# PURIFICATION OF THE CASEINOLYTIC CLP PROTEASE COMPLEX

Bachelor thesis – Final Degree Project

Faculty of Mathematics and Natural Sciences

Eberhard Karls University of Tübingen

**Hosting group:** AG Brötz-Oesterhelt of the Interfaculty Institute of Microbiology and Infection Medicine (IMIT)

**Director**: Prof. Dr. Heike Brötz-Oesterhelt

**Home institution**: University of Zaragoza

Submitted by

**Laura Pedrós Manzanares**

Tübingen, July 2022

# DECLARATION OF AUTHENTICITY

I, Laura Pedrós Manzanares, hereby declare

- that I wrote this work myself and did not use any other sources and ais than those specified.

- that all statements taken literally or analogously from other works are marked as such,

- that the work submitted has not been the subject of another examination procedure, neither in full or in essential parts, or has already been published.

Tübingen, 14.07.2022

Laura Pedrós Manzanares

# INDEX

## SUMMARY

The caseinolytic protease P (ClpP) is an essential protein for bacteria and bacterial derived organelles. It belongs to the AAA+ family of proteases and has proteolytic activity, thereby, it plays an important role in protein digestion. This protease is involved in essential processes such as, cellular regulatory mechanisms, protein homeostasis, responses to environmental stimuli and host infections.

The ClpP complex is formed by two principal components. In one hand, two heptameric rings of ClpP forms the proteolytic core, with catalytic activity. The second major component is the Clp-ATPases, which forms an hexameric ring in both edges of the complex. The function of this ATPase is selecting the substrate for degradation. There are different Clp-ATPases depending on the organism, and each of them recognise a different substrate. For a further specificity, the Clp-ATPase interacts with adaptor proteins.

In humans, the only Clp-ATPase interacting with ClpP is ClpX. The human Clp system plays its role inside the mitochondrial matrix, therefore, a transport of both ClpP and ClpX to the mitochondrial matrix is required for the function of the protease. For this transport to happen, both proteins are first translated as premature proteins, with a Mitochondrial Target Sequence (MTS). This sequence will be then recognised by the cell machinery to transport the proteins inside the mitochondrial matrix. Afterwards, the MTS sequence will be cleaved when the proteins pass into the matrix.

In this study, the proteins of the human Clp system, as well as the Clp protease of *S. aureus*, were purified through the Äkta Column, using the Strep-tag purification technique. Prior to the purification, *E. coli* cells were transformed with a pET plasmid containing the gen of interest, and then the expression of the protein was induced with IPTG. Later, the purification through the column was performed.

Finally, after the purification, the protein concentration was measured, and the proteins were stored at -80°C for further in vitro assays. Afterwards, the purification results were also confirmed by doing an SDS-PAGE Electrophoresis.

As a conclusion, this study fathom in the understanding of Clp protease system expression in the cells, in order to use this knowledge in future studies with this complex.

# ABBREVIATONS

| | |
|---|---|
| °C | Degree Celsius |
| β | Beta |
| ADEP | Acyldepsipeptide |
| Amp | Ampicillin |
| ATP | Adenosine triphosphate |
| *B. subtilis* | *Bacillus subtilis* |
| ClpP | Caseinolytic protease P |
| *E. coli* | *Escherichia coli* |
| HClpP | Human Caseinolytic protease P |
| IPTG | Isopropyl β-D-1-thiogalactopyranosid |
| kDa | Kilodalton |
| MPP | Mitochondrial Processing Peptidase |
| MSF | Migration Stimulation Factor |
| MTS | Mitochondrial Target Sequence |
| NOA1 | Nitric oxide-associated protein 1 |
| OD | Optical Density |
| OXPHOS | Oxidative phosphorylation |
| RNA | Ribonucleic acid |
| ROS | Reactive Oxygen Species |
| rpm | Revolutions per minute |
| SaClpP | *Staphylococcus aureus* Caseinolytic protease P |
| *S. aureus* | *Staphylococcus aureus* |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| *sec* | Staphylococcal Enterotoxin C |
| Ser | Serine |
| Sle1 | Systemic lupus erythematosus susceptibility 1 |
| TAP | Tandem Affinity Purification |
| Tim | Translocase of the Inner Mitochondrial Membrane |
| Tom | Translocase of the Outer Mitochondrial Membrane |
| *tst* | Staphylococcal toxic shock toxin |
| UV | UltraViolet |
| xg | Relative Centrifugal Force |
| ZBD | Zinc-binding domain |

# 1. INTRODUCTION

The caseinolytic protease P (ClpP) system is a highly conserved serine protease present in bacteria and mitochondria. It has been demonstrated to be responsible for maintaining protein homeostasis and protein quality control, as well as virulence regulation in some bacteria (Illigmann et al., 2021).

This enzyme belongs to the family of ATP-dependent proteases. The functional ClpP protease is formed by a tetradecameric proteolytic core with catalytic activity and a hexameric Clp-ATPase bound to each end of the complex. The latter is involved in substrate specificity (Illigmann et al., 2021).

In recent studies, it has been shown that ClpP machinery is important for some types of cancer cells. Therefore, this protease could be a potential target for cancer treatment. The ClpP protease is also involved in other human diseases (Ishizawa et al., 2019).

On the other hand, protein purification is a fundamental step to study individual proteins, but also protein complexes, like Clp system. Through this process it is possible to obtain a high concentration of protein for further studies of any kind (Ferrer-Miralles et al., 2015).

Nowadays, there are several techniques to purify a protein. In this project, the technique used was Strep-tag purification, and it is based in the interaction between the sequence Strep-tag II, which is fused to the protein of interest, and the affinity column.

## 1.1. GENERAL INTRODUCTION TO CLP PROTEASE SYSTEM

The Clp protease system is a multi-component machinery that plays an essential role in protein homeostasis and protein quality control, as well as in targeted proteolysis of transcriptional regulators. It is present in prokaryotic cells, and it is also conserved in prokaryote-derived organelles of eukaryotic cells (Illigmann et al., 2021).

ClpP forms an heptameric structure, while the Clp-ATPase forms an hexameric ring. A functional Clp protease complex is formed by two components (Figure 1). The first one is a tetradecameric proteolytic core ClpP (two heptameric ClpP rings), which consists of a serine protease with peptidolytic activity. The core ClpP needs a second component, a

hexameric energy-dependent Clp-ATPase for the protein hydrolysis. There are different ATPases e.g., ClpX, ClpA, ClpE, ClpC (Illigmann et al., 2021). These ATPases are responsible for the substrate specificity and this control is enhanced by the interaction of the ATPases with adaptor proteins (Kirstein et al., 2009).

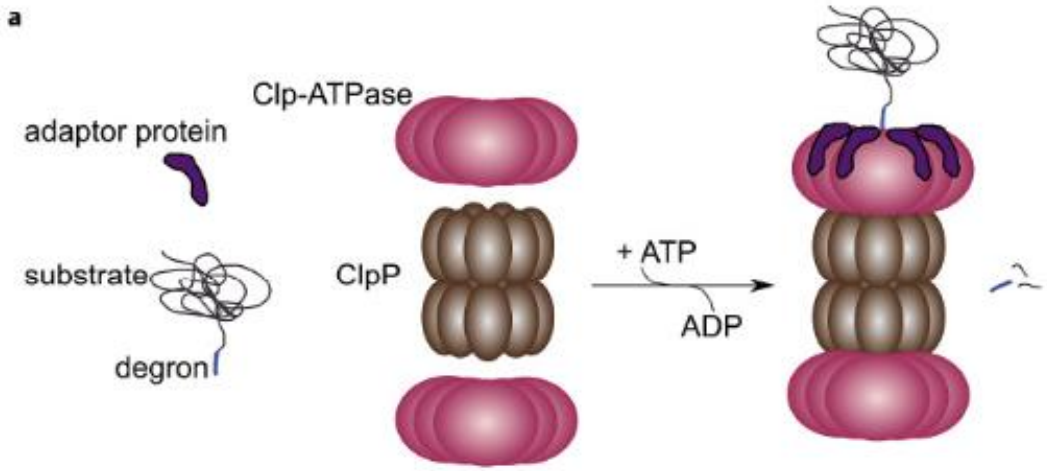


Figure 1 Operation mode and structure of the Clp protease complex. Clp-ATPase-mediated substrate degradation by the proteolytic core ClpP is fuelled by ATP hydrolysis. Further substrate specificity is obtained by interaction with adaptor proteins (Illigmann et al., 2021).

The ClpP is a dynamic molecular machine. In several crystal structures, for example from *E. coli* (Kimber et al., 2010), it has been revealed in three different barrel conformations: compressed, compact and extended. The compact state is a stable intermediate and the other two are the endpoints of the dynamic transition (Wong et al., 2018). The conformation affects to catalysis. Only in the extended conformation, the active sites residues (S, H and D) are in the proper distance and spatial orientation to form hydrogen bonds, which are needed for peptide hydrolysis. The flexibility of the complex is also important for the product release (Geiger et al., 2011).

The Clp-ATPases are bound to hydrophobic pockets in each apical face of ClpP. The interaction with Clp-ATPases stabilizes the active extended state of ClpP.

## 1.2. CLP PROTEASE IN BACTERIA

Bacteria depend on the Clp system (Illigmann et al., 2021). The ability of adaptation to different environments and changes implies a correct regulation of the bacterial proteome. To do that, is important the control and activation of protein expression, but

also a targeted proteolysis of regulatory factors. There is a diversity in the Clp system is between the species (Illigmann et al., 2021).

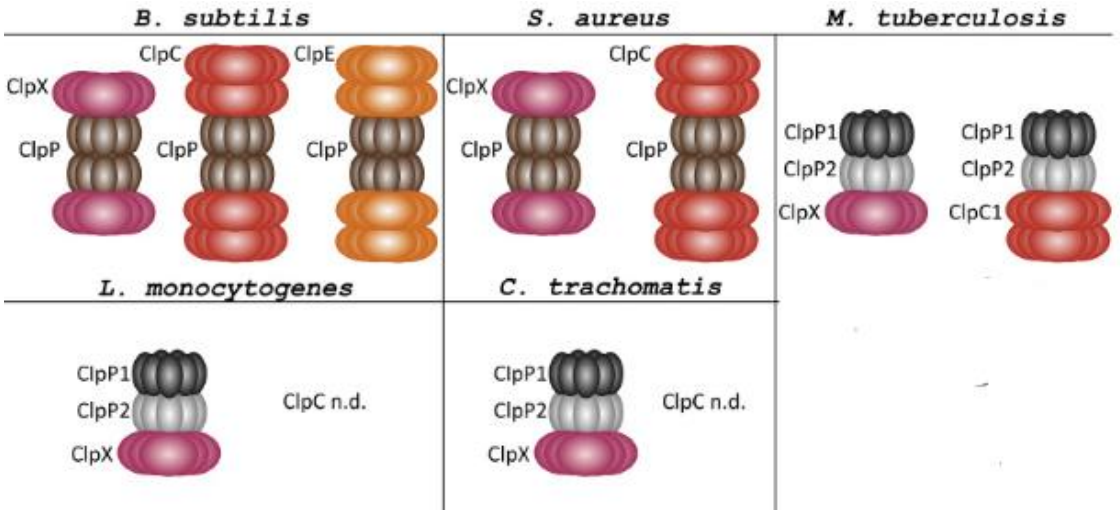


Figure 2. Operation mode and structure of the Clp protease complex in different bacterial species. In *B. subtilis* and *S. aureus*, the Clp-ATPases ClpX, ClpC, and in *B. subtilis* also ClpE can bind to both sides of the tetradecameric barrel. Contrariwise, in *L. monocytogenes*, *M. tuberculosis*, and *C. trachomatis*, comprising a heteromeric ClpP1P2 core, ClpP2 acts as the exclusive interaction partner for the Clp-ATPases ClpX and ClpC1 in *M. tuberculosis*, ClpX in *L. monocytogenes*, and ClpX in *C. trachomatis*. The composition of ClpCP in *L. monocytogenes* and *C. trachomatis* has not been determined (n.d.) so far. (Illigmann et al., 2021).

### *1.2.1. B. subtilis*

In *B. subtilis*, the Clp system has 4 main functions: adaptation to external stress, migration to a favourable environment, allow natural competence, and positively regulates sporulation. The Clp-ATPases that can bind *B. subtilis* ClpP proteolytic core are ClpC, ClpX and ClpE (Figure 2) (Illigmann et al., 2021).

As it has been mentioned before, Clp machinery is essential for protein quality control, specially under stress conditions. ClpP and ClpC also participate in protein turnover under non-stressed conditions. Clp proteases become essential during the stationary phases or when cells are grown in minimal media conditions. Responses like degradative enzyme synthesis, or motility are regulated by ClpP (Illigmann et al., 2021).

In summary, Clp machinery in *B. subtilis* is essential for the regulation of a variety of process, therefore, is important for *B. subtilis* surveillance.

### *1.2.2. S. aureus*

*S. aureus* also requires the presence of the Clp system for general stress response but also for regulation of virulence factors secretion (Illigmann et al., 2021). It has been shown that lack of ClpX ATPase facilitates growing at hight temperatures, and the reason could be that the increase binding between ClpC and ClpP leads to improve protein quality control (Frees et al., 2003). However, ClpX is important for growing at low temperatures. The absence of ClpX decrease the growth at low temperatures. ClpX also plays a major role in cell division, due its involvement in septum formation (Jensen, Bæk, et al., 2019; Jensen, Fosberg, et al., 2019).

Growing at decreased temperatures is not only supported by ClpX, but also by ClpXP protease. This protease is important for regulating the stability of cell wall hydrolyses, which allows a control of the cell wall metabolism (Feng et al., 2013).

As it has been said before, Clp system is also essential for regulation of virulence factors in *S. aureus*. It was shown that mutants lacking *clpP* and *clpX* were unable to cause local skin abscess in mice. During infection, there is an upregulated expression of genes as *clpP* or *clpX*, for *S. aureus* to adapt to the host conditions. Another method for adaptation is iron scavenging from host haemoglobin, which involves ClpXP (Frees et al., 2003).

ClpP and ClpX are essential for the pathogenicity of *S. aureus* due its involvement in the production of a wide variety of virulence factors, as α-haemolysin (Frees et al., 2003; Lowy, 1998). In addition, a functional ClpXP protease is required for the induction of *sec*, *sed* a *tst*, which are important staphylococcal superantigens, responsible for severe diseases (Etter et al., 2020).

Treatment of diseases caused by strains like methicillin-resistant *S. aureus* (MRSA), is really difficult due to the high β-lactam resistance. ClpXP also controls this resistance by degradation of Sle1, and cells lacking ClpXP protease activity are β-lactam hyper-resistant (T et al., 2014).

Comparing both proteases' subtypes (Figure 2), ClpXP is more involved in controlling the *S. aureus* pathogenicity, while ClpCP is more important for protein quality control (Illigmann et al., 2021).

Clp protease also affects biofilm formation by *S. aureus*. Deletion of *clpP* increases the formation, while *clpX* and *clpC* deletion decrease it. In addition, ClpCP was shown to degrade antitoxins in *S. aureus* (P et al., 2010).

Taken all mentioned above together, Clp system is fundamental for *S. aureus*, because it promotes colonisation and infection in the host. As it was mentioned, both proteases have different roles: ClpCP is involved in protein quality control, and ClpXP is more important for pathogenicity. There are two additional Clp-ATPases in *S. aureus*, ClpB and ClpI, but they lack the motif that interacts with ClpP (Frees et al., 2004; P et al., 2010). Therefore, they act as independent chaperones.

## 1.3. Clp IN EUKARYOTIC CELLS: MITOCHONDRIAL Clp

ClpP is not only essential for bacteria, but also crucial for eukaryotic cells, due its importance in mitochondria and chloroplasts. The mitochondrial Clp protease is located in the mitochondrial matrix and is involved in the protein quality control and homeostasis, and in the regulation of oxidative phosphorylation (OXPHOS), mitochondrial ribosome biogenesis, various stress responses and signalling cascades (Valera-Alberni & Canto, 2018; Voos, 2013). On the other hand, in chloroplasts is essential for plastid biogenesis and plant survival, and in *Plasmodium falciparum* for survival to the malaria parasite (el Bakkouri et al., 2010).

Mitochondria have several functions that are essential for eukaryotic cell survival. To work properly, a protein homeostasis is required, which is maintained by Clp protease among other proteins.

The loss of HClpP has been related to infertility and sensorineural hearing loss, while overexpression of HClpP has been linked to several carcinomas (Larkin & Byrd, 2015).

