Arab American University
Faculty of Graduate Studies
Department of Health Sciences
Master Program in Molecular and Cellular Biosciences

Biophysical Characterization of Phospholipase C (PlcB) in Pseudomonas aeruginosa

Asmaa Yousef Saeed Kmail
202020368

Supervision Committee:
Prof. Hilal Zaid
Prof. Jörg Labahn
Dr. Siba Ismael
Dr. Ramzi Shawahna

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Thesis Approval

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This thesis was defended successfully on 05.02.2025 and approved by:

Thesis Committee Members:

Name	Title	Signature
1. Prof. Hilal Zaid	Main Supervisor	
2. Prof. Jörg Labahn	Co- Supervisor	Jörg Lab
3. Dr. Siba Shahnak	Member of Supervision Committee	Siba
4. Prof. Ramzi Shawahni	Member of Supervision Committee	Ramzi

Palestine, 2/2025

Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is substantially my own work and has not been submitted for any other degree at the Arab American University or any other institution.

Student Name: Asmaa Yousef Saeed Kmail

Student ID: 202020368

Signature:

Date of Submitting the Final Version of the Thesis: 25.05.2025

Dedication

I would like to dedicate my thesis to my parents, who always were there for me. You are the reason where I am now. Thanks to my amazing fiancé Mahmoud, who always supports me, encourages me and always has a good tip for me. Thanks to my sisters, and brother who always have an open ear and listen to me. To my partner in crime, who was with me during the nine months in Germany, Heba, your support and warming words, your care, and every single detail that we both shared together had such a big impact on me until now. Thank you for sharing this hard but amazing time with me. I am grateful that I got to know you during that time and that I gained a new close friend, who I see as an important member of my family. Last but not least, I would like to thank my best friend Dina, who was always a good example to a loyal friend, where even distance didn't change our friendship.

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Student’s name: Asmaa Yousef Saeed Kmail

Prof. Hilal Zaid

Prof. Jörg Labahn

Dr. Siba Shanak

Dr. Ramzi Shawahni

Abstract

Pseudomonas aeruginosa (P. aeruginosa), a bacteria considered by the World Health Organization (WHO) as a high-priority pathogen. It mainly affects people with a weak immune system, especially Cystic Fibrosis (CF) patients, caused by a mutation in the Cystic Fibrosis Transmembrane conductance Regulator gene (CFTR). This leads to the formation of mucous in various organs, plugging the airways and the secretion of enzymes (pancreas). Pseudomonas aeruginosa occupies the mucus in the lungs of CF patients, causing lung infections, which can lead to lung damage and even death. Also, P. aeruginosa is known for its ability to resist many Antibiotics, making treatment hard. Its ability to express virulent factors gives P. aeruginosa an advantage in being virulent. Phospholipases are one of those factors, supporting bacteria’s severity on human beings. They are divided into groups, one of them is Phospholipase C (PlcB). The aim of my project is to study the biophysical characterizations of PlcB. This is achieved by cloning PlcB in a plasmid, then finding the right temperature and expression strain that expresses PlcB in its soluble state. Afterward, purification using Ni-NTA, and checking functionality using an activity assay. Then, starting with biophysical characterizations, opening up many doors allowing understanding its structure. In this study, expression of PlcB in different expression strains and temperatures ended up in inclusion bodies formation. Different expression conditions were tried to overcome this problem, like the co-expression of PlcB with its chaperone, lowering the temperature or the concentration of used inducer. In conclusion, I could isolate and purify PlcB using the denaturation-refolding process that was done using urea. SDS PAGE showed that no impurities were isolated during purification. Activity was tested by a kit, confirming protein’s activity. This will allow proceeding to the step of structural analysis. Further experiments must be done to solve the structure of PlcB, by applying experiments that belong to biophysical characterization like Nuclear Magnetic Resonance (NMR), Circular dichroism (CD) and X-ray crystallography. This research was done in Hamburg-Germany, from October 2021 to June 2022.

Keywords: Pseudomonas aeruginosa, cystic fibrosis, X-Ray crystallography

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List of Definitions of Abbreviations

Abbreviations	Title
CD	Circular dichroism
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
Cryo-EM	Cryo-electron microscopy
DAG	Diacylglycerol
EOP	Environmental Organic Pollutants
EPS	Extracellular Polymeric Substances
ER	Endoplasmic Reticulum
e-DNA	Extracellular DNA
FDA	Food and Drug Administration
HIV	Human Immunodeficiency Virus
ICU	Intensive Care Unit
IDP	Intrinsically Disordered Proteins
IL	Interleukine
IPC	In-Process Control
IP3	Inositol triphosphate
IPMK	Inositol Polyphosphate Multikinase
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
NMR	Nuclear Magnetic Resonance
PDB	Protein Data Base
PKC	Protein kinase C
PLC	Phospholipase C
PI	Phosphatidylinositol
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatididylethanolamine

PH	Pleckstrin Homology
QS	Quorum Sensing
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SM	Sphingomyelin
TAT	Twin-arginine translocation system
TIM	Triosephosphate isomerase
TRPC	Transient Receptor Potential Canonical
WHO	World Health Organization

Chapter One: Introduction

The main goal of this study is to analyze and understand the three dimensional structure of an enzyme called PlcB, expressed by a virulent bacteria called Pseudomonas aeruginosa, especially virulent to people with a weak immune system, where its infections can lead to chronic lung damage and can even be lethal, leading to death. Understanding the three dimensional structure will be done by applying biophysical characterization. In order to reach this point, the protein must be purified and isolated in its pure state. Then, the final structure can be analyzed by applying X-ray crystallography and other different techniques like NMR and cryo-EM etc. There is no single research gap that we can mention here, because they are many gaps, since it is a novel protein with much lack of research and previous experiments. Any new outcome should be able to fill a gap.

Importance of the study:

P. areuginosa can be very harmful and even lethal for people with a weak immune system. Mostly affected and threated by its infections are people with CF. One of the main reasons why P. aeruginosa is so virulent is its ability to express various different exogenous (like phospholipases) and endogenous (like Fimbriae and pilli) virulent factors. In order to save those patients from severe infections, increasing their expected life-rate and turning their life into a more normal life, studying those virulent factors and their mechanism of action is a big must. The studied protein PlcB is part of P. aeruginosa’s expressed virulent factors. Since it is a protein, understanding the three dimensional structure of it will lead to a proper understanding of its mechanism of action, opening many doors toward treatment by for example designing drugs inhibiting its virulent function.

Research Problem:

The main problem is the structure of PlcB is not solved yet. Also this enzyme has been lately discovered, so there are not many papers and studies done on this particular protein.

Aim of the study:

The aim of this study was to clone, express and purify PlcB in order to be able to apply different biophysical characterizations. As a result of applying different biophysical characterizations, new doors toward finding out the specific three dimensional structure of PlcB will be opened. This big finding is extremely important because it will help scientists to gain a better understanding of PlcB's function and action of mechanism, making the step of drug-design more doable and possible.

Research Questions:

The main research question of this study is what is the three dimensional structure of PlcB. This question came up because of the importance of understanding the three dimensional structure of proteins in order to be able to understand the function, since in general understanding the structure and functions helps designing drugs against protein, decreasing or even vanishing the virulent effect caused by such virulent proteins. The sub-questions are whether PlcB has similarity and shared domains or motifs with other zinc ion binding phospholipases expressed by other different bacterial strains. In addition to that, what are the amino acids at the binding interface of the protein and how are they involved in its mechanism of action.

Research Hypothesis:

The research hypothesis is that the expressed phospholipase PlcB is a soluble protein that has some structural similarity with other zinc-dependent phospholipases.

Research Limitations:

As in every research, limitations are a major part of the journey. This research had also limitations that I had to deal with, that sometimes made everything more difficult and confusing. One of the limitations I faced from the beginning was that this phospholipase is considered as a novel discovered protein, that lacks enough previous research and literature review that I could have used and that could have helped me at some point. This made it all a little bit more challenging and tricky while working in the lab. Another very important critical point was the limitation with the time I had available in Germany, since this protein needed many different conditions during expression that had to be tried and changed, especially because with every new expression-experiment, a new bacterial culture must be started which takes time, so it was impossible to apply biophysical characterizations during this short period of time. In conclusion, this type of research, especially this type of proteins that are not easily expressed by bacteria expression strains needs more time than only nine months in order to complete the project with strong and marvelous results and findings.

Keywords:

Cystic Firbrosis: a common genetic disease caused by a mutations in the CFTR gene on chromosome number seven. Different organs like the airways, pancreas, male genital system, intestine, liver, bone, and kidney are involved. The lack of CFTR causes fat malabsorption and chronic pulmonary infections leading to lung damage (Castellani et al., 2017).

Pseudomonas aeruginosa: gram-negative bacilli of the genus Pseudomonas, which are common inhabitants of different environments like soil, fresh water, and marine environments. Attention was more received since it is also an opportunist pathogen, causing severe human diseases (Michel-Briand et al., 2002).

Antibiotic-resistant: infections caused by P. aeruginosa are hard to treat because of the presence of its resistance to many different antibiotics (β-lactam and penem group of antibiotics). The mechanism used for its survival is mainly biofilm formation, quorum sensing, horizontal gene transfer and enzyme promiscuity, giving it the perfect combination to resist strong antibiotics (Pachori et al., 2019).

Phospholipase C: many different pathogenic bacteria produce phospholipases C and since the discovery in 1944 that a bacterial toxin (Clostridium perfringens alpha-toxin) possessed an enzymatic activity, there has been considerable interest in this specific class of proteins. The toxic phospholipases C can interact with eukaryotic cell membranes, causing cell lysis (Titball et al., 1993).

Biophysical characterization: applying different methods like X-ray, NMR, cryo-EM, in order to understand the three dimensional structure of proteins, making the mechanism of protein-based therapy and drug design possible (Kamerzell et al., 2011).

Chapter Two: Literature Review

4.1 Pseudomonas aeruginosa, general informations and characteristics:

Pseudomonas aeruginosa is a gram-negative, rod-shaped, aerobic, nosocomial bacteria that belongs to the class γ-proteobacteria and Pseudomonadaceae family. There is no single specific location where you can miss finding P. aeruginosa, in other words, P. aeruginosa is found almost everywhere and anyplace at anytime, starting from aquatic to terrestrial locations, cold to hot environments and different hosts, ranging from plants to mammals. P. aeruginosa is seldom considered as a member of the normal microbial flora in humans. Rate of colonization for specific sites in humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat, and 2.6 to 24% for fecal samples (Lister et al., 2009). One of the interesting properties found in P. aeruginosa is the ability to adapt to new environmental conditions and other environmental challenges, which is due to the enormous large genomic composition found in P. aeruginosa with around 6000 genes, making P.aeruginosa an extremely flexible bacteria for its own advantage (Thi et al., 2020 and Kunz et al., 2022). Pseudomonas aeruginosa is not considered as a harmful bacteria to people with a normal and stable immune system, but is considered as harmful and maybe even lethal for people with a weak immune system, like people who suffer from cancer, Human Immunodeficiency Virus (HIV), infections, burn wounds, surgical wounds and diabetic ulcers, as well as CF patients which are categorized as people with an extremely high risk of getting an infection by P. aeruginosa. The first person who referenced P. aeruginosa is a French physician called Charles-Emmanuel Sédillot, who noticed a green-bluish substance (aeruginosa) on surgical dressings, that comes due to the production of two substances called pyocyanin and 2-aminoacetophenone with a sweet grape odor, which did lead to this specific naming of this bacteria (Rosales-Reyes et al., 2021). Pseudomonas aeruginosa could be finally successfully isolated in pure culture in 1882, when Carle Gessard showed in a publication entitled "On the Blue and Green Coloration of Bandages" the growth of the organism from cutaneous wounds of two patients with bluish-green pus (Lister et al., 2009). The pathogen’s ability to evade both the innate and adaptive immune system gave it the ability to affect immunosuppressive patients in several different ways, like biofilm formation and producing a wide variety of different exogenous and endogenous virulent factors, including phospholipases. It is estimated that the mortality rate of P. aeruginosa infections can exceed to 300,000 worldwide each year (M. À. Sastre-Femenia et al., 2023). According to the WHO, P. aeruginosa is one of the most life-threatening bacteria and is counted as a high-priority or even critical pathogen for Research and Development of antibiotics (Thi et al., 2020 and Tuon et al., 2022). A study showed that 16.2% of Intensive Care Unit (ICU) patients acquired a P. aeruginosa infection, considering it as a high percentage, which shows the need and importance of studying and understanding its infection mechanisms and treatment in a most effective way (Reynolds et al., 2021). Pseudomonas aeruginosa was also the second leading cause of nosocomial pneumonia (14 to 16%), third most common cause of urinary tract infections (7 to 11%), fourth most frequently isolated pathogen in surgical site infections (8%), and seventh leading contributor to bloodstream infections (2 to 6%). Data from more recent studies continue to show P. aeruginosa as the second most common cause of nosocomial pneumonia, health care-associated pneumonia, and ventilator-associated pneumonia (Lister et al., 2009). What makes P. aeruginosa as critical as described by the WHO, is the capacity to be resistant to an extensive wide range of different antibiotics, which is achieved by multiple different mechanisms. There are mainly two different mechanisms. The first mechanism is called the intrinsic resistance, consisting of high restricted membrane permeability (12-100 times lower than Escherichia coli), the presence of antibiotics efflux systems and the production of endogenous antibiotic-inactivating enzymes. The second mechanism is called the acquired resistance mechanism, which results from horizontal gene transfer or mutations resulting in high expression of efflux pumps or β lactamase, which is an enzyme produced by bacteria in order to hydrolyze antibiotics that hold the specific β-lactam ring, deactivating the antibiotics’ molecular properties (Wood et al., 2023). Despite all the various negative impacts resulted by P. aeruginosa, it was found that Pseudomonas species have a positive impact on the environment by decomposing and degrading toxic materials like pesticides, metals, plastics, dyes and Environmental Organic Pollutants (EOP). This is accomplished by the secretion of biosurfactants, which are natural compounds that are produced by microbes, facilitating the degradation of hydrophobic EOP, by making them more bioavailable. Accumulation of those compounds in our food leads to cancer and mutagenesis in both animals and plants, so decomposing those compounds is an important step for maintaining our planet, environment and animals' health (Asiandu et al., 2012). One of the most critical challenges that our world is still facing until now is the accumulation of plastic, especially because it is a very cheap and durable synthetic polymer that takes thousands and thousands of years to degrade and vanish. In addition to that, it is used worldwide almost everywhere in an extremely extensive way in many different fields like industrial and medical fields. A solution would be to find a way that gets rid of plastic, but mostly important in an environmentally friendly way. During the past few years, several different ways have been invented and tried in order to get rid of plastics. It was discovered that several different bacterial species and strains have the ability to break down plastics, by releasing both intracellular and extracellular enzymes that break down plastics, without being toxic to the environment, by releasing microbial biomass and gases. One of the effective plastic-degrading species are Pseudomonas species. It has been reported that the effectiveness to degrade reaches a total of 35 different types of plastics, including most of the mass-produced polymers. In general, Pseudomonads are considered to be adaptable, versatile, and ubiquitous in the environment (Gambarini et al., 2021). This is accomplished by four main steps: biodeterioration, depolymerization, assimilation and mineralization. Biodegradation is the cooperation of bacteria with other abiotic substances, breaking down plastic into smaller pieces. The formation of biofilms accelerates the process of breaking down plastic polymer by the production of Extracellular Polymeric Substances (EPS), that help breaking down plastic surfaces. Depolymerization includes extracellular and intracellular enzymes that break down the polymer complex into much smaller molecules, that will be dissolved in water easily and further absorbed by microbial semipermeable membranes and also used as energy and a source of Carbon. Assimilation is the process of entering small, broken molecules into the cytoplasm of the microbe, producing energy. The last metabolic way is Mineralization, which is achieved by converting biodegradable biomass into minerals, salt, water, and gases. The released gases include nitrogen components and methane. Mineralization is ended when all biodegradable compounds have been consumed by the microorganisms and all carbons are converted to carbon dioxide (Barker et al., 2004).

2.2 Pseudomonas aeruginosa and Cystic fibrosis:

Cystic fibrosis is a complex autosomal recessive genetic disease that is located on chromosome number 7 on the long arm of the chromosome (7q31.2). The expressed protein is a 170 kilodalton (kDa) large protein with 1480 Amino acids, organized into 27 exons. They are many different variants of this mutation, over 1600 mutations of the CFTR gene have been described over the past years. The most common mutation is the deletion of phenylalanine at codon number 508 (phe508del, until recently known as ΔF508), which occurs in about 70% of patients with CF. Different mutations result in different phenotypes of this disease. Some mutations result in a milder forms of this disease, but the problem is that there is not much enough evidence about these rarer mutations to counsel and inform the patients about their condition. This genetic disease affects Caucasians more than any other ethnic groups, like African, Asian or Hispanic groups. The risk of having an affected child with CF in Caucasians is one in 2500 to 3500 children, while it is in 4000 to 10’000 children in Hispanics (Li et al., 2023). Cystic fibrosis is caused by a point mutation in the CFTR gene. A transmembrane conductance regulator is a chloride and bicarbonate anion transport channel that contributes to the absorption and secretion of ions across epithelial surfaces in the body. In summary, the primary function of the CFTR protein is the regulation of liquid volume on epithelial surfaces through chloride secretion and inhibition of sodium absorption. Reduced or loss of function leads to obstructed exocrine glands. This mutation does not affect only one organ but many other different organs, making it a multiorgan disease. For example: the lungs, where defective CFTR disturbs the transport of chloride and sodium ions, NCO_3 and water across the cell membrane of the lungs' airways. This distribution leads to the formation of sticky, thick and dehydrated mucus that clogs the airways, leading to the formation of bacterial infections, inflammations, breathing problems and lowers the function of the lungs. Pseudomonas aeruginosa's lung infections in cystic fibrosis patients are a critical causative agent for patient mortality and death. It was found that P. aeruginosa is the most frequent and the most long-lasting bacteria that occupies CF patients' lungs. The formation of chronic infections is a critical point since chronic infections can develop in up to 70% of CF patients. Infections start by the adhesion of P. aeruginosa to the epithelial cells of the lungs, by the binding of the adhesins (mainly by flagella) with the host's receptors leading to structural changes in the lungs, which can develop until reaching the last station-death. One of the most critical points resulted by P. aeruginosa's infections is the ability to form biofilms, which fastens death by reducing the proper function of the lungs. Another highly affected organ is the pancreas since the accumulation of mucus prevents the secretion of digestive enzymes, which can end up in Malnutrition. In addition to that, CF patients suffer from steatorrhea, which is due to the accumulation of mucus in the pancreas, so the digestive enzymes don't reach the small intestine, preventing proteins and lipids from being digested and broken down, leading to steatorrhea. Therefore, both early detection and treatment became major steps in health care (Reynolds et al., 2021, Moreno et al., 2021). The most trusted and available diagnostic test for CF is the sweat test. This test works by measuring the chloride concentration of the sweat. The concept of that test is that sweat is induced by pilocarpine iontophoresis, which is using a mild electrical current in order to allow pilocarpine (drug) to stimulate the sweat glands by activating muscarinic receptors, causing them to produce sweat, which is collected on a gauze. Normally, functional CFTR reabsorbs the chloride, making the sweat hypotonic. In contrast to that, CF patients have a defected CFTR, so re-absorption of the chloride will not occur, resulting in a high-concentrated chloride in the sweat (De Boeck et al., 2017). Mutations and changes in the genetic material allow P. aeruginosa to survive in the anaerobic environment of CF patients.

