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Exploration of an alginate lyase targeting the biofilm in *Pseudomonas aeruginosa*

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Biotechnology

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Master's Thesis

By

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"Kanskje verden er litt stri,
men når det gråner kan du si,
at du har hatt en bra studentertid.»

Mildrid Angard Hoff
May 2022, Ås

Abstract

Pseudomonas aeruginosa is an opportunistic gram-negative bacterium capable of colonizing immunosuppressed patients such as those with cystic fibrosis. A *P. aeruginosa* infection could lead to mortality since some strains of the bacterium can change morphology by overproducing the exopolysaccharide alginate, becoming a mucoid variant that is more resistant to host defense and medical treatment. Many of the enzymes connected to the alginate machinery have already been characterized. However, the putative polysaccharide lyase (PL) family 7 alginate lyase PA1784 is poorly described in the literature, and new treatment methods for mucoid biofilm might be discovered by testing enzymes associated with alginate. Therefore, PA1784 (renamed *Pa*Aly7a) was first purified, and then activity assays were conducted to test the activity against substrates like sodium alginate, Psl and Pet in biofilm, and purified alginate from *P. aeruginosa*. In addition, the sequence to *Pa*Aly7a was analyzed by bioinformatics tools, and the predicted structure was compared to the PL7 alginate lyase, PA1167. The results from the activity assays showed no signs of activity. Nevertheless, a STRING analysis showed a connection between *Pa*Aly7a and a DNase, so DNA was tested as a substrate in a DNA-degradation assay. The assay showed signs of DNA degradation, but a DNase was identified in the sample after a proteomics analysis. In addition, the results revealed that *Pa*Aly7a formed inclusion bodies when overexpressed in *E. coli* and that the lack of activity could have been due to improper folding of the protein. However, the melting point analysis of *Pa*Aly7a-P2 showed a melting point, indicating that it might be correctly folded. Furthermore, the predicted structure of *Pa*Aly7a showed that the active site and the area around it were tighter and more negatively charged than the active site in the PL7 alginate lyase PA1167. Despite the lack of activity, these results contribute to further knowledge about *Pa*Aly7a and the goal of finding an alternative treatment for *P. aeruginosa* infections in patients with cystic fibrosis.

Sammendrag

Pseudomonas aeruginosa er en gramnegativ opportunistisk bakterie som er i stand til å kolonisere immunsupprimerte pasienter, slik som de med cystisk fibrose. En *P. aeruginosa*-infeksjon kan føre til dødsfall siden denne bakterien kan endre morfologi ved å overprodusere eksopolysakkaridet alginat. Dermed blir en mucoid variant mer motstandsdyktig mot immunforsvaret og medisinsk behandling. Mange av enzymene knyttet til alginat i *P. aeruginosa* har allerede blitt karakterisert, men PA1784 (referert til som *Pa*Aly7a), som er antatt å være en alginat lyase i polysakkarid lyase familien 7, er dårlig beskrevet i litteraturen. Siden nye behandlingsmetoder for *P. aeruginosa*-infeksjoner muligens kan knyttes opp mot enzymer assosiert med alginat, har *Pa*Aly7a blitt renset og testet. *Pa*Aly7a ble testet ved bruk av forskjellige aktivitetsanalyser og ulik substrater slik som natriumalginat, Pel og Psl i biofilmer og renset alginat fra *P. aeruginosa* ble brukt. I tillegg ble sekvensen til *Pa*Aly7a analysert med forskjellige bioinformatiskeverktøy, og den antatte strukturen sammenlignet med en PL7 alginat lyase, PA1167. Resultatene fra aktivitetsanalysene viste ingen tegn til aktivitet. En STRING-analyse viste at *Pa*Aly7a hadde en forbindelse til DNase, DNA ble derfor testet som et substrat i en DNA-nedbrytningsanalyse. Analysen viste tegn på nedbrytning av DNA, men DNase ble identifisert i prøven ved en proteomikk analyse. I tillegg viste det seg at inclusion bodies ble dannet når *E. coli* overuttrykket *Pa*Aly7a. Feil foldet protein kunne derfor forklare hvorfor proteinet ikke viste aktivitet. Imidlertid indikerte smeltepunktanalysen til *Pa*Aly7a-P2 at proteinet var riktig foldet. Videre viste den predikerte strukturen til *Pa*Aly7a at det aktive setet og området rundt var trangere og mer negativt ladet, enn det aktive setet til PA1167. Til tross for manglende resultat av *Pa*Aly7a aktivitet, bidrar disse resultatene til en økt kunnskap om *Pa*Aly7a og målet om å finne en alternativ behandling for *P. aeruginosa*-infeksjoner hos pasienter med cystisk fibrose.

Abbreviations

Å	Ångström (0.1 nanometers)
$A_{275}/A_{280}/A_{540}$	Absorbance of ultraviolet light at 275/280/540 nanometers
ABC/AmBic	Ammonium BiCarbonate
ACN	Acetonitrile
BHI	Brain Heart Infusion
BL21	One Shot® BL21 Star™ (DE3) Chemically Competent *E. coli*
bp	Base Pair
CAZy	Carbohydrate-Active Enzymes database
CF	Cystic Fibrosis
CFU	Colony forming units
CIP	Calf Intestinal alkaline phosphatase
Da	Dalton
DEH	4-deoxy-L-erythro-hex-4-enepyranosyluronate
dH_2O	distilled water
DHB	Dihydroxybenzoic Acid
DNS	3,5-Dinitrosalicylic Acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
eDNA	Extracellular Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extracellular Polymeric Substance
g	Relative centrifugal force
HIC	Hydrophobic interaction chromatography
His-tag	Polyhistidine tag
IAA	Iodoacetamide
IEX	Ion exchange chromatography
IMAC	Ion Metal Affinity Chromatography
IPTG	Isopropyl b-D-1-thiogalactopyranoside
LB	Lysogeny Broth
LC-MS	Liquid chromatography–mass spectrometry
LDS	Lithium dodecyl sulfate

m/z	Mass-to-charge
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
mAU	Millie Arbitrary Units
MSA	Multiple sequence alignment
MWCO	Molecular Weight Cut-off
nm	Nanometers
OD	Optical density
OMVs	Outer membrane vesicles
PA	Phosphoric Acid
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PL	Polysaccharide Lyases
PMSF	Phenylmethylsulphonyl fluoride
PSA	Pairwise sequence alignment
QS	Quorum-Sensing
RND	Resistance-Nodulation-cell Division
rpm	Revolutions per minute
RT	Room temperature
S.O.C.	Super Optimal Broth
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEC	Size-Exclusion Chromatography
TAE	Tris-Acetate-EDTA
TB	Terrific Broth
TFA	Trifluroacetic acid
TGS	Tris-Glycine-SDS
T_m	Protein melting point
TOP10	One Shot® TOP10 Chemically Competent *E. coli*
Tris-HCl	Trizma base hydrochloric acid
TSB	Tryptone soya broth
UV	Ultraviolet

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1 Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was first described in 1872 by Schroeter (Hugh & Leifson, 1964). According to Hugh and Leifson (1964), Schroeter did not isolate the bacteria, but he described and named the organism *Bacterium aeruginosum*. Schroeter called the microorganism *aeruginosum* since the bacterium created a green-blue color on a specific media. The name *aerūgō* means copper rust or verdigris, and the color produced by the organism was very similar to copper rust (Etymologia: *Pseudomonas*, 2012; Palleroni, 2010). In 1882 Carle Gessard isolated and described this bacteria for the first time (Gessard, 1984). He had been given bandages with blue and green discolorations, where he found the discoloration fascinating and ended up isolating the microorganism creating this discoloration. In his notes, he described the bacterium as colorless, globular, from 1 to 1.5 thousandths of a millimeter, aerobic, and very mobile. However, Gessard did not name the bacteria *Pseudomonas aeruginosa*. *Pseudomonas* was first classified as a genus in 1894 by Walter Migula (Palleroni, 2010). Migula described the genus as a group of bacteria that could move with polar organs, and some of them were able to produce spores. This was not a very specific definition of a genus, and therefore many bacteria were wrongly classified as *Pseudomonas*. It was later proven that *Pseudomonas* could not create spores. A couple of years later Migula set *P. aeruginosum* as the type-specie of the genus *Pseudomonas* and renamed it to *P. aeruginosa* (Palleroni, 2010).

P. aeruginosa is a Gram-negative ubiquitous rod bacterium. *P. aeruginosa* can derive energy from 75 different carbon sources and use nitrate as an electron acceptor to survive in an anaerobic environment (Iglewski, 1996). Furthermore, the bacteria *P. aeruginosa* also has a significant natural resistance against high salt concentrations, weak disinfectants, and many antibiotics. This makes the bacteria capable of surviving in numerous environments and on abiotic and biotic surfaces (Gellatly & Hancock, 2013; Hardalo & Edberg, 1997; Iglewski, 1996; Russotto et al., 2015). This ability to survive and utilize different substrates is partly due to a large genome and a large variety of conserved genes (Moradali et al., 2017; Stover et al., 2000).

