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PURIFICATION AND CRYSTALLIZATION OF RelA MUTANTS

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Contents

Resum 3
Resumen 4
Abstract 5
Introduction 6
Aim of the project 10
Materials and methods 11
Materials 11
Methods 12
Plasmid purification 12
Transformation protocol 12
Bacterial cultures 12
Purification protocol 13
Cell lysis 13
Ni-NTA column 13
SDS-PAGE Gels 14
FPLC Chromatography 14
Size-exclusion/Gel Filtration Chromatography (SEC) 15
Ion Exchange Chromatography 15
Nanodrop 16
Crystallization 16
Ethics and sustainability 17
Results 18
RelA Δ2 in *E. coli BL21(DE3)* has low expression rates. 18
RelA Δ2 from *Lemo21* improves expression rates and crystallization results 21
RelA Δ5 failed crystallization attempts. 25
A promising complex: RelA-G-His and Nanobody 27
Discussion 31
Conclusions 32
References 33

Resum

La resposta estricta bacteriana és el mecanisme que els bacteris tenen per respondre a situacions d'estrès, com ara limitació d'aminoàcids, de fosfats o d'àcids grassos, carboni o fins i tot situacions de xoc tèrmic. Quan això té lloc, la resposta estricta és activada per una molècula petita anomenada (p)ppGpp. L'acumulació d'aquest compost dóna lloc a l'atenuació de l'activitat cel·lular bacteriana, i actua de forma que el sistema d'expressió cel·lular es centri en expressar components de resistència per tal de sobreviure.

Aquest petit compost (p)ppGpp és sintetitzat per la proteïna RelA, qui catalitza la reacció entre GTP o GDP i ATP per tal de formar-lo. Aquest guanosin-tetra o pentafosfat activa la via per tal de modificar l'acció de la RNA polimerasa i canviar els sistemes d'expressió cel·lular.

La proteïna RelA té 5 dominis. Des de N-terminal: Hidrolasa, Sintetasa, el domini ThrRS, GTPase i SpoT (TGS), dit de Zinc i el domini de reconeixement de RNA. Al 2016, es va publicar l'estructura de la proteïna RelA unida al ribosoma. Aquesta estructura mostrava que RelA s'embolcallava al voltant del lloc A del ribosoma amb els dos últims dominis C-terminal, tot i que existeixen regions que a dia d'avui són desconegudes, principalment degut a la seva flexibilitat.

L'estudi presentat ha consistit en purificar i cristal·litzar la proteïna RelA i mutants d'aquesta. Es presenten els protocols per tal de purificar i cristal·litzar RelA i 2 mutants (RelA Δ2 i RelA Δ5). En RelA Δ2, la proteïna purificada es va acabar obtenint fent un canvi en la línia cel·lular, i fent servir diferents mètodes de FPLC (Fast Protein Liquid Chromatography). Per RelA Δ5, seguint el mateix protocol, va permetre obtenir una gran quantitat de proteïna purificada. Es van preparar diferents plaques de cristal·lització per ambdues proteïnes, i tot i obtenir resultats significatius, no es va poder optimitzar els cristalls. En RelA-G-His (la construcció sencera de la proteïna), es va seguir una estratègia diferent. Es va intentar formar un conjunt amb nano-anticossos, i fent servir aquests com a xaperones per tal de cristal·litzar, però per falta de temps no es van arribar a completar. Tot i això, es presenta una nova estratègia per futures cristal·litzacions, degut a l'exitosa combinació de les proteïnes purificades.

Resumen

La respuesta estricta bacteriana es el mecanismo que tienen las bacterias para responder a situaciones de estrés, como pueden ser la limitación de aminoácidos, fosfatos o ácidos grasos, carbono o situaciones de choque térmico. Cuando estas situaciones ocurren, la respuesta estricta es activada por una pequeña molécula llamada (p)ppGpp. La acumulación de este compuesto da lugar a la atenuación de la actividad celular bacteriana, y actúa de forma que el sistema de expresión celular esté centrado en expresar componentes para la supervivencia.

Este compuesto (p)ppGpp es sintetizado por la proteína RelA, la cual cataliza la reacción entre GTP o GDP y ATP para formarlo. Este guanosín-tetra o pentafosfato activa la vía para modificar la RNA polimerasa y cambiar los sistemas de expresión celular.

La proteína RelA tiene 5 dominios. Desde N-terminal: Hidrolasa, Sintetasa, el dominio ThrRS, GTPase y SpoT (TGS), dedo de Zinc y el dominio de reconocimiento del RNA. En 2016, se reveló la estructura de la proteína RelA unida al ribosoma. Esta estructura mostraba que RelA se envuelve alrededor del sitio A del ribosoma con los dos últimos dominios C-terminales, aunque existen regiones que a día de hoy todavía son desconocidas, principalmente debido a su flexibilidad.

El estudio presentado ha consistido en purificar y cristalizar la proteína RelA y mutantes de esta misma. Se presentan los protocolos para purificar y cristalizar RelA y 2 mutantes (RelA Δ2 y RelA Δ5). En RelA Δ2, la proteína purificada se obtuvo con un cambio en la línea celular, y con diferentes métodos de FPLC (Fast Protein Liquid Chromatography). Siguiendo el mismo protocolo para RelA Δ5, permitió obtener gran cantidad de proteína purificada. Se prepararon diferentes placas de cristalización para las dos proteínas, pero, aunque se obtuvieron resultados significativos, no se optimizaron los cristales. En RelA-G-His (la construcción entera de la proteína), se siguió una estrategia diferente. Se intentó formar un conjunto con nano-anticuerpos, utilizándolos como chaperonas para la cristalización. No obstante, debido a la falta de tiempo no se pudo completar la cristalización. Sin embargo, se presenta una nueva estrategia para futuras cristalizaciones, debido a la exitosa combinación de las proteínas purificadas.

Abstract

The bacterial stringent response is the mechanism that bacteria have to respond to stress conditions such as limiting amino acids, limiting phosphate or fatty acids, carbon limitation or heat shock. When this happens, the stringent response is activated by the small alarmone (p)ppGpp. The accumulation of this compound causes two main situations: the bacterial cellular activity is mostly turned off and the expression systems are focused in expressing survival components.

(p)ppGpp is synthesised by RelA enzyme, which catalyses the reaction between GTP or GDP and ATP in order to form (p)ppGpp. This guanosine tetra or pentaphosphate, activates the pathway to modify the action of RNA polymerase and the cellular expression systems.

RelA has 5 domains: The N-terminal Hydrolase, Synthetase, ThrRS, GTPase and SpoT domain (TGS), Zinc-finger domain and RNA recognition motif. In a recent study (Brown, Fernández, Gordiyenko, & Ramakrishnan, 2016), the ribosome-bound structure has been revealed, and it shows that RelA wraps around the ribosomal A-site with the last two C-terminal domains, although it has some flexible parts which remain unknown.

The present study aimed to purify and crystallize RelA and RelA mutants. Purification and crystallization protocols for RelA and two RelA mutants (RelA $\Delta 2$ and RelA $\Delta 5$) are presented. For RelA $\Delta 2$, the purified protein was obtained by changing the cell line and using different FPLC (Fast Protein Liquid Chromatography) systems. For RelA $\Delta 5$, the same purification strategy was followed, and lead to a high amount and pure protein. Crystallization trays were set for both proteins, but no significant results were shown. In RelA-G-His (full construction of the protein), a different strategy was followed. The plan has been to form a complex between a nanobody and RelA to stabilize RelA, thus making the crystallization possible, but for a matter of time it could not be completed. Despite this, a new approach for future crystallization attempts is presented, because the complex was successfully bound using the two purified proteins.

Introduction

When bacterial cells are faced with a stress environment, they activate the so called stringent response, in order to rapidly change their cellular processes and survive under stress conditions. Limiting phosphates, fatty acids, limitation of carbon, iron or amino acids can stop the synthesis of DNA and stable RNAs in favour of synthesising crucial components which promote bacteria to survive (Potrykus & Cashel, 2008). The stringent response controls the adaption to nutrient deprivation, and it is broadly conserved through many bacterial species (Boutte & Crosson, 2013).

More than 50 years ago, in 1961, a group of scientists of the Medical Research Council Unit for Molecular Biology in Cambridge, firstly described the response of bacterial cells when are under stress conditions. A genetic locus was found containing repressors of the RNA synthesis or either an operator governing the functions of an operon (Stent & Brenner, 1961). A few years after, it was observed that the cause for the stringent response was due to the appearance of one or two phosphorylated compounds in *Escherichia coli* amino acid limited extracts (Cashel, 1969; Potrykus & Cashel, 2008). These two molecules, guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'diphosphate (pppGpp), are responsible of the activation of the stringent response.

(p)ppGpp, as collectively written, arise when bacteria detect a harsh environment, and its accumulation is caused by the activation of a family of enzymes called RSH (RelA SpoT Homologue). RSH genes are found in one or more variants in every sequenced genome of eubacteria and plants (Potrykus & Cashel, 2008). These enzymes are the responsible for (p)ppGpp synthesis or breakdown.

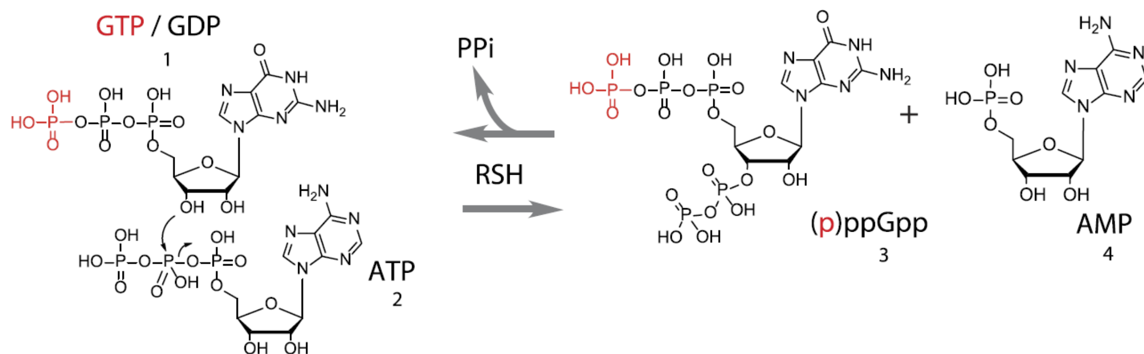

Figure 1: (p)ppGpp synthesis and degradation by RSH enzymes (Beljantseva et al., 2017). RSH enzymes synthesize (p)ppGpp using ATP and GTP/GDP as substrates. Hydrolysis of (p)ppGpp regenerates GTP/GDP, accompanied by release of pyrophosphate (PPi).