Treatment can be achieved by a combination of two intravenous antibiotics with activity against P. aeruginosa, weakening different bacterial strains found in P. aeruginosa affected patients in combination with P. aeruginosa, being the most important pathogen (Reynolds et al., 2021). Due to the development and revolution in knowledge, science and technology, the survival rates of CF patients increased largely. This is approved by a study published in 2021, which showed that the median-predicted survival time reached 65.6 years. (Li et al., 2023, Ong et al., 2023). One of the newest treatments is CFTR modulators, which promote the opening of the channel, like ivacaftor or correctors, such as lumacaftor. Due to the accumulation of sticky mucous affecting proper breathing and lung function, some different treatments are being introduced to CF patients in order to try to clear their lungs from the mucus, fluidize mucosal secretions and improve mucociliary clearance. One of those important treatments is physiotherapy, which should be applied as soon as possible, especially in children. It is mainly achieved by vibration, which consists of rapid compressions applied on the chest wall during expiration or the clapping technique, which involves rhythmically clapping on the chest wall over the area being drained to force secretions into larger airways expectoration. In addition to that, there are dry powders that can be inhaled, like mannitol (Bronchitol®), a naturally occurring polyalcohol, which is a way to treat airway clearance. Its exact mechanism is unknown, but it is thought that it has an osmotic effect, increasing the hydration of the lungs’ surface (Li et al., 2023). Antibiotics can be given systemic (intravenous, oral) or as inhaled substances. Systemic treatment needs a high drug concentration in order to penetrate the formed biofilms, leading to toxicity. Due to that, using inhaled antibiotics, which give a more rapid response and have a reduced systemic toxicity is more favorable than systemic antibiotics and is considered as a standard therapy. An example of two inhaled antibiotics that have been approved by the Food and Drug Administration (FDA) are tobramycin and aztreonam. Tobramycin inhibits bacterial protein synthesis, reduces the density of P. aeruginosa and reduces the rate of hospitalization. On the other hand, Aztreonam is a synthetic beta-lactam that changes the synthesis of the cell wall by blocking peptidoglycan crosslinking. As supposed by anyone who suffers from a disease -whatever kind of disease- a balanced, nutrient-rich food uptake should be standard in order to maintain a healthy, stronger immune response and support the body’s immune system in a right way. Unfortunately, the worst-case scenario would be the need for a lung transplantation, so regular monitoring by planning regular doctoral visits is required. Newer approaches such as gene and small molecule based treatments may have more capacity to stop the disease from further progression. (Vetrivel et al., 2021, Davies et al., 2007).

2.3 Pseudomonas and its virulent factor, Biofilms:

Pseudomonas aeruginosa is known for its antibiotic resistance, making drug development extremely tricky and complex. Biofilm formation is one of the produced virulent factors that counts as one of the reasons how and why P. aeruginosa is extremely resistant to antibiotics. Its ability to form Biofilms by colonization due to cell-to-cell communications allows it to withstand harsh conditions like nutrient deficiency and changes in the external environment (Figure 2.1). Biofilms mainly consist of polysaccharides, extracellular DNA, lipids and proteins (Thi et al., 2020). There are several reasons why antibiotics fail to work properly against biofilms. One of the reasons is the poor response of antibiotics, due to the lack of oxygen in the biofilms, making the effect of antibiotics that function under an aerobic atmosphere almost not effective. Another reason is the lack of Reactive Oxygen Species (ROS) production in the anaerobic biofilm environment since reactive oxygen species count as one of the molecules that can kill bacteria by the formation of Hydroxyl radicals (• OH) (Billón et al., 2023). Also, the mechanism of drug penetration in biofilms is more difficult and complex than when dealing with bacteria that do not produce biofilms, because in bacteria that produce biofilms, the concentration of the proper antibiotic must be increased to be able to solve the problem with penetration. As a result of this increased antibiotic concentration, systemic toxicity can occur. A study showed that the addition of lubricant consisting of perfluorinated liquids prevented the formation of biofilms (Yin et al., 2022). Another way to prevent Biofilm formation is coating surfaces with antimicrobial surface-coating that would prevent the formation and adhesion of Pseudomonas aeruginosa, thus preventing the formation of Biofilms (Yin et al., 2022). Biofilm components, called EPS, are the substances in the biofilm which give P. aeruginosa a strong protection against both chemical and physical stresses, whether they come from the host itself, or the given antibiotics. Biofilms produced by P. aeruginosa consist of different 8 main components: Alginate, pel, psl, e-DNA, mucin, Oxygen, water and ions. Alginate is an unbranched, negatively-charged polymerized polysaccharide with high molecular weight, that gives stability and protection to the three-dimensional structure of the Biofilm, while pel and psl are polysaccharides that function as main, primary and structural-scaffold. Alginate, pel and psl are all synthesized by a second messenger called bis-(3-5)-cyclic dimeric guanosine monophosphate (c-di-GMP), so increased activation of c-di-GMP increases biofilm production. In contrast, decreasing the activation increases the motility. In addition to that, e-DNA (extracellular DNA) is an anionic, double helix that is a nutrient source for embedded bacteria, controls the size of P. aeruginosa colonies, prepares the surface before biofilm formation and inactivates antibiotics by acidification and chelating. Mucin is a high molecular weight, polymeric glycoprotein that is the most important protein in the mucus. It contains a high amount of water and it reduces drug activity due to interactions. Oxygen concentration in Biofilms is low, reducing the antibacterial activity of antibiotics, since P. aeruginosa mostly grows under hypoxia conditions. Heterogenous ions form an osmolarity gradient in cystic fibrosis lungs, from high concentration in the sputum to low inside biofilm, affecting the viscosity of sputum (Guillaume et al., 2022, Hu et al., 2023). An important intercellular signaling system found in various bacteria, both gram-positive and gram-negative (including P. aeruginosa) is called Quorum sensing (QS). It is introduced as a mechanism that prevents cell density from reaching a threshold level, in order to control the density of the population. Small molecules, called autoinducers, bind to specific receptors, which causes a control in the expression of specific genes, leading to an increase or decrease in the size of the formed Biofilm. A study made in 2019 showed that alginate lysate (AlyP1400), a group of alginolytic enzymes purified and isolated from marine bacteria Pseudoalteromonas species, decreased the P.

aeruginosa biofilms after just 24 hours of incubation by 69% and could serve as a specific therapeutic strategy when used in combination with conventional antibiotics. More advanced and optimal methods and techniques are required in order to find an effective and efficient antibiofilm agent that treats P. aeruginosa (Guillaume et al., 2022).

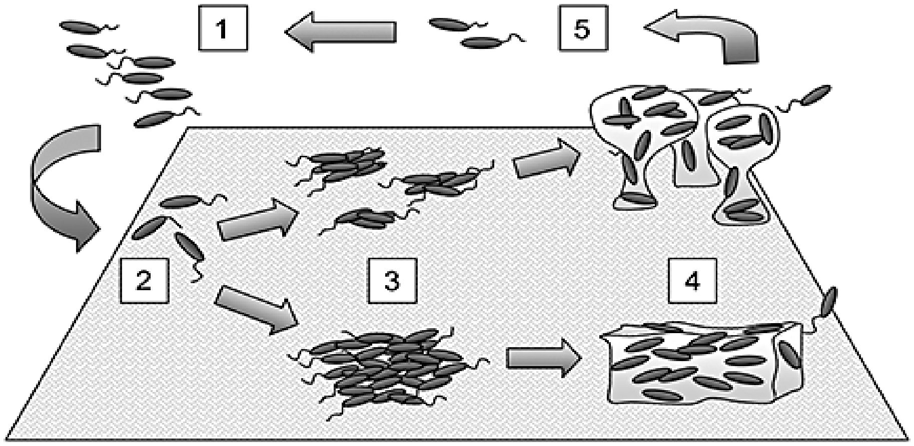

Figure 2.1. Biofilm formation. (Environmental Microbiology, DOI: (10.1111/j.1462-2920.2008.01792.x)

2.4 Pseudomonas aeruginosa and quorum sensing:

Quorum sensing is an important mechanism for P. aeruginosa. The importance of QS and understanding its proper mechanism of actiongoes back to the idea that it plays a critical and important role in the capability of virulent bacteria in inducing the formation of several different virulence factors, starting from biofilm formation until the production of several various virulent proteins like phospholipases. At low bacterial concentration, the concentration of the auto-inducers is low in the environment, furthermore they don’t bind to their specific receptors, so as a result of that, QS-controlled genes remain suppressed and expression does not occur. In contrast, when the bacterial cell density is high, the concentration of the autoinducers increases until a specific reached threshold, which will lead to the binding to special receptors, activating the transcription translation mechanism of specific genes that are responsible for the production of those virulent factors, supporting P. aeruginosa's virulent effect on humans. One of the virulent factors that QS controls is the production of several virulent factors that allow P. aeruginosa to cause tissue damage especially in the lungs of CF patients, evading the immune system, like the production of proteases and exotoxins etc. P. aeruginosa counts as one of the bacteria that is known for Biofilm formation, this is mainly regulated by QS, making P. aeruginosa and other biofilm-producing bacteria extremely Antibiotic-resistant, making treatment harder than bacteria that do not have the capacity to form biofilms. Not only does QS regulate the formation of biofilm, but also plays a key role in the expression of efflux pumps that get rid of antibiotics, making P. aeruginosa more and more antibiotic resistant and the step of treatment even harder. Since QS is the reason for the production of virulent factors, biofilm formation and antibiotic resistance, researchers started to see during the past few years that targeting QS is a good potential therapeutic strategy. Because of that finding, researchers started to explore different QS inhibitors that interact and suppress the signaling pathways involved in the QS, as a result preventing QS from initiating the production of the different virulent factors (Pseci et al., 1997).

2.5 Phospholipids, Phospholipases and Phospholipase C:

Phospholipids are important cellular components that isolate the internal environment from the external environment along with glycolipids and cholesterol. Phospholipids do not just play an important role as isolators for the cell but also play an important role in autophagy, cell division, apoptosis and serve as second messengers. Structural variations allow phospholipids to have different functions in the cell membrane and cell signaling. Phospholipids include phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, phosphatidylcholine, phosphoinositides, phosphatidic acid and sphingomyelin. The two main and critical components of phospholipids are phosphatidylcholine and phosphatidylethanolamine. Any changes in them can lead to the development of cancer, which makes understanding its exact mechanism something extremely important (Balboa et al., 2021, Singh et al., 2023, Stoica et al., 2022). In general, the popularity of bacterial enzymes relies on their enormous huge variety, like their thermal stability, large substrate specificity and their high specific activity (Constantino-Teles et al., 2022). Some bacteria, including Pseudomonas aeruginosa, have the ability to express a type of enzymes that are categorized as virulent factors that target those phospholipids, those enzymes are called phospholipases, which are defined as the enzymes that hydrolyze ester bonds in phospholipids, producing byproducts with different effects like cell signaling and cell proliferation. Some phospholipases are very specific, targeting a specific substrate, while others are less specific, targeting multiple different substrates. Phospholipases play a critical role in inducing infection, targeting tissues and promoting bacterial colonization. There are different groups of phospholipases including PLA, PLB, PLC and PLD, each of those groups has a different site of cleavage on the phospholipid (Figure 2.2).

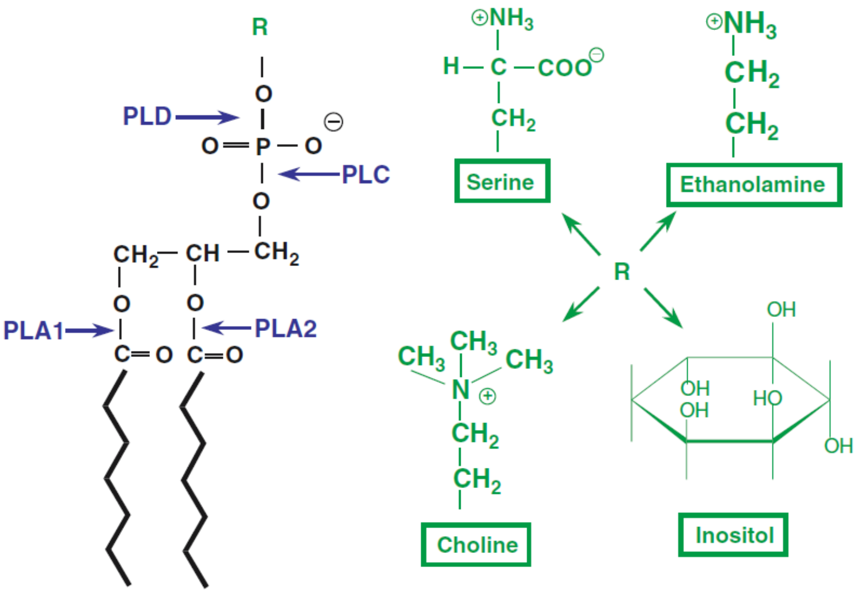

Figure 2.2. Site of cleavage of PLC, PLD, PLA1 and PLA2 (Vasil et al., 2006).

2.6 Mechanism of action in bacterial and mammalian Phospholipase C:

The difference between mammalian and bacterial PLC is that mammalian PLC works for the cell by serving the cell rather than to harm, since it is important for the action of normal cellular functions. In contrast to bacterial PLC, which is considered to be virulent, by breaking down the phospholipid bilayer, resulting in cell lysis. In addition to breaking down the phospholipid bilayer, bacterial phospholipase C also interact with host's immune system, triggering the release of cytokines like IL-8, TNF-α that cause inflammation, supporting tissue damage. To summarize the mechanism of mammalian PLC, PLC hydrolyze ester bond(s) between the glycerol backbone and the phosphate group, producing two second messengers: Diacylglycerol (DAG) and Inositol triphosphate (IP3). Diacylglycerol regulates the activation of Protein Kinase C (PKC), which phosphorylates proteins involved in cell growth, differentiation, and gene expression. Whereas, Inositol triphosphate causes the release of Calcium ions from the endoplasmic reticulum into the cytosol, which causes the activation of various calcium-dependent enzymes and processes. Bacterial phospholipases can activate pathways similar to those in mammalian cells, including the activation of PKC through the production of diacylglycerol. This activation leads to increased phospholipase activity and the secretion of matrix metalloproteinases, resulting in tissue destruction. Additionally, phospholipases can stimulate the arachidonic acid cascade, producing eicosanoids like prostaglandins and leukotrienes that modulate inflammation and vascular responses (Figure 2.3).

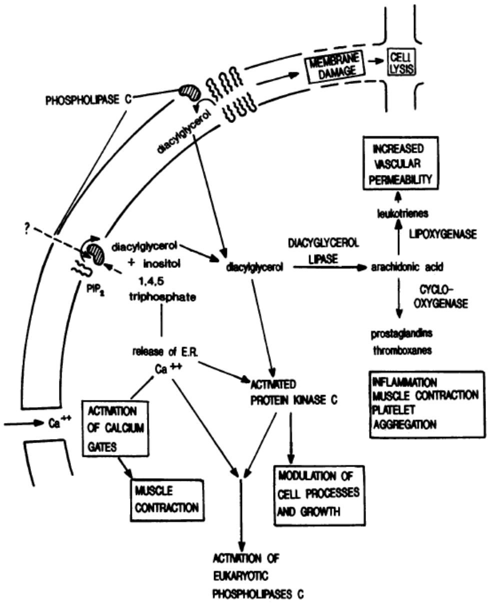

Figure 2.3. Effect of bacterial PLC on Eukaryotic cells (Titball et al., 1993).

In summary, both bacterial and mammalian phospholipases break down phospholipids, with the difference of having a different effect on the cell. Bacterial phospholipases act as pathogenic virulent factors. In contrast, mammalian phospholipases are not pathogenic and virulent, but vital for the cells (Schmiel et al., 1999). Pseudomonas aeruginosa expresses three extracellular PLCs: PlcH, which is a hemolytic phospholipase, PlcN, a non-hemolytic phospholipase and PlcB. Bacteria have two cytoplasmic secretory systems, both of them secrete proteins from the cytoplasm of the bacteria to the periplasm. The first secretory system is called the Sec secretory system, which is the general secretory system that secretes proteins in their unfolded state, while the TAT, which secretes the proteins in their native, folded state. Analysis of TAT mutant led to the discovery of a novel, undiscovered phospholipase called PlcB. PlcH and PlcN are both secreted by the twin-arginine translocase secretory system, while PlcB is secreted by the Sec secretory system. What specifies PlcB from other Phospholipases in Pseudomonas aeruginosa is that they can all hydrolyze Phosphatidylcholine (PC), while only PlcB can hydrolyze

Phosphatididylethanolamine (PE) (Stoica et al., 2022, Balboa et al., 2021, Singh et al., 2023). Another special property found in PlcB, but not in PlcH and PlcN is the ability of PlcB to twitch motility toward specific phospholipids. This is called chemotaxis, which shows the specificity of PlcB toward the specific phospholipids. PE is found to be the first phospholipid that has a chemoattractant property for a prokaryotic organism. Cystic fibrosis patients have an increased level of PE in the bronchoalveolar lavage fluid compared to control groups without cystic fibrosis, so it is highly possible that this increased levels of phospholipids could serve as a chemoattractant supporting chemokinesis of Pseudomonas aeruginosa. It is thought that DAG generated by PlcB is directly integrated into the membrane of P. aeruginosa and affects the activity of a membrane protein that plays an important role in the signaling processes needed for chemotaxis. PlcB expressed by the PA0026 gene has shown to have limited but important similarity over a short region to a class of zinc-dependent prokaryotic PLCs (Obeng et al., 2020, Li et al., 2022). There are two important adjacent genes to the PlcB gene (PA0026). The first is called the PA0027 gene, which is a rotamase (i.e. peptidyl prolyl cis-trans isomerase) with a TAT signal sequence, indicating that it might be secreted through the TAT secretion system. The second gene is called the PA0028, which has a signal sequence that is also constantly found in lipoproteins. Novel studies showed through mutagenesis that those two genes are important due to their role in supporting the secretion process of the PlcB protein. With regards to PlcB and its homologs in other bacteria, like C. perfringens, it seems like they have evolved from a common ancestor. Even though there is some kind of divergence, it is clearly known that certain amino acids and structural features important for metal binding and catalytic activity are pretty much conserved. An interesting point is the fact that outside the catalytic region of PlcB, there is no homology with any other protein found in the NCBI database, even in the organisms that are very close to P. aeruginosa, like for example Pseudomonas fluorescens (Barker et al., 2004). In contrast to PlcN and PlcB, PlcH has been studied in a more detailed way. The main reason for calling it a haemolytic phospholipase is for its ability to lyse red blood cells and showing haemolytic activity. This phospholipase hydrolyses Phosphatidylcholine and Sphingomyelin, which results in the formation of diacylglycerol and ceramide, which are both involved in signal transduction cascades that can lead to inflammation and even cell death. Both PlcB and PlcN do not have on Sphingomyelin an activity, making it a unique substrate specificity for PlcH. Research on animal models (mice) showed that the hydrolysis of PlcH increased the secretion of chemokines and cytokines like Interleukine-6 (IL-6), IL-1β, and Tumor necrosis factor-α. In addition to that, PlcH is cytotoxic to macrophages and can stop the activity of human neutrophils, supporting bacterial survival in the lungs. The exact role of both PlcN and PlcB in the pathogenesis and disease formation of respiratory infections is still an unsolved puzzle. However what is known for sure is that both PlcN and PlcB play a big role in the formation of Biofilms, which counts as one of the extreme virulent factors and survival strategies for Pseudomonas aeruginosa (Maveyraud et al., 2020).