In 2000, Stover et al. published the entire genome sequence of the *P. aeruginosa* strain called PAO1 (Stover et al., 2000), a laboratory strain originally isolated from a wound in Melbourne, Australia (Hare et al., 2012; Holloway, 1955). The results from the sequencing gave insights

into the genetic complexity of *P. aeruginosa*. One mechanism discovered was *P. aeruginosa's* extensive regulatory capability of genes (Stover et al., 2000). Due to fast gene regulations, the bacteria could adapt to drug resistance and new carbon sources faster than other bacteria with smaller genomes. *P. aeruginosa* has the highest percentage of genes that control and command systems like environmental sensors and transcriptional regulators ever observed in a bacterial genome (Stover et al., 2000). In addition, *P. aeruginosa* has 300 different cytoplasmic membrane transport systems which can transport essential and unessential solutions in and out of the cell. The transport systems include an iron-siderophore uptake system, multiple protein secretion systems, and a resistance-nodulation-cell division (RND) efflux system (Stover et al., 2000). Of these 300, *P. aeruginosa* has four different multidrug systems, and all four are a part of the RND family (Nikaido, 1998). In addition, *P. aeruginosa* has many undescribed drug efflux systems, which yield high resistance to antibiotics and fast adaption to a new environment (Stover et al., 2000).

1.1.1 Infections and Pathogenic mechanisms

Pathogenic bacteria are defined as bacteria that can cause damage to the host cell (Fischetti et al., 1999). Virulence factors are, for example, inherent components which lead to damage in the host, molecules, or structure. Factors like adherence factors, make it possible for the microorganism to evade or modulate the immune system (Johnson, 2018). *P. aeruginosa* is classified as an opportunistic pathogen, meaning that the bacterium can cause an infection under certain conditions (Brown et al., 2012). Patients with underlying diseases or a weak immune system represent high-risk groups for infection of this bacterium. Especially patients with prolonged hospital stays, patients with burn wounds, and patients with cystic fibrosis (CF) are likely to be infected by *P. aeruginosa* (Stover et al., 2000; Trubiano & Padiglione, 2015).

A *P. aeruginosa* infection can be either acute or chronic, and the infection can develop from acute to chronic. This development is regulated by quorum-sensing (QS) dependent gene expression (Moradali et al., 2017). QS is defined by Miller and Bassler (2001) as "the regulation of gene expression in response to fluctuations in cell-population density." Bacteria with QS will produce and release chemical signal molecules that affect the gene expression of specific genes (Miller & Bassler, 2001). In *P. aeruginosa*, QS mainly regulates the early stages of infection, biofilm formation, and the genes involved in virulence factor production (Davies et al., 1998; Smith & Iglewski, 2003; Venturi, 2006). Since the QS network in *P. aeruginosa* can react and

adapt to stress signals, a change in the environment or a host factor can cause a QS-based response. This response can lead to the transcription of virulence factors (Lee & Zhang, 2015).

Almost every *P. aeruginosa* strain has a single polar flagellum, which gives the bacteria motility (Iglewski, 1996). Under a *P. aeruginosa* infection, a host's immune system will detect motile *P. aeruginosa* due to the flagellum. Amiel et al. (2010) discovered that the loss of flagellar function would make the bacterium resistant to phagocytosis. *P. aeruginosa* will therefore lose its motility, due to a nonfunctioning flagellum (Amiel et al., 2010), attach to a surface, and go into a more steady state as a biofilm, which causes less stress to the bacteria (Moradali et al., 2017). The biofilm also protects the bacteria from the immune cells by making both polymorphonuclear neutrophils and macrophages less effective (Watters et al., 2016), and polymorphonuclear neutrophils and macrophages are two of the most critical immune responses during infection (Prame Kumar et al., 2018).

By analyzing bacterial samples from CF patients with chronic infections, Doggett et al. (1966) discovered that most *P. aeruginosa* isolates from the lungs showed a mucoid phenotype. *P. aeruginosa* with a mucoid phenotype is defined as bacteria with an overproduction of the exopolysaccharide alginate. This overproduction causes the biofilm to change its morphology to a more viscous texture, as shown in **Figure 1**, and this change makes the bacteria more resistant to host defense and medical treatment (Breidenstein et al., 2011; Chanasit et al., 2020). Bacteria with a mucoid biofilm phenotype are almost impossible to remove and difficult to treat, and for patients with CF, an *P. aeruginosa* infection is the leading cause of morbidity and mortality (Moradali et al., 2017). The mucoid phenotype is also classified as the hallmark of a chronic infection, especially for CF individuals (Malhotra et al., 2018).

Figure 1. A non-mucoid and a mucoid strain. *This figure shows the phenotype of a mucoid and a non-mucoid strain spread on a plate. (A) To the left in the figure, a non-mucoid wild-type PAO1 strain is spread. (B) To the right in the figure, the mucoid CF isolate FRD1 (mucA22) is spread. Picture obtained from (Ramsey & Wozniak, 2005).*

In 1993, Martin et al. (1993) discovered that *P. aeruginosa* with a mucoid phenotype often had a frameshift mutation in *mucA*. MucA is an anti-sigma factor bound to the alginate synthesis sigma-factor AlgU. A frameshift mutation in *mucA* would result in a defective MucA. In a nonmutant, MucA is bound to AlgU, and this binding hinders the transcription of the alginate biosynthesis (**Figure 2 A**). A defected MucA cannot bind to AlgU, and AlgU will therefore promote the alginate biosynthesis permanently, which causes overproduction of the extracellular polysaccharide alginate in the biofilm (**Figure 2 C**) (Hay et al., 2014; May et al., 1991; Nivens et al., 2001). *P. aeruginosa* without a mutation in MucA can also overproduce alginate due to damage to the bacterial envelope (envelope stress) (Hay et al., 2014). This damage affects the outer membrane proteins, which misfolds and react with proteins connected to MucA (**Figure 2 B**). MucA will eventually be degraded, and AlgU released. AlgU will then activate the alginate operon causing a mass production of alginate. By DNA typing, it is now known that the mucoid strain is a clonal variant of the non-mucoid strain (May et al., 1991).

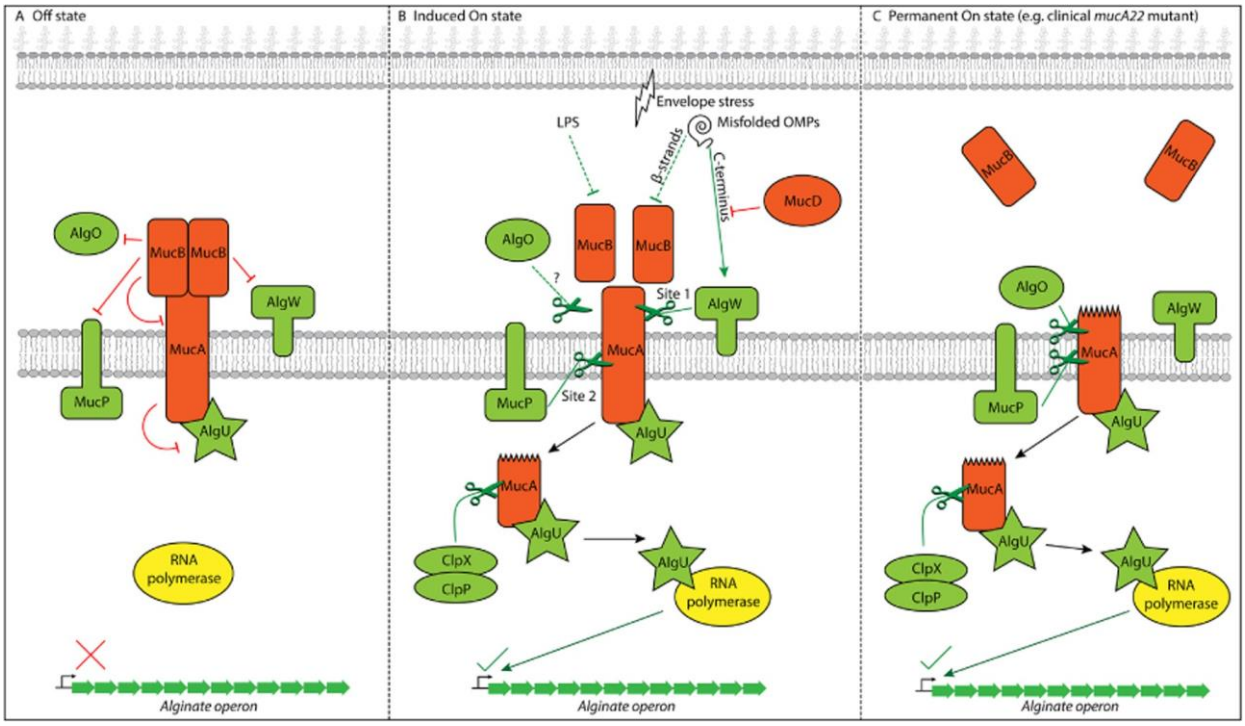

Figure 2. The regulation of the alginate operon. *The figure shows how the alginate promotor AlgU function during "Off" state (A), induced "On" state (B), and permanent "On" state due to mutation in* mucA (C). *The different proteins and reactions are color-coded. The green proteins and lines positively affect alginate production, while the red proteins have a negative effect on alginate production. The figure is obtained from (Hay et al., 2014).*

1.2 Biofilm

Biofilms are defined as "Aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface" by IUPAC (Vert et al., 2012). This biofilm will protect the bacteria by being a physical barrier as well as an enhancing environment for social cooperation. Bacteria in a biofilm treated with antibiotics have a higher chance of survival than free-living bacteria cells (Flemming et al., 2016). This is also observed in *P. aeruginosa* (Høiby et al., 2001).