RSH enzymes control the concentrations of (p)ppGpp, and as this regulatory pathway is present in a large number of pathogens, it has been proposed as a potential anti-bacterial target, as there are genetic knockdowns already done showing a decrease in animal infection (Dean, Ireland, Jordan, Titball, & Oyston, 2009; Gao et al., 2010; Haralalka, Nandi, & Bhadra, 2003; Müller et al., 2012; Wilkinson, Batten, Wells, Oyston, & Roach, 2015)

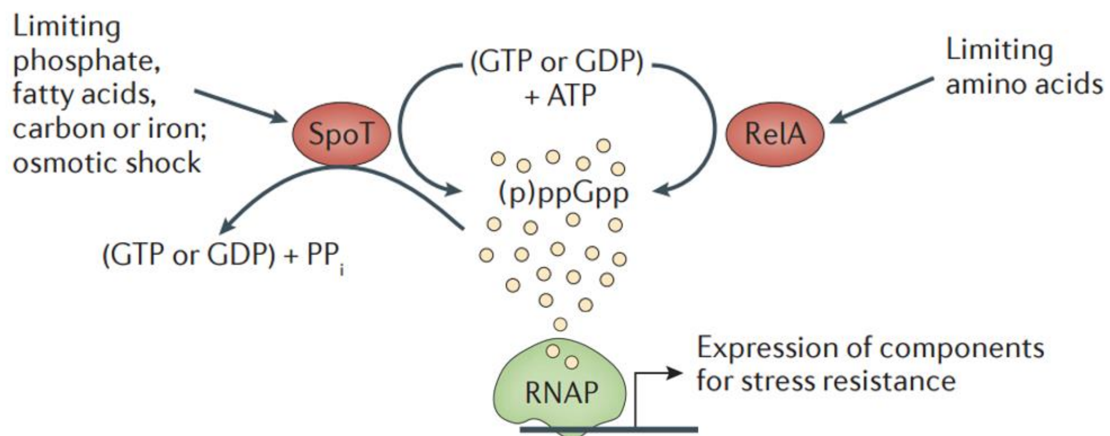

Figure 2: In particular stresses, SpoT and RelA catalyse pyrophosphoryl transfer from ATP to GTP or GDP to synthesize the signalling nucleotides (p)ppGpp. (p)ppGpp together with DksA (not shown) directs transcription through direct interaction with RNA polymerase. (Dalebroux & Swanson, 2012) (Adapted)

These enzymes have different domains, but the most conserved are the Hydrolase and the Synthetase domains. In *E.coli*, the reaction is catalysed by RelA, a multidomain pyrophosphate transferase (Atkinson, Tenson, & Hauryliuk, 2011; Brown et al., 2016). RelA has 5 domains: Hydrolase, Synthetase, TGS (ThrRS, GTPase and SpoT), ZFD (Zinc-Finger Domain) and RRM (RNA Recognition Motif) (Brown et al., 2016). Even though RelA has the hydrolase domain, it shows a synthetase activity only (Potrykus & Cashel, 2008).

Recently, the Cryo-EM structure of RelA bound to the bacterial ribosome got revealed. The structure shows that RelA binds to the ribosome with a multi-domain architecture that folds around the A-site tRNA, which also supports a model in which RelA and ribosome association activates the synthesis of (p)ppGpp (Brown et al., 2016).

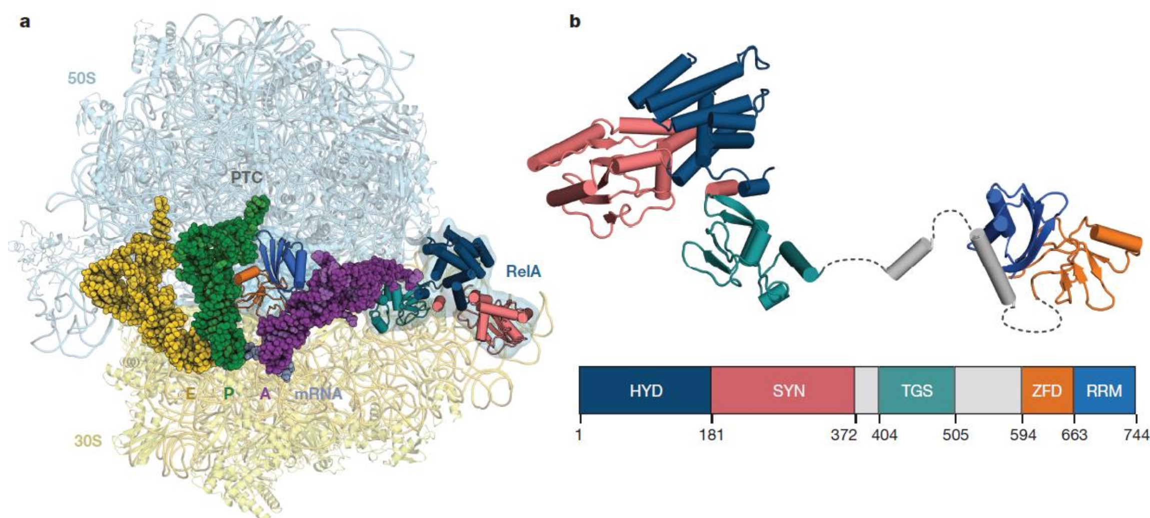

Figure 3: Structure of RelA bound to ribosome (Brown et al., 2016). A) General view of RelA:Ribosome complex, with an uncharged tRNA in the A-site. The E, P and A site of the ribosome are shown. B) Detailed structure of the RelA domains.

Although the study solved the structure with an overall resolution of 3.0 Å, there are some parts which the researchers have not been able to determine the structure due to the flexibility. These parts are the linker parts between the Synthetase and TGS domains, and between the TGS and ZFD.

There have been many studies based on biochemical studies of RelA using *E.coli* as a model (Beljantseva et al. 2017; Hauryliuk et al. 2015; Steinchen et al. 2015), but the *Franscisella tularensis* RelA sequence has some similarities, except from a truncated C-terminus region (Wilkinson et al., 2015).

Nowadays, there are more than 120 000 entries in the Protein Data Bank (PDB), and Structural Biology is a rising field all over the world. In the last years, there has been tremendous efforts to increase the number of deposited molecules (Gavira, 2016). Most of the macromolecular structures in PDB are from X-ray crystallography.(Dessau & Modis, 2011). To obtain protein crystals of high quality, the sample has to be purified in a homogenous state to produce crystals that diffract to high resolution (McPherson, 1999).

The process of crystallization is a rational and empirical process with the objective of producing crystals. Once the protein is purified, the first step is to set initial crystallization screens with a primary screen. Initial screens are usually broad, and have wide range of conditions, such as different salts, polymers, organics, buffers, pH, … Up to 40% of the samples screened will produce some results, but only 10% will be suitable for X-ray

analysis. Once the initial screen has been performed, and some results are obtained, 75% of the proteins require optimization (Hampton Research Corp., 2017)

Crystal optimization is the process where increasing the size or quality of the crystals takes place. It can be done by increasing the volume, change of pH or buffer conditions, precipitant conditions, etc.

Moreover, the use of chaperones as auxiliary tools for crystallizing has risen over the last few years. Binding partners like ions, small molecule ligands and peptides have been used to stabilize the protein and reduce the conformational heterogeneity (Koide, 2009). A new approach is the use of nanobodies for crystallization. Nanobodies are small and stable single domains with full antigen binding capacity. They come from the antibodies with only the heavy chain present in camelids(Pardon et al., 2014).

I have worked with two mutants of *F. tularensis* RelA, RelA Δ2 and RelA Δ5. The first one, contains the first 3 N-terminal domains, while the second one contains the same 3 first domains and 58 amino acids of the linker part. I have tried to purify the mutants and crystallize them. Also, I have done some experiments with the RelA whole construction, with the use of nanobodies.

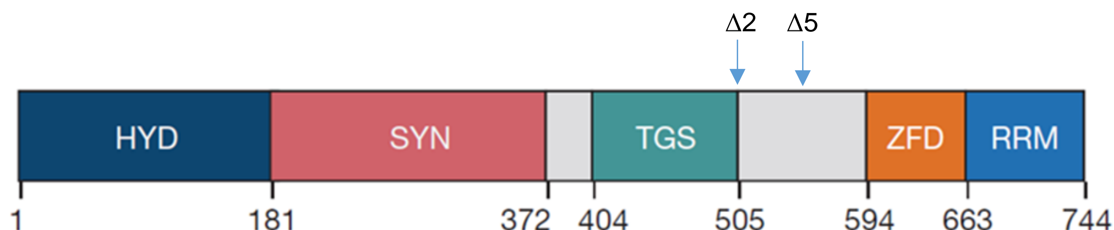

Figure 4: Representation of the different RelA mutants. (Brown et al., 2016). Arrows mark the different lengths of RelA mutants, respectively.

By designing the mutants, we wanted to know in which length RelA is inactive, meaning that a RelA mutant could be autoinhibited, not bound to the ribosome and not synthesising (p)ppGpp. In conclusion, not activating the stringent response.

I have done this project in Ditlev E. Brodersen lab, inside the Centre of Structural Biology (CSB), as part of the Science and Technology faculty from Aarhus University. The work I have done was assisted by the PhD student Cemre Manav.

Aim of the project

The main aims of the project are the following:

- Understand the function, domains and structure of RelA.
- Understand the autoinhibition function of RelA by designing RelA mutants.
- Understand new theoretical and practical concepts related to the Structural Biology field, particularly purification and crystallization approaches.
- Learn the different protocols related to purification of proteins: Techniques, systems, …
- Identify the key points in crystallization, such as the different methods, different sizes and shapes of the crystals.
- Learn the structure and working environment of a structural biology research lab.

And these objectives will try to be accomplished by these procedures:

- Purification of RelA and RelA mutants, RelA ∆2 and RelA ∆5.
- Crystallization of RelA and RelA mutants, RelA ∆2 and RelA ∆5.

Materials and methods

Materials

All materials were provided by Ditlev E. Brodersen lab.

Table 1: List of buffers used during purification.

Purification buffers	
Lysis buffer pH=8.5	50 mM Tris pH 8.5, 500 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 3 mM BME, 10 mM imidazole
Lysis buffer pH=6	50 mM MES-NaOH, 500 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 3 mM BME, 10 mM imidazole
Gel Filtration Buffer	30 mM Tris pH 8.5, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM BME
Ion Exchange Buffer A	50 mM Tris pH 8.8, 5 mM $MgCl_2$, 5 mM BME
Ion Exchange Buffer B	50 mM Tris pH 8.8, 5 mM $MgCl_2$, 5 mM BME, 1 M NaCl
Elution Buffers for Ni-NTA	50 mM Tris pH 8.5 or 50 mM MES-NaOH pH=6, 500 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 3 mM BME and 30/50/75/150/300 mM imidazole
5X Tris-Glycine-SDS running buffer	In 5000 mL of ddH_2O: 150.10 g of Trizima Base, 720 g of Glycine, 25 g of SDS

SDS-PAGE Gels and Buffers: Tris-Glycine Gels were manually casted in the laboratory. Tris-MOPS Gels and Tris-MOPS-SDS running buffer were provided by GenScript and prepared using the instructions provided.

Crystallization buffers: JCSG (Joint Centre of Structural Genomics) Plus crystallization screen was provided by Molecular Dimensions and PegIon Screen was provided by Hampton Research.

All buffers except the running SDS buffer and MOPS buffer were stored at 4ºC during the experiments, and filtered and degassed by a Frisenette Montanil 0.45 µM filter.

Buffers with 2-mercaptoethanol (BME) and imidazole were kept for no longer than 15 days. After that fresh buffers were made.