2.7 PlcB, its effect on host cells and comparison with other phospholipases

Only little is known about the effect of PlcB on host cells, since it is a novel-discovered protein. The main thing known for sure is its ability to form Biofilms, supporting antibiotic resistance of P. aeruginosa against various drugs. Due to the homology of phospholipases, it could be possible that PlcB plays other roles than only Biofilm formation, like targeting host's immune cells and lysing red blood cells, as shown in PlcN and PlcH (Barker et al., 2004). Comparing PlcB with other phospholipases from other bacteria, PlcB produced by Listeria monocytogenes, a gram-positive bacteria has a solved crystal structure (PDB: 2PBL) that hydrolyzes phosphatidylcholine and phosphatidylethanolamine and its pathogenecity is that it allows Listeria monocytogenes to spread between the cells of the host (Marquis et al., 1995). Another phospholipase produced by another bacteria is an alpha toxin called Plc produced by Clostridium perfringens with a solved structure (PDB: 1CA1). It hydrolyzes phosphatidylcholine and sphingomyelin and is an important toxin in gas gangrene, causing cell lysis and tissue destruction (Naylor et al., 1998).

2.8 Metal ions binding sides and Phospholipases with Zinc ion binding sides:

In general, metal ions play a crucial role in keeping proteins functioning properly. Their function is so vital that they are even called the “life metals”. Those metals consist of Sodium (Na), potassium (K), Magnesium (Mg), Calcium (Ca), Manganese (Mn), Iron (Fe), Cobalt (Co), Zinc (Zn), Nickel (Ni), Vanadium (V), Molybdenum (Mb) and Tungsten (W). There are three main general functions of metal ions in proteins: enzymatic control, regulatory control and structural control. Not only are metal ions important for enzymatic, regulatory and structural control, but there are also many biological events that rely on metal ions in order for those biological events to happen. A proof that metal ions are extremely important is the fact that a whole quarter of the proteins that can be found in the protein data bank have a metal ion binding side, starting with zinc, iron and magnesium. The most important biological metal ions are divided into two groups, transition and non-transition elements. Transition elements are the elements that have filled electron shells (Mn, Fe, Co, Cu, Mo, W). On the other hand, non-transition elements are elements that do have completely filled electron shells (Na, K, Mg, Ca, Zn). In proteins, Calcium, Magnesium, Sodium and Potassium are always bound to Oxygen, but not Nitrogen or Sulfur ligands, which is an electrostatic interaction without preferential directions. On the other hand, Zinc and transition ion metals interact with nitrogen, oxygen and sulfur ligands, formed in preferential directions because of their partially covalent character (Merkulyeva et al., 2021).

2.9 Biophysical characterizations for studying proteins’ structure:

In order to be able to treat and understand the pathology and pathway of a protein that might be the causative or one of the causative agents of a disease, a proper and precise understanding of how this protein functions is required. This is only doable when we reach a deep understanding of the structure of that protein. Although to reach this one point, biophysical characterizations need to be done, which will give us a way to be able to understand the structure and opens up a door to analyze the probable, predicted structure. There are many different methods that serve us in order to reach a certain and deeper understanding of the structure, like for example: X-ray crystallography, NMR, mass spectrometry, CD, lately cryo-electron microscopy (cryo-EM) and other related methods and techniques. Starting with X-ray crystallography, it is mostly used in drug discovery, which is a great tool in designing a ligand/drug by inhibiting or activating the function of the protein of interest. One of the requirements, in order to be able to perform X-ray crystallography, is the need to transform the protein of interest into a crystal. Highly flexible molecules will be associated with poor electron density and will not be visible in the final model. The occupancy of an atom, which is the measure of the fraction in which the atom occupies this location and is measured in numbers between 0.0 and 1.0. This occupancy can be divided into three types, the first is the occupancy that is seen as the fraction of time the atom spends at this position, the second is the fraction of unit cells in the crystals where the atom is found at this position and third is a combination of both. The interaction of an X-ray beam with a three-dimensional crystal will produce a diffraction pattern where X-ray waves are diffracted in specific directions with specific amplitudes and phases. Those amplitudes and phases represent the molecular structure of the crystallized molecule. The positions of the diffracted spots on the detector, depend on the three vectors that define the periodicity of the crystal, which defines the unit cell of the crystal. Also, the phases are not experimentally measurable and specific phasing methods are needed to recover this information. Molecular replacement will always give phase information if a homologous structure is already available through the Protein Data Bank. In case there is no homologous structure available, then experimental phasing is required, which is a good thing due to the unbiased phase information but will require hard, long and sometimes frustrating experiments. Seeding is the process of introducing crystal fragments or microcrystals into an equilibrated crystallization drop. Crystallization conditions that allow for cooling of the crystal to 100 Kelvin (K) without the need for previous use of cryoprotection will facilitate the crystal harvesting step. It is important to take into consideration that the binding site of the protein should not be blocked by crystal contacts or by another molecule coming from the crystallization solution, due to the possibility of them being able to compete with ligand binding. Soaking experiments are easy since a very small amount of ligand is needed. A single crystal is immersed in a 1 µl drop of a 0.1 to 1 mM solution of ligand, ranging from several seconds up to a few days. One of the limiting factors that could be faced is the solubility of the ligand. Mostly, the binding affinity is not known, so different concentrations of the ligand should be used, ranging from 0.1 to 1 mM and from 20 to 50 mM for high-molecular-weight ligands. It is highly possible that soaking might cause small conformational changes, co-crystallization is used to get over that, especially if large changes are expected upon ligand binding. In the case of co-crystallization, the protein of interest must be conditioned with the ligand right before crystallization. An important point to consider is that the presence of the ligand could change the conditions of crystallization, so it is important to screen new crystallization conditions. A very common thing is that soaking might make the crystal fragile, so to overcome this issue, cross-linking of the crystal can be done right before soaking. Recently, automation of the soaking procedure has been developed, which makes the whole process more time and work-effective, since several hundreds of soaking experiments can be done within just a few minutes (Vedel et al., 2023). A second widely used instrument for studying the protein's three-dimensional structure is NMR, which is not just applied to proteins but also to polysaccharides, since NMR is one of the most essential and useful techniques for the structural identification of polysaccharides. NMR opens the door to analyze proteins that do not have a stable three-dimensional structure, which are called Intrinsically Disordered Proteins (IDPs) (Khalil et al., 2023). In addition to that, NMR is a useful qualitative and quantitative technique. Furthermore, this technique is able to identify impurities, drug substances and residual solvents of In-Process Control (IPC) samples (Kanemaru et al., 2023). Knowing the three dimensional structure by biophysical characterizations is the key toward drug design. Principally, knowing the 3D-structure means knowing the important active sites of the protein, making development of inhibitors that target this site easier. In addition to that, using computational methods like docking by trying different molecules, blocking the active site, shows the importance of knowing the structure or at least having a prediction, since without this all, drug design and inhibiting the protein’s pathogenicity is not possible. For example, specific inhibitors were able to target a virulent factor produced by P. aeruginosa called LasB protease, reducing its pathogenicity and biofilm formation (Cathcart et al., 2011).

2.10 Using pET plasmids for protein expression in bacterial host cells:

The pET series are preferable expression plasmids used in Escherichia coli for recombinant protein production. Due to the extremely high protein expression yield, the recombination of both Escherichia coli and pET vectors has become the most international, favorable efficient and inexpensive expression system. Many recombinant proteins have been produced in Escherichia coli expression systems for academic and clinical uses, which shows the importance and effectiveness served by the combination of pET vectors with E. coli worldwide. Choosing the correct and suitable bacterial expression strain can be very critical and challenging and even confusing. For example, E. coli-K12 strains have the ability to produce acetate as a by-product, which can be harmful to bacterial cell growth and protein expression. Due to that, those strains are more used for propagating recombinant DNA library clones. On the other hand, E. coli B strains produce acetate in a low and harmless amount, which makes them favorable for protein expression. Many of those expression E. coli strains own a polymerase called the T7 RNA polymerase, but because the T7 RNA polymerase leads to leaking, 1% of glucose (w/v) is usually added to the main expression system, in order to prevent leakage. In addition to the critical point of choosing the right host cells for protein expression, the right temperature that should be used in the main culture can be tricky too, since some proteins are not soluble at high temperatures (37°C), trying to reduce the temperature could be the solution. In addition to that, the right concentration of the inducer IPTG and the right induction time must be figured out in order to get the protein of interest in its most soluble and folded form (Shilling et al., 2020). Until 2020, more than 40,000 articles about pET28a were published, showing that pET28a is the most popular expression plasmid used worldwide (Permyakov et al., 2021).

2.11. Avoiding inclusion bodies formation using solubility tags:

One of the limitations during recombinant protein expression in bacterial strains is the formation of inclusion bodies. Inclusion bodies are insoluble protein aggregates that aren’t desired. This formation is an indication that there is a problem with its proper folding, especially an issue when aiming to study a protein’s structure, where it is important to get it as properly folded and functional as possible. Mainly the net charge of a protein and its Hydrophilicity play a major role in protein’s solubility. To overcome this problem, many different strategies can be applied, like the co-expression of a chaperone, which supports protein’s folding or preventing overexpression, by reducing the protein’s yield or the addition of specific tags or small peptide-tags when cloning the desired gene in its plasmid, which are responsible for increasing protein’s solubility, with the probability of being able to overcome the problem of inclusion bodies formation. What strategy to choose is always protein dependent and should never be generalized (Ventura et al., 2006). For example, fusion tags are common used worldwide. One of the very first fusion tags is the parasite-produced glutathione S-transferase, which has been successfully used in the expression of different mammalian proteins in bacterial expression strains. The most used fusion tag with the biggest solubility effect compared to other tags is the Maltose-binding protein, which has a chaperone-like effect. Another effective fusion tag is a yield-extracted protein called small ubiquitin-related modifier, which has an unknown mechanism of action, but it is thought that it might also act as a chaperone, increasing protein’s solubility. Fusion tags increase the protein’s solubility, but when studying the structure by crystallization, the fusion tag must be removed after protein purification, since it can interfere with the protein’s structure and activity. This removal could decrease the quality of the expressed protein. Another type of tags are short, repetitive polar peptide tags, which have a positive or negative overall net-charge. Due to their small size, they have a soluble effect on the protein of interest without the interaction with the protein’s structure, so an additional step of removing the small peptides after expression and purification is not required, in contrast to the fusion tags. In conclusion, peptide tags are more favorable than fusion tags, with the focus on the charge of the peptide tag that must be similar to the charge of the protein of interest, since opposite charges could lead to protein precipitation (Paraskevopoulou et al., 2018).

2.12. History of another prokaryotic Phospholipases C and the crystallization of a PLC:

During the past few years, the three-dimensional structure of some prokaryotic phospholipases could be further analyzed, providing different phospholipase models. One of them is expressed by Clostridium perfringens, which is a gram-positive, anaerobic, spore-forming bacteria, that is extensively distributed in nature. The expressed phospholipase C (Cp-PLC/alpha-toxin) is one of the major virulent factors that results in food poisoning, non-foodborne diarrhea and the formation of a severe and acute infection, called Gas gangrene. This infection as its name states, produces gas as a byproduct, by the process of the fermentation of sugars into gas. Gas gangrene causes healthy tissue to damage in a rapid way, where only amputation of the affected tissue treats the affected individuals. As also in PlcB, Cp-PLC is a multifunctional metalloenzyme that is Zinc-dependent with a sphingomyelinase and phospholipase C activity. The biological activity of Cp-PLC is the induction of platelet aggregation, hemolysis, increased capillary permeability, myonecrosis and increasing cardiac contractility, which is lethal. Mainly, the toxicity resulted by Cp-PLC is due to both the sphingomyelinase activity and the C-terminal domain of Cp-PLC, which is needed for the calcium dependent interactions with membranes. The enzyme’s substance preference is for phosphatidylcholine and sphingomyelin, which are both two major components of the cell membrane in mammalian cell membranes. This hydrolysis can therefore destroy the integrity of the cell membranes, causing cell lysis. Three-dimensional analysis showed that Cp-PLC is composed of two domains that are joined together by a short hinge region. The N-terminal domain (catalytic domain) consists of ten tightly-packed alpha-helices, while the C-terminal domain consists of four-stranded beta-sheets, which shows high structural similarity with the C2 domain of eukaryotic PLCs and is responsible for the process of binding to phospholipids on membranes (Singh et al., 2023, Flores-Díaz et al., 2003). A phospholipase C expressed by P. aeruginosa that was successfully purified and crystallized is the PlcHR complex. This protein was expressed in an expression system of Pseudomonas and was purified using Ion Metal Affinity Chromatography and the best crystals resulted with a resolution of 2.75 Å (Truan et al., 2013).

Chapter Three: Methodology

3.1 Chemicals and enzymes:

All of the used chemicals and enzymes used in experiments were purchased from the following companies: AppliChem, Sigma, VWR, Carl Roth, Th Geyer, Cube Biotech, Iba-Lifesciences, Roche Applied Science, New England BioLabs and Thermo Scientific.

3.2 Bacterial Strains and plasmids:

Summarized below (Table 3.2).

3.3 Culture growth Media:

- LB medium (Luria-Bertani): 10 g/l Tryptone, 10 g/l NaCl, 5 g/l Yeast extract (pH = 7, adjusted with NaOH.)
- LB agar medium: 10 g/l Tryptone, 10 g/l NaCl, 5 g/l Yeast extract, 15 g/l Agar

3.4 Buffers and solutions:

- 10x Gel electrophoresis Buffer (TAE Buffer): Tris Base, 0.5 mM EDTA pH= 6.1, acetic acid
- SDS loading Buffer (5x): 500 mM DTT, 10% SDS, 50% Glycerol, 0.5% bromophenol blue dye and 250 mM Tris-HCL, pH= 6.8
- 10x SDS electrophoresis Buffer: 0.2501 M Tris-Base, 1.924 M Glycine, 0.03467 M SDS
- Blue Silver Staining solution: 10% Phosphric acid, 10% Ammonium Sulfate, Coomassie G-250, Methanol, MQ water
- Transfer Buffer: 39 mM Glycine, 48 mM Tris base, 20% Methanol
- 10x TBS Buffer: 1.5 M NaCl, 46.2 mM Tris Base, 152.2 mM Tris HCL (pH= 7.6)
- 0.1% TBS-T Buffer: 1.5 M NaCl, 46.2 mM Tris Base, 152.2 mM Tris HCL (pH= 7.6), 0.1% (v/v) Tween Twenty
- Blocking reagent: 5% non-fat milk in TBS-T (w/v)
- Equilibration Buffer: 8 M urea, 150 mM NaCl, 50 mM Tris (pH= 8)

- Washing Buffer: (8 M/ 6 M/ 4 M/ 2 M/ 1 M/ 0 M) urea, 150 mM NaCl, 50 mM Tris (pH=8), 0.1 mM $ZnSO_4$
- Elution Buffer: 350 mM Imidazole, 150 mM NaCl, 50 mM Tris (pH=8), 1mM β-mercaptoethanol

3.5 Molecular weights standards:

- Gene Ruler 1K=kbp DNA ladder: ThermoFisher Scientific

- BLUelf Prestained protein Marker (5-245 kDa): Jena Bioscience

3.6 Kits:

- Plasmid DNA isolation Kit: ThermoFischer Scientific GeneJET Plasmid Miniprep Kit
- Gel extraction kit: thermo scientific GeneJET Gel Extraction kit
- Phospholipase C (PLC) Activity Assay Kit: SIGMA

-Methods:

3.7 Cloning:

3.7.1. Cloning of PlcB in pET28a+:

PlcB was first amplified by using the whole genome of Pseudomonas aeruginosa (PA14, 137.4 ng/µl) and afterwards cloned in the expression plasmid pET28a+, which has an N-terminal six His-tag with a thrombin cleavage site and Kanamycin (KAN) as an antibiotic resistance agent. Two different restriction enzymes were used: NdeI (acting on forward primer) and XhoI (acting on reverse primer). Four different constructs of plcB were cloned: N terminal His tag without signal peptide, N terminal His tag with signal peptide, N and C terminal His tag without signal peptide and N and C terminal His tag with signal peptide. Also a Chaperone (PA310) was cloned in both IBA3C and pET22b+ by using for both plasmids BamHI and XbaI as restriction enzymes. This specific chaperone for PlcB was used in this study in order to co-express it in combination with PlcB, which is expected to help in the folding process of PlcB, with the hope of making PlcB as a more soluble protein, expressed in the soluble, but not pellet lysates.

3.7.2. Primer preparation:

First, primers were received from the company BioTeZ Berlin-Buch GmbH. Primer preparation started by the centrifugation at 10’000 g for one minute. Then, a specific amount of Milli Q water was added. This specific amount was according to the amount written on the paper that came from the company with the primers. Afterwards, the samples were mixed well by a vortex. In addition to that, the primers were centrifuged for one minute. An amount of 10 µl from the prepared stock was mixed with 90 µl of Milli Q water and stored in a new eppendorf tube. This resulted by having two different tubes, one that represents the forward primer and another one that represents the reverse primer. The prepared primers were stored in the freezer at -20°C for further use.

3.7.3. Polymerse Chain Reaction (PCR):

PCR was done according to New England BioLab’s protocol. First, the prepared primers were used in combination with the whole genome of P. aeruginosa PA0014 (137.4 ng/µl) as a template. In addition to the whole genome and the two primers, a cocktail of several different components including 5x GC Buffer, (ThermoScientific), Deoxynucleotide triphosphate (dNTP, New England BioLabs) and Phusion hot start II DNA polymerase (Thermo Scientific) was used in order to start the whole process. Denaturation was done at 98°C for one minute, annealing was done at 64°C for 30 seconds and extension was done at 72°C for one minute. For reassurance that cloning worked out without any complications, the PCR products were then sent to a sequencing lab.

3.7.4. Preparation of 1% Agarose gel:

A total amount of one gram of agarose was weighted for each 100 ml of 10x TAE Buffer and poured into a flask. This mixture was carefully dissolved by using a microwave. The solution was heated until agarose powder was completely dissolved (seen by the naked eyes) and the solution started to boil (prevent over-boiling, since evaporation of solution can happen, changing the solution's concentration). Afterwards, 10 µl of GelRed (Biotium) was added, which is responsible for making the signals of the bands on the agarose-gel more visible under Ultra Violet light. The whole solution was poured slowly into the gel's tray with the suitable combs in the tray. The gels were kept in the trays for around 20 to 30 minutes until the gels completely solidified at room Temperature. Around two microliter of a one kilobase DNA marker (GeneRuler) was used as a ladder for a more precise reference. The samples were mixed with a 6x DNA loading dye (Thermo Fisher Scientific) and loaded carefully into the chambers of the gels. The gels were completely soaked with a Running buffer to cover the surface of the gels, which must be the same type of buffer as the buffer used for dissolving the agarose powder (10x TAE Buffer). Afterwards, the gels were run at around 80-150 Volt and stopped right before the visible dyes migrated completely out of the gels. Results were all seen and analyzed by using the ChemiDoc Imaging System (BioRad). Gels and Running buffer were carefully disposed according to the institution's regultions (Lee et al., 2012).