1.2.1 Formation of biofilm

Biofilm formation can vary based on the substrate available (Shrout et al., 2006). For PAO1 in an environment with limited access to glucose, the biofilm lifestyle is divided into five overall stages (**Figure 3**). In the first stage, planktonic bacteria make a reversible adhesion to a suitable surface. In the second stage, this attachment is made irreversible. The bacteria then start to form different microcolonies in an extracellular polymeric substance (EPS) matrix. In stage three,

these microcolonies get much more extensive and expand at the surface. In addition, the arrangement of the matrix gets more structure. However, the microcolonies are not yet connected, and therefore some areas are still non-colonized. In stage four, the microcolonies are connected, and the entire area is colonized. The biofilm is then simultaneously growing in three dimensions. In the fifth and last stage, bacteria break loose from the biofilm, go into a planktonic state, and start to form colonies on other surfaces (Rasamiravaka et al., 2015).

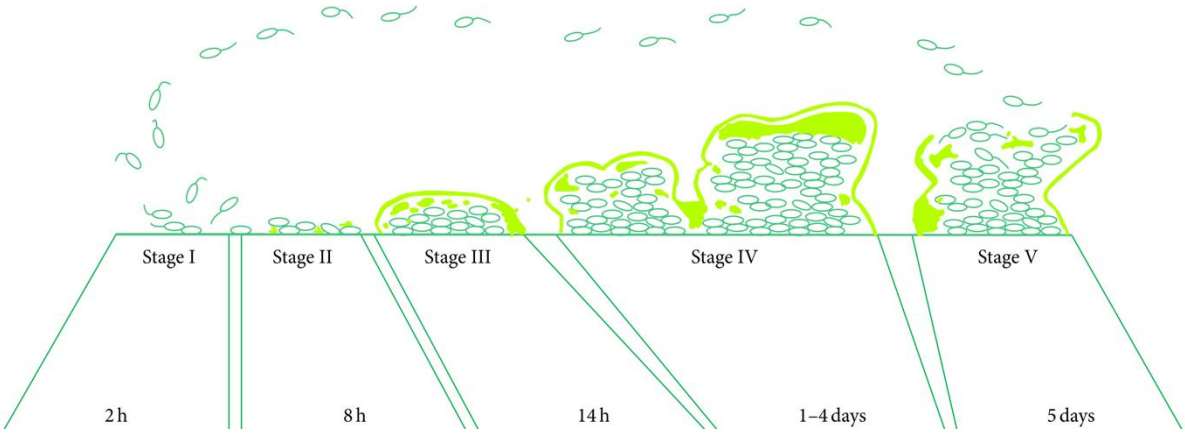

Figure 3. Biofilm formation*. The figure shows the development of biofilm formation with an estimated timeline. The different stages are shown with figures where the EPS is colored in bright green, and* P. aeruginosa *PAO1 as single green bacterium. This figure was obtained from (Rasamiravaka et al., 2015) with some adjustments.*

1.2.2 Extracellular polymeric substance in *P. aeruginosa* biofilm

The biofilm matrix from *P. aeruginosa* can consist of EPSs; alginate (a linear anionic polysaccharide), Pel (a linear cationic polysaccharide), and Psl (a branched neutral polysaccharide), in addition to extracellular DNA (eDNA), protein, and lipids (Ryder et al., 2007; Toyofuku et al., 2012), and it is the exopolysaccharide and eDNA that forms the architecture of the biofilm (Ghafoor et al., 2011). The different EPS is necessary for different stages in biofilm production, and Psl is important in the early stages. Wang et al. (2013) discovered that type IV pili (T4P)-mediated bacteria migration was the source of the Psl fiber matrix. IV pili is also an essential part of the rapid colonization of *P. aeruginosa* (Woods et al., 1980). This is supported by the research done by Iglewski (1996), which stated that clinical *P. aeruginosa* from patients with CF often had pili. Psl also protects the biofilm against several antibiotics and anti-biofilm agents, especially at the beginning of the biofilm formation (Billings et al., 2013). Pel is necessary for the initiation of and maintenance of cell-to-cell communication. Colvin et al. (2011) showed that PA14 would not evolve a biofilm beyond the monolayer stage without Pel. In addition, Pel may also be an essential protector against

antibiotics in PA14. In the mature biofilm from non-mucoid strains (i.e., *P. aeruginosa* strains that do not overproduce alginate), it is strain-dependent whether both Psl and Pel have a vital role structurally (Colvin et al., 2012). According to Colvin et al. (2011), Pel is the most critical EPS in PA14, while Psl is the most critical EPS in PAO1. Alginate is the dominating EPS in the mucoid strains and functions as a structural scaffold as well as a barrier against immune cells and antibiotics (Nivens et al., 2001). For further protection against the immune system, the alginate in *P. aeruginosa* biofilm is O-acetylate. The immune system cannot identify *P. aeruginosa* with O-acetylated alginate (Pier et al., 2001). According to Pier et al. (2001), O-acetylated alginate will not start the one of the immune responses in humans, the alternative pathway of complement, in contrast to non-acetylated alginate. Therefore, the O-acetylation prevents the bacteria from being killed by antibody-independent phagocytosis. Acetylation is also necessary to form microcolonies (Chanasit et al., 2020).

eDNA in *P. aeruginosa* is an essential factor for the structure in the biofilm as it can cross-link with the positively charged exopolysaccharide Pel. These polymers can substitute for Psl in mature biofilms (Jennings et al., 2015). It is well established that eDNA stabilizes biofilms and that eDNA is usually protected from DNase in the biofilm. However, this protection is not complete in the first step of biofilm formation. Whitchurch et al. (2002) tested this by adding DNase I to the culture medium. The result showed that the biofilm was prevented from establishing, and the bacteria could not attach to the surface. In addition, DNase I broke down the biofilm when added to a culture, 60 hours old or less. However, when applied to an 84 hours old culture, the biofilm was more resistant to DNase I. This indicates that the biofilm either has a protecting layer or proteolytic exoenzymes that inhibits DNase I (Whitchurch et al., 2002). eDNA is also essential as a nutrient source for *P. aeruginosa,* and it can create a cation gradient in the matrix, which can lead to a more heterogeneous biofilm (Mulcahy et al., 2008; Mulcahy et al., 2010).

Exopolysaccharides in the biofilm matrix in *P. aeruginosa* have been closely analyzed, while proteins and lipids in the biofilm are not nearly as well studied. Toyofuku et al. (2012) did a proteomic study of the matrix of *P. aeruginosa* PAO1. The findings showed that matrixes consist of extracellular, membrane, and cytoplasmic proteins and that 30% of the identified proteins were outer membrane proteins. Outer membrane vesicles (OMVs) are vesicles with DNA, proteins, lipids, and lipopolysaccharide secreted from gram-negative bacteria with a bilayered phospholipid (Schwechheimer & Kuehn, 2015). The outer membrane proteins

identified are often the same as those in the OMVs. It is well established that OMVs are important in the biofilm matrix, and the experiments done by Toyofuku et al. (2012) further support this.

1.2.3 The role of alginate in *P. aeruginosa* biofilm

The role of alginate in *P. aeruginosa* biofilm has been studied for many decades, but its exact function has not been clear. However, it is well established that alginate is an essential factor in the colonization of the pulmonary tissue since alginate contributes to protecting the bacteria from the immune processes, opsonization, and phagocytosis (Baltimore & Mitchell, 1980; Schwarzmann & Boring, 1971; Simpson et al., 1988), and toxic oxygen radicals (Simpson et al., 1989). Nivens et al. (2001) wanted to test if the alginate was a necessary part of the colonization, adhesion, contribution to biofilm structure, and the role of acetylated alginate. Through a series of experiments testing both mucoid and nonmucoid with and without alginate production, Nivens et al. concluded that alginate is not required for primary adhesion. However, the study showed that alginate has a crucial structural role in biofilm. After 48 hours, nonmucoid *P. aeruginosa* had covered the entire surface with biofilm, while the mucoid biofilm had less surface coverage. However, the mucoid biofilm had a much greater volume, and the biofilm structure was much more profound. The cells in the nonmucoid biofilm were more densely packed than in the mucoid biofilm, which is probably due to the alginate surrounding the cells in the mucoid biofilm. The nonmucoid also showed signs of a more uniform biofilm. However, both biofilms had the same number of bacterial cells per unit surface. Due to the hydrated biofilm of the mucoid type, Nivens et al. (2001) could not measure the microcolonies. Therefore, alginate might be an essential factor in the formation of microcolonies, and the protection from alginate against immune response is probably not as adequate on individual bacterium cells as it is on entire microcolonies (Nivens et al., 2001).

Nivens et al. (2001) also tested the effect and function of deacetylated alginate on mucoid strains compared to O-acetylated alginate. The results from the biofilm production of a mutant *P. aeruginosa* with deacetylated alginate showed signs of inhibition of attachment and growth in a mucoid strain. However, the surface-associated growth was significant only when the mutant turned into a nonmucoid phenotype. The exact mechanism behind the inhibition was unclear. Nivens et al. (2001) hypothesized that since deacetylated alginate is known for being less viscous (Skjåk-Bræk et al., 1989) and the alginate lyase in *P. aeruginosa* AlgL has a higher

affinity for deacetylated alginate (Farrell & Tipton, 2012; Nivens et al., 2001), the matrix was probably not stable enough for microcolonies to form.