HClpP only interacts with HClpX, which is the unique Clp-ATPase present in mammals (Gispert et al., 2013). The genes of human Clp complex are encoded in different chromosomes. *ClpP* gene is encoded in chromosome 19, while *clpX* is encoded in chromosome 15 (Illigmann et al., 2021).

### 1.3.1. Transport of HClpP to the mitochondrial matrix

The transport of human ClpP and human ClpX to the mitochondrial matrix is mediated by the Mitochondrial Target Sequence (MTS) mechanism (Kang et al., 2002).

Firstly, the proteins are translated as precursor proteins with the MTS in the N-terminal. This target sequence is recognised by a cytoplasmatic chaperon called Mitochondria Import Stimulation Factor (MSF). Preprotein unfolding is induced by this factor using ATP, and, in the unfolded state, MSF binds to it. Afterwards, the MSF-preprotein complex binds to a subcomplex of the outer membrane translocation machinery, formed by Tom proteins (Translocase of the Outer Mitochondrial Membrane), specifically. Then, the protein is translocated through the protein translocation channel to the inner membrane (Omura, 1998).

For the protein translocation through the inner membrane, an electrochemical membrane potential is required. The protein translocation machinery in the inner membrane is composed by Tim (Translocase of the Inner Mitochondrial Membrane) proteins. The translocation of the protein to mitochondrial matrix is made in an ATP-dependent manner (Omura, 1998).

Finally, the targeting sequence is removed by the Mitochondrial Processing Peptidase (MPP), when the protein goes to the organelle matrix (Kang et al., 2002).

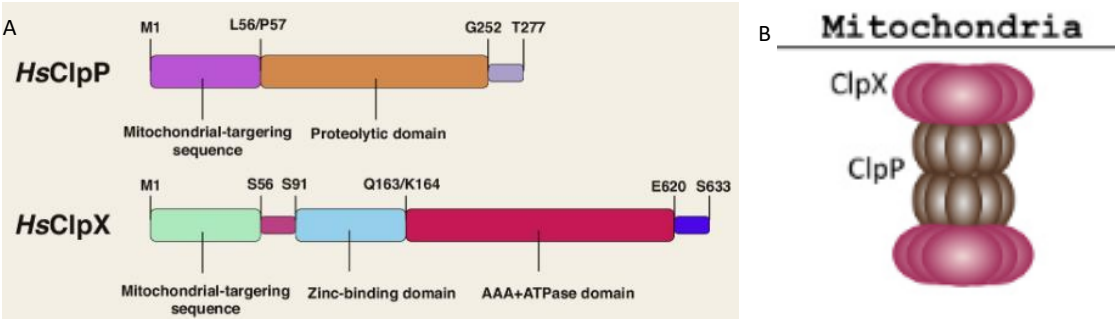


Figure 3 Domain architecture of human caseinolytic protease and structure of hClpP active complex in mitochondria. A. Domain structure of human caseinolytic protease (HsClpP) and human Clp-ATPase (HsClpX) (Luo et al., 2021). B. Structure of the active complex of human Clp system (Illigmann et al., 2021).

### 1.3.2. Clp system structure in humans

In humans there are two forms of HClpP. The premature form contains 277 aminoacids and includes the MTS domain (composed by 56 aminoacids) and a proteolytic domain (Figure 3A). It is in the cytoplasm. The other form is the mature one, which has the MTS removed, therefore, it has about 221 aminoacids (Luo et al., 2021).

On the other hand, HClpX contains 633 residues, including the MTS, a zinc-binding domain (ZBD) and the AAA + ATPase domain (Figure 3A). MTS is responsible of the transport of the protein to the mitochondrial matrix. The ZBD domain recognizes the substrate protein, and the highly conserved ATPase domain binds and hydrolyses ATP (Luo et al., 2021).

HClpP forms an heptameric ring, whereas HClpX forms an hexameric ring. The functional oligomeric state of the complex consists of a double heptameric ring that is capped at each end by an hexameric ring (Figure 3B). The interaction between the rings is stabilized in the presence of ATP. This association depends on the docking of the Clp ATPase through IGF loops. It has been demonstrated that the C-terminus of HClpP is not necessary for the interaction with HClpX or for the enzymatic activity (Illigmann et al., 2021).

The process of degrading proteins is tightly regulated. First, the substrate is recognized and bound HClpX, which is involved in its unfolding in an ATP-dependent way. Then, the unfolded substrate is translocated to the lumen of HClpP, where is degraded into small peptides expelled through the side pores of HClpP (Geiger et al., 2011).

The sensitivity of substrates is species specific. All of the substrates are selected by the ATPase and regulated by adaptors, such as RssB. Furthermore, antiadaptors can regulate the function of adaptors on the substrates and ATPase (Bhandari et al., 2018).

The expression of human mitochondrial ClpP depends on the cell type; a strong expression was shown in skeletal muscle, heart, liver, pancreas and reproductive organs, whereas lung, placenta and kidney reported a lower expression (Illigmann et al., 2021).

### 1.3.3. Heterologous human Clp complexes

Human ClpP is remarkably similar to its bacterial equivalents (Kang et al., 2002). Thanks to these similarities, it is possible to have an interaction between mitochondrial ClpP and *E. coli* ClpX, creating an active complex able to recognise *E. coli*-specific substrates and bound their degradation tags, like the ssrA-tag, in contrast to mitochondrial ClpXP (Illigmann et al., 2021). In that way, substrate recognition appears not to be dependent on a homologous ClpX-ClpP interaction, but it only depends on the Clp-ATPase (Kang et al., 2002). Surprisingly, the other ATPase present in *E. coli*, EClpA, do not bind to HClpP. The inverse heterologous complex, EClpP and HClpX, was also tested, but it could not be formed (Kang et al., 2002).

Mutants of HClpP has been studied in presence of HClpX and EClpX, but they were inactive. It was demonstrated that the HClpP mutant was able to interact with EClpX but did not have proteolytic activity (Kang et al., 2002).

NOA1 is the only substrate verified *in vitro* for the moment. It is an essential mitochondrial GTPase that plays a role in controlling mitochondrial respiration and ribosome biogenesis (Heidler et al., 2011; Kolanczyk et al., 2011). There are also deduced substrates, as SDHB, required for a functional respiratory complex II (Seo et al., 2016).

### 1.3.4. Role of human Clp protease complex in eukaryotes

As it was mentioned before, HClpP complex plays several functions. When a protein stress is sensed, thanks to ClpP, among other proteins, chaperones and proteases encoded in the nucleus are upregulated and imported in the mitochondria to re-establish the homeostasis. ClpXP also plays an important role in mammalian cells since it reduces protein stress in the mitochondrial matrix by degrading defective proteins (Al-Furoukh et al., 2015). The protease also alleviates the accumulation of ROS by inhibiting the function of some subunits of complex I, where the major amount of ROS is produced (Pryde et al., 2016). In addition, ClpXP is involved in activating the autophagy machinery to produce the organelle's death. Furthermore, Clp protease is implicated in mitochondrial fusion and fission dynamics (Illigmann et al., 2021).

HClpX can also play different roles independent of HClpP. For example, it can act as a molecular chaperone, or it can also maintain mitochondrial genome by stabilizing mitochondrial transcription factor A (Al-Furoukh et al., 2015).

## 1.4. ROLE OF Clp PROTEASE HUMAN DISEASES

Given the role of ClpP in different diseases, the protease is considered a potential target for drug development (Bhandari et al., 2018).

### 1.4.1. Role of HClpP in human diseases

Dysfunction of human ClpP can cause non-infectious human diseases. They can be categorised in two groups (Bhandari et al., 2018). The first group includes the genetic disorders caused by mutations that disrupt normal ClpP expression or its function, leading to mitochondrial dysfunction that impacts specific tissues and organs. This is the case of Perrault syndrome, where *clpP* genes are mutated. As a result, there is an unstable ClpP protein that leads to mitochondrial dysfunction (Bhandari et al., 2018; Gispert et al., 2013). Another example is the neurodegenerative disease Friedrich ataxia, whose progression increases due to an ClpP upregulation (Guillon et al., 2009). The second group are oncogenic diseases in which ClpP is vital in sustaining the growth and metastasis of human cancer cells (Bhandari et al., 2018).

#### 1.4.1.1. HClpP in Perrault Syndrome

Perrault syndrome is a rare genetic disorder that is characterized by bilateral, sensorineural hearing loss in both genders in patients. In addition, female patients also suffer from ovarian dysfunction that results in sterility or severe difficulties in conception (Ahmed et al., 2015). Patients might also suffer from neurological defects, such as intellectual disability, as well as motor and sensory peripheral neuropathy. Surprisingly, these phenotypes resemble the phenotypes obtain in a study using ClpP knock-out mice (Gispert et al., 2013).

Perrault syndrome is associated to mutations in five genes, and one of them is *clpP* gene. The primary effect of Perrault mutation in this gene is weaken its structural integrity, leading to an unstable ClpP with a shorter half-life. This leads to mitochondrial dysfunction and the manifestation of the disease (Ahmed et al., 2015). Given that ClpP is highly expressed in ovaries, these organs seem to be affected in this disease.

#### 1.4.1.2. HClpP role in Cancer

Mitochondrial Clp protease is a really interesting protein that is going to be deeply studied in future research due its importance for cancer treatment. It has been shown that HClpP is upregulated in cancer cells, so that by targeting and modulating this protein we might come up with an effective treatment (Zong et al., 2016).

Cancer cells need more energy than normal cells, and the majority of this energy is produced in the mitochondrial OXPHOS. Mitochondria has several roles in tumour cell progression, as protein homeostasis (DeBerardinis & Chandel, 2016; Zong et al., 2016). It is important to remark that cancer cells suffer continuous stress situations due to multiple factors: elevated ROS levels, constant need of nutrients and ATP, or acidosis. One of the ways to solve this stress includes proteases like ClpXP (DeBerardinis & Chandel, 2016).

Some studies have revealed that certain tumours require Clp-XP-mediated protein homeostasis to maintain mitochondrial function and tumour cell survival under stress, and that is needed for the proliferation and metastasis. For example, proliferation of the prostate cancer cells PC3 has been shown to decrease when HClpP is not expressed (Seo et al., 2016). Also, the ability of these cells to invade other tissues is compromised if HClpP is depleted.

Depletion of HClpP has been demonstrated to reduce the rate of proliferation and viability of several lines of leukemic cells, due to the stress situation originated by a decreased OXPHOS and an increased ROS production (Cole et al., 2015). There are heat shock proteins and molecular chaperones overexpressed in cancer cells. These proteins might serve as potential anticancer drug targets, as well as HClpP (Larkin & Byrd, 2015).

## 1.5. CHEMICAL MODULATORS OF hClpP ACTIVITY

### 1.5.1. Inhibitors of HClpP

There are two types of drugs depending on the effect they have in HClpP activity. On one hand, there are the inhibitors of HClpP which are the most direct therapeutic strategy against cancer cells. These compounds covalently modify the 14 catalytic Ser residues (Stahl et al., 2018). This group include β-lactones, like A2-32-01, which is toxic for primary leukemic cells with a high HClpP expression but does not have effects against normal primary hematopoietic cells, which means that it has specific targeting of cancer cells (Hackl et al., 2015). Another group of inhibitors are the phenyl esters, as AV-167, which bases its effect in a nucleophilic attack from the catalytic Ser of ClpP's (Hackl et al., 2015).

### 1.5.2. Activators of HClpP

On the other hand, there are activators of HClpP. In the HClpPX complex, HClpP has the proteolytic function and HClpX has the ATPase activity. Moreover, substrate degradation by HClpP is dependent on the unfoldase activity of HClpX (Stahl et al., 2018). For this reason, it is possible to interference the activity by disabling the gatekeeping function of HClpX. Indeed, some HClpP activators, like ADEPs, have been shown to dysregulate the function of bacterial ClpXP, while keeping ClpP in its activated state (Kirstein, Hoffmann, et al., 2009). This dysregulated activation leads to potent bactericidal effects. Recently, have been identified some analogues of ADEP that can specifically target HClpP, and they have shown anticancer properties (Wong et al., 2018).

## 1.6. PROTEIN PURIFICATION

Protein purification is an essential step for studying proteins and the interactions between them. There are several mechanisms to purify a protein, but all of them share some common steps. First of all, the protein of interest is expressed in the model strain. Secondly, cells are lysed, and then, the real process of purification starts. The protein of interest is bound to a matrix, and afterwards there is a washing to remove the rest of

proteins that did not bind to the column. Finally, the protein of interest is eluted and collected (Ferrer-Miralles et al., 2015).

Proteins are really sensitive molecules to the external conditions, and they easily misfold. Therefore, the conditions applied while working with the protein can play the difference during the purification protocol. One of the most important factors is the strain used. *E. coli* is the most common strain for this kind of protocol, and it is also the strain used in this project (Ferrer-Miralles et al., 2015).

As I mentioned before, there are multiple ways of purifying a protein. The technique used in this project was Strep-Tag purification, which is a Tandem Affinity Purification (TAP) method. It is based in the fusion of a TAP tag, which is a peptide sequence, with the protein of interest. This sequence is then responsible of the protein isolation (Puig et al., 2001).

Prior to the Strep-Tag purification, it is necessary to express the protein in the model strain. One of the most common used strategies for protein expression, and also the one used in this project, is based in the lac promoter of *E. coli*, using as inducer of the expression the IPTG (Marbach & Bettenbrock, 2012).

In this technique, the gen of interest is cloned in a pET plasmid, which has the T7 promoter fused to the gene of interest.

### 1.6.1. Protein expression with pET plasmid adding IPTG

pET plasmid is a common plasmid used to express a protein, and it is based in the functionality of the lac operon. The protein expression is induced by IPTG, which is an analogue of the lactose, so it activates the promoter of the *lac* operon, leading to the expression of the genes in that operon, among which is the one that codes for the protein of interest (Marbach & Bettenbrock, 2012).

For a successful expression of the protein of interest, the vector must have some essential components (Figure 4). First of all, is must carry an origin of replication (*ori*), which is needed for the plasmid to replicate inside *E. coli*. It must also have an antibiotic resistance gene; in this case, AmpR is the resistance gene to Ampicillin, thus, we can

select the cells that have acquired the plasmid by using a medium with Ampicillin. In addition, it must contain the gen for the lac repressor (lacI) to avoid the expression of the protein in the absence of IPTG. The Lac operator site is also essential for the binding of the repressor (Lewis & Bell, 2000). Another key component is the T7 promoter, which is recognised by the T7 RNA polymerase to initiate the expression of the gene of interest when IPTG is added (Dubendorf & Studier, 1991; William Studier et al., 1990). This promoter is fused with the lac operon site, therefore, when the lac repressor is bound to the promoter, the polymerase cannot bind, and the gen of interest cannot be transcribed. The plasmid also contains the gene of interest (in this project is *hclpP* and *hclpX*) fused with the Strep-tag II sequence, which is the responsible of the protein binding to the column while purification.

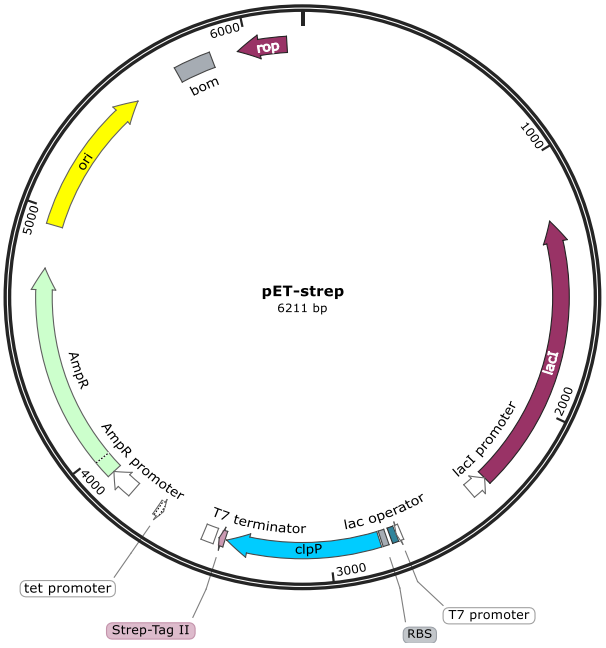


Figure 4. Structure of the pET-11a-Streptag plasmid used in the project. The arrows represent the different genes contained in this plasmid (AG Brötz-Oesterhelt (Unpublished), n.d.).

For this technique to work properly, the expression strain must have the lac promoter followed by the T7 RNA polymerase gene, thereby, IPTG will also induce T7 RNA polymerase expression.