3.7.5. Gel extraction:

DNA was extracted using thermo scientific GeneJET Gel Extraction kit. In simple terms, DNA was extracted from the agarose gel carefully and put in an eppendorf tube. A one to one amount of Binding Buffer (comes with the kit) was added into the eppendorf-tube and placed on a pre-heated thermocycler (BIOER Mixing Block MB-102) with shaking until it was completely one homogenous and completely melted solution. The tube was centrifuged for one minute at 14’000 g and everything was pipetted into a small tube that has a column integrated in the tube (comes with the kit). Again, the tube was centrifuged for one minute and the Flow through was removed and discarded. The column was washed with 700 µl of Washing Buffer and centrifuged again for one minute at 14’000 g. A final centrifugation of the column was done without the addition of any kind of buffers (called dry spin) and pipetted into a new, clean eppendorf tube. Finally, 50 µl of Elution Buffer was added and the final concentration was checked by using the NanoDrop (Thermo Scientific, Version: 1.6.198).

3.7.6. DNA Digestion:

DNA digestion was done according to the Thermo Scientific protocol. In short, the chosen restriction were used on both the plasmid and the insert in combination with 10x Fast Digest Green Buffer (Thermo Scientific) for one hour at 37°C (T100 Thermal cycler BioRad).

3.7.7. DNA ligation into vector:

Ligation was done according to the Thermo Scientific protocol. Briefly, sticky-end ligation was accomplished by using a combination of 10x T4 DNA Ligase (Thermo Scientific) and T4 DNA Ligase Buffer (Thermo Scientific) with the digested plasmid and digested insert at 16°C overnight on the Thermal cycler (T100 Thermal cycler BioRad).

3.7.8. DNA isolation:

DNA was isolated using thermo scientific GeneJET Genomic DNA Purification Kit. Overnight-kept pre-culture was removed from incubator and centriguged at 6000g for 10-15 minutes. Supernatant was removed and the pellet was resuspented with 250 µl of Resuspension Buffer (Buffer stored in the fridge). Slowly, 250 µl of Lysis Buffer was added to the wall of the tube. Gently, the tube was moved up and down for around seven times. A volume of 350 µl of Neutralizing Buffer was added and the tube was moved slowly up and down for around seven times. The tube was centrifuged at 14’000 g for 10 minutes. Supernatant was pipetted into a collection tube with a column and centrifuged at 14’000 g for one minute and the flow through was removed. A volume of 750 µl of washing buffer was added and centrifuged at 14’000 g for one minute. Washing step was repeated and the tube was kept open at room temperature for one minute, in order to allow Ethanol (in washing Buffer) to evaporate. Flow through was removed and dry spinned at 14’000 g for one minute. The tube was put into a new eppendorf tube and 30 µl of Elution Buffer was eluted. Concentration was checked by NanoDrop. Finally, samples were stored in freezer at -20°C for proper use.

3.8 Transformation by heat-shock:

Transformation of genetic material into special bacterial strains by heat shock was performed according to (Hanahan et al., 1983). In general, transformation was applied on heat-sensitive, competent bacterial strains taken from -80°C freezer. Bacterial cells were put as fast as possible into an ice box for about 10 minutes, to allow the bacterial cells to slowly thaw. Then, about one microliter of genetic material of interest (plasmid or ligated construct) that is willing to be transformed was taken from -20°C and pipetted into the tube, containing the competent cells. Afterwards, the tubes were kept in the ice for about 30 minutes. Meanwhile, the thermocycler was turned on at 42°C. After incubation in the ice, heat shock was applied for exactly 90 seconds (thermocycler, BIOER Mixing Block MB-102) and put again back on the ice for five minutes. Around 800 µl of LB media was added to the tube and bacterial growth was allowed to happen by treating them at 37°C for around one hour and 20 minutes on the thermocycler. Afterwards, centrifugation was done at 6’500 g for five to 10 minutes. Next, part of the supernatant was discarded. Around 200 µl of supernatant was kept and the pellet was re-suspended in the little amount of the left supernatant and plated on fresh-prepared LB agar plates with the respective antibiotic. Overnight growth was allowed to happen by keeping the plates in an incubator at 37°C overnight, but for not more than 18 hours. The next day, only bacteria that contained the ligated construct or plasmid of interest with the respective antibiotic resistance gene showed growth and formed a group of pure colonies on the LB agar plates. Plates were stored at 4°C for further use.

3.9 Storage of bacteria:

All E.coli colonies grown on fresh-prepared LB agar plates were kept at 4°C for a maximum of no more than one week.

3.10 Pre-culture preparation:

One single, clear colony grown on fresh LB agar was picked up by an autoclaved micropipette tip and inoculated in a tube, containing around 5 ml of LB Media, supplemented with the respective antibiotic agent. A second tube was used as a control group, including 5 ml of LB Media with the respective antibiotic agent but with the difference of no inoculation of any of the bacterial colonies. Both tubes were kept in a shaker (INFORS HT Multitronat) overnight at 37°C with gentle shaking at around 160 rounds per minute (rpm). The next day, the tubes were directly used for starting the main, big bacterial expression culture or directly put in the cold room (4°C) for later use during the day, preventing overgrowth and cell death (Nallamsetty et al., 2007).

3.11 Expression in different bacterial expression strains:

In order to be able to make biophysical characterisations of my protein of interest, the suitable bacterial strain(s) and temperature(s) that expresse PlcB in the most soluble form must be figured out. First, pre-culture was prepared as described before, but with specific different bacterial expression strains like BL21 Star and C43. The next day, main culture was prepared by taking around 900 µl of the saturated, overnight pre-culture into a 250 ml flask containing 50 ml of LB media with 50 µl of respective antibiotic. Bacterial cultures were allowed to grow by putting the flasks in a shaker at 37°C with shaking at 160 rpm. Optical density (OD) was measured using a spectrophotometer (eppendorf BioPhotometer) each hour to keep the bacterial growth on track and to check the appropriate time at which induction should be made. The cultures were grown until they reached a logarithmic phase of OD 0.4. When OD reached 0.4, one millimolar of Isopropyl ß-D-1-thiogalactopyranoside (IPTG) was induced, in order to allow the bacteria to express and produce my protein of interest. After induction with the inducer, the bacterial cultures were grown for another four hours and then harvested at 6’500 g for 15 minutes. Right before inducing, a two milliliter sample was taken from the culture and labeled as Uninduced (UI). Also another two milliliter samples were taken each two hours after induction and centrifuged at 6’500 g for 10 minutes. Additionally, the samples were kept in the freezer at -20°C until they were ready to be analyzed using a type of gel electrophoresis (SDS PAGE). For cultures grown at 20°C, harvesting was done the next day. As a control, a flask with only LB media and overnight pre-culture was grown exactly in the same way, but without the induction with any inducer. For co-expression of PlcB with the chaperon (PA310_IBA3C or PA310_pET22a+), 200 µg/l of Anhydroustetracyclin was also induced in combination with IPTG at the same time. For preventing leakage to happen, 0.2% of glucose was added to the LB media just before starting the culture (Nallamsetty et al., 2007).

3.12 Opening cells using BugBuster (Sigma) solution:

In order to see if after induction the protein was being expressed in the supernatant (soluble form) or in the pellet (inclusion bodies), the bacterial expression strains expressing my protein must be opened and lysed by a lysis Buffer before running on the SDS PAGE. A commercial lysis Buffer called BugBuster (Sigma) was used in order to open the bacterial cells, following Novagen's protocol. For this procedure, one sample right before harvesting the bacterial cell culture (induced) was taken for lysing with BugBuster. After centrifugation at 6’500 g for 10 minutes, supernatant was discarded, while the pellet was kept. The pellet was weight where for each 0.01 g of pellet, 100 µl of BugBuster solution was slowly added and resuspended. Samples were shaken on the thermocycler at 22°C for 20 minutes. Then, samples were centrifuged at a high speed of 18’000 g /4°C (at cold temperature to avoid protein-degradation) for 20 minutes. After centrifugation, both produced supernatant and pellet were separated from each other and an amount of 20 µl of the supernatant was taken into another eppendorf tube and labelled with “BugBuster-Supernatant” followed by the resuspension with five microliter of 5x SDS, while 1x SDS was added to the pellet and labelled with “BugBuster-Pellet”.

3.13 Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE):

SDS PAGE was performed according to BioRad’s instructions. For my protein of interest, 15% resolving gels and 5% stacking gels were used in all experiments. For the 15% resolving gel: Milli Q water, 30% acrylamide (AppliChem), 1.5 M Tris-Cl (pH= 8.8), 10% SDS (Carl Roth) were mixed in a falcon tube respectively, then 10% ammonium persulfate and TEMED (Th. Geyer) were added and the mixture was resuspended properly, afterwards the mixture got poured into the sandwiched glasses of the gels as fast as possible, since the addition of both ammonium persulfate and TEMED accelarate the polymerization proccess of the gels. On the top of the gels, a very small, thin layer of 70% Ethanol was poured, which was in order to speed up the drying process of the resolving gels. After around 30 minutes, the resolving gels got dry at room temperature and the prepared 5% stacking gels could be poured over the resolving gels right after removing the covering Ethanol. The stacking gels consist of exactly the same components as the resolving gels, but with a difference in the pH of one Molar Tris-Cl (pH= 6.8). The samples to be run were heated before loading at 95°C for 5 minutes on thermocycler (BIOER Mixing Block MB-102) in order to denature the protein’s secondary and tertiary structure. Afterwards, samples were spinnned properly for 30 seconds by using a vortex. Around 12 µl of each heated sample was loaded into each well and as a reference around five microliter of BLUelf Prestained protein Marker (5-245 kDa, Jena Bioscience) was loaded into each first lane of the gels. The gels were first run at a low voltage of around 80 Volt and then after the samples migrated out of the stacking gel, the Voltage was increased to around 140 Volt. The whole gel box (Bio-Rad mini protean tetra cell) was poured with a 1x of SDS Running Buffer, diluted from a 10x SDS Running Buffer (0.2501 M Tris-Base, 0.03467 M SDS, 1.924 M Glycine). The run was stopped right after the blue dye of the samples migrated completely out of the gels. After the run was done, the gels were properly washed by using normal tap water, which will make staining with Silver stain easier, faster and cleaner. After washing with water, gels were then soaked into Silver stain (Water, Phosphoric acid, Ammoniumsulfate, Coomassie G-250, Methanol) over night with gentle shaking at room temperature. The next day, the Silver stain was discarded and the gels were again washed properly with tap water in order to get completely rid of the silver stain. The stained bands could be seen by the naked eyes and pictures were cuptured using ChemiDoc (BioRad).

3.14 Semi-dry Western Blot:

For each gel, one Polyvinylidene difluoride (PVDF, cytiva AmershamTM) and four Whatman filter papers (VWR) were needed. For PVDF's activation, it must be soaked in 100% methanol for arroud 30 seconds. This step must be done by using a forcep, to avoid scratchings on it. All the filters, the PVDF and the gels were soaked in a prepared Transfer Buffer (39 mM Glycine, 48 mM Tris-Base, 20% Methanol) before blotting using a blotting machine (Trans Blot Turbo System, BioRad). Then, all those were sandwiched in the following order: two filter papers, one PVDF, one gel and two filter papers. The membranes were sandwiched bubble free. For 30 minutes, the transfer of the bands occured through the blotting machine. After the run was done, PVDF was carefully removed and soaked in a 5% non-fat dry milk, dissolved in 1x TBS with 0.1% Tween 20 overnight (10x TBS consists of 3 M NaCL, 35 mM Tris base, 165 mM Tris HCL) in a cold room of 4°C with gentle shaking, in order to block unspecific binding. On the next day, PVDF was then washed with 0.1% TBS-Tween 20 (TBS-T) for three times, each time for 10 minutes on a shaker at room temperature. Again, 5% non-fat dry milk was prepared and around 1-2 µl of Monoclonal Anti-poly Histidine Peroxidase antibody (1:10’000, Sigma) was diluted into the non-fat dry milk and kept for around two hours at room temperature with gentle shaking on a shaker. After around two hours, PVDF was again washed with 1x TBS-T for three times, each time for 10 minutes. After it was properly washed, PVDF was soaked in 1x TBS for around one to three hours, for having a clear background. Finally, PVDF was carefully removed with a forecep and placed on a clean plate. Then, two Developing reagents (ECM, Biorad) were used by mixing one milliliter from each of them properly in a two milliliter tube. Afterwards, the mixed homogenous solution was poured over the PVDF and distributed evenly with a blotting roller. This step should be done at dim light. Results were read by using ChemiDoc Imaging System (BioRad).

3.15 Running a Size Exclusion Chromatography (SEC):

Size Exclusion Chromatography was used in order to check the size of PlcB. First, PlcB was concentrated by using an Ultra Centrifugal Filter with a cutoff of 10 kDa (Amicon®, Merck), since PlcB has a size of 36.7 kDa. A 24 ml column (Superdex 200 increase 10/300 GL, ID: 0030: VWR) with Unicorn Manager program was used. The loop was first washed with Milli Q water for several times, afterwards the column was washed with a prepared Sec Buffer (25 mM Tris base (pH=8), 150 mM NaCl, 0.1 mM Zinc Sulfate, 2 mM β-mercaptoethanol). A maximal volume of 500 µl of the concentrated protein was injected into the loop of the column and the run was kept overnight. Samples were run on SDS PAGE gels and Western Blot was done in order to analyze the results resulted by running the SEC.

3.16 Urea purification using Immobilized Metal Affinity Chromatography:

PlcB was cloned with a 6x-His tag terminal, so nickel-charged resin (PureCube 100 INDIGO Ni-Agarose, Cube BioTech) was used in order to allow the protein to bind to the nickel-charged beads, followed by washing my protein with different washing Buffers, each with a different concentration of urea, starting with a high concentartion of eight Molar until reaching zero Molar of urea. Finally, eluting with a proper elution buffer. For urea purification, two diffrent constructs of PlcB were expressed and used for the purification process. The first construct of PlcB had a signal peptide with an N-terminal His-tag (N_pET28a+) while the other construct was without a signal peptide with both N and C-terminal His tags (-NC_pET28a+). Before starting with urea purification, a new and fresh one liter culture of both constructs was made. The two constructs were expressed in Star at 37°C, induced with one milli Molar IPTG at OD= 0.4 and harvested after four hours. One liter of Lysis Buffer (50 mM Tris, 300 mM NaCl, 1mM β-mercaptoethanol, pH=8) was prepared which was used while opening the cells using a homogenizer (EmulsiFlex-C3). A mixture of different protein inhibitors, including: E64 (10 M), Leuptin (10 µM) , Pepstatin (1mM) , AEBSF (0.0001 M) and PMSF (0.001 M) were added to a 80 ml of lysis Buffer. This mixture of lysis buffer was added to the final yield of two grams (2g/1l) of bacterial pellet after harvesting the bacterial cell culture. After resuspending the bacterial cell pellet with the lysis Buffer and opening the cells with an EmulsiFlex-C3, the produced cell lysate was taken and centrifuged at 900 g for 10 minutes at 4°C. The formed pellet after centrifugation included all of the unopened cells, which was not needed, so pellet and supernatant were separated and only the supernatant was taken and centrifuged at 10’000 g for one hour at low temperature of 4°C. After centrifugation, the formed pellet included all of the inclusion bodies. In order to have as much as possible of pure inclusion bodies, a solution of 50 mM Tris Base and 150 mM NaCl was added by gently resuspending the pellet with this solution. After that, centrifugation was again done for 15 minutes at 10’000 g, to end up with pure inclusion bodies. The inclusion bodies found in the pellet were resuspended with 10 ml of eight Molar urea, 50 mM Tris Base and 150 mM NaCl. After resuspension, the mixture was incubated in a cold room with a temperature of 4°C on a shaker for two hours and a sample of 60 µl was taken to run later on SDS gel before loading the whole mixed solution through the column. Four milliliter of Ni-NTA (two column volume) was taken and all the Ethanol was allowed to get out of the column by running the four milliliter of Ni-NTA completely through the column and washing it properly with Milli Q water. The resin was then equilibrated by using an Equilibration Buffer (8 Molar urea, 150 mM NaCl and 50 mM Tris Base (pH=8)). After the resin was equilibrated, the mixture that was on the shaker for two hours was loaded through the resin for four to five times and a sample was collected for further running on SDS gel (called on gel eight Molar). Then, the resin was washed with different concentrations of urea: six, four, two and one Molar, respectively (all prepared in a base-solution of 150 mM NaCl, 50 mM Tris Base (pH=8) and 0.1 mM Zinc Sulfate). Samples were collected after each wash for further running on SDS gel. Afterwards, after the last solution (one Molar urea) was completely run through the resin, the column was closed from both sides and a solution of zero Molar of urea was poured into the closed column and put on a shaker overnight at 4°C, to allow refolding to occur. The next day, the closed column with resin and a mixture of zero Molar urea was removed from the shaker and put back on the column-stand. Zero Molar urea was allowed to run completely out of the column and a sample for running on SDS gel was taken. Then, six milliliter of Elution Buffer (350 mM Imidazole, 50 mM Tris Base, 150 mM NaCl, 0.1 mM Zinc Sulfate, 1 mM Betamercaptoethanol) was loaded through the column to elute the protein bound to the nickel-beads. The eluted sample was collected and the protein concentration was checked by using the NanoDrop.

3.17 Activity assay using Phospholipase C (PLC) Activity Assay Kit:

Phospholipase C activity assay (Sigma) was done in order to see if there is any activity for the phospholipase after running the size exclusion chromatography and having different fractions of PlcB. For this test, a 96 well-plate was used. First, 50 µl of protein from the different SEC fractions were pipetted into each well. After that, 48 µl of a prepared Buffer (25% glycerol, 100 mM Tris Base (pH=8)) was added to each of the wells. Next, two microliter of a one Molar Substrate called p - Nitrophenylphosphorylcholine (NPPC) that comes with the kit was added to each of the wells. After adding all the components, the 96-well plate was incubated in a 37°C incubator for 30 minutes. A yellowish color should be an indication for the presence of the protein's activity (positive result). For more exactness and precise results, both negative and positive controls were made, where the negative control was a composition of the used Buffer in this activity assay (25% glycerol, 100 mM Tris Base (pH=8)) in combination with the 20 mM substrate (NPPC), while the positive control was a combination of the Buffer with the substrate and another phospholipase C, also expressed by Pseudomonas aeruginosa with a known and positive activity, which was taken from my PhD supervisor.

Chapter Four: Results

The aim of this study was to find out the three-dimensional structure of PlcB, by applying biophysical characterizations. In summary, PlcB was cloned in an expression cloning system (plasmid), followed by expression in different bacterial expression strains under different conditions like different bacterial expression strains and different temperatures etc. Afterwards, PlcB was purified by urea purification using IMAC. The size of PlcB was checked using SEC. Finally, the function of the purified PlcB was tested by a colorimetric activity assay specific for only Phospholipases.