1.3 Alginate

Since alginate is the primary EPS produced by the pathogenic mucoid *P. aeruginosa* and its enzymatic degradation is the topic of this thesis, the following chapter is dedicated to a more in-depth description of alginate structure and synthesis.

1.3.1 Chemical structure

Alginate is a linear polysaccharide consisting of β-D-mannuronic acid (M) and its C5 epimer, α-L-guluronic acid (G), connected through an α-1,4-glycosidic bond (Skjåk-Bræk & Draget, 2012). The polysaccharide structure varies between regions of M called M-blocks, regions of G called G-blocks, and a random mix of both M and G structures (**Figure 4**). These blocks vary in length and frequency based on the organism producing the alginate. In addition, some alginate variants have a higher amount of acetylation than others. For example, alginate formed by *P. aeruginosa* is highly acetylated and consists of random M-blocks and MG-blocks while alginate produced by brown algae has almost no acetylation (Davidson et al., 1977; Skjåk-Bræk et al., 1986). The acetylation in alginate produced by *P. aeruginosa* is located on the mannuronic acid mainly on the C-2 and some at C-3 hydroxyl groups (Skjåk-Bræk et al., 1986).

Alginate, as an anion, can form a gel when added to divalent cations such as Ca^{2+}. Based on the amount and structure of the different block types, as well as the length of the alginate, the polysaccharide can create a gel formation with different viscosity (Mørch et al., 2007). For example, it is believed that G-block is the only part of alginate creating a linkage to cations. Therefore, alginate with a more significant amount of G-block creates the stiffest gels (Lee & Mooney, 2012).

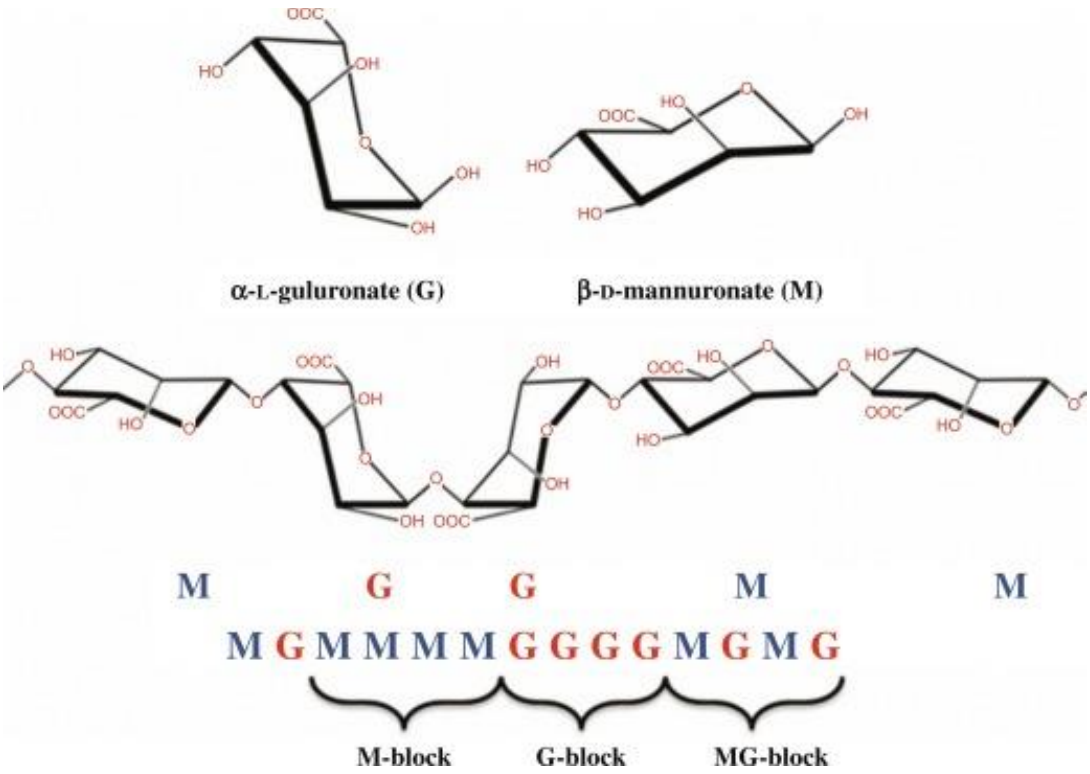

Figure 4. The alginate structure. *The figure shows the structure of α-L-guluronate (G) and β-D-mannuronate (M). The figure also shows the structure and binding for M-block, G-block, and MG-block. This figure is obtained from (Angra et al., 2021).*

1.3.2 The alginate synthesis pathway in *P. aeruginosa*

The synthesis of alginate in *P. aeruginosa* requires twelve proteins. These proteins are likely a synthesis complex from the inner membrane through the periplasm to the outer membrane (Chanasit et al., 2020). The proteins, AlgD, Alg8, Alg44, AlgK, AlgE, AlgG, AlgX, AlgL, AlgI, AlgJ, AlgF, and AlgA, are necessary for alginate polymerization, modification, and secretion (**Figure 5**) (Moradali et al., 2015). The alginate synthesis pathway in *P. aeruginosa* is highly studied, although there are still some unknown steps (Ramsey & Wozniak, 2005). The first step in alginate polymerization is to synthesis the alginate precursor guanosine diphosphate (GDP)-mannuronic acids. This precursor is controlled and produced by the enzymes AlgA, AlgC, and AlgD. After producing GDP-mannuronic acid, the polysaccharide is polymerized by Alg8 and Alg44 and transported across the inner membrane to the periplasm (Moradali et al., 2015; Ramsey & Wozniak, 2005). In the periplasm, the alginate is modified by acetyltransferase AlgX, some of the mannuronate is epimerized to guluronate by the C-5-epimerase AlgG, and some are acetylated at C-2 or C-3 by AlgF, AlgJ, and AlgI. After the modification step, the alginate is transferred out of the periplasm and through the outer membrane through AlgE

(Chanasit et al., 2020; Franklin & Ohman, 1996; Franklin & Ohman, 2002; Ramsey & Wozniak, 2005).

Chanasit et al. (2020) recently did an experiment where mutants of the alginate overproducing strain PDO300 had a deletion of *algI*, *algJ*, or *algF*. Compared to the other mutants and the wild-type, PDO300 Δ*algF* produced significantly less alginate. However, some alginate production did occur with each of the mutants. The proteins, AlgI, AlgJ, and AlgF, were not considered necessary for alginate polymerization. However, these enzymes are crucial for the O-acetylation of alginate since neither of the mutants produced O-acetylated alginate. In addition, the mutants gave an indication of the protein structure and location in the inner membrane and periplasm. AlgF is estimated to be in direct contact with AlgI and AlgJ, while AlgI is estimated to have an interaction with Alg8 (**Figure 5**) (Chanasit et al., 2020).

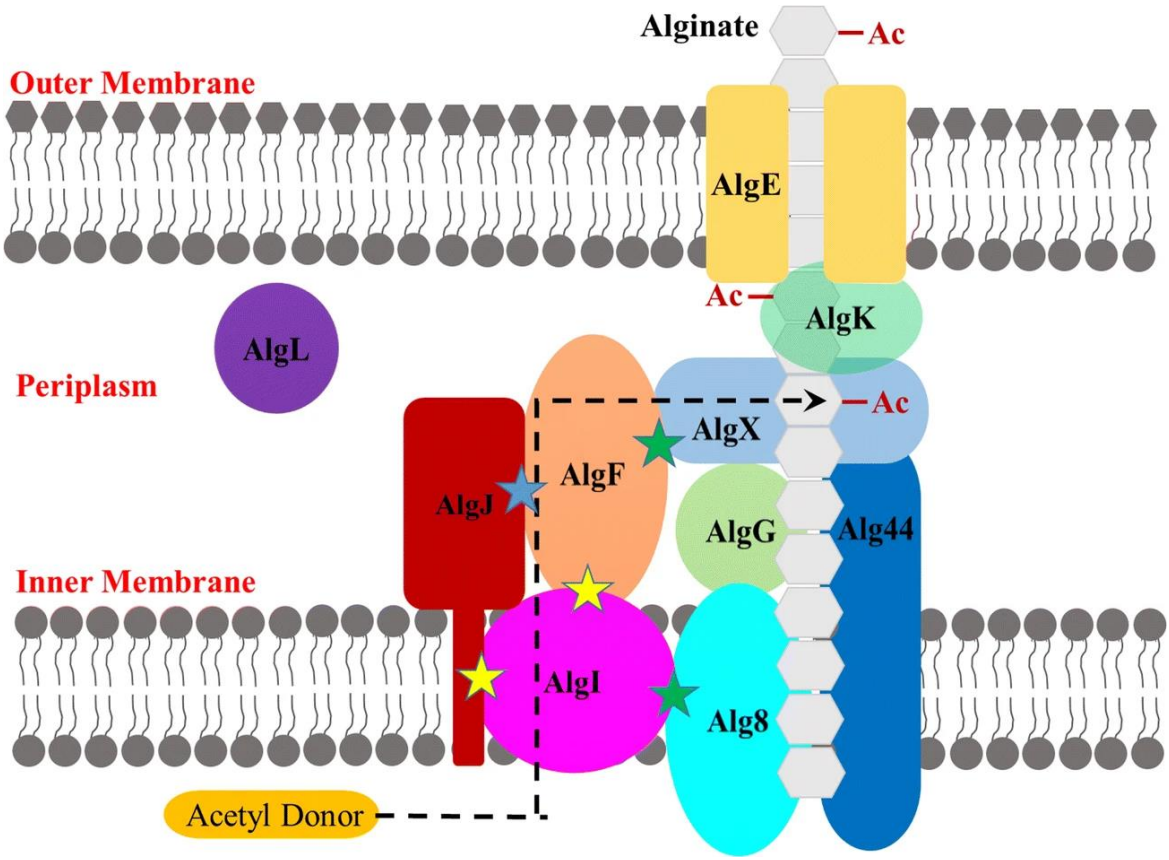

***Figure 5. Potential structure of the O-acetylation complex in* P. aeruginosa**. *The figure shows the proposed structure of the O-acetylation complex of alginate in* P. aeruginosa *and the location of the different proteins involved. AlgI-AlgJ-AlgF is believed to form a complex attached to the alginate polymerization/secretion multiprotein complex. The grey hexagons represent alginate in the figure. Illustration obtained from (Chanasit et al., 2020).*