All buffers were prepared by weighing out the appropriate amounts on a Buch and Holm GX-2000 precision weight. Buffers’ pH was measured by Radiometer Copenhaguen pHM 92.

Methods

Plasmid purification

Plasmid purification was done with the ThermoScientific GeneJET Plasmid Miniprep Kit. Transformed cells were resuspended, lysed and neutralised. Then plasmids were purified from chromosomal DNA using the ThermoScientific GeneJET Spin Column included in the kit, washed and eluted. All steps were done following the manufacturer's instructions.

Transformation protocol

Transformation is the process in which foreign DNA is introduced into a competent cell. The transformation step was done following the next protocol: 50 µL of the competent cells used (NovaBlue *E. coli* BL21, Lemo21 (DE3), XL-10 Gold) and 2 µL of the ligation product were incubated for 5 minutes in ice. After that, the mixture was incubated at 42 ºC using a Techne Dri-block for 30 seconds, and then back in ice for 2 minutes. Finally, 150 µL of LB sterile media were added, and the mixture was incubated for 1 h at 37 ºC 150 rpm.

After 1 h, agar plates treated with Kanamycin were set with the mixture and left at 37ºC overnight.

A control plate was set only with the digested vector without the PCR product.

Bacterial cultures

Overnight culture: 250 mL of sterile LB broth were mixed with 250 µL of Kanamycin 50 mg/mL in a 1 L flask. 2 or 3 colonies were picked with a plastic tip and left inside the flask. The mixture was incubated overnight at 37 ºC 150 rpm in a IKA KS 4000i control incubator.

LB media for growth: 6 flasks of 5 L were filled with 2 L of ddH_2O and 40 g of LB Broth powder (20 g per L - following the manufacturer instructions). After mixing, they were autoclaved.

Bacterial growth: In each 2 L LB media, 2 mL of Kanamycin and 40 mL of the overnight culture were added. The cultures were incubated in a New Brunswick Innova 44/44R incubator at 37 ºC and 130 rpm, until the OD_{600} of the cultures was around 0.6-0.7. Then, after a short cool down on ice, protein expression was induced by 1 mL of 1 M IPTG, and left overnight at 21 ºC 130 rpm or 3 h at 30 ºC 130 rpm. The optical density was measured by a VWR UV-1600PC Spectrophotometer.

All steps that involved possible contamination of the cultures were performed under a sterile fume hood.

Harvesting cells: After the induction time was finished, the cultures were centrifuged for 15 minutes at 4 ºC 6000 rpm in a ThermoScientific Sorvall Lynx 6000, with F9-6x1000 Lex rotor. Supernatant was discarded, and pellet was recovered with ice-cold ddH_2O in falcon tubes.
An extra centrifugation step to remove remaining media or water was performed for 30 minutes at 4 ºC 4500 rpm in a BeckmanCoulter Allegra25R centrifuge with TS-5.1-500 rotor. Supernatant was discarded and cells were stored at -20 ºC.

Purification protocol

Cell lysis

Cell pellet previously stored in falcon tubes at -20 ºC were thawed in ice. 40 mL of lysis buffer pH 8.5 for all RelA-related constructions and pH 6 for the nanobodies purification were added to the mixture, followed by the immediately addition of 400 µL of 50 µM PMSF and 40 µL of DNAse I 10mg/mL. These 2 last compounds were added to inhibit proteases and avoid nucleic acids contamination, respectively.
The mixture was vortexed until there were no cell clumps remaining. If necessary, the addition of lysis buffer and PMSF was repeated.
The cells were decanted into a glass bottle, and kept in ice since the following step. Then cellular lysis was done by sonication, into a Bandelin Sonoplus HD 2200 sonicatior.
Cells were sonicated for 30 minutes 5 cycles in 10 minutes' intervals. Between intervals, the cells were lightly mixed.
After that, cell debris was spun down using SS34 tubes in the BC Allegra25R centrifuge for 45 minutes at 4 ºC 14000 rpm.
Supernatant was filtered into a 0.8 µM Advantec membrane filter before the next step, and stored in ice.

Ni-NTA column

Ni-NTA 1 mL affinity columns were obtained from GE Healthcare. The column was coupled to a Gilson Minipuls 3 using plastic tubes. The general protocol was the following.
First the column was washed with ddH_2O for 10 column volume (C.V.) to remove the ethanol. After that, it was equilibrated with the Lysis Buffer used (either pH = 8 or pH=6) for 10 C.V. Then, the sample was loaded and flow through (FT) was collected. When all the sample was loaded, a wash step with the same Lysis Buffer used was done to remove all unbound proteins. Wash 1 and wash 2 samples were collected, for about 8 mL each in 10 mL falcon tubes.

After that, the elution step with Elution Buffers was performed. Elution fractions from 30, 50, 75, 150 and 300 mM imidazole buffers were collected for about 8 mL each in 10 mL falcon tubes and kept at 4ºC for the next purification step.
At the end, column was washed with ddH_2O for 50 C.V.

SDS-PAGE Gels

The laboratory-made SDS-PAGE gels were casted with the following protocol.
For 12 gels at 15%, the procedure was:
For the Separation Gel: 42.5 mL of Acryl-bis 30:0.8, 10.6 mL of Tris-Cl pH 8.8 3M, 850 µL of SDS 10%, 30.1 mL of ddH_2O, 850 µL of $(NH_4)_2S_2O_8$ 10% and 66µL of TEMED were mixed, and decanted into the BioRad Multicasting-chamber, after putting the gel glass in the correct position and ensure no leaks. Isopropanol was slightly added after the mixture to ensure a flat line on the top of the gel. After 30 minutes, isopropanol was removed.
When the separation gel was polymerized, the Stage Gel was prepared: 5.8 mL of Acryl-bis 30:0.8, 8.75 mL of Tris-Cl pH 8.8 3M, 350 µL of SDS 10%, 19.7 mL of ddH_2O, 350 µL of $(NH_4)_2S_2O_8$ 10% and 52.5 µL of TEMED were mixed and decanted on top of the separation gel, and the BioRad combs for 15 wells were fitted inside. The gels were left for about 30 minutes to polymerize and then were stored at 4ºC.
Procedure for loading the gels:
10 µL of the sample of interest were mixed with 5 µL of 5X SDS Loading Buffer (125 mM Tris-PO_4, 10% SDS, 1% BME, 50% Glycerol + Bromophenol blue) and boiled for 2 minutes at 95ºC in a Techne Dri-block. After this 2 minutes samples were ready to load.
5 µL of the Fermentas PageRuler Prestained Protein Ladder was included in each gel.
Samples were loaded into either a commercial GenScript ExpressPlus PAGE Gel, 10x8, 4-20% or a laboratory-casted SDS-PAGE gel, made with the procedure above.
Non-comercial gels were ran at 200V, 100mA, for 45-55 minutes, and GenScript gels at 140V, 100mA for 45-55 minutes. The gels were ran using a GE Electrophoresis Power Supply – EPS 301. Once the gels had finished, were stained for 2 minutes with a Coomassie Staining solution (For 1L, 100 mL dH_2O, 500 mL Ethanol 96%, 400 mL acetic acid 99.8% and 2g of Coomassie Blue R-250) at 80ºC.
Then, the gels were destained with a destaining solution (For 1L, 900 mL dH_2O and 100 mL of acetic acid 99.8%) at 80ºC. The gels were left with destaining solution until they were completely destained.

FPLC Chromatography

All FPLC purification experiments were carried out on a GE Healthcare ÄKTA Purifier at 4ºC with the UNICORN software.

Size-exclusion/Gel Filtration Chromatography (SEC)

Size-exclusion Chromatography is a process in which molecules are separated by their size as they pass through a packed SEC medium. The smaller the molecules they are, the later they elute, as they can enter into the pores of the resin, while the biggest molecules would elute first, as they cannot enter the pores.

Size-exclusion / Gel Filtration Chromatography were carried out by a Superdex200 10/300 GL and a Superdex200 Increase 10/300 GL columns from GE Healthcare. Both columns have a void volume (V_0) of 8.5 mL and a total volume (V_t) of 24 mL. The protocol was the following: The column was washed with water in order to remove the ethanol and then was equilibrated with Gel Filtration Buffer. Then, the 500 µL loop from GE Healthcare and the Hamilton Company 500 µL Syringe 1750 RN were washed with water and then with Gel Filtration Buffer. Once the loop was installed to the ÄKTA Purifier system, 500 µL of the Gel Filtration Buffer were injected in order to check if there was any leak into the system.

The sample of interest was concentrated to 500 µL using a VIVASPIN 20 from Sartorius with a 50 kDa molecular weitght cut off for RelA constructions and 10 kDa for nanobody constructions.

Once our sample had been concentrated to 500 µL, it was transferred to a Eppendorf 1.5 mL plastic tube, and spun down for 30 seconds at maximum speed in a Eppendorf minispin F-45-12-11 centrifuge. After that, sample was loaded into the syringe and injected into the ÄKTA system. The syringe was left connected during the procedure in order to avoid air getting into the system. Fraction tubes were 1.5 mL fresh Eppendorfs tubes at the coupled Fractionator-950 from GE Healthcare.

Then, the program was started.

Ion Exchange Chromatography

Ion Exchange Chromatography is a process in which molecules are separated by their total charge. This technique involves the separation of size-similar molecules with different pI, by changing the buffers' pH. If the pH of the buffer is lower than the protein pI, the protein total charge will be positive, and the resin to separate them would be negative, so a Cation exchange chromatography will be used, whereas if the pH of the buffer is greater than the protein pI, the total protein charge will negative, and the resin to separate them would be positive, so a Anion exchange chromatography has to be used.

Ion Exchange chromatography were carried out by a 1 mL SourceQ (anion exchange with laboratory-packed SourceQ resin), 1 mL MonoQ (anion exchange with commercial

packed resin), 1 mL SourceS (cation exchange with laboratory-packed SourceS resin) and 1 mL MonoS (cation exchange with commercial packed resin). All components and resins were from GE Healthcare.
The column first was washed with water to remove the ethanol. Then it was washed with 100% of buffer B (high salt concentration) to remove all remaining proteins. After that, an equilibration step with 5% buffer B was performed. The sample of interest was diluted to 5% of Buffer B, filtered with a Q-Max Syringe 0.45 µM filter from Frisenette and loaded into a previously washed and equilibrated (with buffer A) GE Healthcare 50 mL Superloop. Once the Superloop was connected into the system, and fresh fractionation 1.5 mL Eppendorf tubes and a 50 mL falcon tube from Labsolute for collecting the flow through were placed into the Fractionator-950 coupled to the system.
After that, the program was started, to load the sample and elute with a gradient from 5% buffer B to 80% in a 35 C.V. length of elution.

Nanodrop

The measurement of the concentration of the proteins was carried out on a Nanodrop ND-1000. The program used was ND1000 3.8.1., with the feature Measuring Proteins A280.
The protocol was the following. Once the program started, 2 µL of ddH_2O water were loaded to initialize the Nanodrop. Then the drop was cleaned with Linen Paper. After that, 2 µL blank sample was loaded (Most of the times Gel Filtration buffer). The drop was cleaned and the 2 µL of the sample were loaded and the protein concentration was measured at OD_{280} Nanodrop was cleaned with ddH_2O after usage.