Given all this vector components, the protein expression can be regulated, and the process is as showed in Figure 5. In normal conditions, while the expression culture is growing and there is no IPTG, the lac repressor is bound to the lac operator, so the expression of the gene of interest is inhibited, as well as the expression of T7 RNA

polymerase. After the expression has growth until it reaches a particular OD, IPTG is added, so the repressor is removed. This will have two consequences. In one hand, T7 RNA polymerase is expressed in the expression strain. As a result, T7 promoter of the plasmid is activated, and together with lacI removal, the gene of interest is transcribed. Finally, the mRNA binds to the ribosome and the protein of interest is expressed (Dwivedi, 2011).

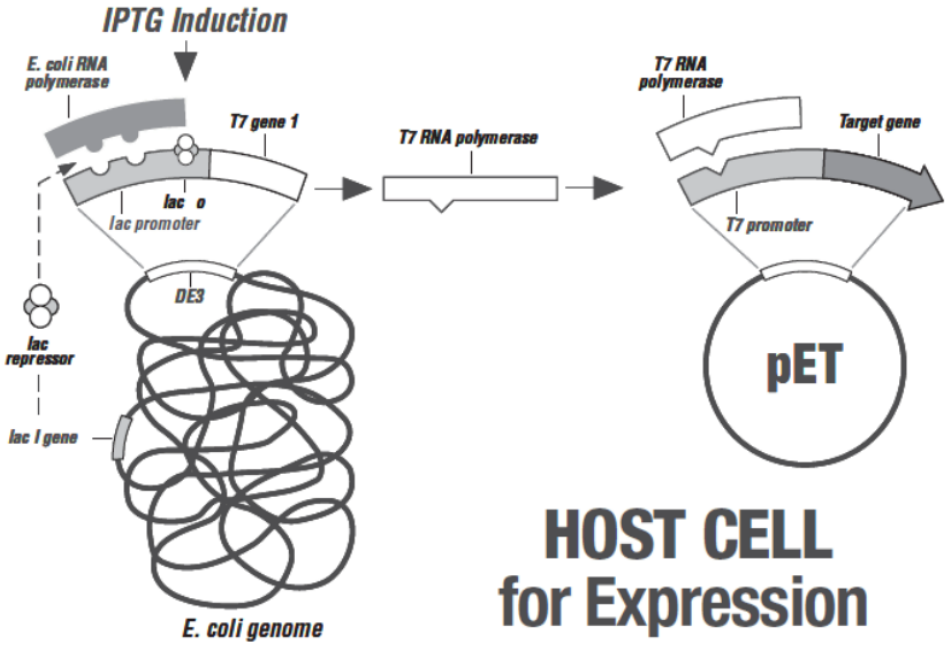


Figure 5. Scheme of the protein expression using pET plasmid and the induction with IPTG. When IPTG is added, the T7 RNA polymerase is expressed in the expression strain. This event, together with the removal of lacI from the lac operator in the T7 promoter of the pET plasmid, leads to the expression of the gene of interest (Dwivedi, 2011).

### 1.6.2. Strep-tag purification technique

The basis of the Strep-tag purification protocol is the binding affinity between biotin and streptavidin. The column has streptavidin, and the protein of interest is a recombinant protein that is fused with a peptide capable of binding in the biotin binding pocket of streptavidin (Strep-tag II sequence) (*Expression and Purification of Proteins Using Strep-Tag® or Twin-Strep-Tag® A Comprehensive Manual*, n.d.). In this particular technique, streptavidin has been changed for Strep-Tactin to improve the interaction.

To start the purification, the column is equilibrated. Secondly, the cell lysate with the protein of interest is loaded in the column (Figure 6). When the solution passes through

the column, only the recombinant protein should bind to Strep-Tactin, while the rest of host proteins are washed with buffer. Once all the proteins have been removed, desthiobiotin is added. This molecule is an analogue of the biotin, so it binds to Strep-Tactin in the same place as Strep-tag II, but with more affinity, so the protein of interest is eluted and collected in tubes (Expression and Purification of Proteins Using Strep-Tag® or Twin-Strep-Tag® A Comprehensive Manual, n.d).

Finally, the column is regenerated to remove the binding with desthiobiotin.

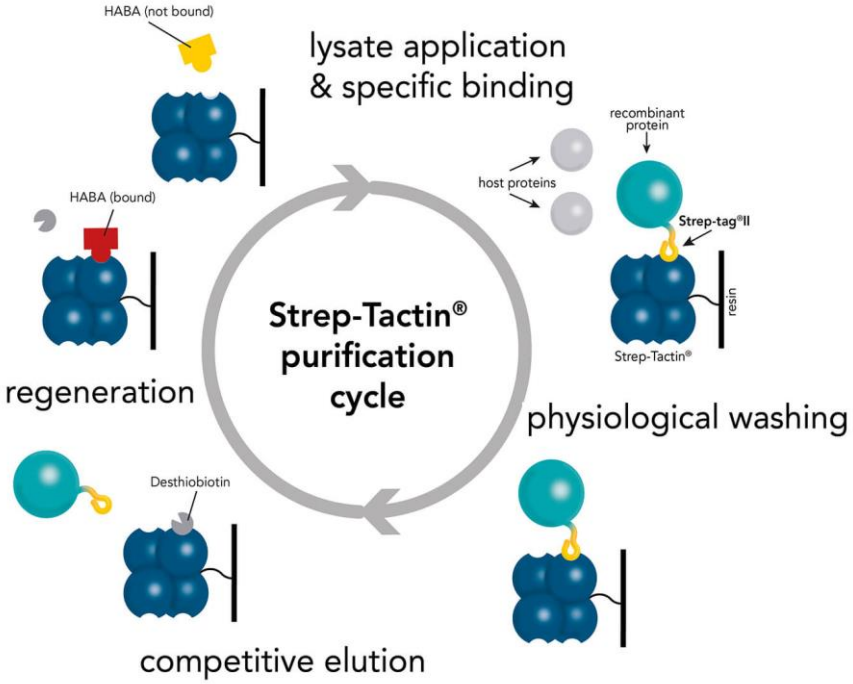


Figure 6. Schematic view of the Strep-Tactin purification cycle. Firstly, the sample is loaded, and the specific binding occurs. Secondly, there is a physiological washing of the host proteins that do not bind to the column. Then, desthiobiotin is added for a competitive elution to collect the protein of interest. Finally, the column is regenerated *(*Expression and Purification of Proteins Using Strep-Tag® or Twin-Strep-Tag® A Comprehensive Manual*, n.d.)*.

#### 1.6.2.1. Strep-tag II sequence

The Strep-tag II sequence is composed by 8 residues, and it is essential for Strep-Tag purification technique, because it is fused with the protein of interest to separate them from the rest of host proteins. This small peptide binds to core streptavidin with high specificity and has a remarkable intrinsic stability (Schmidt & Skerra, 2007).

Given that the binding with streptavidin is reversible, this peptide is a powerful tool for protein purification on affinity column with immobilized streptavidin. The elution is

made by competition with a peptide that also binds to the same streptavidin site, but with higher affinity, such as desthiobiotin (Schmidt & Skerra, 2007).

The Strep-tag II sequence can be easily fused to the gen of interest, and it does not affect the protein function. In addition, it has high resistant to cellular proteases (Schmidt & Skerra, 2007).

## 1.7. PROJECT OBJECTIVES

The aim of this project is to purify the different proteins of the human Clp system for further in vitro assays, as well as comparing these results with the ones obtained in the purification of *S. aureus* ClpP, which is supposed to be an easier purification.

The goal is to understand how the expression of this system occurs in cells by using *E. coli* as model organism for the experiments.

To reach this goal, the project will be structured in different steps (Figure 7). Firstly, the *E. coli* strain is transformed with the pET plasmid that carries the gen of interest, and this pre-culture is grown overnight. The following day, the pre-culture is grown in a bigger flask until it reaches a particular OD, and afterwards, the protein expression is induced with IPTG.

Next day, the purification is performed using the Äkta Column. Finally, the results obtained in the chromatogram are confirmed with an SDS-PAGE Electrophoresis.

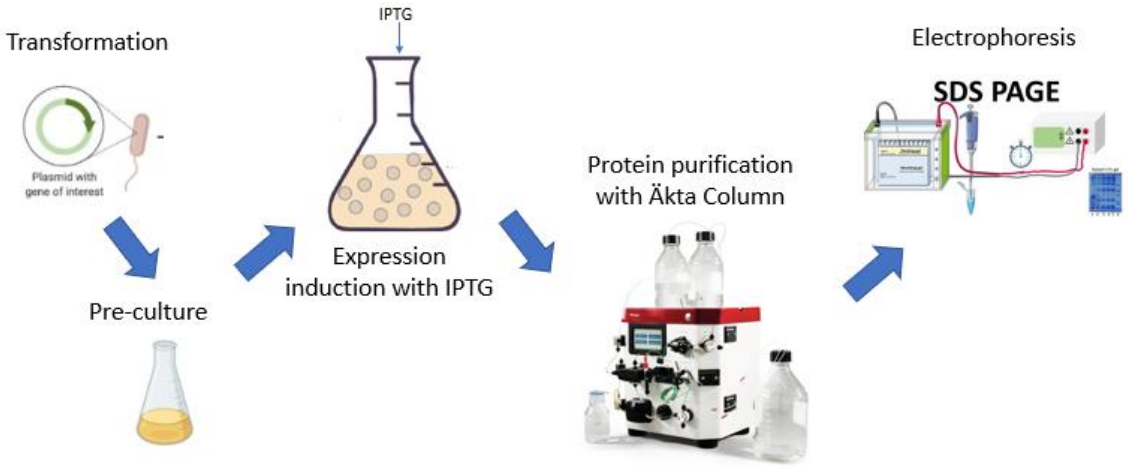


*Figure 7. Scheme of the purification protocol followed in the Project. First is the E. coli transformation. Secondly, the pre-culture is grown. The following day the expression culture is grown until it reaches an OD and afterwards, the expression of the protein is induced with IPTG. Then, the protein is purified through the Äkta Column. Finally, the results are confirmed with an SDS-PAGE Electrophoresis.*

# 2. MATERIALS AND METHODS

## 2.1. MATERIALS

### 2.1.1. Equipment

| Equipment | Type | Company |
|---|---|---|
| Precision balance | Weight | Ohaus Adventurer |
| pH Meter Calibration | Calibrator | Mettler Toledo |
| Magnetic stirrer C-MAG HS7 | - | IKA |
| 300 Volt peqPower | Electrophoresis | PEQLAB |
| Bolt 4-12% Bis-Tris Plus | Electrophoresis gel | Thermo Scientific |
| Heraeus Multifuge X3R | Centrifuge | Thermo Fisher Scientific |
| Ecotron (Incubation Shaker) | Incubator | INFORS HT |
| Biowave CO8000 | Cell density meter | WPA |
| PreCellys Evolution Homogeneizer | Lysis | Bertin Technologies |
| PreCellys Lysing Kit | Lysis | Bertin Technologies |
| Sorvall LYNX 6000 Super Speed Centrifuge | Centrifuge | Thermo Fisher Scientific |
| Äkta Pure | Chromatography | |
| Filtropour S 0,45 | Filtration | SARSTEDT AG & Co. KG |
| Amicon 10000 MWCO filter tube | Filtration | Thermo Fisher Scientific |
| Amicon 30000 MWCO filter tube | Filtration | Thermo Fisher Scientific |
| Syringe Omnifix Luer Solo | Sterile filtration | BRAUN |
| Eppendorf Biospectrometer Basic | Spectrometer | Eppendorf |

### 2.1.2. Chemicals and Buffers

| Chemical | Company |
|---|---|
| Ampicilin | ROTH |
| IPTG, dioxane free | Thermo Scientific |
| PageRuler unstained protein ladder | Thermo Scientific |
| miliQ water | - |

| | |
|---|---|
| Storage Buffer (20 mM Tris-HCl pH7 + 100 mM NaCl + 10% v/v Glycerol) | |
| Strep-tag Washing Buffer (100 mM Tris-Cl pH 8.0 + 150 mM NaCl + 1 mM EDTA) | |
| StrepE Buffer (100 mM Tris-Cl pH 8.0 + 150 mM NaCl + 1mM EDTA + 2,5 mM desthiobiotin) | |
| Strep Regeneration Buffer (100 mM Tris-Cl pH 8.0 + 150 mM NaCl + 1 mM EDTA + 1mM HABA + 0,5 M NaOH) | |
| Desthiobiotin | Iba |
| 10X Bolt Sample Reducing Agent | Novex |
| 4X Bolt LDS Sample Buffer | Novex |
| 'Der blaue Jonas' single step Coomassie blue protein dye | |

### 2.1.3. Bacterial strains, plasmids and medium

| |
|---|
| *E. coli (SG1146 ΔclpP ΔclpX ΔclpA)* (Pan et al., 1996) |

| Plasmid | Provided by |
|---|---|
| pET-11a- Strep Tag – HClpP | AG Brötz-Oesterhelt |
| pET-11a- Strep Tag – HClpP+MTS | AG Brötz-Oesterhelt |
| pET-11a- Strep Tag – HClpX | AG Brötz-Oesterhelt |
| pET-301-Strep Tag - SaClpP | AG Brötz-Oesterhelt |
| Difco LB Broth, Miller (Luria-Bertani) | |

## 2.2. METHODS

### 2.2.1. Expression of *E.coli* with pET plasmid and induction with IPTG

The main goal of this step is to express the protein of interest in an *E. coli* culture. The cells in this culture are knock-out for *ΔeClpP, ΔeClpA* and *ΔeClpX* genes, so they do not interfere with the expression of the protein of interest, which are HClpP and HClpX.

#### 2.2.1.1. Growth of the expression strain

First of all, the *E. coli* cells are inoculated in LB Media with an Ampicillin concentration of 100 µg/ml and are grown in shaking at 37°C and 190 rpm. This will be the preculture.

The following day, OD of the overnight culture is measured. Afterwards, the culture is transferred to fresh LB medium with an Ampicillin concentration of 100 µg/ml and is grown at 37°C and 150 rpm. When the culture reaches an OD between 0.5-0.8, it is induced with IPTG, in order to promote the expression of the protein of interest.

#### 2.2.1.2. Protein expression with pET plasmid adding IPTG

As it was already said, in this project I used pET plasmids for the protein expression. This vector has different components and each one fulfils a function (Figure 4).

When the cells have reached the required OD, IPTG is added to induce the expression of the protein of interest. The induction occurs in shaking conditions at 150 rpm and 20°C, for around 20 hours.

As I mentioned in the introduction, during this process the lac repressor is removed from the lac operator, therefore the promoter is activated and initiates the transcription of the gen of interest.

### 2.2.2. Purification of the protein of interest through Strep-Tag purification.

#### 2.2.2.1. Preparation of the sample to load it in the affinity column.

Once the protein has been expressed, the culture is centrifuged at 4900 rpm during 30' in order to get all the cells in the bottom. The supernatant is discarded, and the cells are stored at -20°C.

The following day, the cells are melted down with StrepW Buffer and afterwards, they are mechanically disrupted with Precellys Homogenizer, using a protocol that consists of three rounds of 30'' at 6400 rpm centrifuge with breaks in between of 20''. This protocol should be repeated three times.

The next step is filling the disrupted cells into ultracentrifuge tubes and ultracentrifuge them at 32.000 xg for 30' at 4°C. This step is necessary in order to get rid of the solid materials from the cells after the lysis and other contaminants. After the centrifugation, the supernatant is poured in new ultracentrifuge tubes and centrifuge again in the same

conditions. Then, the supernatant with the protein of interest is poured in a Falcon tube. Finally, the solution with the protein is filtered through a 0.45 µm pore in a different Falcon tube.

### 2.2.2.2. Strep-Tag purification

Afterwards, the Strep Tag purification is performed.

Firstly, the column is equilibrated using 20% ethanol, miliQ water and StrepW Buffer. Then, the sample is loaded in the column (Figure 6). During this process, the protein of interest should bind to the column thanks to the Strep tag II sequence, while the rest of proteins are washed with buffer during the washing of the column step. Afterwards, the recombinant protein is eluted with Buffer E that contains 2.5 mM desthiobiotin.

Finally, the column is regenerated with water, NaOH and ethanol.