1.4 Alginate lyases

1.4.1 Function and mechanism

Alginate lyase catalyzes the depolymerization of alginate by cleaving the α-1,4-glycosidic bond by the β-elimination reaction. This reaction creates a new reducing end and unsaturated uronic acid called 4-deoxy-L-erythro-hex-4-enepyranosyluronate (DEH) on the nonreducing end (**Figure 7**) (Gacesa, 1987). An elimination reaction creates a new π-bond by cleaving a σ-bond and releasing a nucleophilic group (**Figure 6**) (Clayden et al., 2012). β-elimination is called elimination since a nucleophile is removed and β since it is the σ-bond between Cβ (carbon-β) and a nucleophile that breaks to create a new π-bond (Reddy et al., 2016). In **Figure 6**, the σ-bond between carbon-β and a nucleophile and the new π-bond is marked with a black arrow.

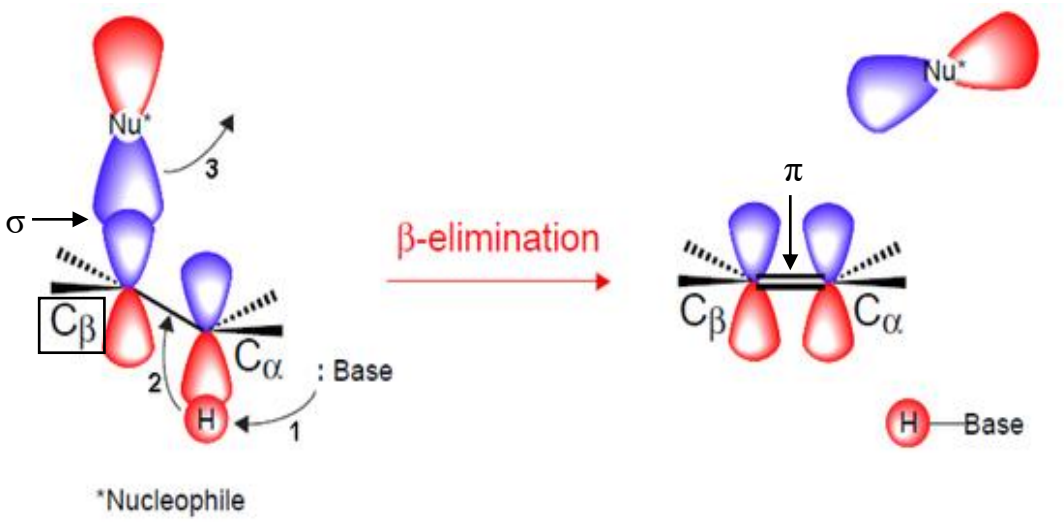

***Figure 6. Illustration of the β-elimination reaction**. The σ-bond between Cβ and a nucleophile is cleaved in the reaction, and a new π-bond is created. The Cβ, σ-bond, and the new π-bond are marked with arrows or with a box. Illustration obtained from (Reddy et al., 2016) with a few modifications.*

Alginate lyase can work either endolytic or exolytic. Exolytic enzymes will break down the substrate by cleaving off monomers at the ends of the polymer, while an endolytic enzyme will cleave the glycoside bond of an internal site of the polymer, creating un-saturated oligo alginates with different lengths (**Figure 7**) (MacDonald et al., 2016). Most alginate lyases are endolytic (Vuoristo et al., 2019). Endolytic proteins often have a wide-open active site center, while exolytic proteins can have an active site with one blocked site, like a pocket (Xu et al., 2018).

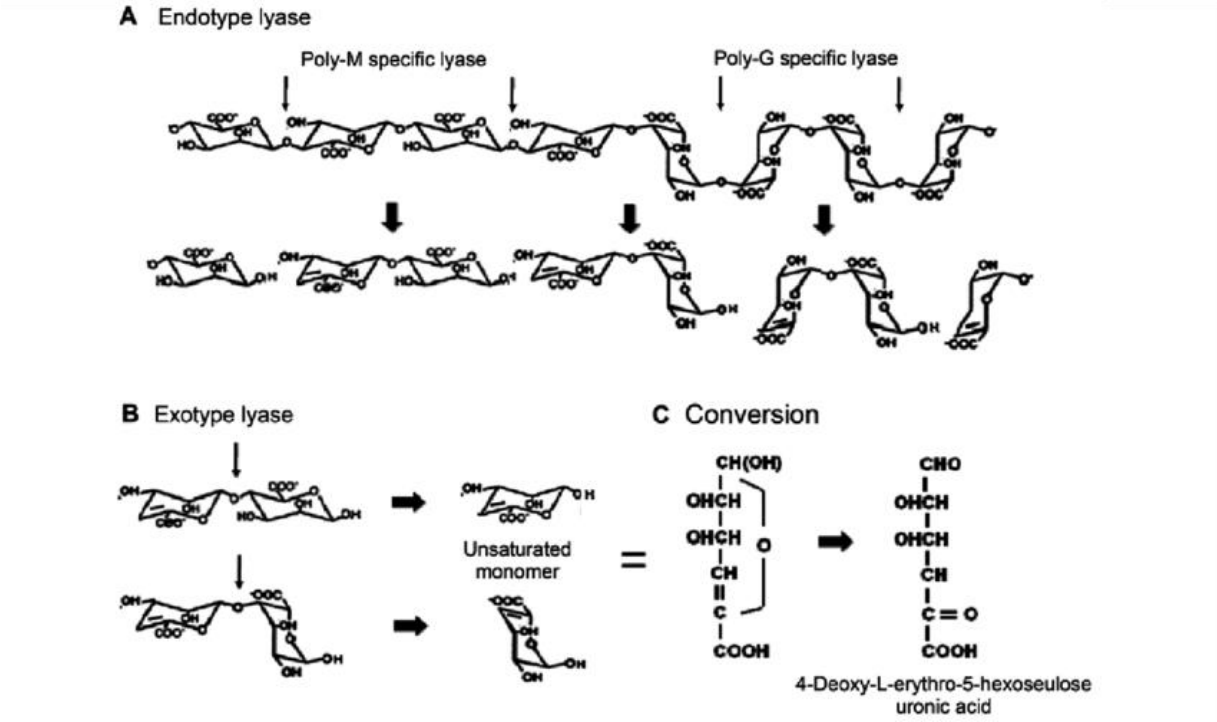

Figure 7. Degradation of alginate by endotype lyase and exotype lyase. *The figure shows the degradation of alginate to disaccharides and DEH. (A) shows how endolytic lyases degrades alginate. The figure shows both polyM specific lyase and polyG specific lyase. (B) shows how exotype lyase degrades alginate to DEH. The figure is obtained from (Kim et al., 2011)*

1.4.2 Distribution and classification

In the Carbohydrate-Active Enzymes database (CAZy), Carbohydrate-Active Enzymes are divided into different families based on sequence similarity and molecular mechanism (CAZy; http://www.cazy.org/) (Drula et al., 2021). In addition, the CAZy database describes the different families. All families in CAZy are defined with at least one biochemical characterized enzyme, and based on this characterized enzyme has every family an expected function (http://www.cazy.org/) (Drula et al., 2021). A family can also be divided into different subfamilies, and a subfamily consists of enzymes that only correspond to each other (http://www.cazy.org/) (Drula et al., 2021). Alginate lyases are categorized and divided into eight different polysaccharide lyase (PL) families based on their amino acid sequence. Alginate lyases are found in PL family 5, 6, 7, 14, 15, 17, 18 (Huang et al., 2019), and a group of unknown enzymes (Ertesvåg, 2015). Most endolytic alginate lyases are classified into either PL5 or PL7, while most exolytic are part of PL15 and PL17 (Zhu & Yin, 2015).