4.1 Cloning

4.1.1. Polymerase Chain Reaction (PCR):

The studied gene PA0026 is located in the genome of Pseudomonas aeruginosa and encodes a Phospholipase C called PlcB, which consists of 328 amino acids. In this research, four different constructs of the PlcB gene (PA0026) were designed and cloned in pET28a+ expression plasmid. Two of the four were cloned with a signal peptide, where one of them had both N and C- terminal His tags and the other one only N-terminal His tag. The other two were without a signal peptide, where one of them had both N and C-terminal His tags while the other one only an N-terminal His tag (Figure 4.1).

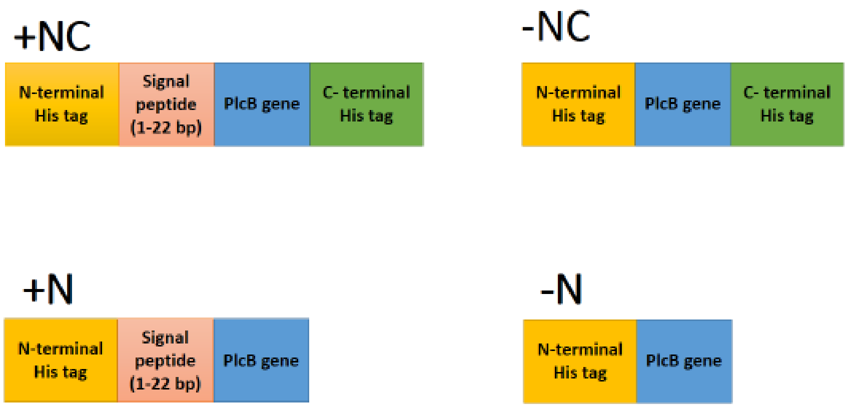

Figure 4.1. Four different constructs of PlcB, two with a signal peptide (left), one of them with both N and C- terminal His tags and the other one only with N-terminal His tag. The other two were without a signal peptide, one with both N and C-terminal His tags, the other with only an N-terminal His tag (right).

The signal peptide was removed as previous research show that this can increase the expressed protein's solubility. In addition to that, co-expression of PlcB with its chaperon was applied and since the chaperon had no signal peptide, which means it should stay in the cytoplasm and will not be secreted. I needed them to physically interact so another way to make them come together had to be found out, which ended up by removing the signal peptide of PlcB to avoid the secretion from the cytoplasm and allow it to come together with the chaperon and bind to it in the cytoplasm. In my project, it came out that the process of removing the signal peptide was not as effective as expected and no changes in the solubility could be detected when Western Blot was done for both with and without signal peptide, since results showed positive expression in the pellet fraction, but no expression in the supernatant for the PlcB protein. For the PCR reaction, a total of 100 µl was prepared and then divided into 10 tubes, each of them with 10 µl from the main mixture. The amounts of each of the PCR components is shown below (Table 4.1). The PCR products were run on 1% agarose gel (Figure 4.2). The gel showed right bands in their right molecular weight for each of the genes when compared to the ladder, so extraction of the bands from the gel was done. The concentration for each of them was checked using the NanoDrop (Table 4.2).

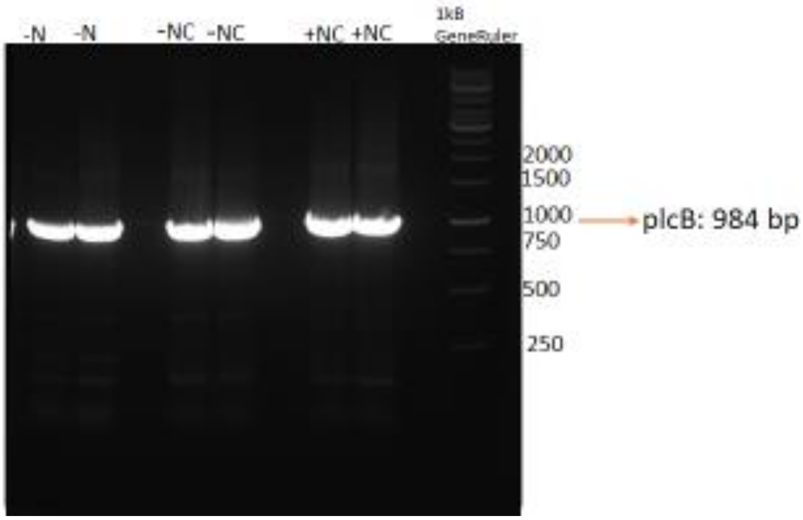

Figure 4.2. 1% agarose of PCR product of –N, -NC and +NC.

4.1.2. Digestion:

After gel-extraction of the PCR products, two different restriction enzymes (NdeI, XhoI) were used on both the genes (-N, -NC, +NC) and the plasmid (pET28a+) in order to be able to ligate them together and producing new constructs. The exact components and amounts used shown below (Table 4.3, Table 4.4). After digestion was done, the samples were run on a 1% agarose gel as shown (Figure 4.3). As a control for my plasmid, undigested (coiled) pET28a+ was also run, where it was expected to show a lower band than the digested, since coiled and round plasmids run faster than linear ones through the porous agarose gel. The bands were extracted and concentration was checked using the NanoDrop (Table 4.5).

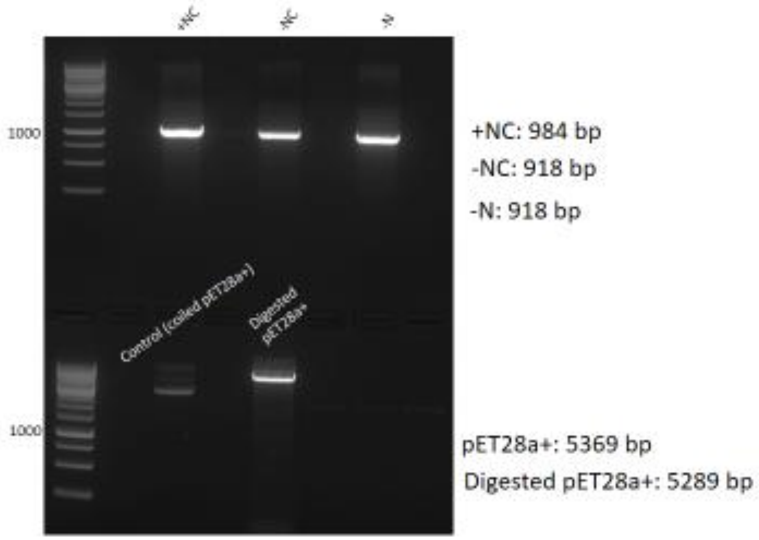

Figure 4.3. Digestion of pET28a+, -N, -NC and +NC on 1% agarose gel.

4.1.3. Ligation:

Ligation of the digested genes (-N, -NC, +NC) and digested plasmid (pET28a+) was done at 16°C overnight. The amount of each components is as shown below (Table 4.6). The next day, the ligated products were transformed into Top 10 competent cells. This step was done in order to isolate the constructs in order to increase the amount. This was done by using the ThermoFischer Scientific GeneJET Plasmid Miniprep Kit.

4.1.4. Digestion to check and confirm for proper ligation:

To ensure that ligation occurred without any problems, the ligated products were digested at 37°C for one and a half hour with the two previously used restriction enzymes (NdeI, XhoI) as shown below (Table 4.7). Then, the products were run on a 1% agarose gel (Figure 4.4, fig 4.5). The gels confirmed that ligation occurred, since the lanes showed two bands, the upper bands representing the plasmid pET28a+ with a molecular weight of 5289 base pairs and the lower bands represent my gene of interest which is for without signal peptide 918 base pairs and 984 base pairs for with signal peptide. So having two bands for each in the correct molecular weight was a positive result, showing that ligation was successful. For –NC, all lanes showed positive results. For +NC, lane number two and four showed a negative result and for –N only lane number five showed a negative result.

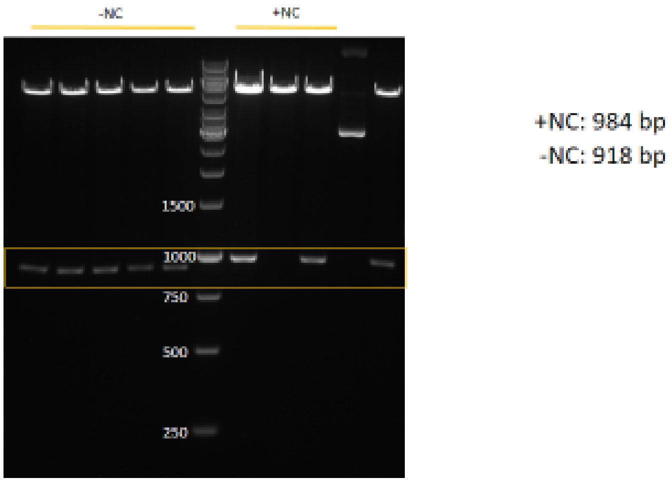

Figure 4.4. 1% agarose gel of digested –NC_pET28a+ and +NC_pET28a+ to confirm ligation.

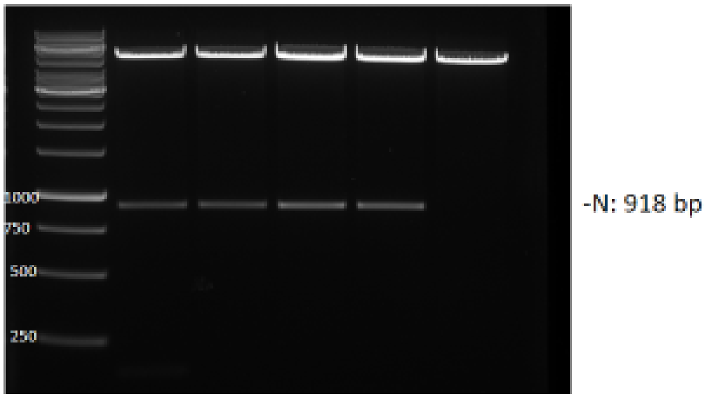

Figure 4.5. 1% agarose gel of digested –N_pET28a+ to confirm ligation.

4.1.5. Checking for correct ligation by PCR using T7 primers:

Using T7 primers was possible since we used in our cloning system a pET plasmid. In order to reassure in another second way that ligation happened in a correct way (other than digestion as showed above), T7 primers were used in another new PCR run (Table 4.8). Denaturation was done at 98°C for one minute, annealing for at 56°C for 30 seconds and extension at 72°C for 90 seconds. This all was repeated for whole 34 cycles (34x). Since those primers will yield in the addition of another 233 base pairs, it was expected to see on the agarose gel higher bands than expected and this is what I could see on the gel as shown (Figure 4.6). For –N lane number one was a little higher than the other bands and lane five showed a negative result (also in digestion lane number 5 for –N was negative). For –NC all lanes showed a positive result and for lane +NC, lane number two and four showed a negative result (also in digestion they were negative). Due to the similar results found in both digestion and PCR by using T7 primers, it can be concluded that ligation occurred in a correct way in the ones that gave for both ligation and PCR by T7 primers a positive result. After reassurance by digestion and PCR using T7 primers, the constructs that gave a positive result were sent to a sequencing lab for a final reassurance.

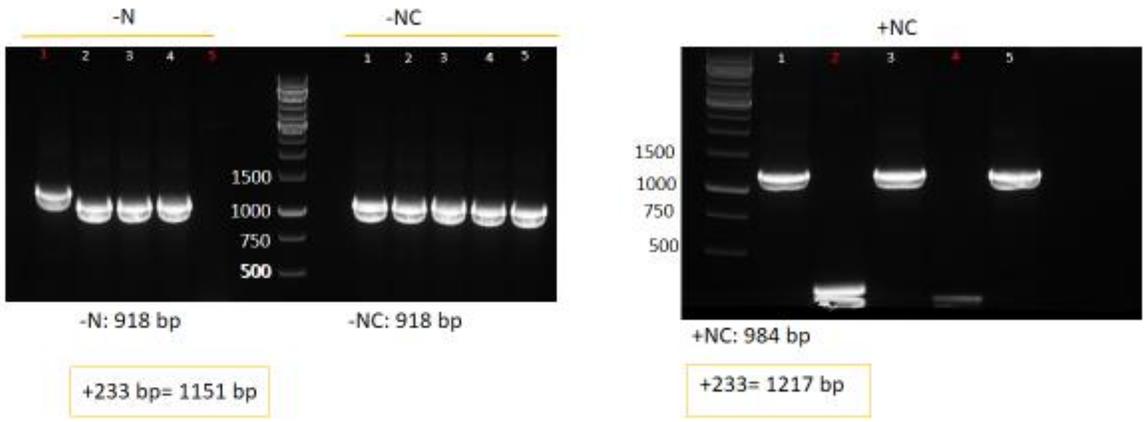

Figure 4.6. Using T7 primers to reassure proper ligation.

4.1.6. Cloning of chaperone:

The chaperone, expressed by the gene PA0027 that is adjacent to the gene of my protein of interest (PlcB) was cloned in a pET vector (pET22b+). The main reason for this step was because at the beginning of this project, the chaperone was cloned in IBA3C plasmid (by my PhD supervisor), which needed Anhydrous Tetracycline as an inducer during protein expression, but since PlcB protein needs IPTG (cloned in pET28a+), it was thought that uniting the type of inducer could give better results, instead of using two different inducers during co-expression. For PCR, the whole genome of Pseudomonas aeruginosa strain 14 was used as a template and both NdeI and XhoI were used as Restriction enzymes, respectively as shown below (Table 4.9). The selected annealing temperature was 66.4°C. Correct, positive bands could be observed on 1% agarose gel (1284 base pairs). A special type of gel (analytical gel) was applied to get more accurate results because it helps to determine the exact size due to the lower volume loaded into each well (Figure 4.7). After running the samples, the bands were extracted using GeneJet extraction kit with a final concentration of 212.1 ng/µl (NanoDrop).

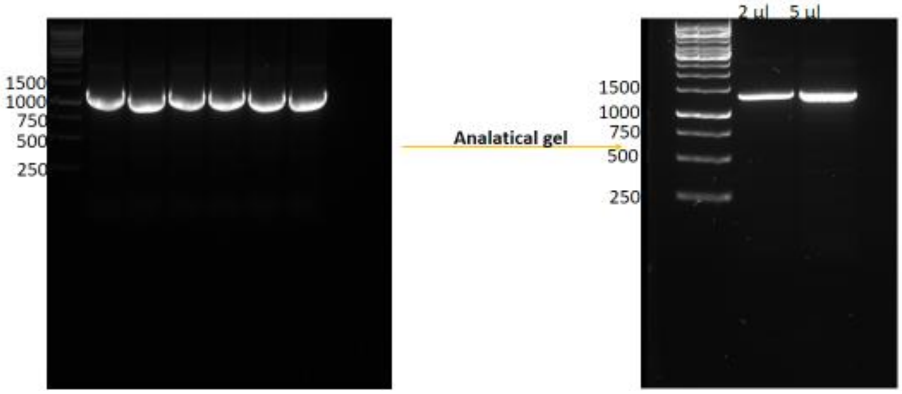

Figure 4.7. 1% agarose gel of chaperone PA310 after running PCR (left) and analytical gel (right) of PA310.

4.1.7. Digestion of the chaperone and pET22b+:

Digestion of both pET22b+ and the chaperone was done for one and a half hours at 37°C on thermocycler (Table 4.10, Table 4.11). Afterwards, the samples were run on 1% agarose gel. As a control, undigested pET22b+ was loaded. The band of the digested pET22b+ was higher than the control (5493 bp), which means that digestion worked. The chaperone showed a band in the right size. (1284 bp). Also, analytical gel was run to see the bands in a finer way (Figure 4.8). After digestion, both plasmid and chaperone were extracted from the agarose gel in order to be able to start with ligation. The concentration after extraction of pET22b+ was 6 ng/µl and 63.6 ng/µl for the chaperone.

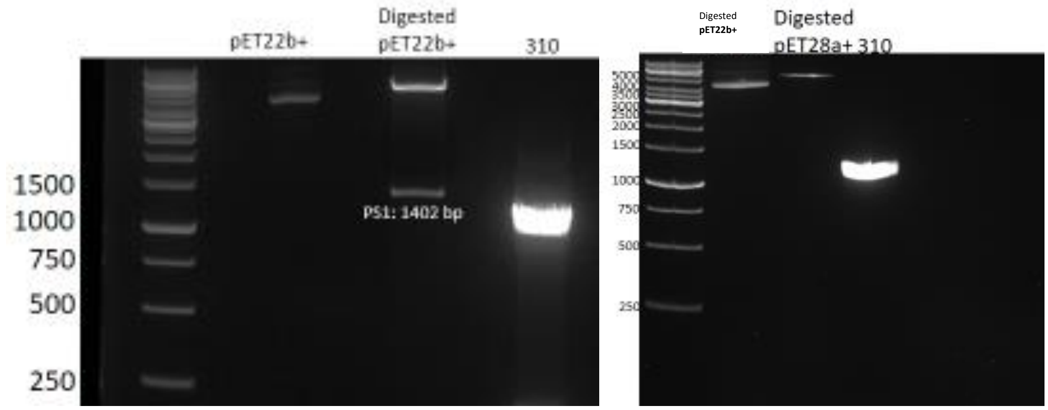

Figure 4.8. 1% agarose gel of digested pET22b+ and digested PA310 (left) and analytical gel of digested pET22b+ and digested PA310 (right).

4.1.8. Ligation of chaperone and pET22b+:

Ligation in a one to three (1:3) ratio of both the chaperone and pET22b+ was done at 16°C overnight (Table 4.12). To check if ligation worked out, the ligated construct was digested with both NdeI and Xho1 at 37°C (Table 4.13). Afterwards, run on 1% agarose gel. The formation of two bands is an indication that ligation worked out (Figure 4.9). Lane two, four, five and eight showed two bands on the agarose gel, so the ligated pET22b+_310 from those lanes was sent for sequencing for a final reassurance.

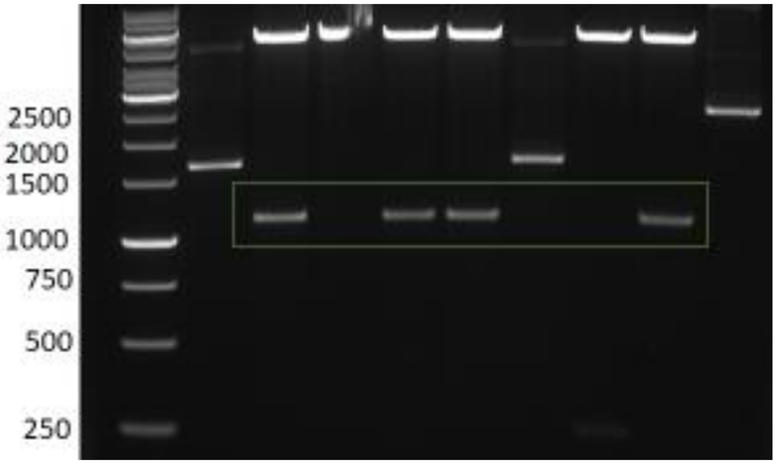

Figure 4.9. 1% agarose gel after digestion of pET22b+_PA310 with NdeI and XhoI.