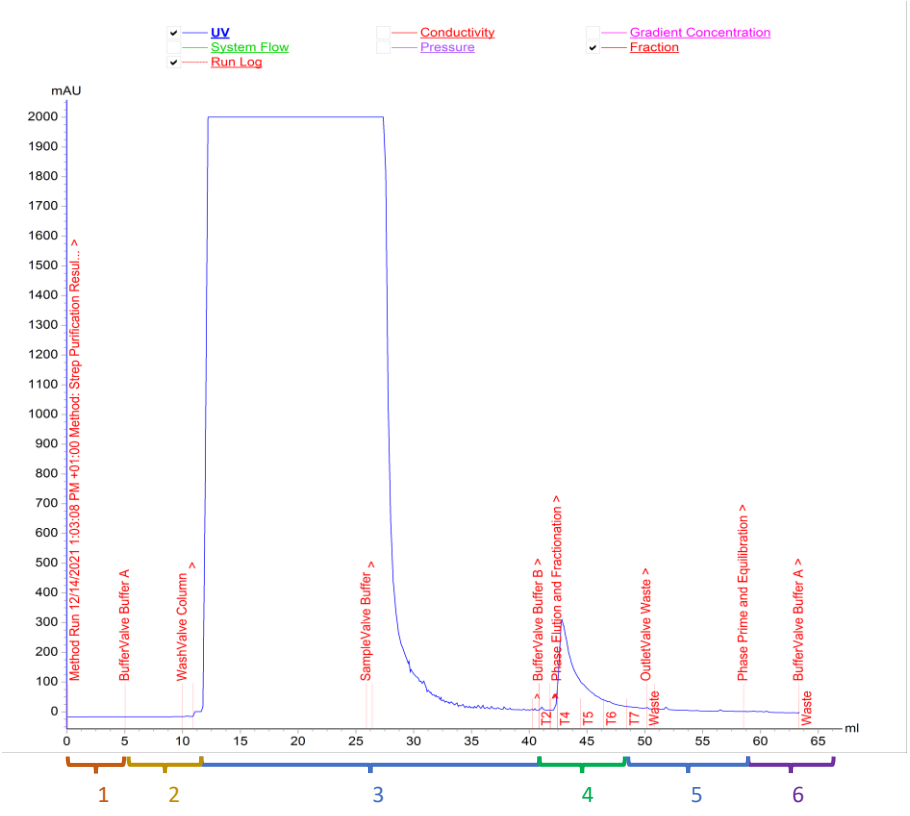


Figure 8. Representation of an example of the Äkta column chromatogram. Each number corresponds with one step of the purification process. Step 1 is the wash of the column. Step 2 is the sample load. Step 3 matches with the washing of all the proteins that did not bind to the column. Step 4 is the elution and fractioning step, when desthiobiotin is added. Step 5 is a new wash of the column. Step 6 is the prime and equilibration step.

After the purification, the results are visualized in the Chromatogram (Figure 8). The peak of UV seen in the graph during the elution step matchs with the major amount of protein eluted, which is expected to be the protein of interest due it is supposed to be eluted when desthiobiotin is added. Therefore, the fractions in the peak are collected.

#### 2.2.2.3. Buffer exchange and protein concentration measurement

Then, a Buffer Exchange is performed by using an Amicon 10000MW and 30000MW filter tube for HClpP and HClpX, respectively. To do that, the solution with the protein is loaded in the tube, and it is centrifuged until the sample is only at 500 µl. The aim of this step is to eliminate the old buffer and add new Storage Buffer, where the protein is more stable. In addition, this step also pretends to concentrate the protein of interest. After the first centrifugation, the tube is filled up with Storage Buffer (new Buffer) and it goes to centrifuge again until the sample is at 200-500 µl.

Afterwards, the protein is collected in Eppendorf's in aliquotes of 25 µl. The aliquotes are measured with Nanodrop in order to know the protein concentration.

Lastly, the aliquotes are shockfreezed with liquid nitrogen and stored at -80°C for further in vitro assays.

### 2.2.3. SDS-PAGE Electrophoresis

After Strep-tag purification process and before collecting the protein of interest to perform the Buffer Exchange, a sample of 20 µl of each fraction obtained after the chromatography is collected in eppendorfs tubes and storage at -20°C to do an SDS-PAGE electrophoresis afterwards and check the quality of the purification.

SDS-PAGE electrophoresis is a method to separate the proteins according to their polypeptide chain length and molecular weight, due to the fact that the proteins are unfolded by SDS (Matsumoto et al., 2019). Smaller proteins will run faster in the gel. In this case, the gel used is 4%-12%, which makes the separation of the proteins better.

The gel has two parts: concentration gel and separator gel. The separation is produced in the separator gel, but before the proteins enter in that gel, they pass through the

concentration gel, which avoids protein dispersion and makes sure that all proteins enter in the separation gel at the same time and speed. Then, the proteins are separated according to their molecular weight (Menor-Salván, 2019).

To do the electrophoresis, the first step is preparing the electrophoresis mix, which is made with Bolt sample reducing agent and a Bolt LDS Sample Buffer in a proportion 2:1. 6 µl of this mix are added to each sample and then, they are heated at 70 °C for about 15'. While the samples are being heated, the gel is prepared, and the electrophoresis buffer is added (MOPS Buffer).

When the Eppendorfs tubes are heated, they go to centrifuge for 1', and then they are ready for the electrophoresis. First of all, the marker is loaded. The marker used in the electrophoresis was a PageRuler Unstained Protein Ladder. It has 14 markers, and the size goes between 10 to 200 kDa. The bands correspond with the different molecular weight: 200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15, 10 kDa

After that, each sample is loaded in a different lane. Finally, the electrophoresis is run at 150V for 1 hour and 15'.

Once the run has finished, the gels are stained while they are lightly agitated with 'Der blaue Jonas' single step Coomassie blue protein dye, which is a special fast type of Coomassie blue. Finally, 'Der blaue Jonas' is substituted by miliQ water, and after 30' approximately, the gels can be seen in the Gel Doc XR+ Gel Documentation System.

# 3. RESULTS

## 3.1. PROTEIN PURIFICATION

Prior to the purification of the proteins of interest, which are the ones forming the human Clp protease complex (HClpP, HClp+MTS and HClpX), the functionality of the transformation with pET in *E. coli* and the late Strep-tag purification was checked using *S. aureus* ClpP, which is supposed to give more efficient results.

During the purification, the sample was collected in different fractions of 5 ml, thereby, the fractions containing the protein of interest can be stored and used in further studies.

### 3.1.1. Checkerboard: Purification of SaClpP protein

As I said before, in order to check the functionality of the protocol, ClpP of *S. aureus* was purified. This protocol is similar to the one used in HClpP, premature HClpP and HClpX, but changing some details as the plasmid, which was pET-301-Streptag. Furthermore, the chromatography column used was different, but the essence of the processes is the same.

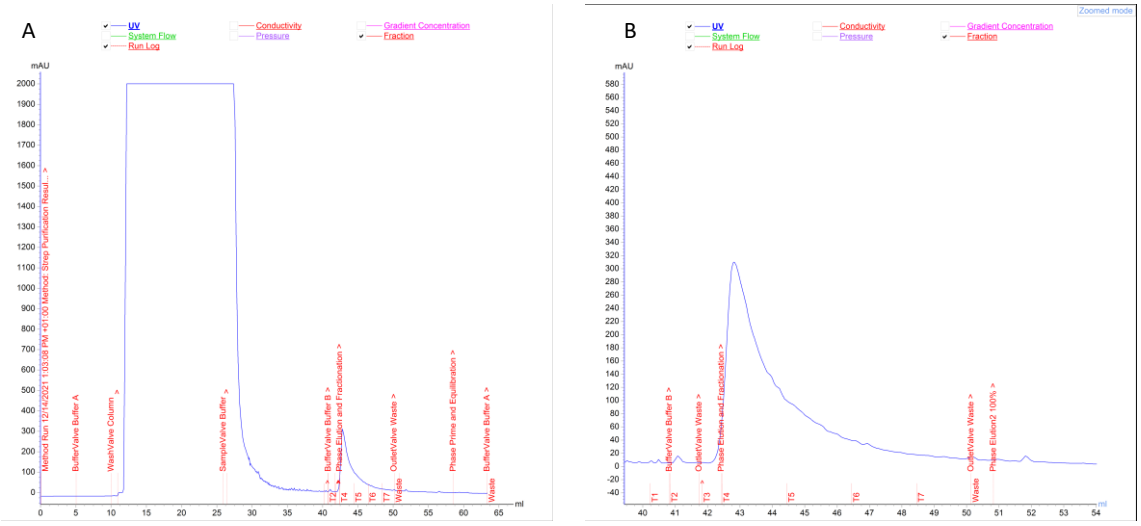


Figure 9. Chromatogram of S. aureus ClpP Strep-tag purification. The blue line is the UV signal. Written in red are the names of the different steps of the purification protocol. The different fractions are named as the letter T followed by the number of the fraction. B. Zoom in of the region where the UV peak is, around 310 mAU

In the chromatogram (Figure 9), the different steps of the purification protocol appear. During the wash of the column, the UV signal is really high because all the proteins that

are not from interest, are running through. Then, the signal decreases when the sample application starts.

Around 40 ml, when the elution step begins, it appears a peak of UV signal, corresponding with the protein of interest. This peak occurs in fraction 4 and reaches 310 mAU. This is a pretty good signal, meaning that the amount of SaClpP purified is pretty high. After that, the signal decreases while the elution is happening until it goes to 5 mAU. Finally, after this step, the UV reaches 0 mAU and this is maintained until the purification finishes.

Afterwards, the concentration of the protein collected in fractions 4, 5 and 6 was measured with Nanodrop, and the result obtained was 1,37 mg/ml, which is a pretty good concentration.
As it was mentioned, the SaClpP Strep-tag purification is quite easy to perform and obtain good results, as we can see in the graph (Figure 9).

### 3.1.2. HClpP Strep-tag Purification

After checking the effectiveness and functionality of Strep-tag protein purification, the protocol was performed with human Clp protease complex.

HClpP was purified three times. The start culture was the *E. coli* strain mentioned at the beginning, transformed with pET-11a-Strep tag plasmid, which contained *hclpP*.

First of all, I will mention the expression results and then the purification results of each attempt. After every purification, an SDS-PAGE was performed to confirm the results obtained in the chromatogram, with the exception of the SaClpP purification and the first HClpP purification.

#### 3.1.2.1. First try of HClpP purification

In the first try, the OD of the *E. coli* preculture after letting it grow overnight was 2 in both of the flasks. Before inducing with IPTG, the OD in the Expression flasks were 0,54 and 0,60.

Regarding the purification results of the first try. As it can be seen in the chromatogram (Figure 10), the elution of HClpP starts in fraction 4 (T4), where is the peak. In this process, the desthiobiotin is removing the binding between Strep-Tactin and HClpP. This time, the fractions expected to contain the protein of interest are T4 and T5, as they are the ones with more UV signal. After that peak, there is supposed to be no more HClpP, as the UV signal is near 0 mAU. In the Zoom zone in chromatogram (Figure 10B), it is possible to see that in T4 the UV signal reaches 140 mAU, and then it decreases until 20 in fractions 6 and 7. It is a lower UV signal than the one seen in SaClpP chromatogram.

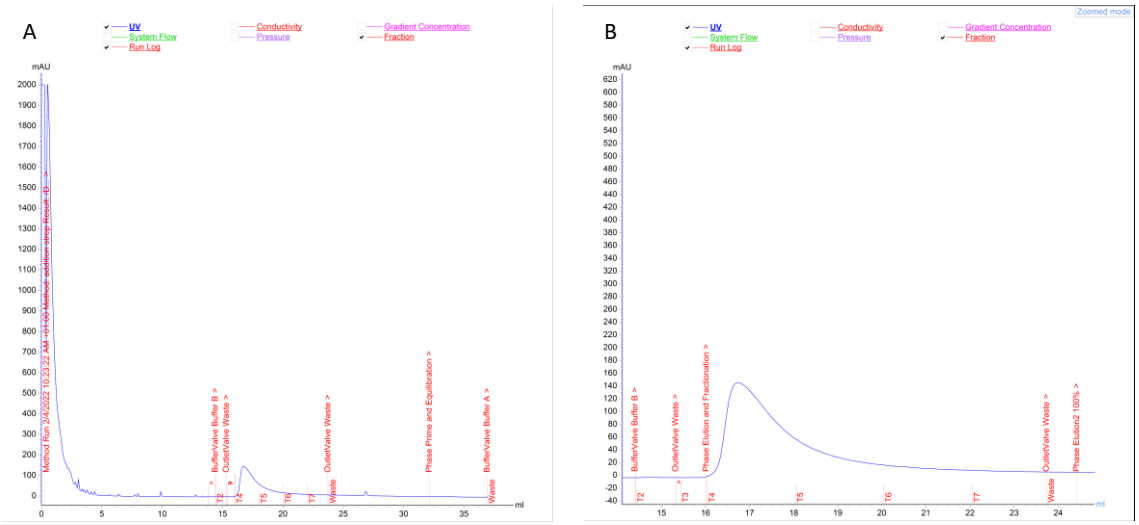


Figure 10. Chromatogram of first human ClpP Strep-tag purification. A.The blue line is the UV signal. Written in red are the names of the different steps of the purification protocol. The different fractions are named as the letter T followed by the number of the fraction. B. Zoom in of the region where the UV peak is, which reaches around 140 mAU.

The concentration of the protein in the fractions T4 and T5 was measured with Nanodrop. In the first try, the protein concentration obtained was 0,73 mg/ml.

### 3.1.2.2. Second try of HClpP purification and electrophoresis

Expression results in the second try were a bit different than the first ones. The OD of the *E. coli* preculture was 2,30 in both flasks, which means the cells have grown more overnight than in the first try, while the OD of the expression flask before inducing with IPTG were 0,78 and 0,65. As the cells were kept more time in the media compared to the previous attempt, it is coherent they got a bigger OD.

In the second HClpP purification, the results were also quite similar to the first ones (Figure 11). As usual, the first part of the chromatogram (until T11) corresponds to the wash of the column. After fraction T11, the elution of HClpP starts. In this case, the fractions with higher UV signal are T14 (around 100 mAU) and T15 (around 45 mAU), so these fractions are the ones expected to contain the protein of interest (Figure 11).

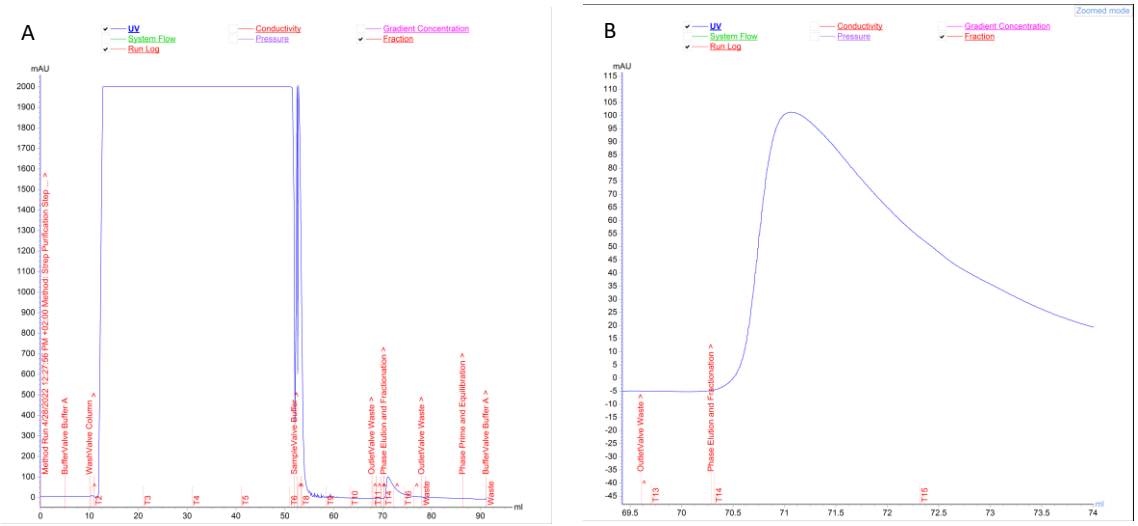


Figure 11. Chromatogram of second human ClpP Strep-tag purification. A. The blue line is the UV signal. Written in red are the names of the different steps of the purification protocol. The different fractions are named as the letter T followed by the number of the fraction. B. Zoom in of the region where the UV peak is, which reaches around 100 mAU.

It can be seen that the peak of UV signal in the second purification (Figure 11B) is a bit lower than the one obtained in the first purification (Figure 10B), as it reaches an UV signal near 100 mAU.

The protein concentration was measured at first in fractions T14, T15 and T16, and the amount obtained was 0,58 mg/ml, which makes sense due to the fact that a lower UV signal was obtained in the chromatogram comparing with the first try.