Crystallization

Crystallization is the process of the formation of a protein crystal. Under supersaturated conditions, proteins can become crystals and be susceptible of being analysed in a X-ray source in order to get the diffraction results. The growth and optimization of the crystals remains largely empirical in nature, and there is no theory or guide.
There are lots of methods to achieve the supersaturation condition and try to crystallize. Vapor diffusion, sequential extraction, pH induced or controlled evaporation are a small example of the multiple techniques to crystallize.

The crystallization protocol, in this case, was prepared by using the sitting drop vapor diffusion technique, and the use of crystallizations screens was to identify a hit.
Sitting drop is a technique in which a drop containing a mixture of the sample and the reservoir is placed in vapor equilibrium with the reservoir. To achieve the equilibrium,

water vapor evaporates from the drop to the reservoir, and the drop increases its supersaturation state. When the concentration of the reagent is approximately the same in the drop and the reservoir, equilibrium is reached.

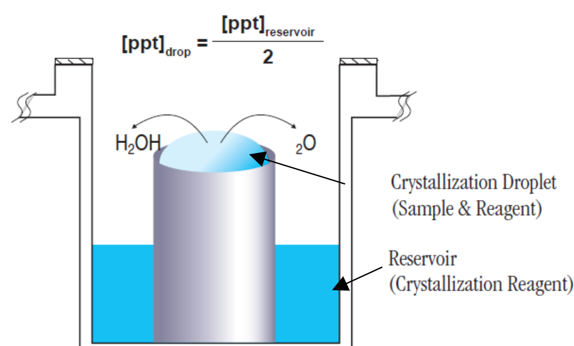

Figure 5: Principles of the Sitting drop crystallization technique (Hampton Research Corp, 2017)

Crystallization experiments were held in a SwissCi MRC 96 well plate with 2 sitting drops. The protocol was the following:

Having the crystallization screen in a 96 deep-well plate, 70 µL of each buffer were put into the reservoir position from a SwissCi MRC 96 well plate with 2 sitting drops with a ThermoScientific Multichannel pipette. Then, the plate was covered with tape in order to avoid evaporation until the next step. After that, tape was removed, and 200 nL of the buffer were put in the 1st drop and mixed with 200 nL of the reservoir, and 200 nL of the protein were put in the 2nd drop and mixed with the respective 200 nL of the reservoir. The experiments were carried in a Mosquito TTP robot from LabelTech or an ORYX4 robot from Douglas Instruments. Once the robot had finished the whole plate, it was covered with Crystal Clear Sealing Tape from Hampton Research.

Also an experiment with bigger crystallization drops was carried: In a Hampton Research 24-well Cryschem Plate M, where 400 µL buffer were put in the reservoir position, and 1 µL of the buffer and 1 µL of the protein were mixed in the crystallization droplet.

The plates were stored at 19 ºC or 4ºC.

To observe the results, a Molecular Dimensions Crystal Light 100 UV Source coupled to a computer and a Leica M165C microscope was used.

Ethics and sustainability

Aarhus University and the Science Park has a system to collect chemical waste. (Aarhus University, 2017). During this year, I have learnt the importance of waste management on a research laboratory, and applying sustainability criteria in order to reduce the amount of waste and the correct strategy to manage with biochemical residues.

Results

RelA Δ2 in E. coli BL21(DE3) has low expression rates.

The construction RelA Δ2, was the first mutation to work with. This construction, contains the 3 first domains of the RelA gene. It contains the Hydrolase, the Synthetase and the TGS domain. Its molecular weight is 58.8 kDa, and its pI=6.6. This mutation was tried because of the flexibility of the linker parts between the TGS and ZFD (Zinc Finger) domains. Mutation at this part of the protein was thought to confer more stability and so, more facilities at the time to crystallize.

RelA Δ2 first attempt to purification was done following a strategy which had worked previously in other RelA/SpoT type enzymes by the PhD student, Cemre Manav, who leaded the project.

Firstly, RelA Δ2 was purified by a Ni-NTA 1 mL column. Then, an SDS gel was performed with the different fractions, as showed in Figure 6.

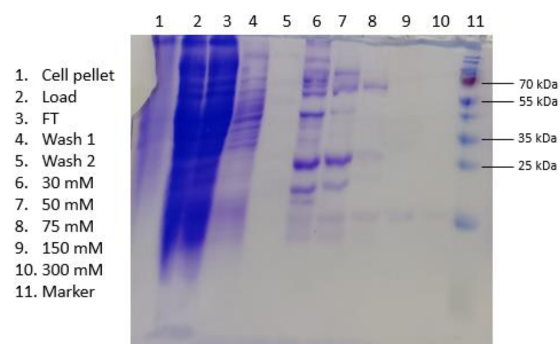

Figure 6: SDS-PAGE Gel with the Ni-NTA fractions. FT: Flow through. 30-300 mM are the different imidazole eluates from Ni-NTA affinity column.

After that, a Gel Filtration chromatography with a Superdex200 (Figure 7) was performed with the fractions 30, 50 and 75 mM eluates from the NiNTA affinity column.

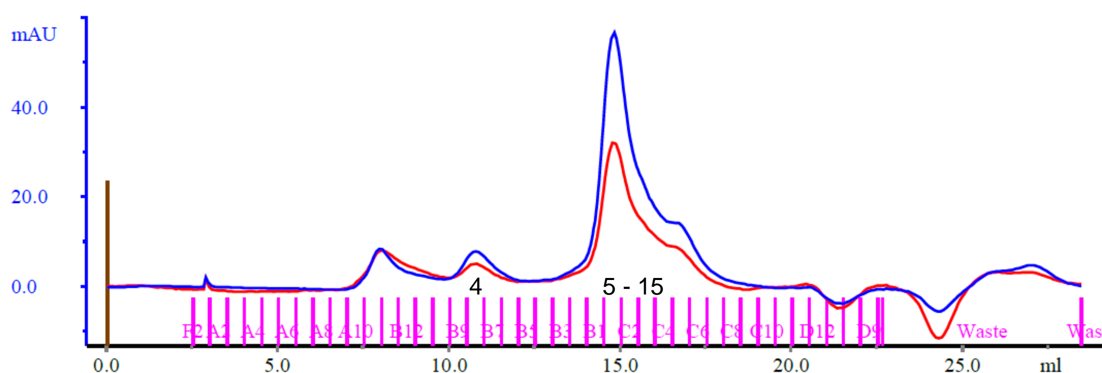

Figure 7: Gel Filtration Superdex200 chromatogram. Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm. Numbers under the peaks are the lanes in SDS-Gel (Figure 8).

After the Gel Filtration, an SDS-PAGE gel was performed in order to verify if the peaks were the protein of interest (Figure 8). The SDS-PAGE gel confirmed it. The fractions from B3 to C8 had the RelA Δ2 protein (58.8 kDa), although there was not a lot of protein, a further purification step was done. An Ion Exchange Source Q was ran using the fractions mentioned before (Figure 8). The results were not the expected as the amount of protein present was useless, and not a single peak was observed.

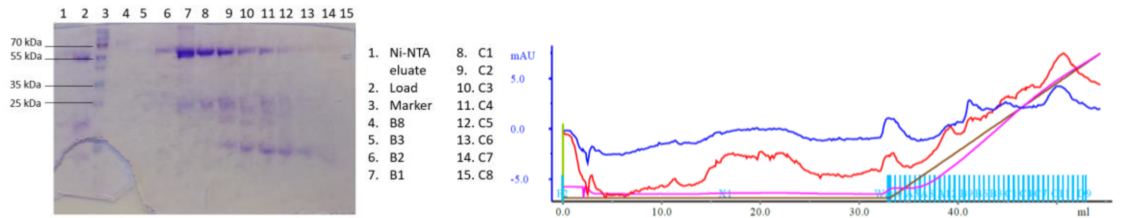

Figure 8: Left: SDS-PAGE Gel After Superdex200. Lanes are also marked in the Chromatogram from Picture 7. Right: Ion Exchange SourceQ Chromatogram. Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm. Pink curve: Conductivity. Brown curve: Concentration of buffer.

Following the same strategy, another batch was purified. Ni-NTA affinity column and then Gel Filtration Superdex200. After this step, there was a peak eluting at 10.9 mL which seemed to be the “tetramer-like” protein, as the molecular weight in Gel Filtration columns is just an estimation.

After that, protein analysis was performed on a SDS-PAGE gel with the fractions of that “tetramer-like” peak. The gel showed the fractions contained a mostly pure protein, so crystallization trays were set up (Figure 9 and 10).

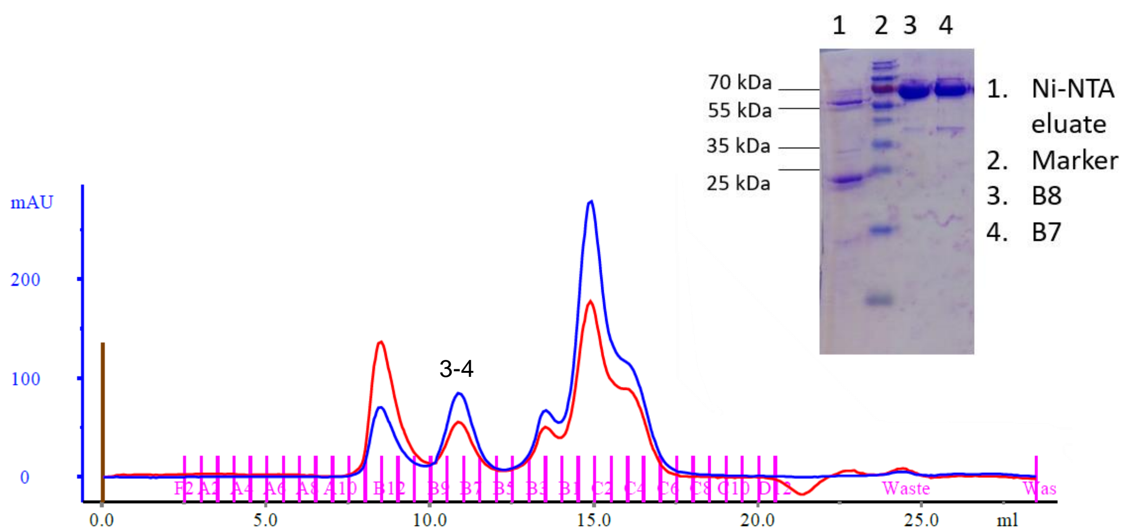

Figure 9: Gel Filtration Superdex200 Chromatogram (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm) and SDS-PAGE Gel with the fractions showed.

The fractions were concentrated to 100 µL and then the protein concentration was measured at the nanodrop.

Protein	Concentration (mg/ mL)
RelA Δ2 (*E.coli* BL21(DE3)) – “tetramer-like”	7.2

The initial trays were set with the JCSG crystallization screen at 19ºC but unfortunately no hits were shown, as mostly all wells were either precipitated, phase separated or clear drops.