The enzymes can also be grouped by specificity. Alginate lyases with specific binding to M-units are classified as mannuronate-specific alginate lyases (EC 4.2.2.3), while enzymes with

specific binding to G-units are called guluronate-specific alginate lyases (EC 4.2.2.11). Most mannuronate-specific alginate lyases (polyM lyase) and guluronate-specific alginate lyases (polyG lyases) can degrade other homopolymers to a certain extent. Oligoalginate lyases, also called exo-alginate lyases, are enzymes capable of degrading oligosaccharides by exolytic elimination (EC 4.2.2.26). Some alginate lyases are also classifieds as mannuronate & guluronate-specific alginate lyases (EC 4.2.2.-) (http://www.cazy.org/) (Drula et al., 2021). The polysaccharide lyase family (EC 4.2.2.-) consists of enzymes capable of degrading polysaccharides containing uronic acid through β-elimination (Lombard et al., 2010). It is most commonly that the alginate lyases are M-specific and endolytic (Wong et al., 2000).

One way to determine substrate specificity is to analyze the highly conserved regions of the alginate lyase sequence. Most of the characterized alginate lyases are from the PL7 family, and the structure-function relationship is therefore based on PL7 and the crystal structures available for enzymes in this family. The PL7 alginate lyases have three conserved sequence motifs that are important for activity; (R/E)(S/T/N)EL, Q(I/V)H, and YFKAG(V/I)YNQ, according to Zhu and Yin (2015) and according to Wong (2000) are the conserved motifs for alginate lyase in PL7; R*ELR*ML, VIIGQ(I/V)H, and YFKAG*Y*Q. In these conserved motifs, five residues form the active site; arginine (R), glutamine (Q), histidine (H), and 2x tyrosine (Y), respectively (Yamasaki et al., 2005). In the active site in the PL7 ALY-1 from *Corynebacterium* sp., is it proposed that the four of the five amino acids have important roles. Q117 and Y195 interact with alginate close to the reaction site so the substrate will be oriented correctly for the reaction, R72 interacts with the carboxyl groups on the alginate, and H119 works as a base for the deprotonation in the β-elimination reaction (Osawa et al., 2005). Altogether, these regions form a jelly-roll β-sandwich structure that builds up a cavity believed to bind to the substrate (**Figure 8 B**) (Zhu & Yin, 2015). In addition, Q(I/V)H has a connection to substrate specificity. Enzymes with the sequence QVH are most likely a mannuronate-specific alginate lyase, while enzymes with QIH have a higher specificity against poly G or polyMG. Alginate lyase lost its function when the region YFKAG(V/I)YNQ was deleted. Therefore, this region is likely required to catalyze alginate degradation (Zhu & Yin, 2015).

Based on the 3-dimensional structures of alginate lyases in PL5, 6, 7, 14, and 15, alginate lyases are divided into three families (Hehemann et al., 2014). PL6 is in the parallel β-helix family (Huang et al., 1999), PL5 and PL15 are in the $(\alpha/\alpha)_6$ barrel family (Yoon et al., 2001), and PL7 and PL14 are in the β-jelly roll family (Ogura et al., 2009; Yamasaki et al., 2004). In addition

to these three families, bifunctional alginate lyase in family PL18 has a sandwich structure (Dong et al., 2014; Zhu & Yin, 2015), and chlorella virus in PL14 has two antiparallel β-sheets with a deep cleft showing a β-jelly roll fold (Ogura et al., 2009). In **Figure 8** the predicted structure of AlgL in PL5 and PA1167 in PL7 are shown. AlgL has a distinct (α/α) barrel, and PA1167 has a β-sandwich fold.

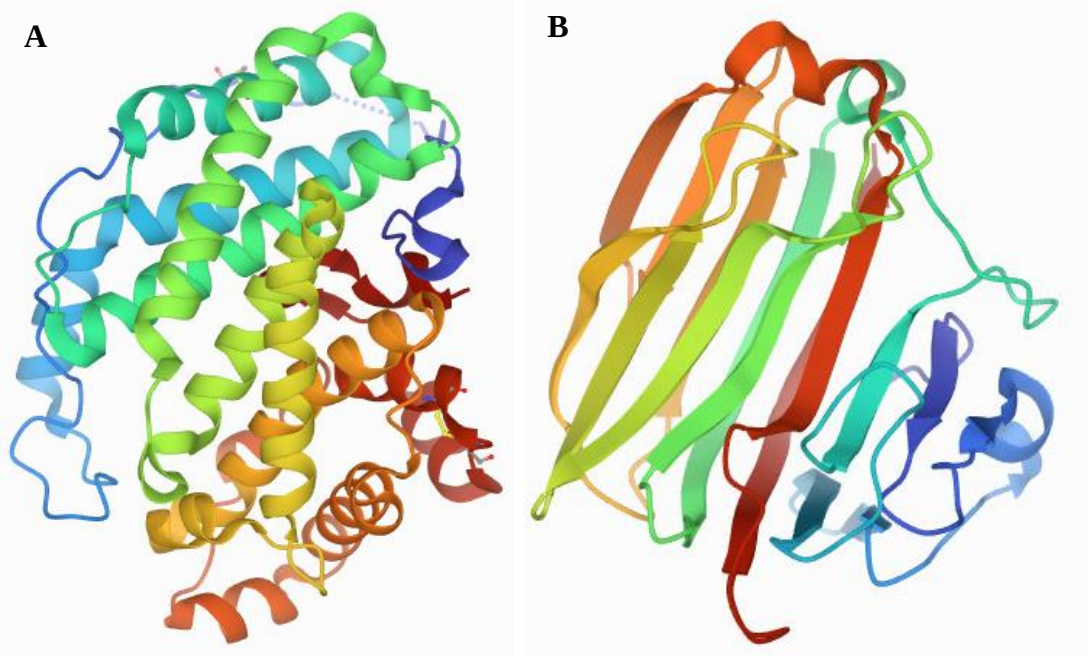

Figure 8. The predicted crystal structure of alginate lyases AlgL and PA1167. *Figure (A) shows a picture of AlgL, an alginate lyase classified in PL5 family. The enzyme has a (α/α)-barrel. The picture is obtained from (Howell et al., 2015). Figure (B) shows a picture of PA1167 classified as a part of the PL7 family. PA1167 has a β-sandwich fold. The picture is obtained from (Yamasaki et al., 2004).*

Alginate lyases are found in the genomes of organisms ranging from prokaryotes to eukaryotes, where they are involved in the synthesis, modification, and degradation of alginates. Macroalgae are the dominant alginate producers and are an essential nutrient source for different marine organisms (Inoue & Ojima, 2019). Several marine invertebrates eat brown algae and degrade the components of brown algae as an energy and carbon source. Therefore, these animals possess several bacteria with the ability to degrade alginate (Ito et al., 2019). Alginate lyase has also been identified in the hepatopancreas in the invertebrates *Aplysia depilans* (Boyen et al., 1990) and *Dolabella auricula* (Nisizawa et al., 1968). Marine bacteria like *Pseudomonas sp.* (Muramatsu & Sogi, 1990), and *Halomonas marina* (Wong et al., 2000), and soil bacteria like *Bacillus* sp. (Nakagawa et al., 1998) produce alginate lyases to degrade alginate and use the M and G units as energy and carbon sources (Wong et al., 2000). Most bacteria that do not produce alginate have only one lyase enzyme. Some marine bacteria

produce both a mannuronate-specific alginate lyase and a guluronate-specific alginate lyase to harness all the energy from alginate (Brown & Preston, 1991). Some marine mussels also produce alginate lyases to degrade alginate as a carbon source (Kim et al., 2011). In addition, viruses have shown sequencing results that indicate alginate lyase activity. This has been found in the *Chlorella* virus (Suda et al., 1999). Alginate lyase in the *Chlorella* virus breaks down the cell wall of the green algae of the genus *Chlorella* (Ogura et al., 2009).

For bacteria that produce alginate, alginate lyase might have a different role than degrading alginate for energy purposes. According to Ertesvåg (2015), no alginate producing bacteria has been shown to use alginate as a carbon source. Still, several of the bacteria produce alginate lyase. Therefore, alginate lyases probably have a different role, and Ertesvåg (2015) suggested that alginate lyase might control the misplaced alginate in the periplasm.

1.4.3 The alginate lyases of *P. aeruginosa*

The *P. aeruginosa* PAO1 genome contains three genes encoding putative alginate lyases: *algL*, *PA1167*, and *PA1784*. So far, the best-characterized enzyme is AlgL, and little data exist for PA1167 and PA1784. The existing knowledge is presented in the following subchapters.

1.4.3.1 AlgL

AlgL is the most studied of the three alginate lyases discovered in *P. aeruginosa*. In the alginate synthesis complex, the alginate lyase AlgL is located in the periplasm. As estimation of the location is shown in **Figure 5**. However, the exact purpose of AlgL in *P. aeruginosa* is still unknown. Boyd et al. (1993) suggested that AlgL was unnecessary for bacteria growth, and that the alginate production was not affected by the lack of AlgL. However, different studies after 1993 have reached a different conclusion. Monday and Schiller (1996), showed that mutations with deletion of *algL* could not produce mucoid biofilm. In a study done by Jain and Ohman (2005), the mucoid *P. aeruginosa* strain FRD1 was used to create an Δ*algL* mutant. This mutant produced alginate but lysed after a few hours. The lysis of the mutant indicated that AlgL was a necessary enzyme for FRD1. The lysis was caused by the accumulation of alginate or the precursor in the periplasm. Based on these results, AlgL was believed to degrade alginate and polyM mislocated in the periplasm. The study also showed that AlgL was not necessary for alginate production. Gheorghita et al. (2022) discovered similar results as Jain and Ohman

(2005). They demonstrated that AlgL degrades alginate, which accumulates in the periplasmic space to maintain homeostasis, and that AlgL was vital for the viability of the bacteria.