With the fractions obtained in the second and third try, an electrophoresis was performed in order to know if the fractions collected had the HClpP completely purified (Figure 12). Each number corresponds to the fractions obtained in the purification.

As it was mentioned, the bands of the marker correspond with different molecular weights: 200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15, 10 kDa.

In the lane where fraction 1 was loaded, it is expected to see nothing, given that fraction 1 corresponds with the washing of the column using StrepW Buffer, so there should be

no bands. As we can see in the gel picture (Figure 12A), that is exactly what it happened because it is completely clean, which is a good signal meaning the buffer used was not contaminated.

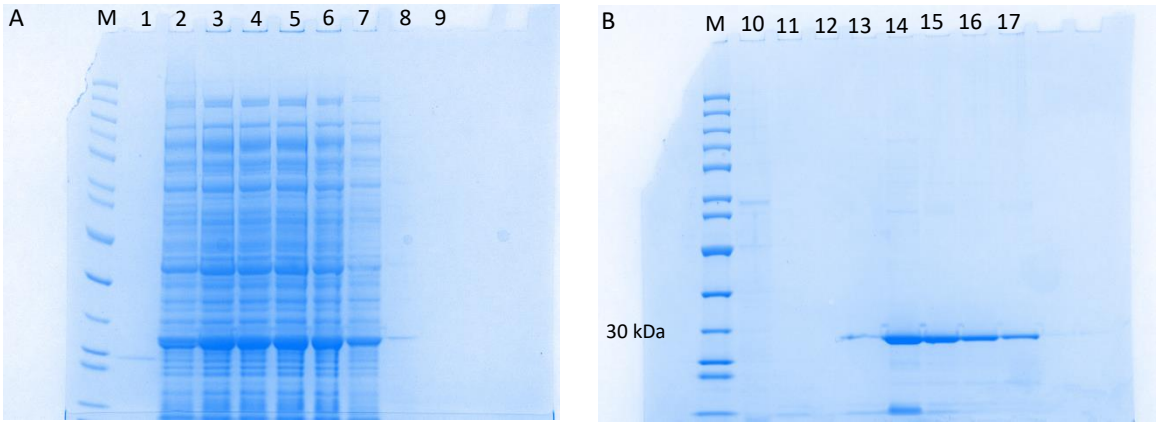


Figure 12. Electrophoresis of second human ClpP Strep-tag purification. Each number corresponds with one fraction. Marker is represented with an M. A. First gel with fractions from 1-9. B. Second gel with fractions from 10-17. The protein of interest is in fraction 14-17.

The lanes with fractions 2-7 correspond with the wash of the column, so they contain every molecule that did not bind to the Strep-Tactin. In these lanes, it is expected to see a lot of bands corresponding with all the proteins that remained in the sample but did not bind to the column, so they are not of interest. When those lanes are analysed with more detail, it is possible to see that lane 7 has a lesser band intensity, which might be because it is the final part of the washing of the column, so there are less proteins to be washed.

Then, in lane with fraction 13 (Figure 12B), and specially in lanes 14, 15, 16 and 17 it is possible to see a strong band under 30 kDa. The expected weight for the mature HClpP protein is 25,5 kDa, but is should be kept in mind that in SDS-PAGE Buffer the protein runs a bit higher, so the band should appear lightly under 30 kDa. Therefore, the strong bands in lanes 14, 15, 16, 17 could match with HClpP. However, in fraction 14 there is also a strong band around 15 kDa, which might correspond with another protein that skipped the purification process.

In addition, as the fraction T17 was not selected at first but it has a band that fits with the protein of interest, the protein concentration in that fraction was also measured, and the result was 0,14 mg/ml. It is reasonable that the protein concentration was lower

than in the other fractions (58 mg/ml) because the band was lighter than the others, which means that the amount of protein is lower.

### 3.1.2.3. Third try of HClpP purification and electrophoresis

In the third and last try, the OD obtained in the preculture was 2 in both flasks, as in the first try. Before inducing with IPTG, the OD of the expression flasks were 0,64 and 0,71.

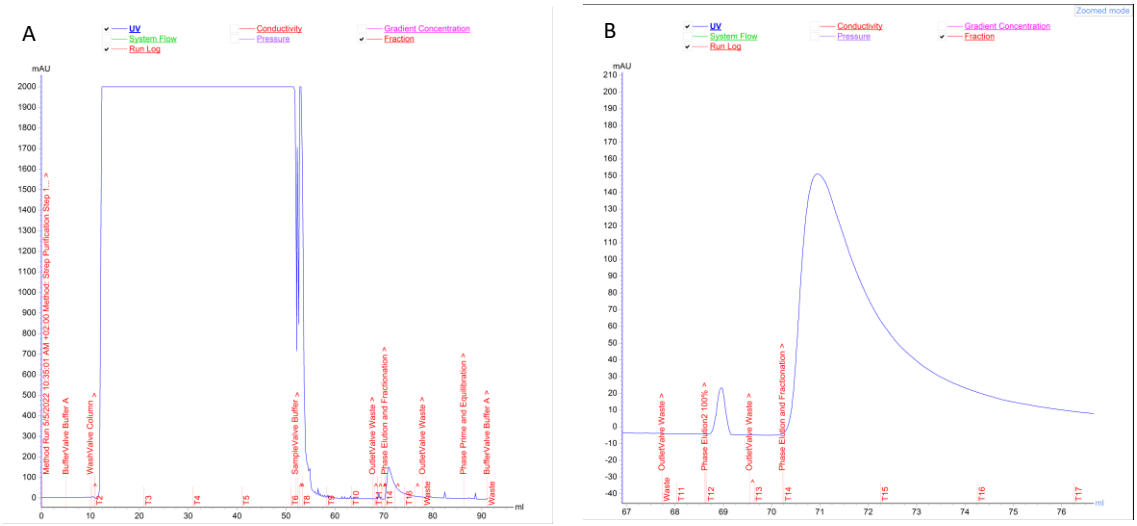


Figure 13. Chromatogram of third human ClpP Strep-tag purification. The blue line is the UV signal. Written in red are the names of the different steps of the purification protocol. The different fractions are named as the letter T followed by the number of the fraction. B. Zoom in of the region where the UV peak is, which reaches around 150 mAU.

In the chromatogram (Figure 13) the high UV signal, matches again with the host proteins that did not bind to the column because they did not have affinity for Strep-Tactin.

The important part is the peak that appears in fraction T14, which indicates the start of the elution step of HClpP. In the Zoom in chromatogram (Figure 13B), is shown that the UV peak reaches 150 mAU, which is the highest signal in the three HClpP purifications, and then it decreases until 20 mAU. This region corresponds with the fractions T14, T15 and T16, which are the ones supposed to contain the protein of interest. T17 might have also a small amount of protein but it will be better seen in the electrophoresis gel.

The concentration of the protein measured in those fractions was 0,74 mg/ml.

Afterwards, the SDS-PAGE electrophoresis was performed. As it can be seen in the first gel (Figure 14A), the first lane is completely clean, as it was expected, because in that fraction there is supposed to be only washing buffer used to wash the column. Then, lanes with fractions 2-7 have several bands because they correspond to the column washing process after the sample is loaded, so there are lots of proteins that do not bind to the column.

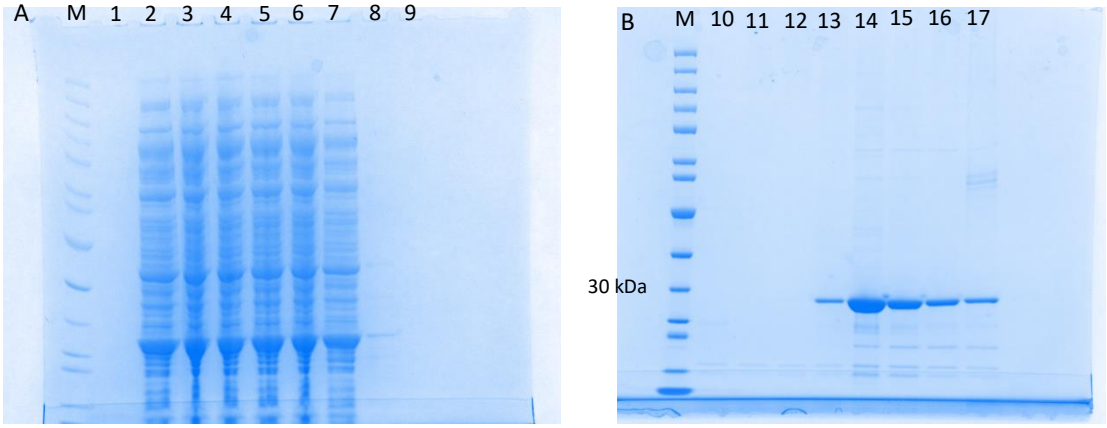


Figure 14. Electrophoresis of third human ClpP Strep-tag purification. Each number corresponds with one fraction. Marker is represented with an M. A. First gel with fractions from 1-9. B. Second gel with fractions from 10-17. The protein of interest in in fraction 14-17.

On the other hand, as it can be seen in the second gel (Figure 14B), fractions 13-17 contain the protein of interest, because they have strong bands under 30 kDa. Fraction 14 is the one with that shows the thickest band on the expected height of HClpP.

The bands appearing in the second gel (Figure 14B) above and under the bands of HClpP might be an impurity of the purification.

### 3.1.3. HClpP+MTS Strep-Tag purification

After the three HClpP purifications, the protocol was tried with the pre-mature HClpP, therefore it still has the Mitochondrial Target Sequence (MTS). For this process, the column used was a different one, but the protocol was the same. The purification was tried twice, but in any of them bands around the expected weight for HClpP+MTS were obtained in the electrophoresis.

It is important to keep in mind that in these purifications, the cells were grown only in one flask, instead of the two flasks used in the HClpP purifications, therefore, the

number of cells is reduced by 50%, and in the same way, the amount of protein expressed.

### 3.1.3.1. First try of HClp + MTS purification and electrophoresis

Regarding the expression results of the first try, the OD obtained in the pre-culture was 2. Before inducing with IPTG, the expression flask had an OD of 0,78.

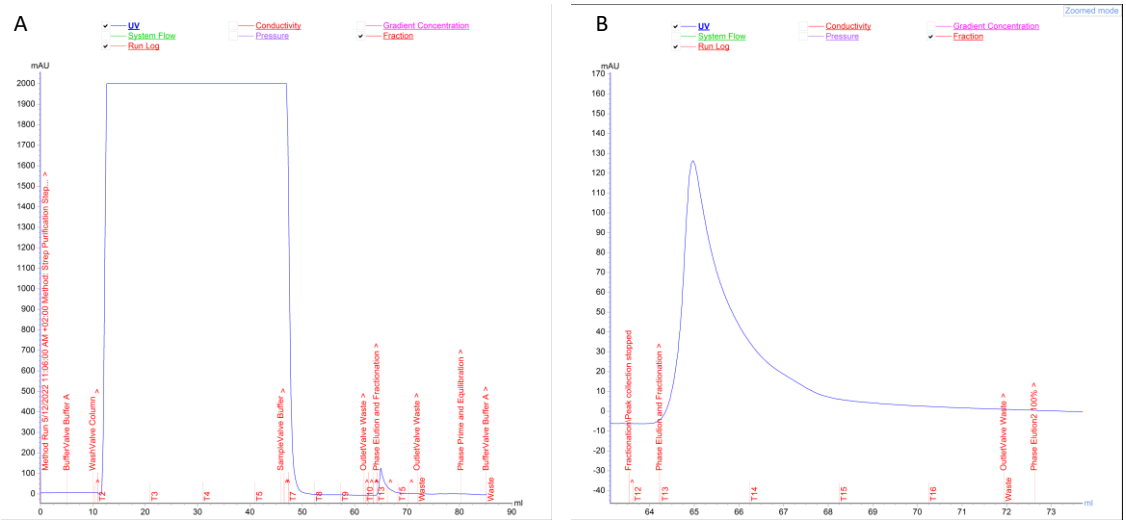


Figure 15. Chromatogram of human ClpP+MTS Strep-tag purification. The blue line is the UV signal. Written in red are the names of the different steps of the purification protocol. The different fractions are named as the letter T followed by the number of the fraction. B. Zoom in of the region where the UV peak is, which reaches around 125 mAU.

In the chromatogram (Figure 15), the UV peak appears in fraction T13, and it reaches around 125 mAU, as it can be seen in the Zoom mode (Figure 15B). This signal means that there is a quite high amount of protein, but it is not possible to assess if it is the protein of interest. The fractions collected to continue the purification were T13 and T14. In fraction 15 the signal is very light, so maybe there is some protein, but the amount will be very low, therefore it is not collected. The protein concentration obtained in fractions 13 and 14 was 0,57 mg/ml.

For the electrophoresis, in addition to the fractions obtained after the purification, three other samples were loaded. One sample was taken after the expression culture grown during the first night (1). Other sample was taken before inducing the cells with IPTG (2). The third sample was taken just before centrifuging the cells after the night of IPTG induction (3).

As it can be seen, the first gel might run correctly (Figure 16A). The lane where fraction 1 was loaded is completely clean, as it was expected, because it should contain only washing buffer. Then, in lanes from 2-5, there are several bands matching with the different proteins that did not bind and were washed during purification. In lane 6, it can also be appreciated some light bands, also corresponding to the washing of the proteins that did not bind.

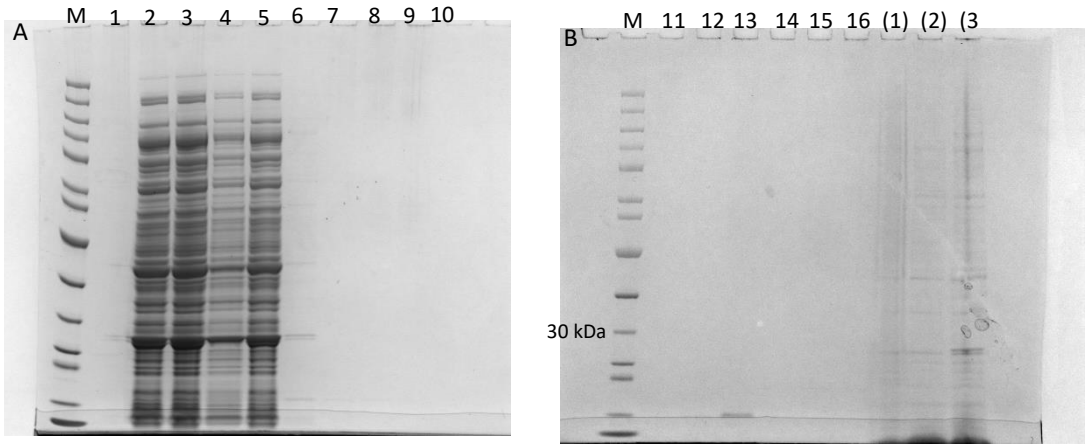


Figure 16. Electrophoresis of human ClpP+MTS Strep-tag purification. Each number corresponds with one fraction. Marker is represented with an M. A. First gel with fractions from 1-9. B. Second gel with fractions from 10-15. (1) is the sample taken after the expression culture was grown during the first night. (2) is the sample taken just before inducing the cells with IPTG. (3) is the sample taken after letting the IPTG work for one night, just before centrifuging the cells.

The HClpP+MTS band should appear in fractions 13 and 14, at least, as it was shown in the chromatogram. The weight of the premature of HClpP is 30,2 kDa, but, as it runs a bit higher in SDS-PAGE Buffer, the band is expected to appear above 30 kDa. However, by seeing the second gel (Figure 16B), the results were not as expected. There are no bands in the lanes where the fractions with the protein of interest were loaded.

In addition, there is a band in fraction 12 around 15 kDa. This band should not be there, so it is probably a contamination.

### 3.1.3.2. Second try of HClpP+MTS purification and electrophoresis

In the second try, the OD of the preculture after growing overnight was 2. Next day, the expression culture grew until reaching an OD of 0,70. It should be noticed that the cells grew slower than the times before. Afterwards, the cells were inducted with IPTG.

During the purification protocol, there was an error connection with the column, so the process was divided in two parts, thereby, there are two chromatograms (Figure 17). The first part of the process, before the error, is represented by the Figure 17A, while the second part is represented in Figure 17B. The fractions collected before the error are named by the number of the tube, while the ones collected after the error are named by the number of the tube and *.

The elution and fractioning step occurred in the second part, and as it can be seen in the chromatogram (Figure 17B), a peak that reached around 35 mAU was obtained in fraction 8*.

