.co.jp/catalog/pdf/Superose_6_Increase_Datafile.pdf