This enzyme is characterized as a PL5 mannuronate-specific alginate lyase (Kinoshita et al., 1991), it is most active at pH 6.2, and the optimum temperature is between 20 and 40 °C (Linker & Evans, 1984; Yamasaki et al., 2004). The structure of AlgL is a α/α-barrel, and this enzyme has an expected molecular mass of 41 kDa (**Figure 8**). Farrell and Tipton (2012) wanted to test the substrate affinity for AlgL against acetylated alginate since *P. aeruginosa* produces acetylated alginate. The results showed that AlgL had a higher affinity against non-acetylated alginate. In addition, outside of the periplasmic space, no unacetylated alginate was found (Farrell & Tipton, 2012).

1.4.3.2 PA1167

Yamasaki et al. (2004) presented a study where PA1167 and AlgL were purified and characterized. The result from the characterization showed that PA1167 was most active at 40 °C in a 50 mM Tris-HCl buffer with pH 8.5, and they showed that PA1167 preferred depolymerized polyMG over polyM and polyG. PA1167 also showed activity against alginate biofilm from *P. aeruginosa* 8830. AlgL was also tested and showed many differences from PA1167. PA1167 and AlgL differ in structure, the optimal pH, and molecular mass. Yamasaki et al. (2004) were able to find the novel folding structure of PA1167, as the fourth polysaccharide lyase. PA1167 is a PL7 in subfamily 1 with β-sandwich (**Figure 8**). Compared to AlgL, the activity of PA1167 with polyMG was approximately 24-fold lower than AlgL with polyM. They suggested that PA1167 lacked specificity and activity since AlgL is probably the primary enzyme for the depolymerization of extracellular alginate in *P. aeruginosa.*

Overexpression of PA1167 in the mucoid strains FRD1 and FRD1153 were able to degrade or prevent alginate mucoid biofilm formation. FRD1 produces a mucoid biofilm that consists of M/G blocks and O-acetylated groups, and the biofilm produced by FRD1153 consists of non-acetylated M/G alginate. When the gene was induced, these strains could not produce mucoid biofilm, only non-mucoid biofilm. This was not the case in the mannuronate-producing strain FRD462, which showed no differences between the parent strain and the mutant (Douthit, 2004). This supports recent findings that PA1167 is a polyM/G specific alginate lyase.

1.4.3.3 PA1784 (*Pa*Aly7a)

In the literature, the third alginate lyase in *P. aeruginosa* PAO1 is called PA1784, and the gene obtained from the strain PA14 is called *PA14_41500*. In this thesis, this protein is referred to as *Pa*Aly7a.

Compared to AlgL and PA1167, the information about PA1784 is deficient. However, in a thesis written by Douthit (2004), different mutants with and without PA1784 and PA1167 were tested in the mucoid strains FRD463, FRD1, and FRD1153 and PAO1. In one of the experiments, knockout mutants of PA1784 in strains FRD463, FRD1, and FRD1153 were constructed. FRD463 Δ*PA1784* and FRD1 Δ*PA1784* did not show any difference between the knockout and the parent strain, while FRD1153 Δ*PA1784* produced more fluid alginate than the parent strain. PAO1 with overexpressed PA1784 and PAO1 Δ*PA1784* were made to test if the strain could utilize alginate. The result indicated that PAO1 could not utilize alginate as a carbon source, with or without PA1784. Douthit (2004) also wanted to test how PA1784 would affect the forming of mucoid biofilm in FRD463, FRD1, and FRD1153 by cloning in inducer-controlled PA1784. However, the overproduction of PA1784 did not affect the production of mucoid biofilm in the strains FRD1 and FRD1153. Based on these results, PA1784 does not show activity as an alginate lyase. Douthit (2004) also compared PA1784 with other PL7 alginate lyases. Based on the amino acid identity, specifically the conserved regions YFKAG*Y*Q and RSELR, PA1784 was classified as a guluronate-specific lyase in PL7 subfamily 1 (http://www.cazy.org/) (Drula et al., 2021).

1.4.4 AMOR_PL7A

AMOR_PL7A is a PL7 alginate lyase found in *Saccharina latissima* and the gene is collected from the metagenomic data set from the Arctic Mid-Ocean Ridge (AMOR). This alginate lyase was characterized by Vuoristo et al. (2019). Their experiment discovered that AMOR_PL7A was an endolytic mannuronate-specific alginate lyase, which could also degrade sodium alginate. The optimum temperature and pH for this enzyme was 65 °C and pH 6. It also had the highest activity in seawater when the protein was tested with different salt concentrations. In addition, this enzyme was proven to have high stability without substrate present. Based on these results, AMOR_PL7A was used as a positive control in this thesis with the optimum conditions, buffer 50 mM NaAc + 500 mM NaCl, pH 6, at 65 °C.

1.4.5 Alginate lyase as a treatment of mucoid biofilm

The role of alginate lyase in the treatment of mucoid biofilm is disputed. Several studies have tested the effect of alginate lyase and antibiotics on biofilm produced by *P. aeruginosa*. Blanco-Cabra et al. (2020) showed that polyM/G alginate lyases, A1-II' and Alg2A from *Sphingomonas* sp., with and without the antibiotic ciprofloxacin could degrade nonmucoid and mucoid biofilm. In comparison, polyM and polyG alginate lyases did not show activity against the biofilm, with and without ciprofloxacin. Lamppa and Griswold (2013) tested the activity of A1-III-His from *Sphingomonas* sp. on biofilm, and the results showed that the alginate lyase was more effective than the antibiotic tobramycin alone. However, the same results were shown when inactive proteins were added to the biofilm. Therefore, Lamppa concluded that it was not the activity of the protein which caused the dispersion of biofilm, but rather the enzyme itself. In another study, Alkawash et al. (2006) were able to find a co-administration between alginate lyase from *Bacillus circulans* ATCC 15518 and the antibiotic gentamicin which led to a higher elimination rate of mucoid biofilm. The biofilm was grown under conditions similar to the respiratory tract of patients with CF. These studies show that alginate lyases have potential as a possible treatment of mucoid biofilm produced by *P. aeruginosa*. However, the mechanism behind this treatment is still discussed.

1.5 Aim

P. aeruginosa is an opportunistic pathogen that can lead to deadly infections. One of the leading causes of chronic, life-threatening infection is *P. aeruginosas* ability to change to a mucoid strain. Mucoid strains overproduce the exopolysaccharide alginate forming a very viscous biofilm. Alginate is believed to have an essential role in this, and so has the enzymes alginate lyases. From *P. aeruginosa*, three proteins have been identified, but only one has been thoroughly characterized. Douthit (2004) tested if PA1784 could degrade alginate using microbiological methods, but PA1784 has never been tested biochemically. The aims of this thesis are to determine the properties of PA1784 and investigate its putative activity toward alginate and other relevant substances. By doing so, the results may indicate if the enzyme later can be used as a part of the treatment of *P. aeruginosa*, especially for people suffering from CF.

2 Materials

Appendix A - Table A 1 shows an overview of the equipment used throughout this study with the suppliers. In **Appendix A - Table A 2** the chemicals used throughout this study are listed with the suppliers.

2.1 Plasmids

Table 1 shows the plasmids used in this study.

Table 1. Plasmid*. The table shows the plasmids used in this study, along with the supplier.*

Plasmid	**Supplier**
pUC19 *PA14_41500*	GenScript®, The plasmid was kindly provided by Per Kristian Edvardsen.
pNIC-CH	Addgene (Gift from Opher Gileadi)
pET (pET-28a(+)-TEV)	GenScript®
pBAD (pBADHisChitEF2863)	Invitrogen

2.1.1 pUC19

PA14_41500 was ordered from GenScript® in the transport vector pUC19. The plasmid included a resistance gene against the antibiotic kanamycin. When growing *E. coli* with pUC19, kanamycin 50 μg/mL was added to the plates to separate the bacteria with the vector from the other bacteria. The plasmid was kindly provided by Per Kristian Edvardsen.

2.1.2 pNIC-CH

The pNIC-CH vector was used to create recombinant plasmids. The target gene, *PA14_41500*, was inserted into the vector. Then the vector was further transformed into One Shot® TOP10 Chemically Competent *E. coli* (TOP10). pNIC-CH+*PA14_41500* was isolated from TOP10 and transformed into the protein-producing One Shot® BL21 Star™ (DE3) Chemically Competent *E. coli* (BL21).

pNIC-CH has a C-terminal 6x polyhistidine tag (His-tag) just before the target gene, making it possible to isolate the protein from other proteins in *E. coli* by IMAC. The antibiotic kanamycin

50 μg/mL was added to the plates to separate the *E. coli* with pNIC-CH+*PA14_41500* from the other bacteria, since the plasmid included a resistance gene against kanamycin.

2.1.3 pET

The pET vector was used to create recombinant plasmids. The target gene, *PA14_41500*, was inserted into the vector, and then the vector was further transformed into TOP10. pET+*PA14_41500* was isolated from TOP10 and transformed into the protein-producing BL21.

pET has a C-terminal 6xHis-tag just before the target gene, making it possible to isolate the protein from other proteins in *E. coli* by IMAC. The antibiotic kanamycin 50 μg/mL was added to the plates to separate the *E. coli* with pET+*PA14_41500* from the other bacteria, since the plasmid included a resistance gene against kanamycin.