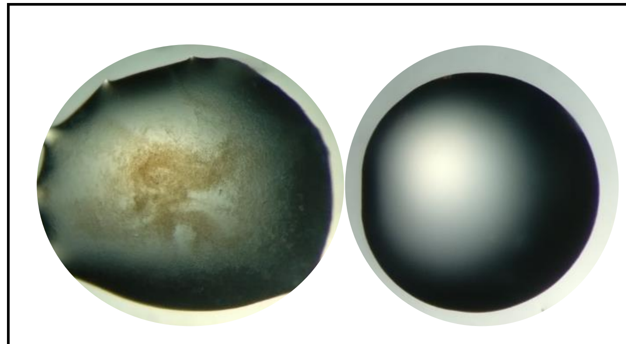

Figure 10: Examples of two pictures of crystallization drops with RelA Δ2 from *E.coli* BL21(DE3). Only precipitated (left) and clear drops (right) were obtained.

The experiment was repeated in order to get a higher amount of protein and try more crystallization screens or different concentrations. In order to do that, a new batch of BL21(DE3) transformed cells were grown and induced to express the mutated protein. The general procedure to open the cells was performed (Cell lysis and sonication) and the same strategy to purify the protein. An early NiNTA column, size-exclusion, an ion exchange chromatography and a final size-exclusion.

In the second step of the purification protocol, the Gel Filtration with the Superdex200, different results were shown. (Figure 11)

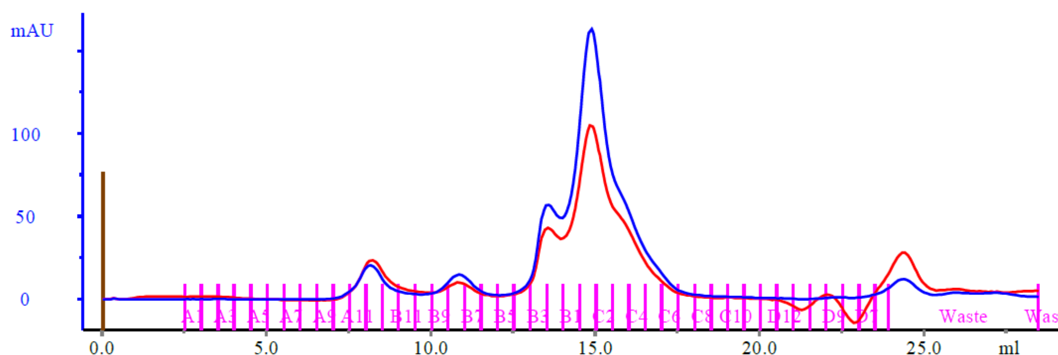

Figure 11: Superdex200 Chromatogram after Ni-NTA affinity column. Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm.

This time, the “tetramer-like” peak eluting at 10.9 mL had not the enough amount of protein to be significant, so the fractions were discarded. Then, the “monomer-like” peak eluting at 14.8 mL was further purified, so an Ion Exchange Chromatography was performed (Figure 12).

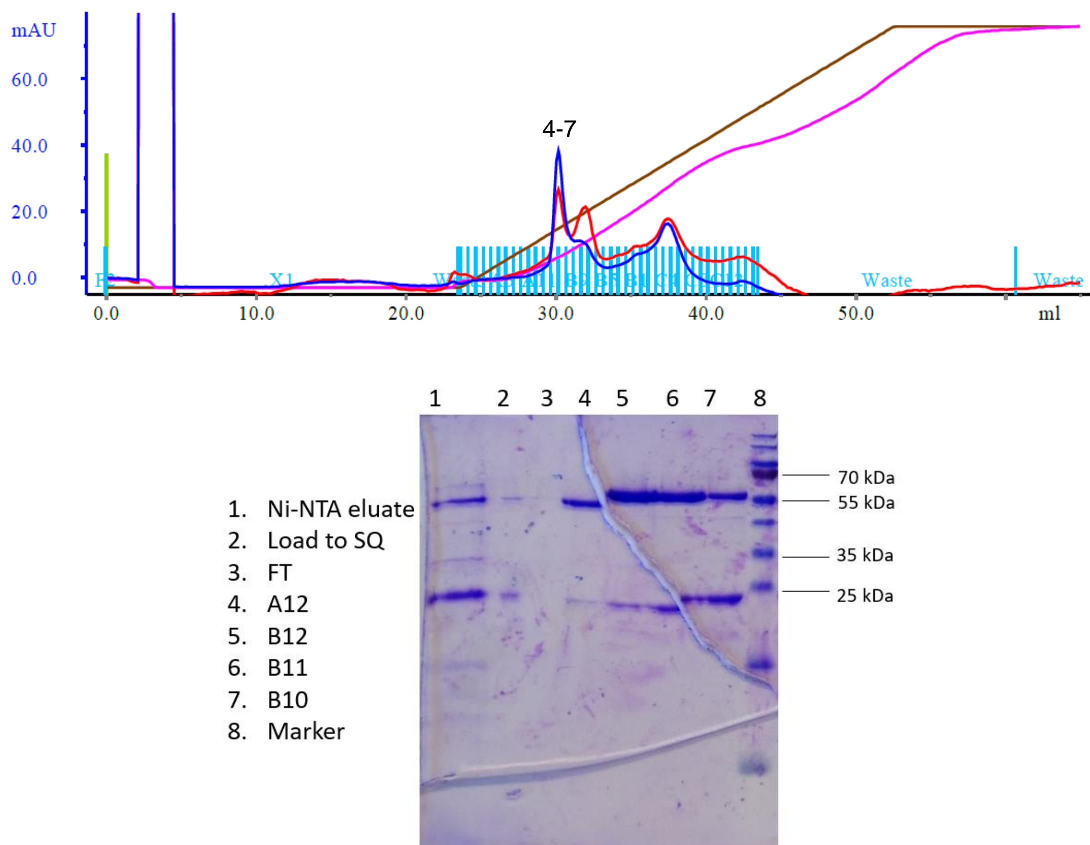

Figure 12: Ion Exchange SourceQ Chromatogram (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm. Pink curve: Conductivity. Brown curve: Concentration of buffer) after Superdex200. SDS-PAGE after SourceQ with the fractions mentioned. Lanes from the SDS-PAGE are marked on the chromatogram

The procedure was discarded, because the amount of protein was not enough to set up crystallization trays and it was not pure enough as a band of 25 kDa was also found in SDS-PAGE.

Finally, the strategy of purifying RelA Δ2 from BL21(DE3) was discarded, as the experiment was repeated 3 times and no promising results were shown. As a solution, different cell lines were tried, in order to get better expression levels.

RelA Δ2 from Lemo21 improves expression rates and crystallization results.

After not ending with any decent strategy to purify RelA Δ2 in *E. coli* BL21(DE3), it was decided to try different *E. coli* cell lines. XL10 Gold and Lemo21(DE3) were tried, and

purified with Ni-NTA column to see the expression levels. Although XL10 Gold cells did not get any level of expression of RelA Δ2, Lemo21(DE3) showed great expression.

The cells were opened by the same procedure, cell lysis and sonication. After filtering, a Ni-NTA affinity column was performed (Figure 13).

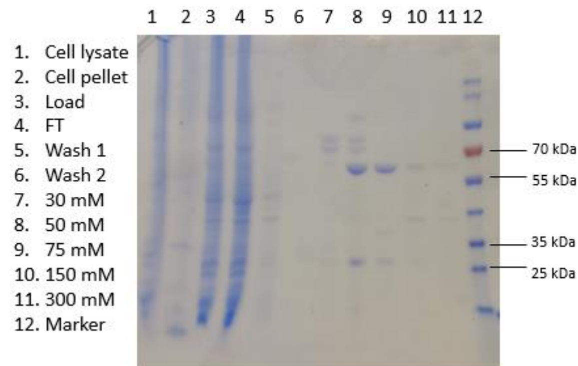

Figure 13: SDS-PAGE Gel After Ni-NTA affinity column. FT: Flow through. 30-300 mM are the different imidazole eluates from Ni-NTA affinity column.

After that, the fractions which showed the protein of interest were up concentrated to 500 µL and then loaded into a Gel Filtration Superdex200, and after that, a SDS-PAGE was performed to further protein analysis (Figure 14).

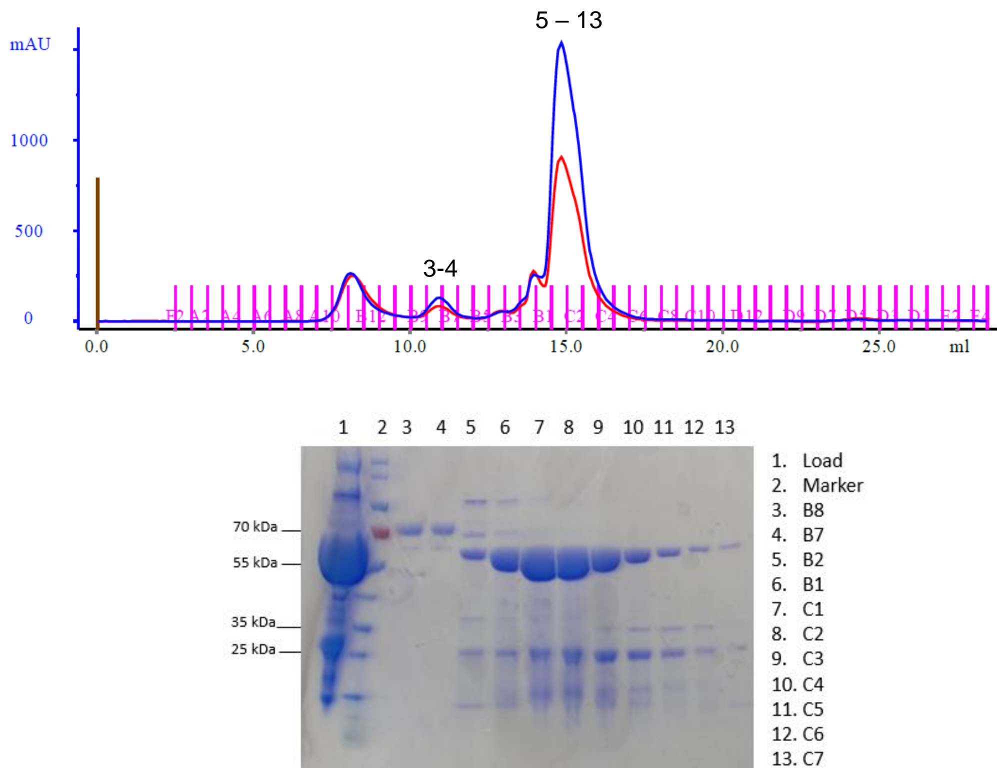

Figure 14: Gel Filtration Superdex200 Chromatogram (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm) after Ni-NTA column. SDS-PAGE after Superdex200 with the fractions mentioned. Lanes from the SDS-PAGE are marked in the chromatogram.

After the Gel Filtration, it was decided to do a Ion Exchange SourceQ to get a higher level of purification (Figure 15).