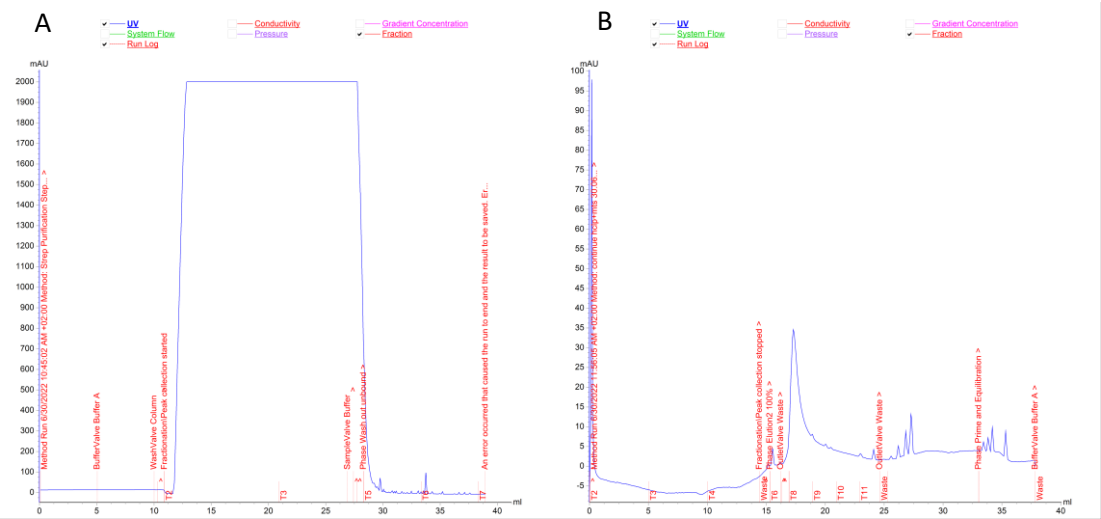


Figure 17. Chromatogram of second human ClpP+MTS Strep-tag purification. The blue line is the UV signal. Written in red are the names of the different steps of the purification protocol. The different fractions are named as the letter T followed by the number of the fraction. A. First part of the purification, before the connection error. B. Second part of the purification, after the connection problem. In this part, the elution phase occurred, so the protein was collected. The peak appears in fraction T8* and reaches around 35 mAU.

To continue the protocol and finally measure the protein concentration, fractions 8*, 9* and 10* were collected. The protein concentration was measured in the different eppendorfs where the protein was stored, and the results obtained were between 0,18 and 0,25 mg/ml. This low concentration fits with the small peak obtained in the chromatogram.

However, in the electrophoresis no bands around the expected weight for HClpP+MTS were obtained. It can be seen in Figure 18.

The expectations were to see a band above 30 kDa in lanes where fraction 8* and 9* were loaded representing HClpP+MTS and matching with the peak seen in the

chromatogram (Figure 18B). In contrast, there is no band in those lanes. However, two bands are observed around 15 kDa in lanes 7* and 8*. These bands may represent a contamination collected in those fractions (Figure 18B).

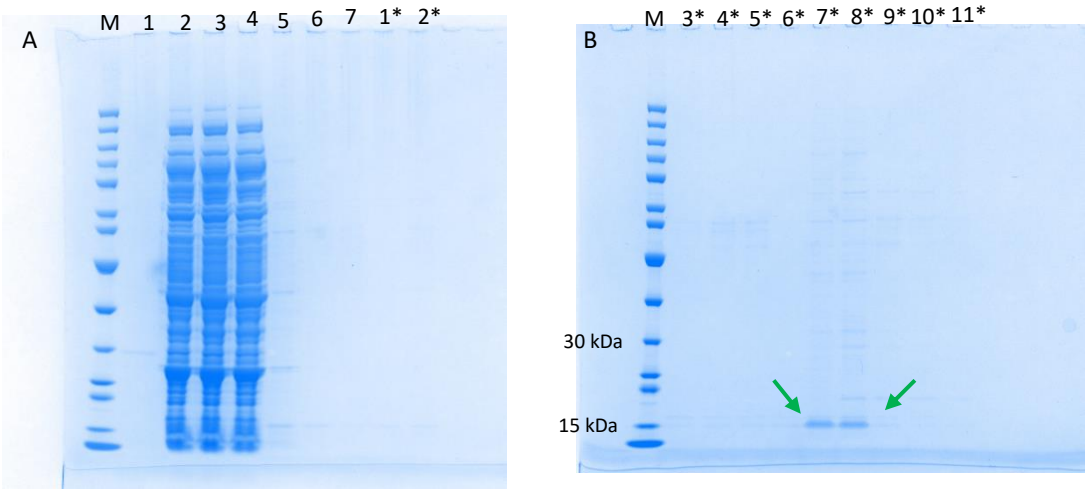


Figure 18. Electrophoresis of second human ClpP + MTS Strep-tag purification. Each number corresponds with one fraction. Fractions collected during the second part of the process, after the connection problem are represented by the number of the fraction followed by *. Marker is represented with an M. B. There are two bands around 15 kDa in fractions 7* and 8* that do not match with the protein of interest.

### 3.1.4. HClpX Strep-tag purification

Finally, HClpX, which is the ATPase of the human Clp complex, was also tried to purify twice. As it happened with HClpP+MTS purification, the number of cells grown were reduced by 50% compared to HClpP and SaClpP purifications, so the amount of protein is expected to be lower also.

It is important to keep in mind that HClpX is very difficult to purify because it is very low expressed in the cells. Knowing that, I would mention that in any of the cases the purification succeeded.

#### 3.1.4.1. First try of HClpX purification and electrophoresis

In the first try, the OD reached in the preculture was 2. On the other hand, the OD in the expression flask before the induction with IPTG was 0,61.

During the purification, there was another problem with the connection, so there are again 2 chromatograms, each of them matching with a part of the process (Figure 19). This time, the problem occurred at the beginning, during the sample application, so the

fractions in the first chromatogram are not important because they only contain washing particles.

In the chromatogram of the second part of the process (Figure 19B) it is really difficult to differentiate any UV peak in the elution and fractioning step corresponding with the protein of interest. Nevertheless, by doing some zoom in that particular step (Figure 19C), there is a really small UV peak in the fraction T10*, which might correspond with HClpX, but it cannot be assessed. This peak is really small, it barely reaches 5 mAU.

In spite of this small peak, fractions T10 and T11 were collected to continue with the purification with the buffer exchange step.

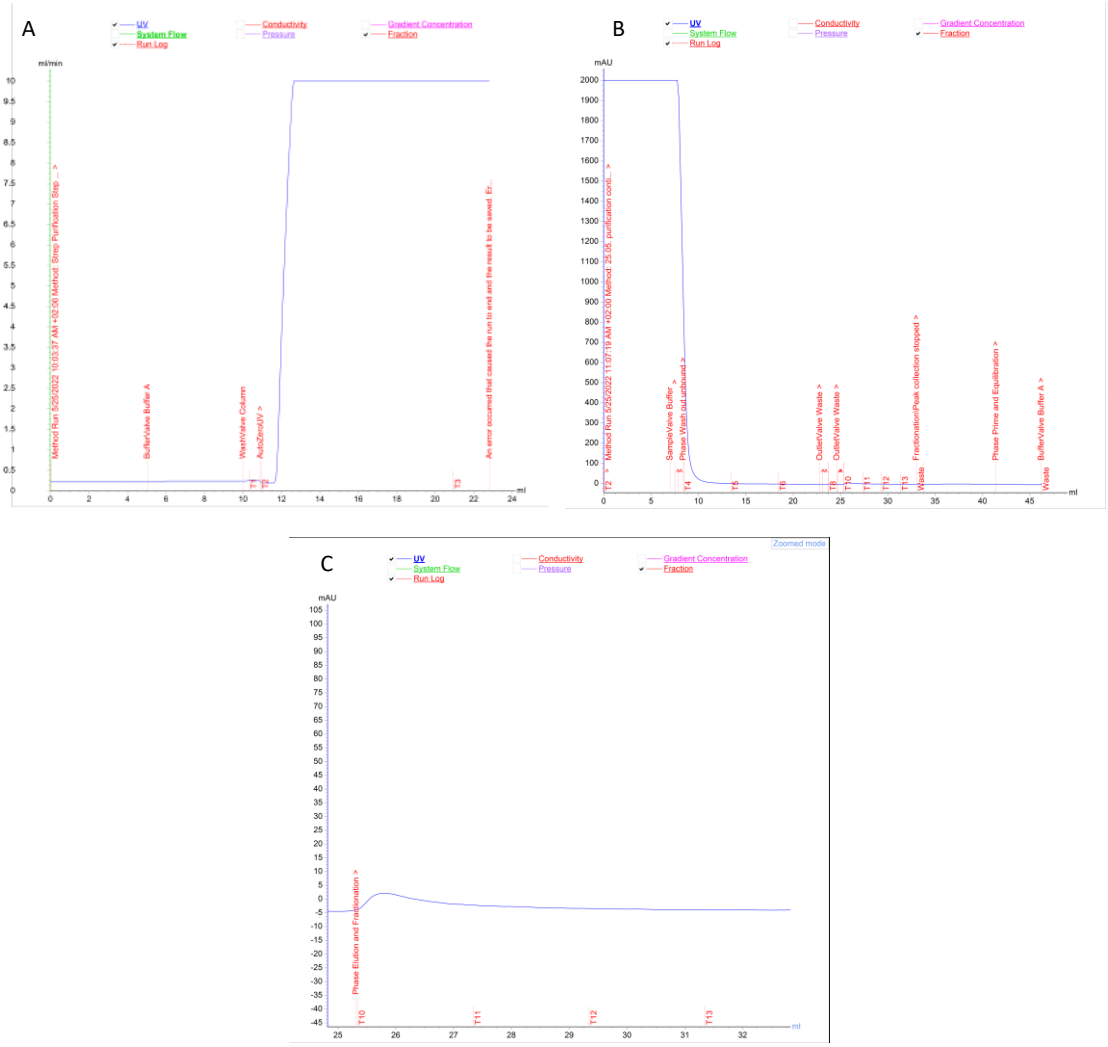


Figure 19. Chromatogram of first human ClpX Strep-tag purification. The blue line is the UV signal. Written in red are the names of the different steps of the purification protocol. The different fractions are named as the letter T followed by the number of the fraction. A. First part of the purification before it stopped due to the error. The process is stopped in the washing step, in fraction T3. B. Second part of the purification after the error. C. Zoom in the peak zone during the second part of the purification, although is really small. The peak barely reaches 5 mAU in fraction T10*.

Finally, the concentration of the protein was measured in some eppendorfs. The protein concentration was between 0,20 and 0,35 mg/ml. Although this is a very low concentration, is higher than I expected for that small peak.

Afterwards, the SDS-PAGE Electrophoresis was performed (Figure 20). The only bands that could be seen were in the first gel (Figure 20A), and were the ones corresponding to the fractions 2, 3, 4, 5 and 6, which include the proteins washed at the beginning of the process. In the second gel (Figure 20B) HClpX was expected to appear, but it did not.

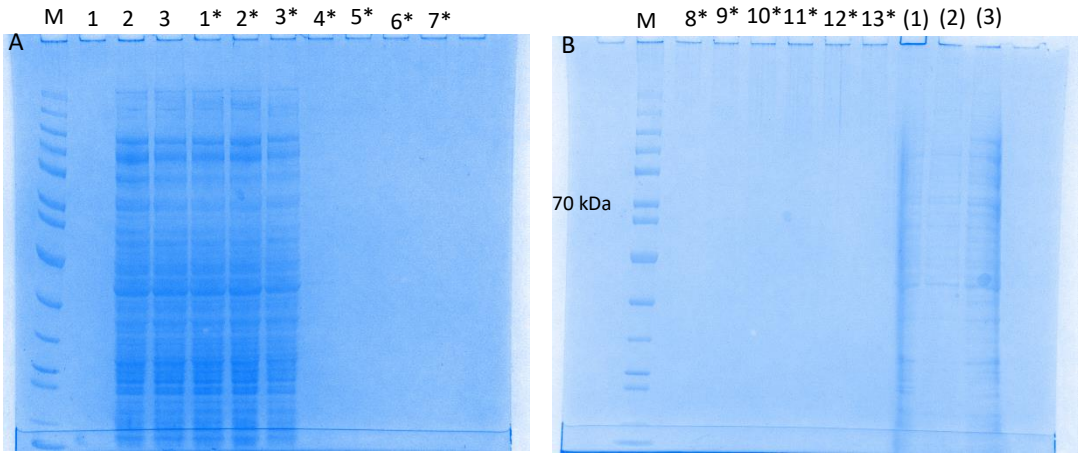


Figure 20. Electrophoresis of first try of human ClpX Strep-tag purification. Each number corresponds with one fraction. Fractions collected after the connection error are named by the number of the fraction followed by *. Marker is represented with an M. B. Second gel with fractions from 10-17. (1) is the sample taken after the expression culture was grown during the first night. (2) is the sample taken just before inducing the cells with IPTG. (3) is the sample taken after letting the IPTG work for one night, just before centrifuging the cells.

### 3.1.4.2. Second try HClpX purification and electrophoresis

The results of the second time HClpX was purified were very similar to the ones obtained in the previous try, which means the purification did not work either.

First of all, the OD obtained after letting the preculture grow overnight was 2, as usual. On the other hand, the OD of the expression culture was 0,55, but it took a lot of time for the cells to grow until that OD. That signal means that something is going wrong.

The purification process through the column was stopped twice due to two connection problems. As the second part of the process, between the errors, only affected the sample application process, there is no change in the chromatogram, thereby I am not

including that graphic in the figure (Figure 21). Fractions collected after the two problems are named with the number and **.

If there were protein in the sample loaded in the column, there was supposed to be a peak when the elution and fractioning step started, so in fraction T10**. As it can be seen in the Zoom figure of the chromatogram (Figure 21C), there is no peak, meaning that no protein was eluted, or at least, the amount of protein was too small to be detected.

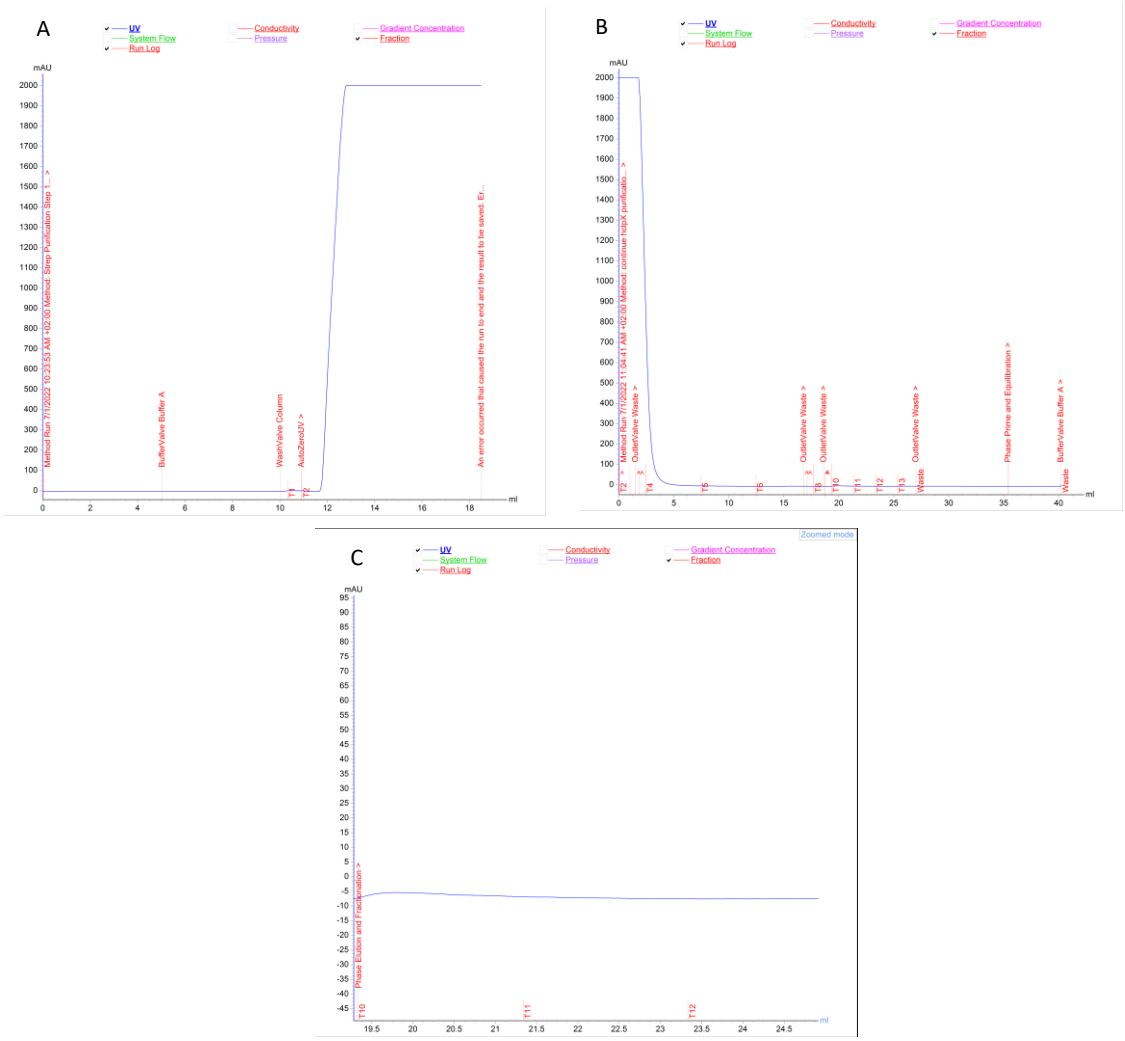


Figure 21. Chromatogram of second human ClpX Strep-tag purification. Blue line is UV signal. In red it appears the names of the different steps of the purification protocol. The different fractions are named as the letter T followed by the number of the fraction. A. First part of the purification before it stopped due to the first error. The process is stopped in the sample application step, in fraction T2. B. Third part of the purification after the second error. C. Zoom where it was supposed to be the peak during the third part of the purification, but there is no peak.