4.2 Expression in different expression systems:

Different bacterial expression strains were tested in order to see which bacteria has the ability to perform expression of my protein in its most soluble state. Expression was done at high temperature (37°C) and at low temperature (20°C). Low temperature (20°C) was tried, since it is advised to reduce the temperature during expression in order to reduce the protein's transcription and translation rate, lowering the risk of protein aggregation. In summary, results of running SDS PAGE and Western Blot analysis showed that no bacterial strain could express my protein of interest in its soluble state, always giving positive SDS PAGE and Western Blot signals in the pellet fractions. Also, lowering the temperature to 20°C did not change the solubility and showed positive signals in the pellet fractions too. My protein of interest is known to have a chaperon that is expressed by an adjacent gene, so co-expression was tried to investigate if the chaperone will bind to my protein and making it more soluble. The results showed that co-expression of the protein with its chaperon did not change the solubility at all. Two constructs without signal peptide were cloned, since the chaperone that I have (PA310_pET22b+) does not have a signal peptide, which means that it is expected to not leave the cytoplasm, but my protein has a signal peptide that responds to the Sec secretory system, so removing the signal peptide to allow my protein to bind to the chaperon by staying in the cytoplasm and not leaving the cell's cytoplasm. For that reason, another two constructs without signal peptide were cloned (with N-terminal His tag and with both N and C-terminal His tags) and also expressed in expression strains at different temperatures, but this too showed no change in the solubility, which means that whether with or without signal peptide, no positive bands appeared in the supernatant fractions, indicating that the protein's solubility could not be reached in the mentioned conditions (PlcB has a molecular weight of 36.7 kDa, the chaperone 48.8 kDa).

4.2.1. Expression of PlcB with signal peptide N-terminal His-tag (+N_pET28a+) and N and C-terminal His-tag (+NC_pET28a+) in Star (DE3) and C43 at 37°C and 20°C:

The constructs with signal peptide were expressed in both Star (DE3) and C43 at both 37°C and 20°C. Signals were clearly seen in the pellet fractions when harvesting after 4 hours at an OD of 0.4 (Figure 4.10, fig 4.11 and fig 4.12).

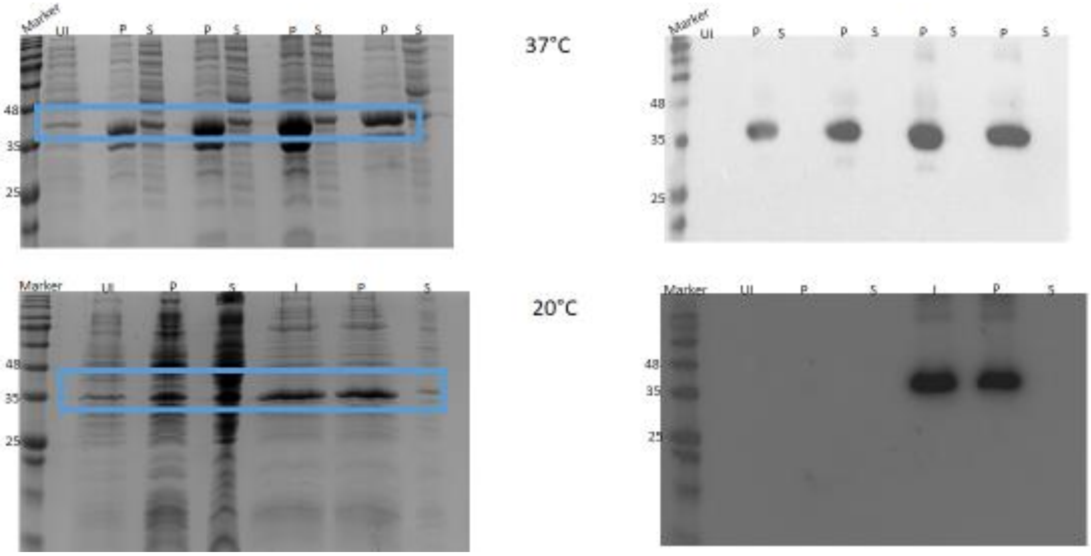

Figure 4.10. 15% SDS PAGE (left) and His-tag Western Blot (right) after expression of +N_pET28a+ in Star (DE3) at 37°C (up) and 20°C (down).

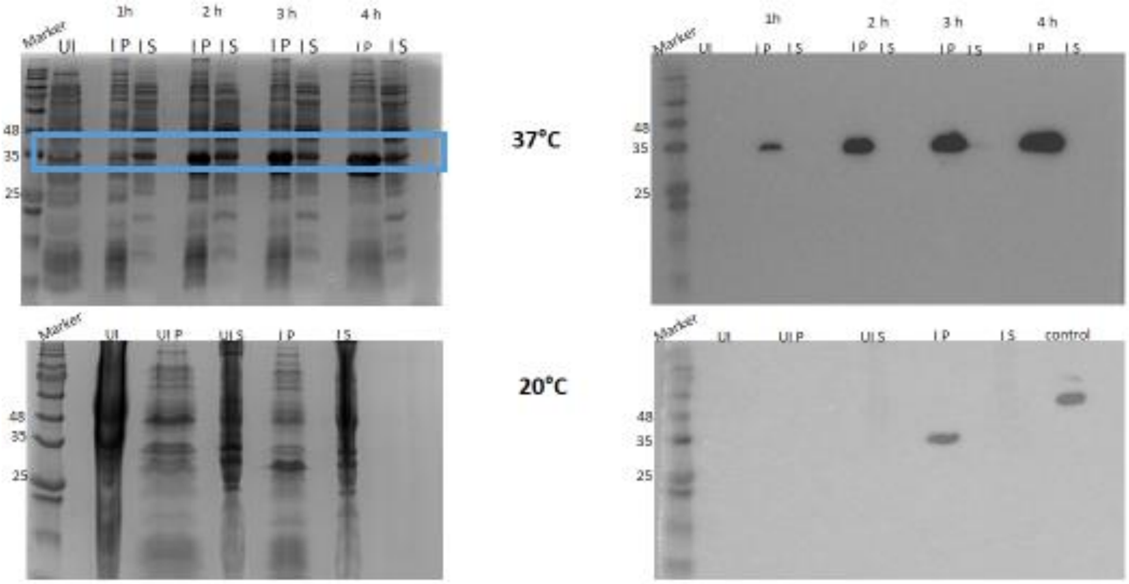

Figure 4.11. 15% SDS PAGE (left) and His-tag Western Blot (right) after expression of +N_pET28a+ in C43 at 37°C (up) and 20°C (down).

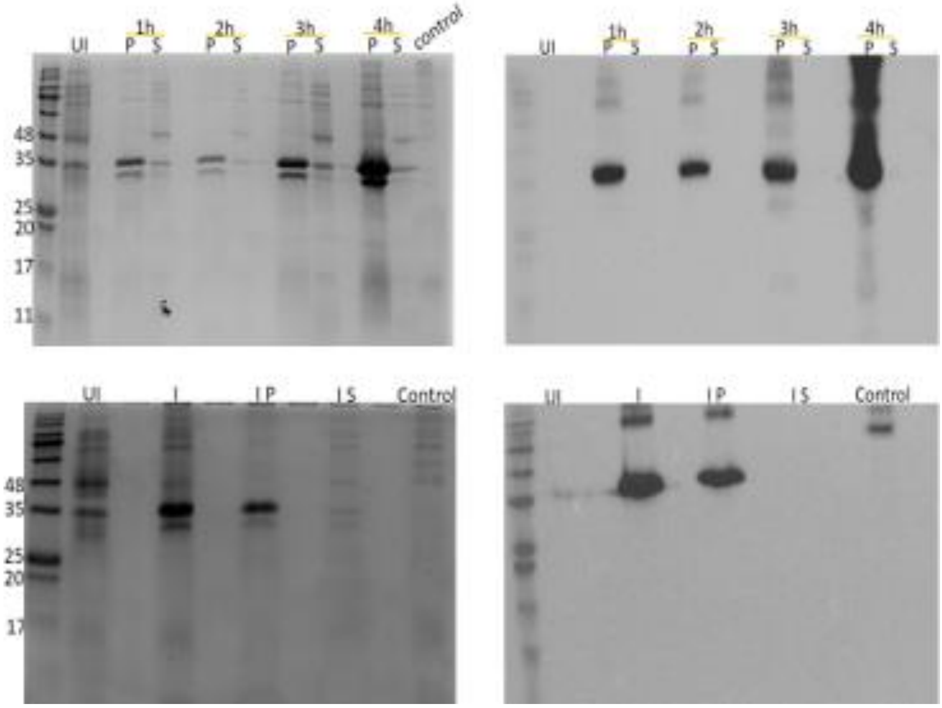

Figure 4.12. 15% SDS PAGE (left) and His-tag Western Blot (right) after expression of +NC_pET28a in Star (DE3) at 37°C (up) and 20°C (down).

4.2.2. Expression of PlcB without signal peptide N terminal His tag (-N_pET28a+) and without signal peptide N and C-terminal His-tag (-NC_pET28a+) in Star at 37°C and 20°C

Expression of PlcB without signal peptide was done at both 37°C and 20°C. SDS gels and His-tag Western Blots showed that the protein ended up in the pellet fractions. This means that removing the signal peptide did not make PlcB more soluble (Figure 4.13, fig 4.14).

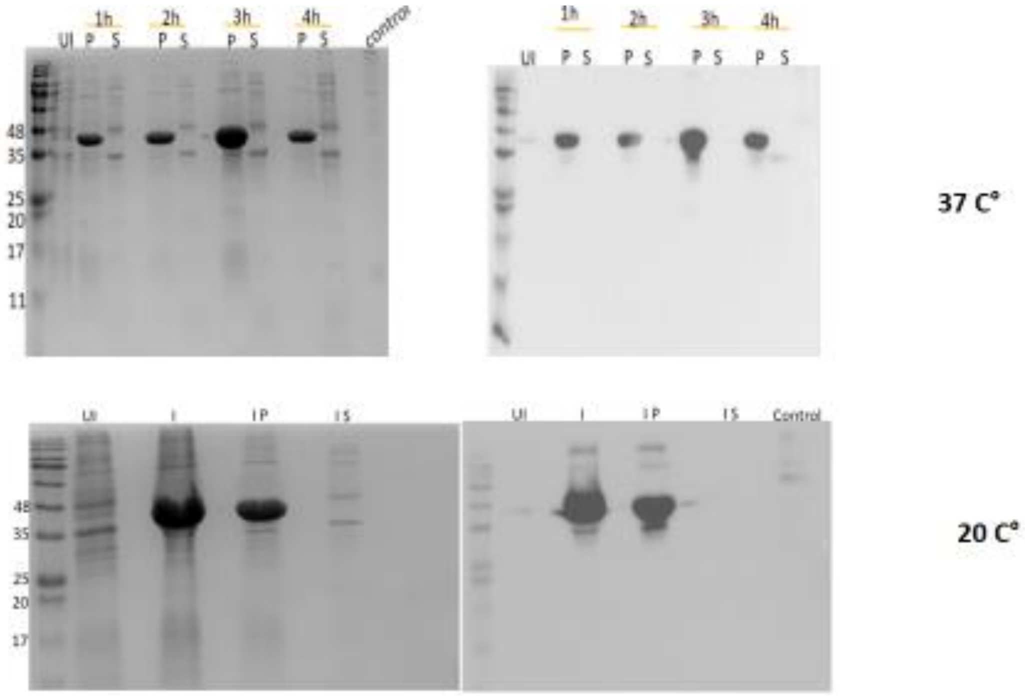

Figure 4.13. 15% SDS PAGE (left) and His-tag Western Blot (right) after expression of -N_pET28a+ in Star (DE3) at 37°C (up) and 20°C (down).

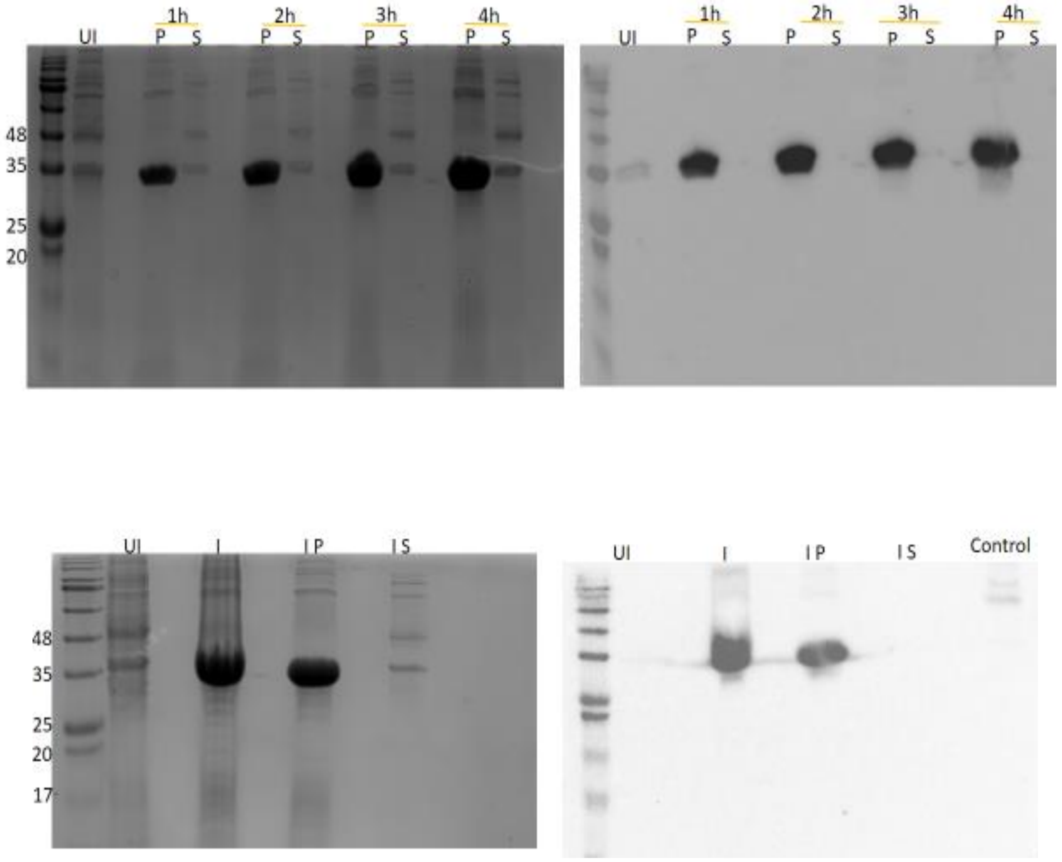

Figure 4.14. 15% SDS PAGE (left) and His-tag Western Blot (right) after expression of – NC_pET28a+ in Star at 37°C (up) and 20°C (down).

4.2.3. Inducing at high Optical Density (OD):

After trying to express at both high temperature and low temperature, it was obvious through both SDS PAGE analysis and several Western Blots that the protein (in all four constructs) whether with or without signal peptide, it always ended up in the pellet fractions. This result was an indication that my protein might be aggregated and always ended up as inclusion bodies in the pellet fractions. One possible strategy to avoid this from happening is to induce at high Optical Density (OD). The main point of inducing at high OD is that the cells stop from growing rapidly after the bacterial cells reach a very high density, which means that lesser protein is being expresses, which will lead to a lower expression yield. This means that there is a possibility that at high OD, proteins could be expressed at lower yield, preventing the protein from aggregation and the formation of inclusion bodies. Expression of +N_pET28a+ was done in Star (DE3) at 37°C with the induction of IPTG when reaching an OD of 1.5, which is considered as a high OD. Samples after one and two hours were taken. The sample that represented the 2 hours was opened up by BugBuster lysis buffer to see if the protein ended up in the supernatant or still in the pellet fraction. As the results show, expression at high OD with a lower expression yield did not avoid the aggregation issue and the protein was still being expressed in the pellet fractions, but compared to when induced at lower OD as shown before, the bands when induced at high OD were thinner, due to the lower yield at high OD. A sample from the LB Media alone was taken to see if my protein was being pulled out of the cells, but no positive signal appeared (Figure 4.15). For the expression of +N_pET28a+ in C43 at 37°C at high OD, no positive bands could be seen at all in any of the fractions (Figure 4.16).

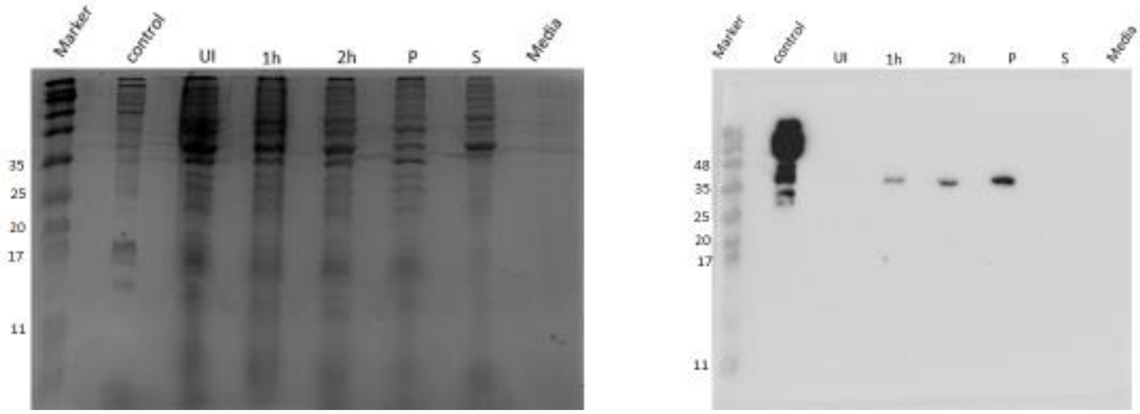

Figure 4.15. 15% SDS PAGE (left) and His-tag Western Blot (right) after expression of +N_pET28a(+) at 37°C in Star (DE3) and inducing at OD 1.5

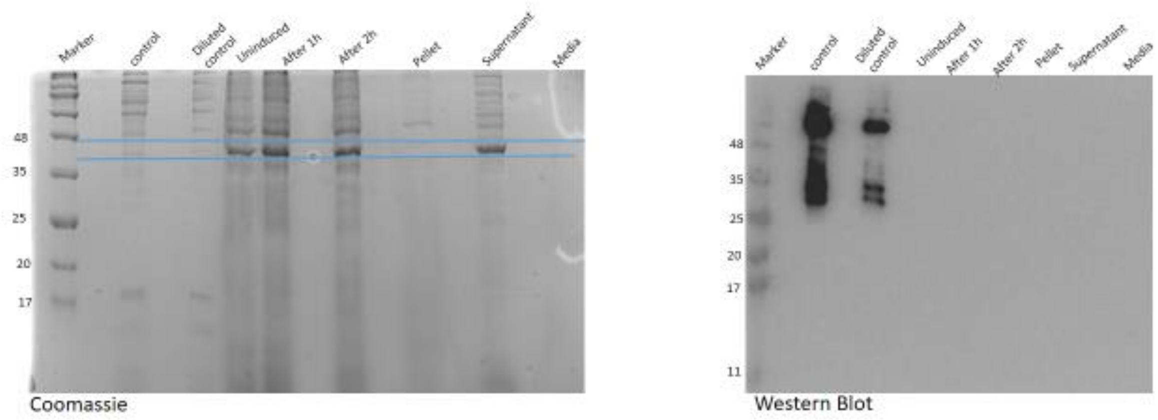

Figure 4.16. 15% SDS PAGE (left) and His-tag Western Blot (right) after expression of +N_pET28a(+) at 37°C in C43 and inducing at OD 1.5

4.2.4. Inducing at a lower concentration of IPTG:

One of the advised ways in order to get a protein more soluble is to lower the concentration of the inducer (IPTG). In the previous expression cultures, the used concentration of IPTG was always one millimolar, so the concentration was lowered from one millimolar to 0.2 mM of IPTG. Both constructs with and without signal peptide were expressed in the bacterial expression strain Star, where induction was done when OD reached 0.4 at 20°C. No change could be observed, since the positive signals still were seen in the pellet fractions, but not in the supernatant. In conclusion, lowering the IPTG concentration still showed the formation of inclusion bodies in the pellet fractions (Figure 4.17, fig 4.18).