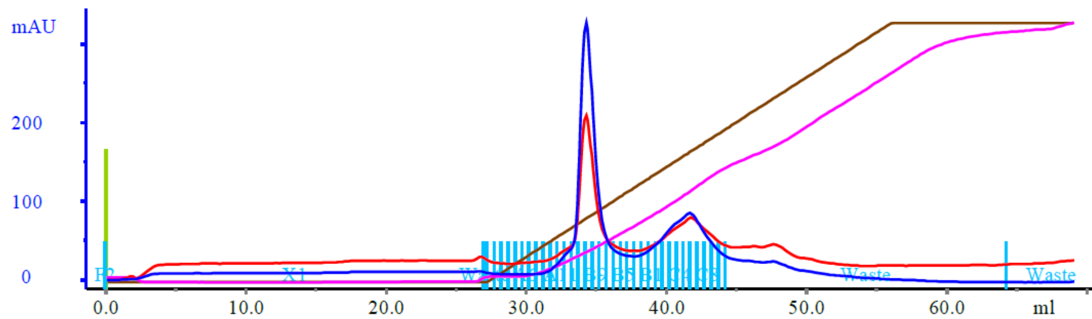

Figure 15: Ion Exchange SourceQ Chromatogram (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm,. Pink curve: Conductivity. Brown curve: Concentration of buffer B) after Superdex200.

After the Ion Exchange, a Final Gel Filtration Superdex200 was performed. An SDS-PAGE was performed to protein analysis with the fractions of the peak (Figure 16).

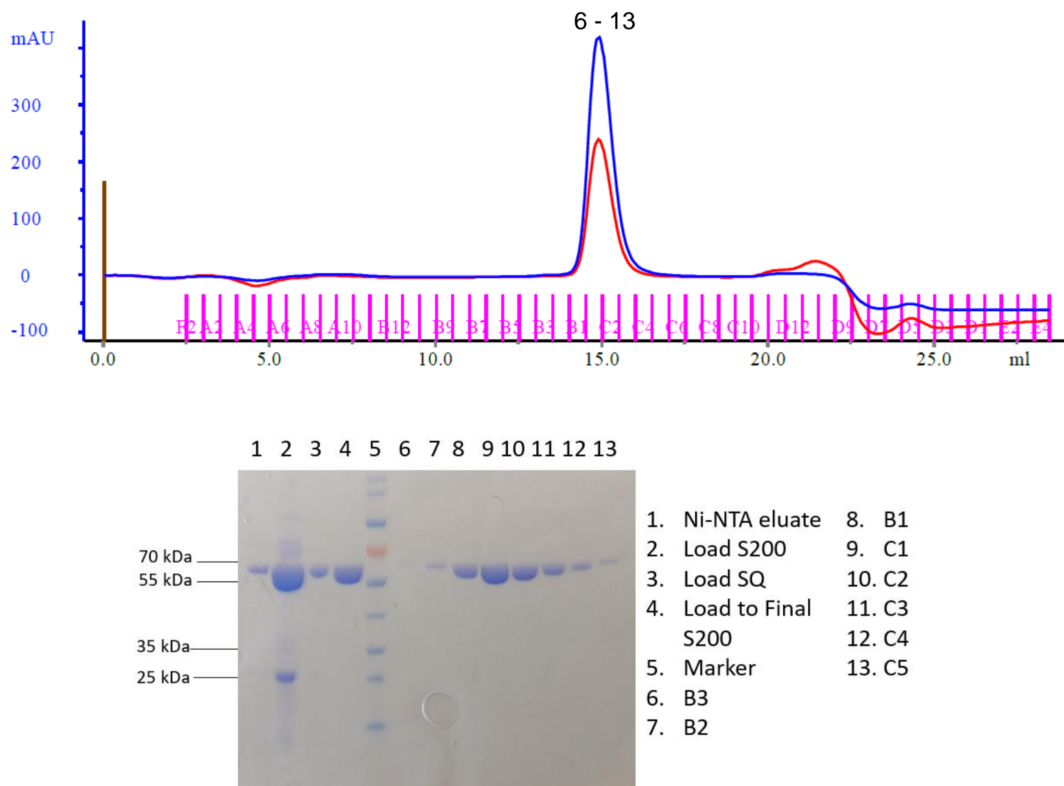

Figure 16: Final Gel Filtration Superdex200 Chromatogram (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm) after Ni-NTA column. SDS-PAGE after Superdex200 with the fractions mentioned. Lanes from the SDS-PAGE are marked on the chromatogram.

As it can be seen in Figure 16, the protein was properly purified at the end, and we got enough amounts of recombinant protein without any other band. Hence, the 4 steps of

purification allowed to obtain high amount of pure protein, which is a required step previously to protein crystallization. Initially, crystallization trays were set up with PegIon and JCSG crystallization screens. While most of the wells were precipitated, clear drops or phase separation, 2 wells of the PegIon screen showed promising results (Figure 17).

Protein	**Concentration (mg/ mL)**
RelA Δ2 (Lemo21(DE3))	10.33

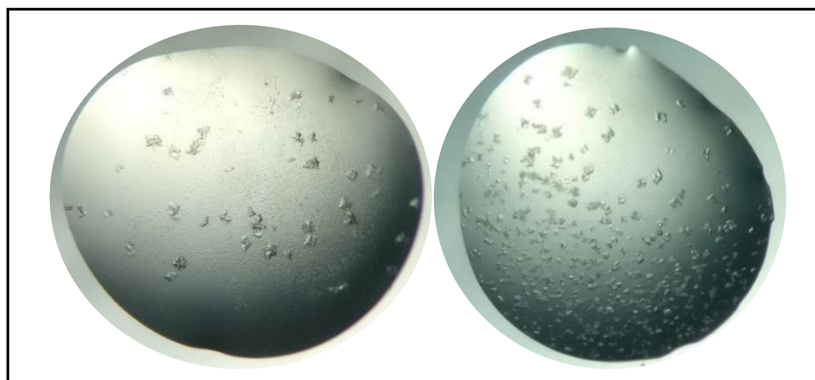

Figure 17: Pictures of the crystallization drops in a 96-well plate with RelA Δ2 from E.coli Lemo21(DE3). Left: PegIon E6: 0.2 M Sodium Malonate pH=6, 20 % (w/v) PEG3350 19ºC Right: PegIon F4: 8% v/v Tacsimate pH = 7 , 20 % (w/v) PEG3350 19 ºC

Then, as the results obtained were quite promising, it was tried to optimize the crystals obtained in the *PegIon* E6 well, by designing an optimizing screen (Figure 18).

PEGIon E6: 0.2M Sodium Malonate pH=6 , 20% (w/v) PEG3350
RelA Δ2 Lemo cells

	1	2	3	4	5	6	7	8	9	10	11	12
A	Original Buffer	Original Buffer	Original Buffer	Original Buffer	Original Buffer	Original Buffer	Home-made	Home-made	Home-made	Home-made	Home-made	Home-made
B	0.2M 2%	0.2M 4%	0.2M 6%	0.2M 8%	0.2M 10%	0.2M 12%	0.2M 14%	0.2M 14%	0.2M 16%	0.2M 16%	0.2M 18%	0.2M 18%
C	0.2M 20%	0.2M 20%	0.2M 22%	0.2M 22%	0.2M 24%	0.2M 26%	0.2M 28%	0.2M 30%	0.2M 32%	0.2M 34%	0.2M 38%	0.2M 40%
D	0.02M 20%	0.02M 20%	0.05M 20%	0.05M 20%	0.1M 20%	0.1M 20%	0.15M 20%	0.15M 20%	0.2M 20%	0.2M 20%	0.25M 20%	0.25M 20%
E	0.3M 20%	0.3M 20%	0.35M 20%	0.35M 20%	0.4M 20%	0.4M 20%	0.45M 20%	0.45M 20%	0.5M 20%	0.5M 20%	0.6M 20%	0.6M 20%
F	0.05M 10%	0.05M 10%	0.1M 15%	0.1M 15%	0.15M 20%	0.15M 20%	0.2M 22%	0.2M 22%	0.25M 25%	0.25M 25%	0.3M 30%	0.3M 30%
G	0.3M 5%	0.3M 5%	0.25M 8%	0.25M 8%	0.2M 10%	0.2M 10%	0.15M 15%	0.15M 15%	0.1M 20%	0.1M 20%	0.05M 30%	0.05M 30%
H	0.05M 25%	0.05M 25%	0.1M 20%	0.1M 20%	0.15M 18%	0.15M 18%	0.2M 15%	0.2M 15%	0.25M 10%	0.25M 10%	0.3M 8%	0.3M 8%

Figure 18: Grid design of the 96-well plate for optimizing the conditions for PegIon E6. Original buffer stands for PegIon E6 comercial buffer, and Homemade stands for the same buffer conditions as PegIon E6, but prepared in the lab.

The optimizing screen did not improve the quality of the crystals neither its size. It also was carried out a bigger well experiment, in order to increase the size of the crystals (Figure 19).

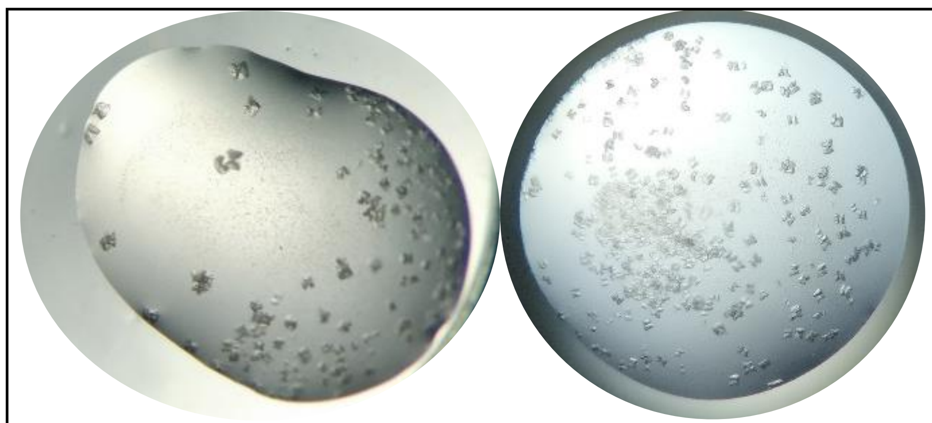

Figure 19: Pictures of the crystallization drops in a 24-well plate with RelA Δ2 from E.coli Lemo21(DE3). Left: PegIon E6: 0.2 M Sodium Malonate pH=6, 20 % (w/v) PEG3350 19ºC Right: PegIon F4: 8% v/v Tacsimate pH = 7 , 20 % (w/v) PEG3350 19 ºC

As the crystals did not improve either in size or quality, the project was discarded and no further attempts were made with RelA Δ2.

RelA Δ5 failed crystallization attempts.

The second construction of the project was RelA Δ5. This mutation consists of the 3 first N-terminal domains (Hydrolase, Synthetase and TGS) of the RelA and a little part of the linker. This linker is a very flexible part, and the structure is still unrevealed.

The strategy to purify RelA Δ5 was exactly the same as the RelA Δ2 construction, but only with the *E. coli* BL21(DE3) cell line. The plan was to open the cells as described in the methods section, and then use a Ni-NTA affinity column, Size-exclusion chromatography, Ion exchange chromatography and finish with another Size-exclusion.

In the first Ni-NTA affinity column, the RelA Δ5 eluted mainly in the 30, 50 and 75 mM of imidazole elution fractions. Then, these fractions were concentrated to 500 µL and were loaded into a Superdex200 Gel Filtration column (Figure 20).