Despite not obtaining a peak in the chromatogram, fraction 10** was collected to continue with the buffer exchange step. At the end, the protein concentration was

measured in the different Eppendorf tubes where the protein was collected to be stored. The protein concentration obtained was 0,15 mg/ml.

Later on, and as it was expected due to the small amount of protein obtained, the electrophoresis showed no band matching with HClpX (Figure 22).

If the purification had been successful, a band was supposed to appear around 70 kDa in some fractions in the second gel corresponding with HClpX (Figure 22B). In contrast, there is no band around 70 kDa in those fractions.

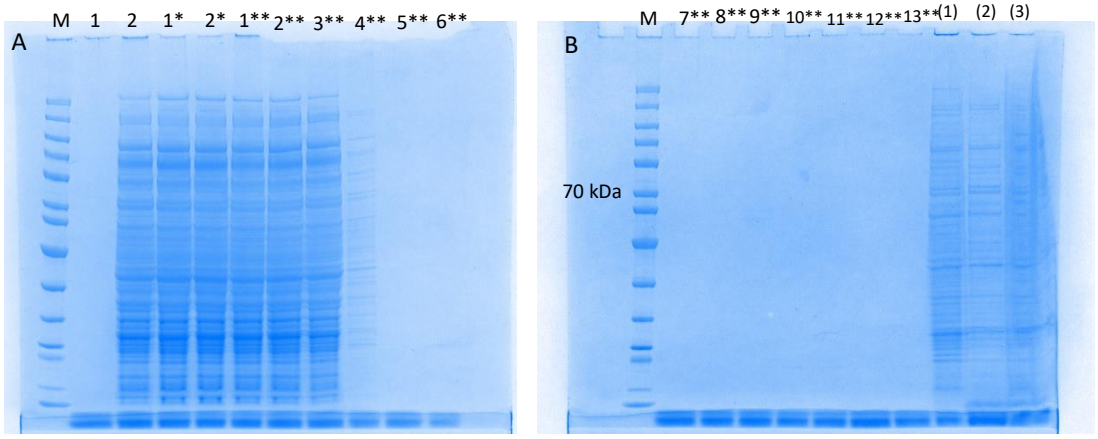


Figure 22. Electrophoresis of second try of human ClpX Strep-tag purification. Each number corresponds with one fraction. Fractions collected after the connection error are named by the number of the fraction followed by *. Fractions collected after the second connection problem are named with the number of the fraction followed by **. Marker is represented with an M. B. (1) is the sample taken after the expression culture was grown during the first night. (2) is the sample taken just before inducing the cells with IPTG. (3) is the sample taken after letting the IPTG work for one night, just before centrifuging the cells.

The only bands that can be appreciated in the gels are the ones corresponding to the proteins washed before eluting the protein of interest, in fractions 2-3** (Figure 22A), and the proteins expressed in the cells before breaking them, represented in lanes (1), (2) and (3). As we can see in those lanes, there is a small band around 70 kDa, which might represent that HClpX expressed inside the cells.

# 4. DISCUSSION AND CONCLUSIONS

In this part of I would like to discuss the results obtained in the different purifications, as well as to give the conclusions I obtained after doing this project.

The aim of the project was to purify the different proteins forming the human Clp protease system, but the results obtained were different depending on the protein purified in each case.

## 4.1. DISCUSSION OF THE RESULTS

The materials and methods used in the purification protocol of this project had been already used by my supervisor Yvonne Thoma during her research for the PhD. The strain and the plasmids were the same, as well as the proteins purified.

Although I used the same protocol for every purification, the results were different in any case, to the point that some of them worked, while others did not succeed.

### 4.1.1. SaClpP Purification

First of all, the results of SaClpP purification were consequent with what could be seen in the chromatogram. The peak obtained reached 320 mAU, and the protein concentration measured was 1,37 mg/ml. It should be said that it is not difficult to obtain this protein concentration because the protein is high expressed in the cells, and it is easy to purify.

In addition, both the peak and the concentration measured, might correspond with SaClpP protein, but it is not possible to assess that only using the purification experiment. To ensure that those results match with SaClpP, they should be analysed in an SDS-PAGE electrophoresis or a similar assay.

After this checkboard assay, it is possible to conclude the protein expression using pET plasmids in *E. coli*, the subsequent induction with IPTG, and the later purification using the Äkta column is working. Therefore, this protocol can be tried with the proteins of real interest, which are HClpP, the premature for of HClpP (HClpP+MTS) and HClpX.

### 4.1.2. HClpP Purification

HClpP protein purification was tried three times. In all the cases the results were quite similar, which makes sense, given that the design of the experiments was the same. The only difference is that the SDS-PAGE electrophoresis was performed only in the second and the third tries.

#### 4.1.2.1. Purification results

Looking to the overall results, HClpP was successfully purified. In every try there was a peak where the protein of interest was expected to be eluted, being the highest in the third purification, which reached 150 mAU (Figure 13). In addition, the protein concentrations measured were also in harmony with the results obtained in the chromatogram. The higher the UV signal, the higher the protein concentration.

The amount of protein obtained in each try (0,73 mg/ml, 0,58 mg/ml, 0,74 mg/ml) by a volume of 500µl is not very high compared to the ones standardly obtained in other purification tries by other colleague of the research group, which are around 5-8 mg/ml (Yvonne Thoma, 2022).

#### 4.1.2.2. Electrophoresis results

Regarding the SDS-PAGE electrophoresis, the results were also successful because they support the results obtained during the purification. It is possible to see bands in the expected weight for HClpP in the fractions where the protein of interest was supposed to be collected during the purification.

However, the band appearing around 10 kDa in fraction 14 in the second try (Figure 12B), should not be there. This band might be a contamination, but there are other options. There is a small chance that during the purification, HClpP protein was cleaved by a random reason (no protease inhibitors were used in the protocol), making the protein smaller; thereby, that band could be HClpP after losing a part of its sequence. It also can be another protein that was able to bind to Strep-Tactin at the same time as the HClpP because it also appears in fraction 13 and 15.

As I mentioned before, in the electrophoresis gel of the third try (Figure 14B), there are some bands appearing above and under HClpP bands. These bands might be an impurity of the purification and might correspond to proteins that were not washed before the elution during the wash and outbound step, and they finally eluted with the protein of interest. A possible solution to avoid these bands could be washing the column with more CV before the elution process, so it is possible to get rid of them before the protein of interest is collected.

### 4.1.3. HClpP+MTS Purification

To continue with the discussion, I would say that I did not get the expected results in the purification of the premature form of HClpP (HClp+MTS). The protocol design was very similar to the previous purifications, but with two differences.

First of all, the column used was different because it was specific for this protein. The other difference, and for me the most important one, that might be the responsible of not obtaining a complete success in the results was that the population of cells used in these purifications was reduced by 50% comparing to the previous purifications. In the previous experiments I used two big flasks for the expression strain, while in HClpP+MTS expression, I only used one big flask, because it was performed as the same time as HClpX expression, so I used one flask for each protein. As the number of cells is reduced by half, the amount of protein expressed should be lower than in the other purifications.

#### 4.1.3.1. Purification results

The protocol was tried twice. In both cases, the purification process worked, as a peak was obtained. The highest one appeared in the first try (Figure 15), given that it reached 125 mAU. This peak is expected to correspond to the HClpP+MTS, but it cannot be ensured only with this experiment. We need an additional assay, such as the SDS-PAGE electrophoresis.

#### 4.1.3.2. Electrophoresis results

In contrast, the SDS-PAGE electrophoresis did not work in any of the cases.

This is not surprising in the second try, because the amount of protein obtained in the purification was not really high (peak of 35 mAU), so maybe there was not enough protein to give a band in the gel.

Furthermore, the bands obtained in fractions 7* and 8* around 15 kDa (Figure 18B) might be a contamination, but there can be another possibility. Maybe, during the purification process, HClp+MTS was cleaved by a protease, so the molecular weight was reduced. Thereby, those bands around 15 kDa might correspond to the premature form of the HClpP after losing part of its sequence.

On the other hand, in the first try, the amount of protein was high enough to give a band, so I assume that the reason why there are no bands matching with the protein of interest in the gel (Figure 16B) is because I made some mistakes while loading the samples. Another possible reason is that during the time that passed between the purification and the SDS-PAGE electrophoresis (one day), the store conditions of the protein were not correct, so the protein degraded. There is a third reason to explain the absence of the bands matching with HClpP+MTS in the gel. In that gel (Figure 16B) samples takes of the cell lysate were also loaded, so it was expected to see a lot of bands in those lanes, which are named (1), (2) and (3), corresponding with all the protein expressed inside the cells. In contrast, the bands cannot be clearly seen. Thereby, maybe the problem was that the gel was not left enough time in 'Der blaue Jonas' for the bands to stain.

### 4.1.4. HClpX Purification

HClpX purification was designed exactly like HClpP+MTS, so the number of cells expressing the protein was reduced by 50% compared to HClpP and SaClpP purifications. The only difference was the column used and Amicon tube to concentrate the protein after the purification through the Äkta Column.

#### 4.1.4.1. Purification results

This purification did not succeed, even though it was tried twice. There was no peak in any of the purifications.

These results are not surprising, given that HClpX is really low expressed in the cells, therefore, it is difficult to obtain a good amount of protein in the purification.

#### 4.1.4.2. Electrophoresis results

In both tries, the SDS-PAGE electrophoresis did not show any band in the location where HClpX was expected to be. In fact, the absence of the band corresponding to HClpX is not strange, due to the fact that the UV signal obtained in the chromatogram in all the cases was very low, or even did not exist. In the case that there was a little amount of protein, it could have degraded before the electrophoresis, keeping in mind it was performed the following day after the purification.

As this protein is really low expressed in the cells, maybe it is better to check if the protein has been expressed before doing the purification. To do this, I suggest taking a sample of the cell lysate before and after the IPTG induction and let them run in an SDS-PAGE electrophoresis. If there is a difference in the bands appearing around 70 kDa between the two samples, that would mean the protein had been expressed.

### 4.1.5. Troublesshooting

All these differences in the results of the purifications doing the same protocol make me thing that the reason why some purifications did not work is not in the general protocol, but in specific problems, and I would like to mention some of them that, from my point of view, clearly affected the success of the purifications.

After the first purification tries, I decided to let the expression culture reach a higher OD before inducing with IPTG, in order to have more cells able to express the protein. As a result, the amount of protein expressed should be higher than in previous purifications, but only in the third HClpP purification and in the first HClpP+MTS I reached this goal.

One mistake that I was making during the first purifications, but I was not realizing, was the temperature in which the proteins must be kept during some steps of the process. Each time I used the centrifuge, and the protein should stay at a particular temperature during the process, I was not waiting to initiate the centrifugation until the centrifuge

reached the appropriate temperature. The same happened with the shaker used to grow the cultures.

At the beginning, this might not be a big problem, as HClpP and HClp+MTS are very stable and high expressed in the cells, therefore, they may not have degraded. In contrast, when HClpX purification did not work for the first time, I realised that I was making this mistake. Therefore, I corrected it for the last HClpX purification. However, the purification was not successful either.

After searching in the literature, I have found that in other human Clp system purifications using *E. coli* as expression strain, scientist have induced the protein expression with 1mM IPTG (el Bakkouri et al., 2010), while in this project the protein expression in all cases (HClpP, HClpP+MTS, HClpX) was induced with 0,5 mM IPTG. Maybe, if the induction had been done with more IPTG, the amount of protein would have been higher. So, that should be a change I would do for following tries.

On the other hand, the Äkta column was not working properly. It had several connection problems while the protein was running though the column. As these problems started to appear in the second HClp+MTS purification, that is also when the results started to be bad, it might also affect the process. Nevertheless, the only consequence of the connection problem is that the purification process is stopped, so it should not affect the protein purification at all.

Lastly, I would repeat that, particularly HClpX is really difficult to purify as it is very low expressed in the cell. So, I would suggest that for the next time I could try to increase the protein expression by adding more IPTG or using two flasks for the expression culture, instead of one, therefore, there are more cells to express the protein. I also read in the literature that when this protein is purified, it is loaded in three different columns, in order to get a more quality purification (Kang et al., 2002). This can also be an improvement in the protocol for the next time to get better results.

## 4.2. GENERAL CONCLUSION

As it can be seen, there are a lot of aspects to improve for the next time, but the results were not so bad keeping in mind that I was a completely unexperienced student in this kind of protocols.

As I just mentioned, maybe if a higher amount of IPTG had been used to induce the protein expression, or if a higher number of cells had been transformed with the plasmid, more protein would have been obtained.

Furthermore, I would like to remark that no protease inhibitors were used during the protocol. Given that the proteins are susceptible of being degraded by proteases present in the medium during the purification process, I would also suggest for next tries using protease inhibitors, in order to avoid the degradation of the protein of interest before it gets separated when passing through the affinity column (Ryan & Henehan, 2017).

As an overall conclusion I would say that there are lot of differences between the protocol used in this project, and the protocols used in other human Clp system purifications, and there are several improvements that can be made to get better results. However, as I said before, this protocol had been already tried by my supervisor Yvonne Thoma and it succeed.

Also, it must be kept in mind that during the laboratory work, there are several factors that might affect the proteins and some of them are beyond human control, meaning that is very difficult to get perfect results, even though the protocol is perfectly made.

# 5. AKNOWLEDGMENTS

I want to thank Prof. Dr. Heike Brötz-Oesterhelt, the head of the department “Microbial Bioactive Compounds” in the Interfaculty Institute of Microbiology and Infection Medicine (IMIT), for giving me the opportunity to work in their research group doing this project, even though I was an Erasmus student.

I also want to give a special thank you to the PhD student Yvonne Thoma, for being my supervisor during this project and teach me all about protein purification, but also about cell culture. I really appreciate the effort of being with me during the first weeks, helping me anytime I needed and answering me every question I had.

Furthermore, I would like to thank you all the research group, for again, helping me with every problem or question I had, and for creating such a friendly environment to work in the laboratory.

Finally, I save a special thank you to Prof. Patricio Ferreira, from the University of Zaragoza, in Spain, for the help he had offered during the whole year, and for supervising this year I have spent abroad.

# 6. REFERENCES


AG Brötz-Oesterhelt (Unpublished). (n.d.). *IPTG induction of HClpP expression*.