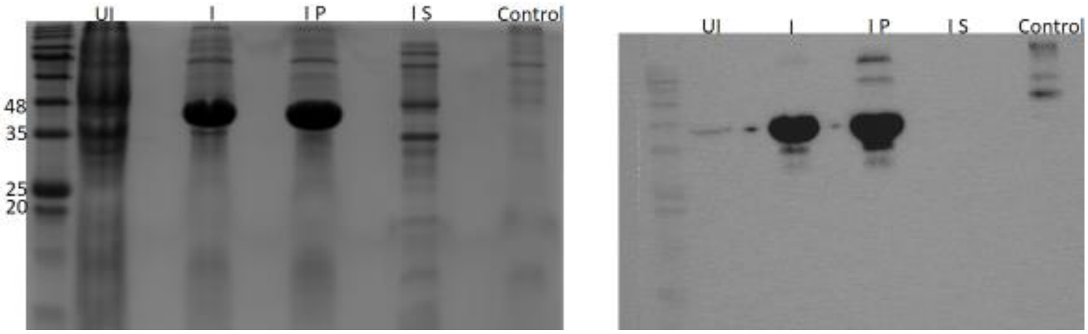

Figure 4.17. 15% SDS PAGE (left) and His-tag Western Blot (right) after expression of –N_pET28a+ at 20°C with lower IPTG concentration (0.2 mM).

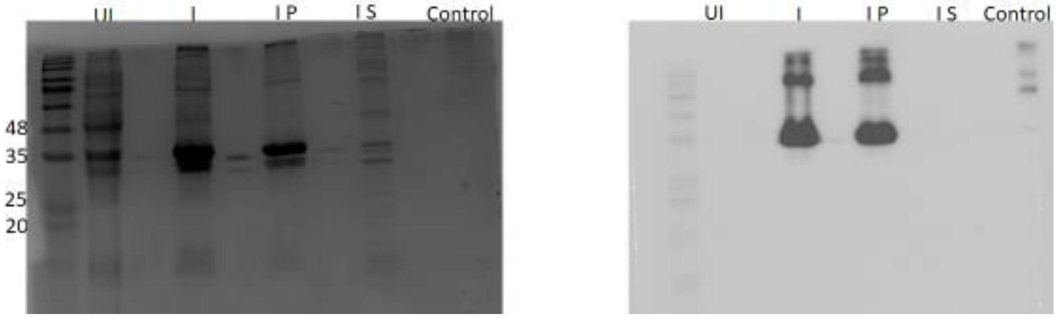

Figure 4.18. 15% SDS PAGE (left) and His-tag Western Blot (right) after expression of +N_pET28a+ at 20°C with lower IPTG concentration (0.2 mM).

4.2.5. Co-expression of PlcB_pET28a+ with chaperone PA310:

Co-expression of PlcB (+N_pET28a+) with its chaperone PA310 was done with the hypothesis that this will help PlcB to fold properly, resulting in a soluble state. Both SDS PAGE and Western Blot analysis showed that co-expression did not produce a signal in the supernatant fraction (Figure 4.19, fig 4.20). According to research, glucose prevents during expression the action of protein-leaking from happening, so a percentage of 0.2% glucose was mixed into the LB culture medium just before starting with the expression-experiment. According to that, co-expression of PlcB with its chaperone with the addition of 0.2% glucose into the media was done, to prevent leaky expression and to give the cells more time to grow. Harvesting was done after two hours of induction (Figure 4.21). In summary, the results show that the addition of 0.2% glucose to the culture media did not give a positive band in the supernatant fraction. Indeed, the addition of glucose did not give a big change compared to when expressing using only LB media without the addition of glucose. Because the cloned chaperone (PA310_IBA3C) was without any signal peptide, it could be possible that the chaperone stayed in the cytoplasm of the cells, which could be the reason why PlcB (+N_pET28a+), which had a signal peptide, and the chaperone did not come together and no proper folding happened of the protein could ever happen, leading to the inclusion bodies formation. The construct –NC_pET28a+ was expressed with the chaperone (PA310_pET22b+) and a little band could be observed in the supernatant of the Western Blot (Figure 4.22). This could be due to the removal of signal peptide in PlcB, which would allow both chaperone and protein to come together in the cytoplasm and fold properly.

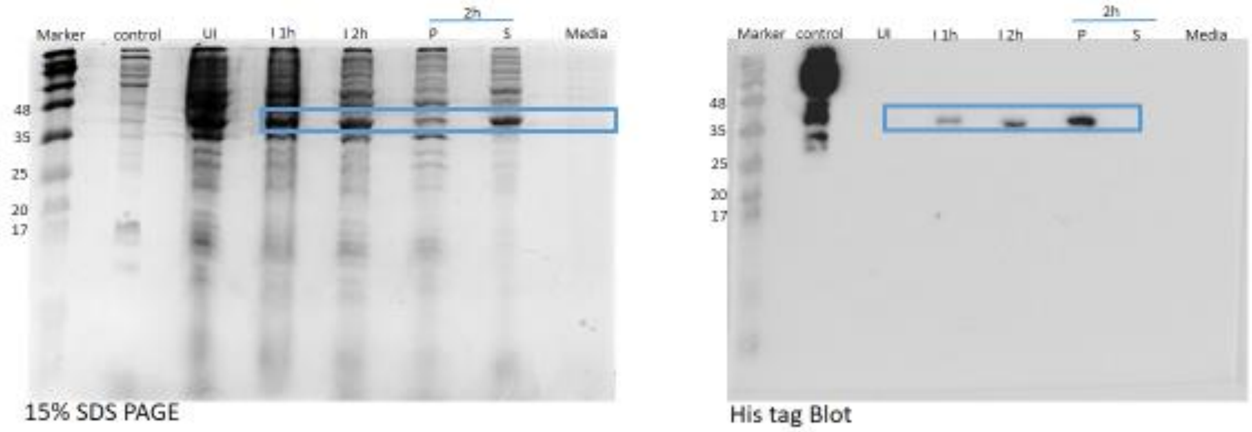

Figure 4.19. Coexpression of +N_pET28a+ with PA310_IBA3C in Star (DE3) at 37°C.

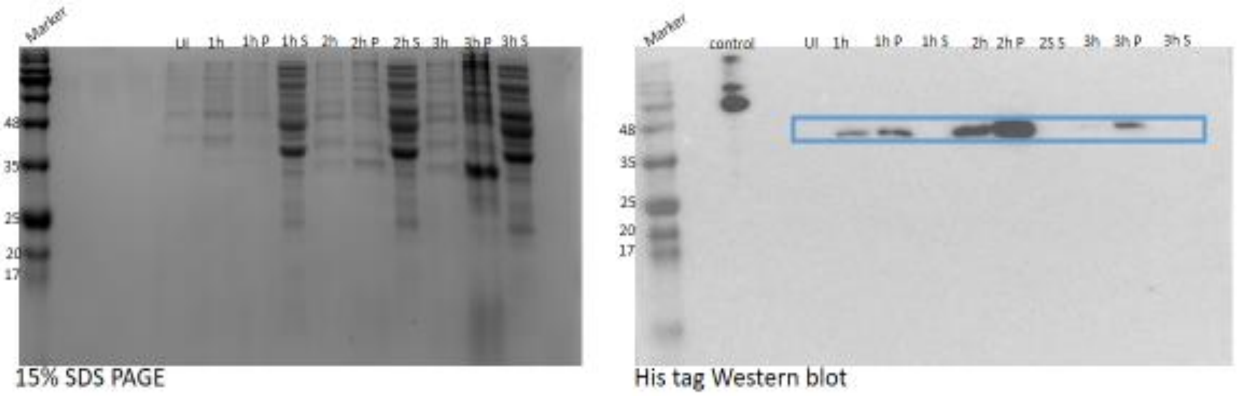

Figure 4.20. Coexpression of +N_pET28a+ with PA310_IBA3C in C43 at 37°C.

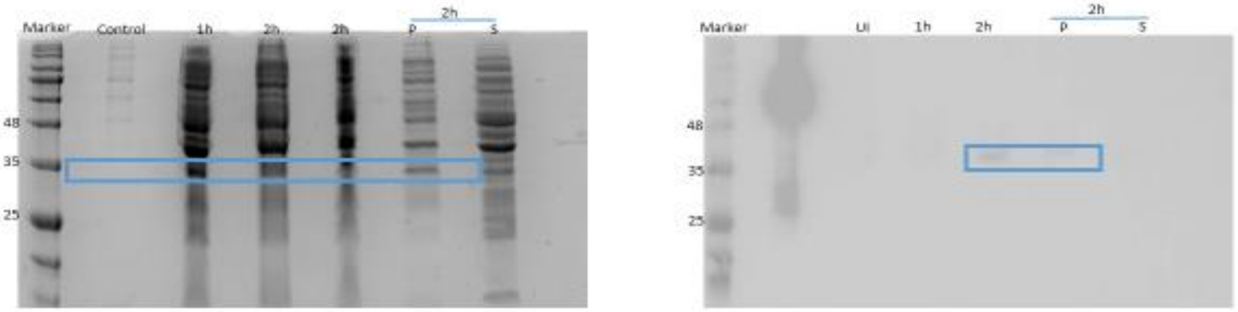

Figure 4.21. Coexpression of +N_pET28a+ with PA310_IBA3C in Star (DE3) with 0.2% glucose at 37°C.

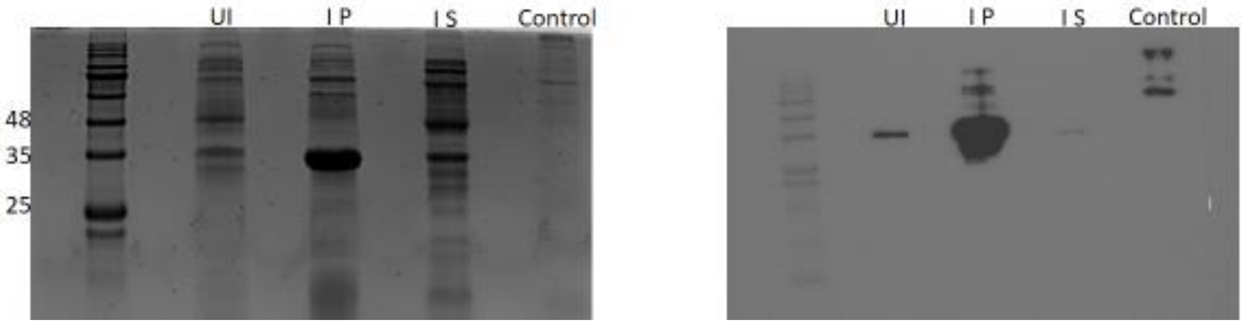

Figure 4.22. Coexpression of -NC_pET28a+ with PA310_pET22b+ in Star (DE3) at 20°C.

4.3 Refolding and purification by using urea:

The continuous formation of inclusion bodies always ending up in the pellet needed a solution. Because of expressing in different bacteria using different conditions did not solve the problem of inclusion bodies formation, a new strategy needed to be done. An article published in 1997 (Tan et al., 1997) showed how another Zinc-dependent phospholipase C from Bacillus cereus could be expressed by using urea purification. First, again both with and without signal peptide constructs were again expressed in the expression strain Star (DE3) at 37°C to reassure expression before starting with urea purification (Figure 4.23). The construct with signal peptide (+N_pET28a+) showed cell death right after induction at OD 0.4 (decreasing in OD), so only the construct without signal peptide (-NC_pET28a+) was used for urea purification shown below (Table 4.14). After inducing with one millimolar of IPTG and harvesting four hours later, the cells were opened using a homogenizer. After running the cell lysate through the column and going through all the steps required for urea purification and finally eluting using an elution Buffer, the protein's concentration could be calculated by using the NanoDrop by applying the Lambert-Law equation ($A = \varepsilon dc$). The coincidence number of PlcB is known to be 91790. So according to NanoDrop's calculations, the final concentration of my protein after urea purification was 0.2 mg/ml in 6 ml of Elution Buffer. Samples of elution fractions from column were analyzed by SDS PAGE and Western Blot to see if urea purification worked out and if there were any impurities with PlcB. A clear band could be seen in the elution buffer fraction in both SDS PAGE and Western Blot, indicating that Purification could be done successfully. In addition to that, no specific impurities could be observed on SDS PAGE (Figure 4.24). During elution, each 500 µl were collected separately in an eppendorf tube, to run on 15% SDS PAGE. Positive bands in the right size could be observed on the gel (Figure 4.25). Also, the concentration of each eluted fraction in each eppendorf tube was checked. The fraction with the highest concentration was found to be in tube number six with a final concentration of 0.371 mg/ml (Table 4.15).

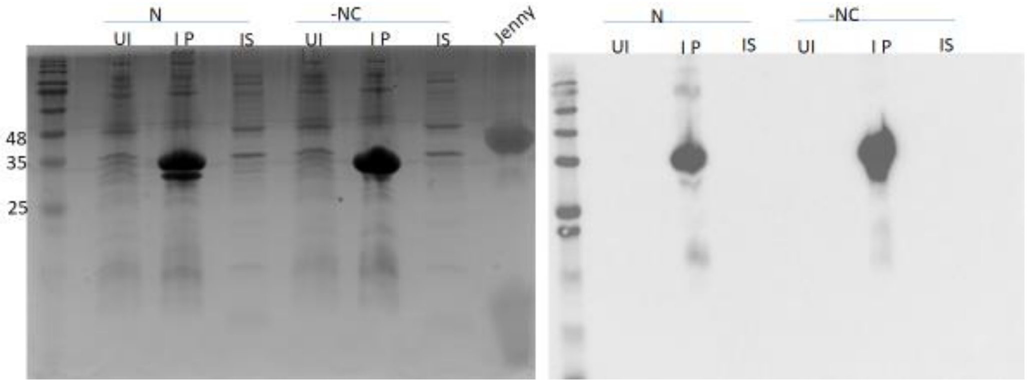

Figure 4.23.15% SDS PAGE (left) and His-tag Western Blot of +N_pET28a+ and –NC_pET28a+ in Star (DE3) at 37°C.

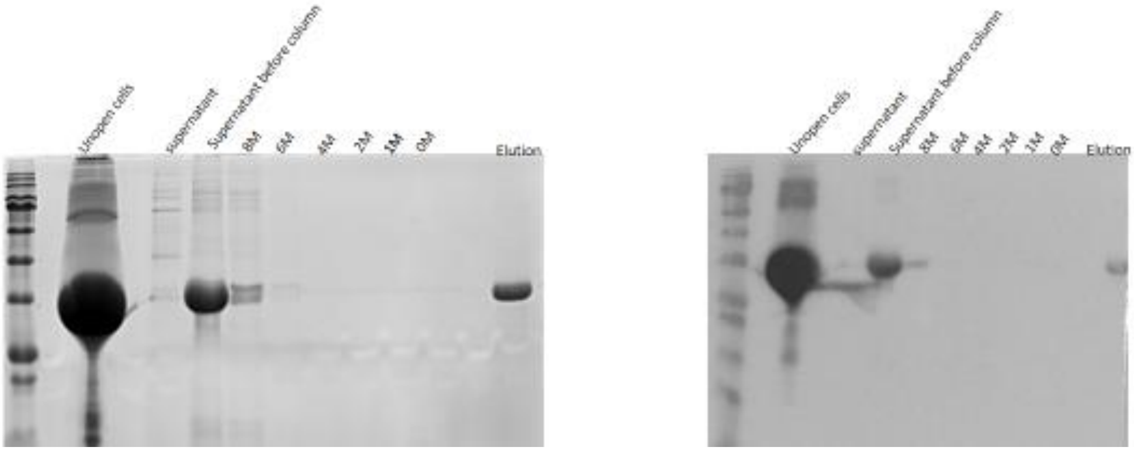

Figure 4.24. 15% SDS PAGE (left) and His-tag Western Blot (right) of fractions after urea purification of –NC_pET28a+.

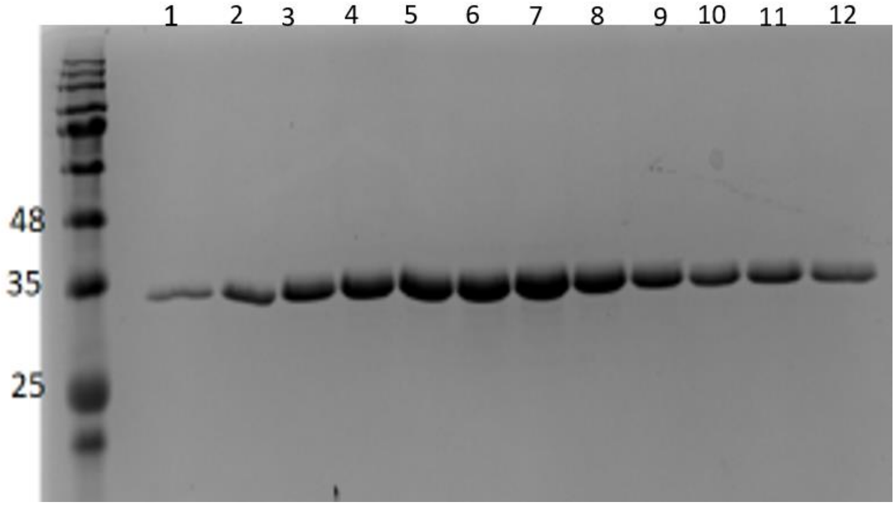

Figure 4.25. 15% SDS PAGE of the eluted fractions from urea purification.

4.4 Size Exclusion Chromatography (SEC), Gel Filtration:

After urea purification, SEC was run in order to determine the size of PlcB. From the formed peak (Figure 4.26), the size of PlcB could be estimated. First, the protein in the six milliliter Elution buffer was concentrated (2.594 g/ml in 500 µl). The resulted 500 µl after concentrating was injected into the loop overnight (Superdex 200 Increase). Mechanism of function of SEC is that large particles can not enter the pores of the stationary phase, so they elute faster than the small particles (low volume). Small particles can enter the pores of the stationary phase, making their distance to travel longer, so they elute later than larger particles (higher volume). With a size of 36.7 kDa, PlcB is considered as a small protein, so it is expected to have a peak at around 17 ml. According to Figure (4.26), there was no peak at 17 ml. A large peak could be observed at 13.66 ml, which is an elution volume smaller than expected (smaller than 17 ml). This could be explained by the prediction that PlcB formed oligomers. This oligomer-formation increased the overall size of the protein 3the column than it was expected.