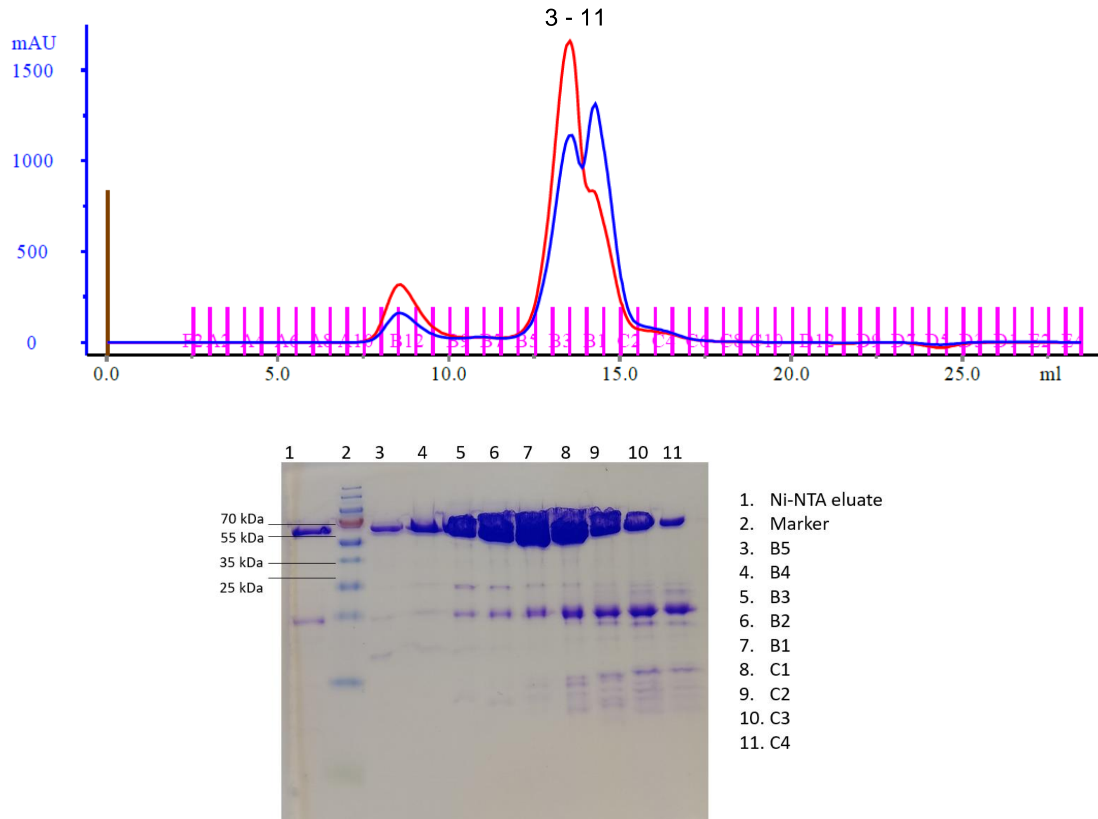

Figure 20: Gel Filtration Superdex200 Chromatogram (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm) after Ni-NTA column. SDS-PAGE after Superdex200 with the fractions mentioned. Lanes from the SDS-PAGE are marked on the chromatogram.

The sample seemed to be contaminated by nucleic acids (260 mAU higher than 280 mAU), but it was decided to continue with a further purification. An Ion Exchange SourceQ followed by a final Gel Filtration Superdex200 were performed to obtain a single peak (Figure 21).

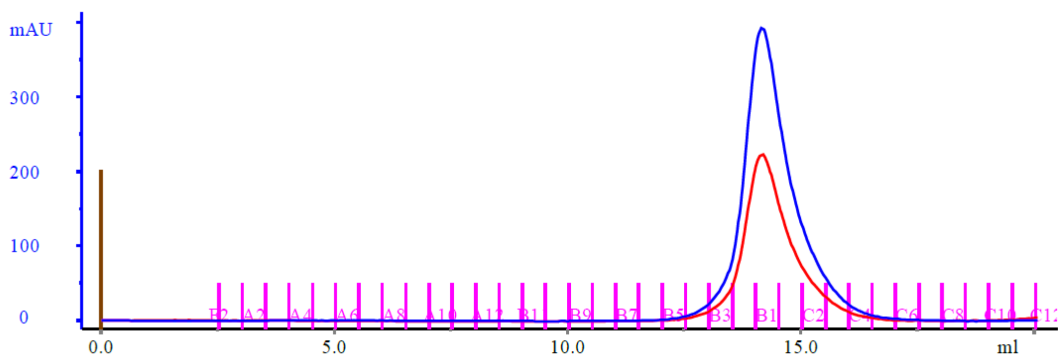

Figure 21: Final Gel Filtration Superdex200 Chromatogram (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm) after Ni-NTA column.

After that, the fractions containing the peak were concentrated to 100 µL and the concentration was measured:

Protein	Concentration (mg/ mL)
RelA Δ5 (*E.coli* BL21(DE3))	5.64

The crystallization trays were set with the JCSG and PegIon screen at 19ºC and at 4ºC. Unfortunately, no hits were shown, and the project was discarded.

A promising complex: RelA-G-His and Nanobody

The last two weeks of the project were focused on try to crystallize the whole construction of RelA. (RelA – Glycine – Hisx6 Tag) The strategy attempted was to crystallize the whole construction of RelA using nanobodies as auxiliary tools.

The strategy was to purify the two proteins separately and then incubate them to let the complex formation. NanoBody4 (NB4) was purified by a Ni-NTA affinity column, followed by an Ion Exchange SourceS and Ion Exchange MonoS (Figure 22) . A final Gel Filtration Superdex200 increase was performed in order to verify the state of our protein (not aggregations, Figure 22). Finally, a SDS-PAGE gel was ran with the final Gel Filtration (Figure 23).

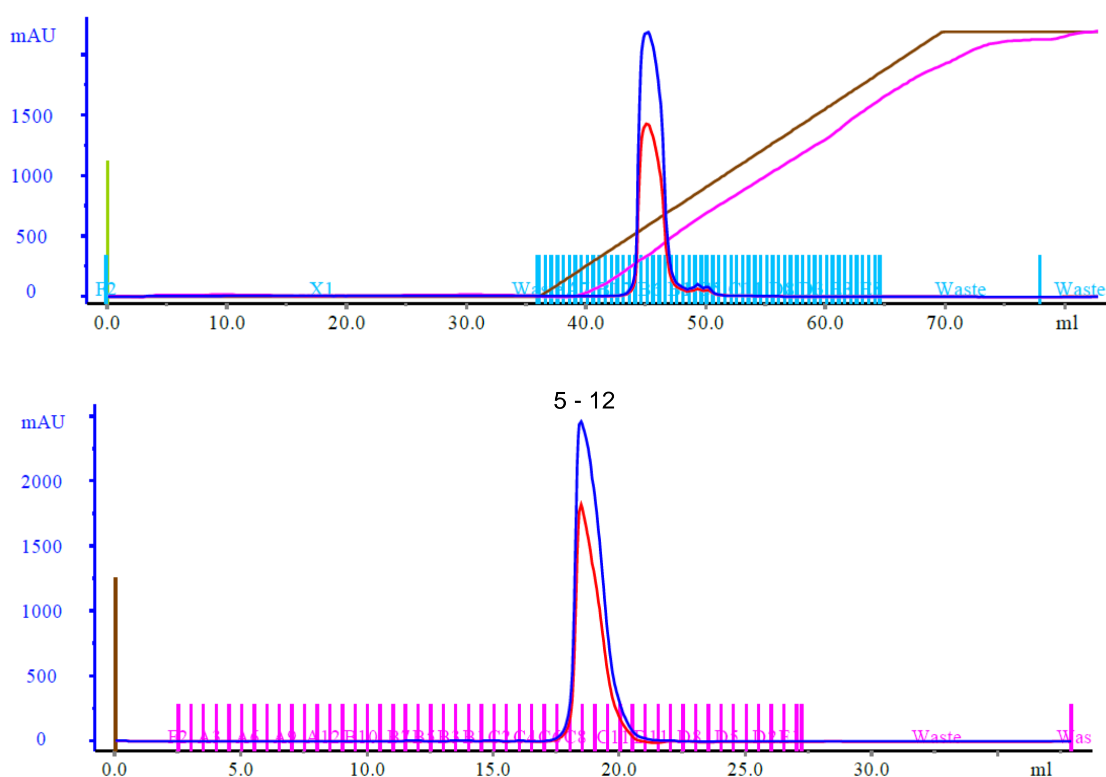

Figure 22: NB4 Purification procedure. Top: Ion Exchange MonoS Chromatogram. (Pink curve: Conductivity. Brown curve: Concentration of buffer B). Down: Final Gel Filtration Superdex200 increase. (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm). Lanes from the SDS-PAGE are marked on the chromatogram.

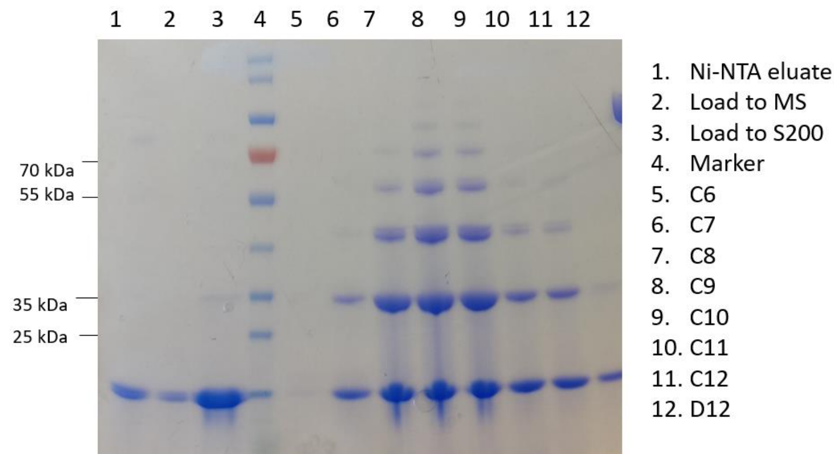

Figure 23: SDS-PAGE Gel showing the Nanobody purification steps. Some oligomeric states appear after the Final Gel Filtration Superdex200 increase (Lanes 5-12).

RelA-G-His was purified by Ni-NTA column followed by a Gel Filtration (Figure 24).

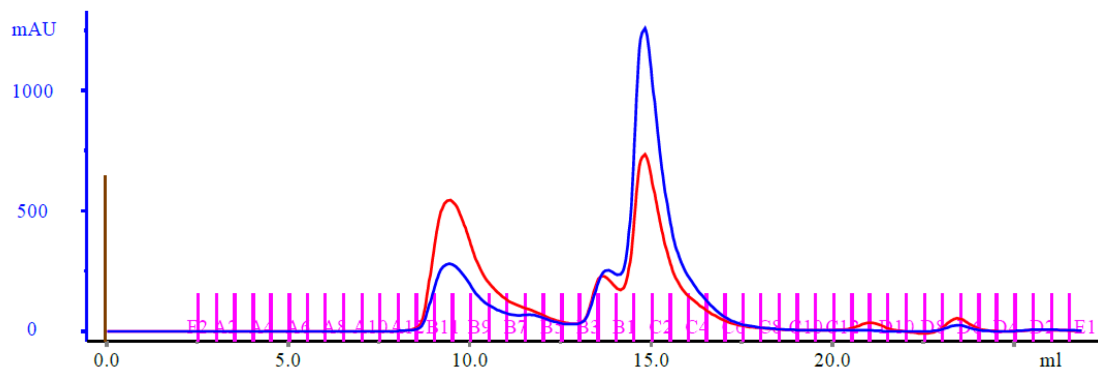

Figure 24: RelA-G-His Purification procedure. Gel Filtration Superdex200 increase. (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm) after Ni-NTA column.