Ahmed, S., Jelani, M., Alrayes, N., Mohamoud, H. S. A., Almramhi, M. M., Anshasi, W., Ahmed, N. A. B., Wang, J., Nasir, J., & Al-Aama, J. Y. (2015). Exome analysis identified a novel missense mutation in the CLPP gene in a consanguineous Saudi family expanding the clinical spectrum of Perrault Syndrome type-3. *Journal of the Neurological Sciences*, *353*(1–2), 149–154. https://doi.org/10.1016/j.jns.2015.04.038

Al-Furoukh, N., Ianni, A., Nolte, H., Hölper, S., Krüger, M., Wanrooij, S., & Braun, T. (2015). ClpX stimulates the mitochondrial unfolded protein response (UPRmt) in mammalian cells. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1853*(10), 2580–2591. https://doi.org/10.1016/j.bbamcr.2015.06.016

Bhandari, V., Wong, K. S., Zhou, J. L., Mabanglo, M. F., Batey, R. A., & Houry, W. A. (2018). The Role of ClpP Protease in Bacterial Pathogenesis and Human Diseases. *ACS Chemical Biology*, *13*(6), 1413–1425. https://doi.org/10.1021/acschembio.8b00124

Cole, A., Wang, Z., Coyaud, E., Voisin, V., Gronda, M., Jitkova, Y., Mattson, R., Hurren, R., Babovic, S., Maclean, N., Restall, I., Wang, X., Jeyaraju, D. V., Sukhai, M. A., Prabha, S., Bashir, S., Ramakrishnan, A., Leung, E., Qia, Y. H., … Schimmer, A. D. (2015). Inhibition of the Mitochondrial Protease ClpP as a Therapeutic Strategy for Human Acute Myeloid Leukemia. *Cancer Cell*, *27*(6), 864–876. https://doi.org/10.1016/j.ccell.2015.05.004

DeBerardinis, R. J., & Chandel, N. S. (2016). Fundamentals of cancer metabolism. *Science Advances*, *2*(5). https://doi.org/10.1126/sciadv.1600200

Dubendorf, J. W., & Studier, F. W. (1991). Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor. *Journal of Molecular Biology*, *219*(1), 45–59. https://doi.org/10.1016/0022-2836(91)90856-2

Dwivedi, Kshitiz. (2011). M. Sc. (2011). *Expression of recombinant insecticidal protein (code: NBRI 12) as a fusion protein in E. coli, its purification, refolding and evaluation of its biological activity*.

el Bakkouri, M., Pow, A., Mulichak, A., Cheung, K. L. Y., Artz, J. D., Amani, M., Fell, S., de Koning-Ward, T. F., Goodman, C. D., McFadden, G. I., Ortega, J., Hui, R., & Houry, W. A. (2010). The Clp Chaperones and Proteases of the Human Malaria Parasite Plasmodium falciparum. *Journal of Molecular Biology*, *404*(3), 456–477. https://doi.org/10.1016/j.jmb.2010.09.051

Etter, D., Schelin, J., Schuppler, M., & Johler, S. (2020). Staphylococcal Enterotoxin C—An Update on SEC Variants, Their Structure and Properties, and Their Role in Foodborne Intoxications. *Toxins*, *12*(9), 584. https://doi.org/10.3390/toxins12090584

*Expression and purification of proteins using Strep-tag® or Twin-Strep-tag® A comprehensive manual*. (n.d.). www.strep-tag.comhttp://www.iba-lifesciences.com/patents.htmloruponinquiryatinfo@iba-lifesciences.comor

Feng, J., Michalik, S., Varming, A. N., Andersen, J. H., Albrecht, D., Jelsbak, L., Krieger, S., Ohlsen, K., Hecker, M., Gerth, U., Ingmer, H., & Frees, D. (2013). Trapping and Proteomic

Identification of Cellular Substrates of the ClpP Protease in Staphylococcus aureus. *Journal of Proteome Research*, *12*(2), 547–558. https://doi.org/10.1021/pr300394r

Ferrer-Miralles, N., Saccardo, P., Corchero, J. L., Xu, Z., & García-Fruitós, E. (2015). *General Introduction: Recombinant Protein Production and Purification of Insoluble Proteins* (pp. 1–24). https://doi.org/10.1007/978-1-4939-2205-5_1

Frees, D., Chastanet, A., Qazi, S., Sørensen, K., Hill, P., Msadek, T., & Ingmer, H. (2004). Clp ATPases are required for stress tolerance, intracellular replication and biofilm formation in Staphylococcus aureus. *Molecular Microbiology*, *54*(5), 1445–1462. https://doi.org/10.1111/j.1365-2958.2004.04368.x

Frees, D., Qazi, S. N. A., Hill, P. J., & Ingmer, H. (2003). Alternative roles of ClpX and ClpP in Staphylococcus aureus stress tolerance and virulence. *Molecular Microbiology*, *48*(6), 1565–1578. https://doi.org/10.1046/j.1365-2958.2003.03524.x

Geiger, S. R., Böttcher, T., Sieber, S. A., & Cramer, P. (2011). A Conformational Switch Underlies ClpP Protease Function. *Angewandte Chemie International Edition*, *50*(25), 5749–5752. https://doi.org/10.1002/anie.201100666

Gispert, S., Parganlija, D., Klinkenberg, M., Drose, S., Wittig, I., Mittelbronn, M., Grzmil, P., Koob, S., Hamann, A., Walter, M., Buchel, F., Adler, T., Hrabe de Angelis, M., Busch, D. H., Zell, A., Reichert, A. S., Brandt, U., Osiewacz, H. D., Jendrach, M., & Auburger, G. (2013). Loss of mitochondrial peptidase Clpp leads to infertility, hearing loss plus growth retardation via accumulation of CLPX, mtDNA and inflammatory factors. *Human Molecular Genetics*, *22*(24), 4871–4887. https://doi.org/10.1093/hmg/ddt338

Guillon, B., Bulteau, A.-L., Wattenhofer-Donzé, M., Schmucker, S., Friguet, B., Puccio, H., Drapier, J.-C., & Bouton, C. (2009). Frataxin deficiency causes upregulation of mitochondrial Lon and ClpP proteases and severe loss of mitochondrial Fe-S proteins. *FEBS Journal*, *276*(4), 1036–1047. https://doi.org/10.1111/j.1742-4658.2008.06847.x

Hackl, M. W., Lakemeyer, M., Dahmen, M., Glaser, M., Pahl, A., Lorenz-Baath, K., Menzel, T., Sievers, S., Böttcher, T., Antes, I., Waldmann, H., & Sieber, S. A. (2015). Phenyl Esters Are Potent Inhibitors of Caseinolytic Protease P and Reveal a Stereogenic Switch for Deoligomerization. *Journal of the American Chemical Society*, *137*(26), 8475–8483. https://doi.org/10.1021/jacs.5b03084

Heidler, J., Al-Furoukh, N., Kukat, C., Salwig, I., Ingelmann, M.-E., Seibel, P., Krüger, M., Holtz, J., Wittig, I., Braun, T., & Szibor, M. (2011). Nitric Oxide-associated Protein 1 (NOA1) Is Necessary for Oxygen-dependent Regulation of Mitochondrial Respiratory Complexes. *Journal of Biological Chemistry*, *286*(37), 32086–32093. https://doi.org/10.1074/jbc.M111.221986

Illigmann, A., Thoma, Y., Pan, S., Reinhardt, L., & Brötz-Oesterhelt, H. (2021). Contribution of the Clp Protease to Bacterial Survival and Mitochondrial Homoeostasis. *Microbial Physiology*, *31*(3), 260–279. https://doi.org/10.1159/000517718

Ishizawa, J., Zarabi, S. F., Davis, R. E., Halgas, O., Nii, T., Jitkova, Y., Zhao, R., St-Germain, J., Heese, L. E., Egan, G., Ruvolo, V. R., Barghout, S. H., Nishida, Y., Hurren, R., Ma, W., Gronda, M., Link, T., Wong, K., Mabanglo, M., … Andreeff, M. (2019). Mitochondrial ClpP-

Mediated Proteolysis Induces Selective Cancer Cell Lethality. *Cancer Cell*, *35*(5), 721-737.e9. https://doi.org/10.1016/j.ccell.2019.03.014

Jensen, C., Bæk, K. T., Gallay, C., Thalsø-Madsen, I., Xu, L., Jousselin, A., Ruiz Torrubia, F., Paulander, W., Pereira, A. R., Veening, J.-W., Pinho, M. G., & Frees, D. (2019). The ClpX chaperone controls autolytic splitting of Staphylococcus aureus daughter cells, but is bypassed by β-lactam antibiotics or inhibitors of WTA biosynthesis. *PLOS Pathogens*, *15*(9), e1008044. https://doi.org/10.1371/journal.ppat.1008044

Jensen, C., Fosberg, M. J., Thalsø-Madsen, I., Bæk, K. T., & Frees, D. (2019). Staphylococcus aureus ClpX localizes at the division septum and impacts transcription of genes involved in cell division, T7-secretion, and SaPI5-excision. *Scientific Reports*, *9*(1), 16456. https://doi.org/10.1038/s41598-019-52823-0

Kang, S. G., Ortega, J., Singh, S. K., Wang, N., Huang, N., Steven, A. C., & Maurizi, M. R. (2002). Functional Proteolytic Complexes of the Human Mitochondrial ATP-dependent Protease, hClpXP. *Journal of Biological Chemistry*, *277*(23), 21095–21102. https://doi.org/10.1074/jbc.M201642200

Kimber, M. S., Yu, A. Y. H., Borg, M., Leung, E., Chan, H. S., & Houry, W. A. (2010). Structural and Theoretical Studies Indicate that the Cylindrical Protease ClpP Samples Extended and Compact Conformations. *Structure*, *18*(7), 798–808. https://doi.org/10.1016/j.str.2010.04.008

Kirstein, J., Hoffmann, A., Lilie, H., Schmidt, R., Rübsamen-Waigmann, H., Brötz-Oesterhelt, H., Mogk, A., & Turgay, K. (2009). The antibiotic ADEP reprogrammes ClpP, switching it from a regulated to an uncontrolled protease. *EMBO Molecular Medicine*, *1*(1), 37–49. https://doi.org/10.1002/emmm.200900002

Kirstein, J., Molière, N., Dougan, D., & Turgay, K. (2009). Adapting the machine: Adaptor proteins for Hsp100/Clp and AAA+ proteases. *Nature Reviews. Microbiology*, *7*, 589–599. https://doi.org/10.1038/nrmicro2185

Kolanczyk, M., Pech, M., Zemojtel, T., Yamamoto, H., Mikula, I., Calvaruso, M.-A., van den Brand, M., Richter, R., Fischer, B., Ritz, A., Kossler, N., Thurisch, B., Spoerle, R., Smeitink, J., Kornak, U., Chan, D., Vingron, M., Martasek, P., Lightowlers, R. N., … Mundlos, S. (2011). NOA1 is an essential GTPase required for mitochondrial protein synthesis. *Molecular Biology of the Cell*, *22*(1), 1–11. https://doi.org/10.1091/mbc.e10-07-0643

Larkin, K., & Byrd, J. C. (2015). Antagonizing ClpP: A New Power Play in Targeted Therapy for AML. *Cancer Cell*, *27*(6), 747–749. https://doi.org/10.1016/j.ccell.2015.05.013

Lewis, M., & Bell, C. E. (2000). A closer view of the conformation of the Lac repressor bound to operator. *Nature Structural Biology*, *7*(3), 209–214. https://doi.org/10.1038/73317

Lowy, F. D. (1998). *Staphylococcus aureus* Infections. *New England Journal of Medicine*, *339*(8), 520–532. https://doi.org/10.1056/NEJM199808203390806

Luo, B., Ma, Y., Zhou, Y., Zhang, N., & Luo, Y. (2021). Human ClpP protease, a promising therapy target for diseases of mitochondrial dysfunction. *Drug Discovery Today*, *26*(4), 968–981. https://doi.org/10.1016/j.drudis.2021.01.007

Marbach, A., & Bettenbrock, K. (2012). lac operon induction in Escherichia coli: Systematic comparison of IPTG and TMG induction and influence of the transacetylase LacA. *Journal of Biotechnology*, *157*(1), 82–88. https://doi.org/10.1016/j.jbiotec.2011.10.009

Matsumoto, H., Haniu, H., & Komori, N. (2019). *Determination of Protein Molecular Weights on SDS-PAGE* (pp. 101–105). https://doi.org/10.1007/978-1-4939-8793-1_10

Menor-Salván, C. (2019, February). *SDS-PAGE: ELECTROFORESIS EN GEL DE POLIACRILAMIDA*.

Omura, T. (1998). Mitochondria-Targeting Sequence, a Multi-Role Sorting Sequence Recognized at All Steps of Protein Import into Mitochondria. In *JB Review J. Biochem* (Vol. 123). https://academic.oup.com/jb/article/123/6/1010/918115

P, D. N., T, T. E., Zhibiao, F., & L, C. A. (2010). Proteolytic Regulation of Toxin-Antitoxin Systems by ClpPC in Staphylococcus aureus. *Journal of Bacteriology*, *192*(5), 1416–1422. https://doi.org/10.1128/JB.00233-09

Pan, C. Q., Finkel, S. E., Cramton, S. E., Feng, J.-A., Sigman, D. S., & Johnson, R. C. (1996). Variable Structures of Fis-DNA Complexes Determined by Flanking DNA – Protein Contacts. *Journal of Molecular Biology*, *264*(4), 675–695. https://doi.org/10.1006/jmbi.1996.0669

Pryde, K. R., Taanman, J. W., & Schapira, A. H. (2016). A LON-ClpP Proteolytic Axis Degrades Complex I to Extinguish ROS Production in Depolarized Mitochondria. *Cell Reports*, *17*(10), 2522–2531. https://doi.org/10.1016/j.celrep.2016.11.027

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., & Séraphin, B. (2001). The Tandem Affinity Purification (TAP) Method: A General Procedure of Protein Complex Purification. *Methods*, *24*(3), 218–229. https://doi.org/10.1006/meth.2001.1183

Ryan, B. J., & Henehan, G. T. (2017). *Avoiding Proteolysis During Protein Purification* (pp. 53–69). https://doi.org/10.1007/978-1-4939-6412-3_4

Schmidt, T. G., & Skerra, A. (2007). The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nature Protocols*, *2*(6), 1528–1535. https://doi.org/10.1038/nprot.2007.209

Seo, J. H., Rivadeneira, D. B., Caino, M. C., Chae, Y. C., Speicher, D. W., Tang, H.-Y., Vaira, V., Bosari, S., Palleschi, A., Rampini, P., Kossenkov, A. v., Languino, L. R., & Altieri, D. C. (2016). The Mitochondrial Unfoldase-Peptidase Complex ClpXP Controls Bioenergetics Stress and Metastasis. *PLOS Biology*, *14*(7), e1002507. https://doi.org/10.1371/journal.pbio.1002507

Stahl, M., Korotkov, V. S., Balogh, D., Kick, L. M., Gersch, M., Pahl, A., Kielkowski, P., Richter, K., Schneider, S., & Sieber, S. A. (2018). Selective Activation of Human Caseinolytic Protease P (ClpP). *Angewandte Chemie International Edition*, *57*(44), 14602–14607. https://doi.org/10.1002/anie.201808189

T, B. K., Angelika, G., G, M. R., Louise, T., Andreas, P., Wilhelm, P., & Dorte, F. (2014). β-Lactam Resistance in Methicillin-Resistant Staphylococcus aureus USA300 Is Increased by Inactivation of the ClpXP Protease. *Antimicrobial Agents and Chemotherapy*, *58*(8), 4593–4603. https://doi.org/10.1128/AAC.02802-14

Valera-Alberni, M., & Canto, C. (2018). Mitochondrial stress management: a dynamic journey. *Cell Stress*, *2*(10), 253–274. https://doi.org/10.15698/cst2018.10.158

Voos, W. (2013). Chaperone–protease networks in mitochondrial protein homeostasis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1833*(2), 388–399. https://doi.org/10.1016/j.bbamcr.2012.06.005

William Studier, F., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990). *[6] Use of T7 RNA polymerase to direct expression of cloned genes* (pp. 60–89). https://doi.org/10.1016/0076-6879(90)85008-C

Wong, K. S., Mabanglo, M. F., Seraphim, T. v., Mollica, A., Mao, Y.-Q., Rizzolo, K., Leung, E., Moutaoufik, M. T., Hoell, L., Phanse, S., Goodreid, J., Barbosa, L. R. S., Ramos, C. H. I., Babu, M., Mennella, V., Batey, R. A., Schimmer, A. D., & Houry, W. A. (2018). Acyldepsipeptide Analogs Dysregulate Human Mitochondrial ClpP Protease Activity and Cause Apoptotic Cell Death. *Cell Chemical Biology*, *25*(8), 1017-1030.e9. https://doi.org/10.1016/j.chembiol.2018.05.014

Yvonne Thoma. (2022). *Personal Communication*.

Zong, W.-X., Rabinowitz, J. D., & White, E. (2016). Mitochondria and Cancer. *Molecular Cell*, *61*(5), 667–676. https://doi.org/10.1016/j.molcel.2016.02.011