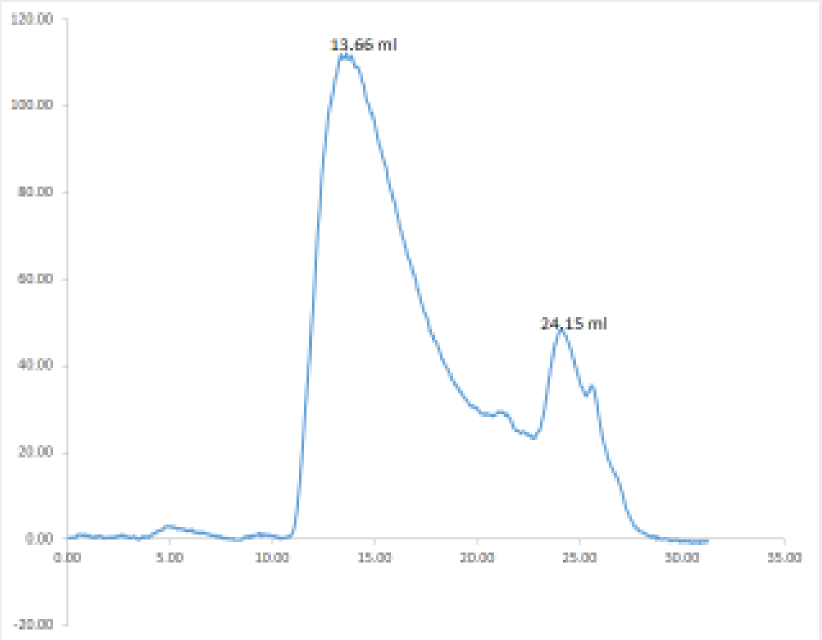

Figure 4.26. Size Exclusion Chromatography profile of eluted fraction after urea Purification (Superdex 200 Increase).

4.5 Activity assay by using Phospholipase activity assay kit:

In Structural Biology, a clear statement says that understanding the proper structure means understanding the proper function of a protein. So in order to see if urea purification could give me my protein in its soluble and folded form, activity assay specific for phospholipases was done. The elution fractions resulted by SEC that formed the highest peak as shown in Figure (4.26) at a volume of 13.66 ml were used in a 96-well plate. A positive result is observed by the naked eye when a color change (colorless to yellow) is seen. After incubating overnight in an incubator at 37°C, a slight change in the color could be seen in some of the wells. This change in the color is a positive result and shows that my protein is functioning. How to be sure that the results are reliable is by using both a positive and a negative control. Even after 24 hours, the negative control stayed colorless, which is a strong indicator that the results are reliable. Both Figures (4.27 and 4.28) included the same Buffer, except for Figure (4.28), which included the addition of one Millimolar $ZnSO_4$. The idea of adding to the Buffer Zinc sulfate is because of our previous knowledge that PlcB is considered as a Zinc dependent enzyme.

No proper difference was found between the addition and the lack of Zinc Sulfate. The highest concentration was found to be in well B12 (0.020 mg/ml) and C6 (0.038 mg/ml) shown (Table 4.16). As a conclusion, when looking at the results of both SEC and Phospholipase activity assay, it can be concluded that PlcB formed oligomers with a slight activity.

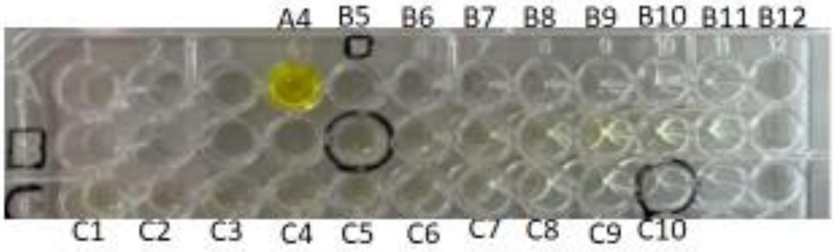

Figure 4.27. Phospholipase activity assay without Zinc Sulfate (B5-C10)
A4: +ve control (PlcH and substrate), B5: -ve control (Buffer and Substrate).

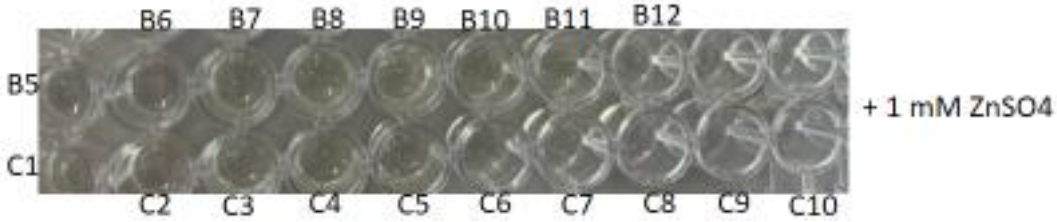

Figure 4.28. Phospholipase activity assay with Zinc Sulfate (C1-C10).

Chapter Five: Discussion

5.1 Restating research question and hypothesis:

In this research, the main general question was what is the three dimensional structure of the novel, cytoplasmic, zinc-dependent PlcB expressed by P. aeruginosa, belonging to the phospholipase C group. This question came up in order to understand the molecular basis of disease, caused by the extreme virulent bacteria, P. aeruginoas. Answering this question will contribute to limit or at least to lower its dangerous, virulent and sometimes deadly effect in immunosuppressive patients, especially and mostly in CF patients. The hypothesis behind this research was that PlcB could be a soluble protein, since it is a cytoplasmic protein and most cytoplasmic proteins are easily expressed in bacterial expression strains (easier to express than membrane proteins). In addition to that, it might have similarity and shared domains with other different phospholipases, especially Zinc-dependent phospholipases, that are expressed in other different bacterial strains.

5.2 Summary of main findings:

It was found that PlcB is not a soluble protein, contrary to what was expected, since it is considered as a cytoplasmic protein and not a membrane protein. PlcB easily formed inclusion bodies and always ended up in the pellet fractions in both SDS PAGE and Western Blot analysis. Many different conditions were tried in order to get PlcB in a soluble state, starting from trying different bacterial expression strains, to lowering temperature, to increasing the OD when harvesting, to co-expression with a chaperone until lowering the concentration of used inducer (IPTG). All different mentioned conditions were tried in order to get PlcB in a soluble state, but in all lysates, it always ended up in the pellet fraction, without any success. An article published in 1997 (Tan et al., 1997) showed how another zinc-dependent phospholipase C from another bacteria (Bacillus cereus) could be produced and isolated by using urea purification. As a result of this finding, urea purification was tried as a last hope. Thankfully, PlcB could be purified in this way too. Both SDS PAGE and His-tag Western Blot analysis showed that PlcB could be purified and isolated properly without the formation of any impurities through other proteins. This could be emphasized through SDS PAGE of the eluted fraction. After urea purification, SEC was run to check the size of PlcB. The resulted plot showed that PlcB formed oligomers, since it was eluted through the Superdex 200 column earlier than expected. An activity assay kit specific for phospholipases could confirm by a slight change in color from colorless to yellow, that the isolated protein was functional, indicating that it is in a more or less proper folding.

5.3 Research Significance and Contribution:

After cloning PlcB in its plasmid, my focus was to get PlcB in its most soluble, folded, active and functional state as possible. This was important in order to be able to start with the biophysical characterizations like to form crystals and to expose the crystals to X-Ray beams. After many trials and many changes in the bacterial-expression conditions, like changing the expression temperature from high to low, lowering the concentration of inducer and co-expression in combination with its chaperon it was found that PlcB was not that easily soluble under “basic” and “common” conditions that are usually applied to cytoplasmic proteins. This finding will be able to open up many doors for future-researchers who aim to express PlcB by saving enormous time, effort and money, since they can skip all those steps of trying to express PlcB in different bacterial expression strains with the different conditions that I used. In conclusion, my research would help researchers who aim working on PlcB by skipping the part of trying different bacterial-expression conditions in order to get PlcB soluble and can directly move forward to urea purification and continue from there.

5.4. Research significance according to PlcB’s virulent effects:

From the name of the enzyme, PlcB breaks down the phospholipid bilayer found in the host cell, causing cell lysis and cell death. Not only does PlcB break down the cells, but it also contributes in other different mechanisms that support the virulent strength of P. aeruginosa on the effected host cells. One of those virulent effects caused by PlcB is Biofilm formation.

When P. aeruginosa reaches a certain high optical density, an auto-inducer called homoserine lactone is expressed, targeting the gene expressing PlcB, activating PlcB production, which will enhance the process of Biofilm formation. This strong layer composed of polysaccharides, lipids and proteins protects P. aeruginosa, since this protective layer can prevent drugs to pass through and dissolve. This allows P. aeruginosa to be that Antibiotic resistant and makes targeting hard and more complicated (Lewenza et al., 2017).

5.4 Future Research Directions:

Because PlcB could be isolated and purified by urea purification and activity could be assured using the phospholipase-specific activity assay, future directions would be to try to get PlcB in its crystal state by trying different Buffers, that will turn PlcB into a crystal, which will make its exposure to X-Ray beams possible. This exposure will show the density of the electrons within the crystal by measuring the angles and intensity of diffracted X-ray, which will give a picture of the chemical bonds and the specific position of the atoms. This will lead to a proper understanding of the three-dimensional structure of PlcB. Furthermore, this will allow researchers to be able to design drugs working against PlcB and reducing the virulence effect of P. aeruginosa. This reduced lowering virulence effect could be a hope, especially for CF patients, by giving them a better life quality and increasing their life-rate. Another direction would be trying to use the cell-free expression system. This system has many advantages, like the prevention of toxicity, due to the lack of using cells, so if the protein causes cell death (as faced), then using a system that does not have cells prevents cell death and the protein is being expressed without any loss in both protein quality and quantity. Another advantage in this system is the easy addition of any co-factors or chaperons, which could help in the folding process of PlcB. In addition to the above mentioned future directions, co-expression of PlcB with the other two proteins found in the same operon (chaperone and the third hypothetical protein), could be a good idea, especially because the chaperone helps in the folding process and the hypothetical protein plays a role in secretion, so having them all work together as one and at the same time could solve the problem of misfolding and inclusion bodies formation. Finally, applying the Michaelis-Menten kinetics law, which determines how efficient PlcB degrades phospholipids. This finding could help us to design specific drugs targeting PlcB based on its kinetics.

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Appendices

Tables:

Table 3.2: Bacterial Strains and plasmids

Strain/plasmid name	**Company**
BL21(DE3)	Sigma
C43(DE3)	Sigma
PA0014: wild type	From Kolbe's group
E. coli Top 10 competent cells	Invitrogen
IBA3C plasmid	Iba Lifesciences
pET28a+	Novagen
pET22b+	Novagen
PlcB_pET28a+: NdeI and XhoI	(this study)
PA310_pET22b+: NdeI and XhoI	(this study)
PA310_IBA3C: BamHI, XbaI	(this study)

Table 4.1: Amount of PCR components for PlcB

PCR components	Amount
MQ	63 µl
5x GC Buffer	20 µl
Deoxynucleotide triphosphate (dNTP)	2 µl
Dimethyl sulfoxide (DMSO)	2 µl
Forward primer	5 µl
Reverse primer	5 µl
Template (PA14 Genome) 137.4 ng/µl	2 µl
Phusion enzyme	1 µl
Total	100 µl

Table 4.2: Concentration of PCR products after agarose-gel extraction (ng/µl)

	Concentration ng/µl
-N	37.9
-NC	27.4
+NC	41.3
pET28a+	28.4

Table 4.3: Digestion of –N, - NC and +NC with NdeI and XhoI

Components	**Amount (µl)**
MQ	5
Green Buffer	5
NdeI	2
XhoI	2
-N, -NC, +NC	45
Total	**60**

Table 4.4: Digestion of pET28a+ with NdeI and XhoI

Components	**Amount (µl)**
MQ	0
Green Buffer	6
Nde1	2
Xho1	2
pET28a+	50
Total	**60**

Table 4.5: Concentration of pET28a+, -N, -NC and +NC after digestion with NdeI, XhoI

	Concentration (ng/µl)
pET28a+	22.6
-N	31.4
-NC	21.3
+NC	27.8

Table 4.6: Ligation of digested pET28a+ with digested –N, -NC and +NC

Components	**Amount**
MQ	4.5
10x Ligase Buffer	1
Digested insert	1
Digested pET28a+	2.5
Fast Ligase	1
Total	**10**

Table 4.7: Digestion of ligated constructs with NdeI, XhoI to confirm correct ligation

Components	**Amount (µl)**
MQ	1
10x Green Buffer	2
Nde1	1
Xho1	1
N_pET28a+, -NC_pET28a+, +NC_pET28a+	15
Total	**20**

Table 4.8: Using primers to reassure proper ligation

Components	**Amount (µl)**
MQ	112
1% DMSO	2
5x GC Buffer	40
dNTP	4
T7 Forward Primer	10
T7 Reverse Primer	10
Ligated vector	20
Phusion enzyme	2
Total	**200**

Table 4.9: PCR components of chaperone PA310

Components	**Amount (µl)**
MQ	189
5x GC	60
dNTP	6
DMSO	6
Forward primer (NdeI)	15
Reverse primer (XhoI)	15
PA14 genome 137.4 ng/µl	6
Phusion enzyme	3
Total	300

Table 4.10: Digestion of chaperone using NdeI, XhoI

Components	**Amount (µl)**
MQ	0
Green Buffer	2
Nde1	3
Xho1	3
Chaperone (310) 212.1 ng/µl	12
Total	20

Table 4.11: Digestion of pET22b+ using NdeI, XhoI

Components	Amount (µl)
MQ	0
Green Buffer	6
Nde1	2
Xho1	2
pET22b+ 20 ng/µl	50
Total	60

Table 4.12: Components of ligating pET22b+ with PA310 (pET22b+_PA310)

Components	**Amount (µl)**
MQ	8.16
T4 Ligase Buffer	2.00
Ligase	1.00
Chaperone PA310 (63.6 ng/µl)	0.54
pET22b+ (6 ng/µl)	8.30
Total	20.0

Table 4.13: Digestion of pET22b+_PA310 with NdeI, XhoI to check proper ligation

Components	**Amount (µl)**
MQ	1
Green Buffer	2
Nde1	1
Xho1	1
pET22b+_PA310	15
Total	20

Table 4.14: Optical Density of expressing –NC_pET28a+ in Star (DE3) at 37°C

Time	**OD**
After 1h	0.060
2h	0.246
2.5h	0.415
After 1h of induction (10x)	1.670
After 2h of induction (10x)	2.300
After 3h of induction (10x)	3.130
After 4h of induction (10x)	2.750

Table 4.15: Concentration of Elution fractions (mg/ml)

Elution Fractions (500 µl)	**Concentration (mg/ml)**
Tube 1	0.055
Tube 2	0.102
Tube 3	0.139
Tube 4	0.190
Tube 5	0.232
Tube 6	0.371
Tube 7	0.289
Tube 8	0.237
Tube 9	0.195
Tube 10	0.105
Tube 11	0.104
Tube 12	0.090

Table 4.16: Concentration of SEC's elution fractions after phospholipase activity assay

Fractions	**mg/ml**
B5	0
B6	0.012
B7	0
B8	0.005
B9	0.012
B10	0.018
B11	0.007
B12	0.020
C1	0.014
C2	0.013
C3	0.021
C4	0.007
C5	00.027
C6	0.038
C7	0.007
C8	0.036

C9	0.001
C10	0

Protocols:

-PCR: https://www.neb.com/en/protocols/2012/09/06/protocol-for-phusion-hot-start-flex-dna-polymerase-m0535

-DNA digestion:
https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011980_DNA_Digestion_UG.pdf

-DNA ligation:
https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011990_T4_DNA_Ligase_5_Weiss_200_Weiss_U_UG.pdf

-Gel extraction protocol:
https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012661_GeneJET_Gel_Extraction_UG.pdf

-Plasmid/ genomic purification protocol:
https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012663_GeneJET_Genomic_DNA_Purification_Kit_UG.pdf

-Novagen's Bug Buster protocol:
https://wolfson.huji.ac.il/purification/PDF/Protein_Expression_Extraction/NOVAGEN_BugBuster_protein_extraction.pdf
-SDS PAGE protocol
https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf

الخصائص البيولوجية-فيزيائية لبروتين بي السي بي لبكتيريا البسيودوموناس ارجنوسا

اسم الطالبة: أسماء يوسف سعيد كميل

أ. د. هلال زيد

أ. د. يورج لبان

د. صبا شنك

د. رمزي شواهنه

ملخص

تعد بكتيريا البسيودوموناس ارجنوسا من البكتيريا التي تُصنّفها منظمة الصحة العالمية ضمن مسببات الأمراض ذات الأولوية العالية. تصيب هذه البكتيريا بشكل رئيسي الأشخاص الذين يعانون من ضعف في جهاز المناعة، وخصوصًا مرضى التليف الكيسي، وهو مرض ناتج عن طفرة في الجين المنظم لدخول و خروج ايونات في الجسم. تؤدي هذه الطفرة إلى تراكم المخاط في عدة أعضاء، مما يسبب انسداد الممرات الهوائية ويؤثر على إفراز الإنزيمات في البنكرياس. تستقر البكتيريا في هذا المخاط الموجود في رئة المرضى المصابين بالتليف الكيسي، مما يؤدي إلى التهابات رئوية قد تسبب تلفًا في الرئة وقد تصل إلى الوفاة. تتميز هذه البكتيريا بقدرتها على مقاومة العديد من المضادات الحيوية، مما يجعل علاجها صعبًا. كما أن امتلاكها لعوامل المقاومة يزيد من شدة خطورتها. من بين هذه العوامل، إنزيمات الفسفوليبيز التي تُساهم في شدة تأثير البكتيريا على الإنسان. تنقسم هذه الإنزيمات إلى عدة أنواع، من بينها الفسفوليبيز (ب).

يهدف هذا البحث إلى دراسة الخصائص البيوفيزيائية للفسفوليبيز (ب). تم تحقيق ذلك من خلال استنساخ الجين في بلازميد، ثم تحديد درجة الحرارة ونوع البكتيريا المناسبة التي تُمكِّن من إنتاج

البروتين في حالته القابلة للذوبان. بعد ذلك، تم تنقية البروتين، وتم التأكد من فعاليته من خلال اختبار مخصص للنشاط الحيوي. أظهرت نتائج التعبير في أنواع بكتيريا ودرجات حرارة مختلفة أن البروتين تم إنتاجه في صورة غير ذائبة داخل البكتيريا. للتغلب على هذه المشكلة، تم تجربة عدة ظروف تعبيرية مختلفة، مثل التعبير المشترك للبروتين مع بروتين مساعد، أو خفض درجة الحرارة، أو تقليل تركيز المادة المحفزة. في النهاية، تمكّنت من عزل وتنقية البروتين باستخدام مادة اليوريا. تبين أن العينة المنقاة خالية من الشوائب كما تم التأكد من نشاط البروتين باستخدام اختبار تؤكد فعاليته الحيوية. هذا يمهّد للانتقال إلى مرحلة تحليل البنية. ولا بد من إجراء تجارب إضافية للكشف عن تركيب الفسفوليبيز (ب)، وذلك باستخدام وسائل التحليل الفيزيائي الحيوي مثل الرنين المغناطيسي النووي، والانحراف الدائري، وتقنية تحليل البنية بواسطة الأشعة السينية.

أُجريت هذه الدراسة في مدينة هامبورغ بألمانيا، في الفترة ما بين شهر تشرين الأول من عام ألفين وواحد وعشرين، وحتى شهر حزيران من عام ألفين واثنين وعشرين.

الكلمات المفتاحية:
بسيدوموناس اريجينوسا, التليف الكيسي, الاشعة السينية