After the Superdex200, the fractions selected were loaded into an Ion Exchange SourceQ column, and a Final Gel Filtration (Figure 25). Then, the fractions containing the peak were kept at 4ºC.

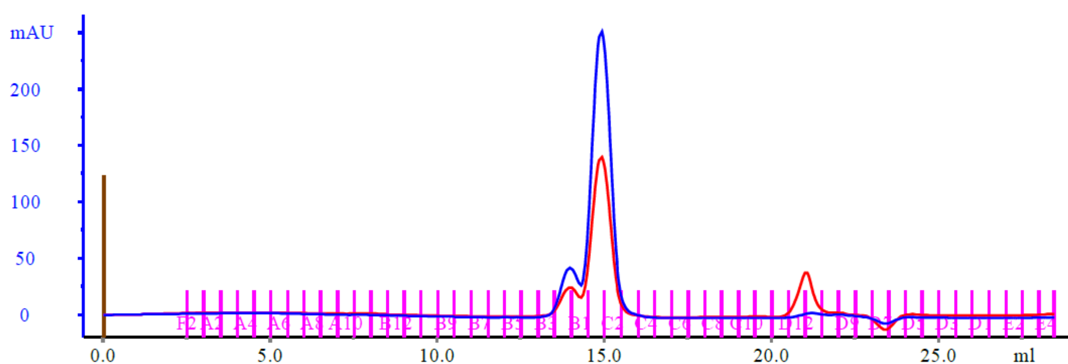

Figure 25: Final Gel Filtration Superdex200 increase with RelA-G-His. (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm) after Ion Exchange SourceQ.

As it can be seen, 2 pure peaks were obtained from the 2 finals Superdex200 increase Gel Filtration (NB4 and RelA; Figure 22 down and 25, respectively). After that, the concentration was measured into the nanodrop, with the procedure mentioned in the methods section.

Protein	Concentration (mg/ mL)
RelA – G – His	3.29
Nanobody (NB4)	70.07

RelA – G – His and the Nanobody were incubated in a 2:1 proportion. The nanobody was diluted to 7 mg/mL and then the reaction was performed into a 100 µL total volume.

Protein	Stock concentration (mg/mL)	Volume (µL)	Final concentration (µM)
RelA – G – His	3.29	45.5	20
Nanobody (NB4)	7	7.5	40
ddH_2O/ Buffer		47	
Total		100	

The mix was left to incubation for 2 hours at room temperature. After the two hours were done, the mix was diluted to 500 µL with Gel Filtration Buffer and a Superdex200 increase was performed (Figure 26).

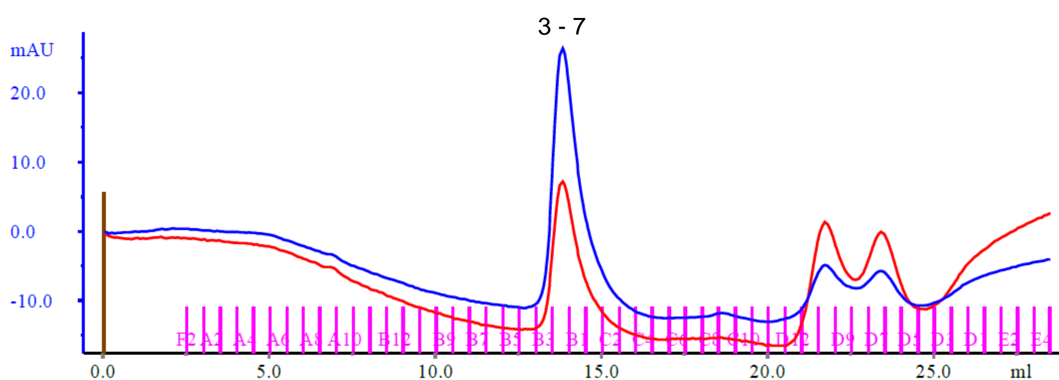

Figure 26: Final Gel Filtration Superdex200 increase of the RelA and NB4 complex. (Blue curve: Absorbance at 280 nm. Red curve: Absorbance at 260 nm)

As it can be seen, the peak is a little shifted, it elutes at 13.8 mL, which could mean that the complex is formed. After that, a SDS-PAGE was performed to verify it (Figure 27).

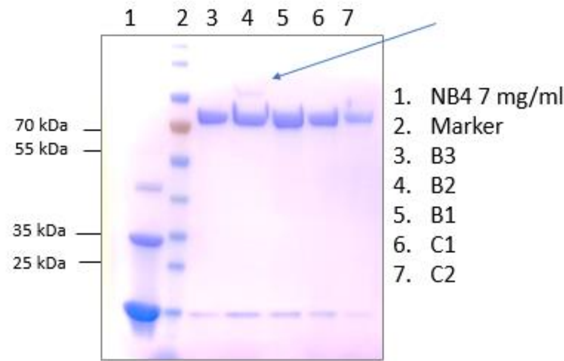

Figure 27: SDS-PAGE Gel showing the RelA+NB4 complex. Due to the little amount of protein the SDS-PAGE procedure was altered, and 100 µL of the sample were taken. They were boiled with the lid open until 20 µL of final volume. Bands around 10kDa are NB4 protein (13 kDa), and around 70 kDa is the RelA protein (75 kDa). A little faint band of the complex is shown in lane 4 (blue arrow). Although SDS-Loading Buffer is able to remove interactions between two proteins, the complex it might be so stable that appears in the SDS-PAGE results. Lanes from the SDS-PAGE are marked on the chromatogram on Figure 26.

An attempt to crystallize the protein was not made due to the lack of a high concentration of the complex.

Then, in order to compare the elution volumes of the Nanobody, the RelA – G – His and the RelA – G – His+Nanobody complex, the chromatograms of the final Gel Filtration Superdex200 Increase (Figure 22 and 25) were compared (Figure 28).

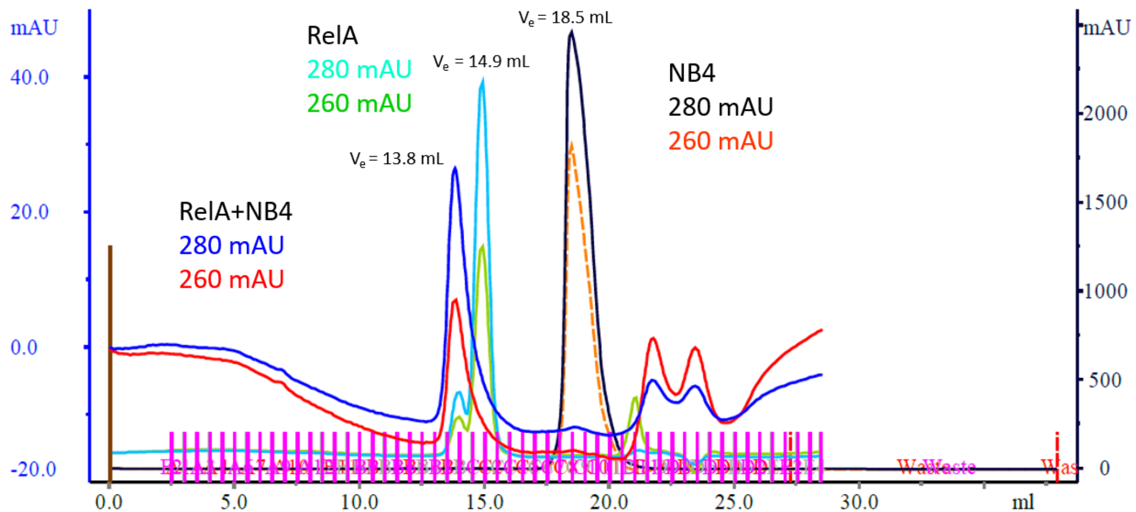

Figure 28: Comparative of the 3 elution volumes (V_e) of the diferent proteins and complex in a Gel Filtration Superdex200 increase. Peaks (more than 20 mAU) from left to right: RelA+NB4 complex, RelA and NB4.

Discussion

RelA Δ2: The protein RelA Δ2 was mutated with the thought that without the linker parts it would confer more stability to the protein, and would be easier to crystalize.

Although several conditions were tried, none of them resulted in high quality crystals. Changing the cell line yielded a higher protein expression, but not an improvement in the quality of the crystals. From the two hits obtained with the initial screens, only one was optimized. Another optimizing screen (PegIon F4: 8% v/v Tacsimate pH = 7, 20 % (w/v) PEG3350 19 ºC) could also had been tried. More initials screens should have been tried in order to identify significant hits.

Moreover, further studies with RelA Δ2 could have been done. Also attempts to crystalize RelA Δ2 with specific nanobodies could help in order to stabilize the protein.

RelA Δ5: With this protein, several attempts were done in order to obtain some significant results, but in each of the 4 initial crystallization trays set up, no results were obtained (Crystallization trays were set with JCSG and PegIon initial screens, at 19 ºC and at 4 ºC). This could be caused by the extreme flexibility of the linker part, which prevents RelA Δ5 to crystallize. Also, an attempt to purify and crystallize the protein with nanobodies as chaperones could have been done.

In this project, only two mutants of RelA have been put into work, and a wider range of mutants could have helped in order to obtain the desired crystals

RelA-G-His: RelA-G-His was the whole construction of the protein. Although in some steps of the purification protocol a high amount of protein was lost, an optimization of the process could help to increase the quantity of protein. The Nanobody purification protocol is defined, as a high amount of protein is obtained and concentrated.

Some oligomeric states of the Nanobody appear after the final Gel Filtration (Figure 23). This could happen because the nanobody is extremely stable, and at high concentrations some oligomeric states could appear, and because SDS-Loading Buffer was kept at room temperature, and it is not as efficient as it should be.

Furthermore, crystallization trays should have been set up with initial screens, but the lack of time prevent it from doing it. Also, another strategy would have been the following: First perform a small scale purification in order to see the elution volume of the RelA – NB4 complex, and then after knowing that start a combined purification. This could have been done by mixing the cell lysates of RelA-G-His and NB4, and then start a co-purification protocol.

Conclusions

The conclusions of the Bachelor Project are the following:

- A strategy to purify RelA Δ2 was found.
  Several *Escherichia coli* cell lines were tried, and finally, Lemo21(DE3) was found to be the one with maximum expression rate.
- Different forms to crystallize RelA mutants were tried.
  For RelA Δ2, crystallization trays were set with two initial screens, and an optimizing screen was performed, but without promising results.
  For RelA Δ5, no hits were shown during the experiments.
- Finally, a strategy was provided to further attempts to crystallize RelA protein, by the use of nanobodies as chaperones. The nanobodies and the complex with RelA was found to be very stable, and further experiments with a higher amount of proteins could be done.

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