2.1.4 pBAD

The pBAD vector was used to create recombinant plasmids. The target gene, *PA14_41500*, was inserted into the vector, and then the vector was further transformed into TOP10. pBAD+*PA14_41500* was isolated from TOP10 and transformed into the protein-producing BL21.

pBAD has an N-terminal 6xHis-tag just before the target gene, making it possible to isolate the protein from other proteins in *E. coli* by IMAC. The antibiotic ampicillin 100 μg/mL was added to the plates to separate the *E. coli* with pBAD+*PA14_41500* from the other bacteria, since the plasmid included a resistance gene against ampicillin.

2.2 Primers

Table 2. shows the primers, their sequence, and a description of the primer used in this study.

Table 2. Primers. *The table shows an overview of the primers used in the study, the primer sequence, and a description.*

Primer	**Description**	**Sequence**
Alg_Lya_PA14_41500_For_AflII	Forward primer for *PA14_41500* with AflII (primer made by Per Kristian Edvardsen)	TTTGTTTAACCTTAAGATGATCGATCTCAGCACCTGG

Alg_Lya_PA14 _41500_Rev_PstI	Reverse primer for *PA14_41500* with PstI (primer made by Per Kristian Edvardsen)	GATGATGGTGCG CTGCAGTGCGAT GGGCGGTGTTCAG
pNIC-for	Forward primer for pNIC-CH (primer made by Per Kristian Edvardsen)	TGTGAGCGGAT AACAATTCC
pNIC-rev	Reverse primer for pNIC-CH (primer made by Per Kristian Edvardsen)	AGCAGCCAACT CAGCTTCC
pET_28a(+)_ TEV_conf_F	*PA14_41500* Forward primer	TGTGAGCGGAT AACAATTCCC
pET_28a(+)_ TEV_conf_R	*PA14_41500* Reverse primer	CCAACTCAGCTT CCTTTCGGG
pBAD_conf_F	*PA14_41500* Forward primer	GCGGATCCTACC TGACGCT
pBAD_conf_R	*PA14_41500* Reverse primer	CAGACCGCTTC TGCGTTCTG

2.3 Proteins and Enzymes

The different enzymes and proteins used in the study are shown in **Table 3**. **Table 4** lists the different alginate lyases used.

Table 3. Protein/Enzyme*. The table shows a list of the proteins and enzymes used in this study with the supplier.*

Protein/Enzyme	**Supplier**
5X In-Fusion HD Enzyme Premix	Takara bio
BenchMark™ Protein Ladder	Invitrogen
Calf Intestinal alkaline phosphatase (CIP)	Sigma-Aldrich
ChiC (BL21 with pNIC-CH+ChiC)	Kindly supplied by Per Kristian Edvardsen
Red Taq DNA Polymerase Master Mix (2X)	VWR
Q5® Hot Start High-Fidelity Master Mix (2X)	New England Biolabs

***Table 4. Protein/Enzyme Alginate lyase**. The table shows a list of the alginate lyases used throughout this study and the supplier.*

Protein/Enzyme	**Supplier**
AMOR_PL7A	Kindly supplied by Nanna Rhein-Knudsen from *S. latissima*
*Pa*Aly7a (*PA14_41500*)	From *P. aeruginosa*

2.4 Kits

Table 5. gives an overview of which kit has been used in this study.

***Table 5. Kits.** The table shows an overview of the kits and their content used in this study with the supplier.*

Kit	**Supplier**	**Contents**
NucleoSpin® Gel and PCR Clean-up	MACHEREY-NAGEL	Binding Buffer NTI Collection Tubes (2 mL) NucleoSpin® Gel and PCR Clean-Up Columns (yellow rings) Wash Buffer NT3
Nucleospin® Plasmid/Plasmid (NoLid) protocol - kit	MACHEREY-NAGEL	Collection Tubes (2 mL) Lysis Buffer A2 Neutralization Buffer A3 NucleoSpin® Plasmid Columns (white rings) Resuspension Buffer A1 Wash Buffer A4
NucleoSpin® Genomic DNA from microorganisms	MACHEREY-NAGEL	Lysis Buffer MG Wash Buffer BW Wash Buffer B5 (Concentrate) Elution Buffer BE Liquid Proteinase K MN Bead Tubes Type B NucleoSpin® Microbial DNA Columns (light green rings) Collection Tubes (2 mL)
In-Fusion® HD Cloning kit	Takara Bio	5X In-Fusion HD Enzyme Premix

		Control vector (PUC19) Control insert (2000bp)

2.5 Substrates

2.5.1 5 % Alginate

Alginate was used as the primary substrate in the activity assay experiments.
Weighted out 5 g sodium alginate and added it to a 100 mL measuring flask. 100 mL of dH_2O was added, and the alginate was dissolved using a magnetic stirrer. The solution was stored at 4 °C. Sodium alginate is purified from brown algae.

2.5.2 1 mM Glucose

Glucose was used as a standard in 3,5-Dinitrosalicylic Acid (DNS) reducing end assay.
Weighted 0.018 g glucose and dissolved it in 100 mL dH_2O in a 100 mL measuring flask. The solution was stored at 4 °C.

2.6 Bacterial Strains

2.6.1 *Escherichia coli*

Table 6. gives an overview of the *E. coli* strains used in this study.

***Table 6.* E. coli**. *The table shows* E. coli *bacterial strains used during protein expression.*

Bacterial Strain	**Supplier**
One Shot® TOP10 Chemically Competent *E. coli*	Thermo Fisher Scientific
One Shot® BL21 Star™ (DE3) Chemically Competent *E. coli*	Thermo Fisher Scientific

2.6.2 *P. aeruginosa*

Table 7. gives an overview of the *P. aeruginosa* strains used in this study.

***Table 7.* P. aeruginosa**. *The table shows* P. aeruginosa *bacterial strains used in this study.*

Bacterial Strain	**Supplier**
PAU2	Kindly provided by Chief Physician Karianne Wiger Gammelsrud
PAO1	Kindly provided by Professor Victor Nizet

PAO1 ΔWspF	Kindly provided as a gift from Professor Matthew R. Parsek
PAO1 ΔWspF ΔPel	Kindly provided as a gift from Professor Matthew R. Parsek
PAO1 ΔWspF ΔPsl	Kindly provided as a gift from Professor Matthew R. Parsek

2.7 Cultivation Media and Agar

Different media were used on various occasions. Lysogeny Broth (LB) liquid media were used for overnight cultures, and LB agar plates were used to grow both *E. coli* and *P. aeruginosa* strains. LB agar plates were also used during transformation. In the production of protein *Pa*Aly7a, the terrific broth was mostly used. Other media were tested to find the optimal media for *Pa*Aly7a expression. Super Optimal Broth with Catabolite repression (S.O.C.) medium was used during *E. coli* transformations.

2.7.1 Lysogeny Broth (LB)

Liquid medium:

- 10 g Bacto™ Tryptone
- 5 g Bacto™ Yeast extract
- 10 g NaCl

The dry reagents were weighed out, added to a 1 L graduated flask, and dissolved with 800 mL dH_2O. The dry ingredients were dissolved by shaking the flask, and then dH_2O was added to a final volume of 1 L. The solution was made sterile by autoclaving the flask for 15 minutes at 121 °C. When the solution was cooled down, it was stored at room temperature (RT).

Agar plates:

- 10 g Bacto™ Tryptone
- 5 g Bacto™ Yeast extract
- 10 g NaCl
- 15 g Agar Powder

The dry reagents were weighed out, added to a 1 L graduated flask, and dissolved with 800 mL dH_2O. The dry ingredients were dissolved by shaking the flask, and then dH_2O was added to a final volume of 1 L. The solution was made sterile by autoclaving the flask for 15 minutes at 121 °C. The flask was placed in a heath cabinet at 50 °C for approximately 30 minutes to lower

the temperature. Agar plates for bacterial cultivation were either added kanamycin or ampicillin to a final concentration of 50 μg/mL or 100 μg/mL, respectively. This was done by adding the antibiotics to the flask with LB agar on a sterile bench. The sterile Petri dishes were filled with 15-20 mL of LB agar and left solidifying. All plates were stored at 4ºC subsequently.

2.7.2 Terrific Broth (TB)

Liquid medium:

TB Stock 1.1x

- 12 g Bacto™ Tryptone
- 24 g Bacto™ Yeast extract
- 4 mL Glycerol (85 % non-sterile)

The dry ingredients were weighed out, added to a 1000 mL graduated flask, and dissolved in 800 mL dH_2O using a magnet stirrer. Four mL of glycerol was added before adjusting the volume to 900 mL with dH_2O, yielding a TB stock solution at 1.1x concentration. The solution was made sterile by autoclaving the flask for 15 minutes at 121 °C. After the solution was autoclaved, 50 mL of K_2HPO_4 0.72 M and 50 mL of KH_2PO_4 0.17 M (see section **2.11.6**) were added for a final concentration of 1X. The medium was stored at RT.

2.7.3 Brain Heart Infusion (BHI)

Liquid medium:

37 g BHI was dissolved and autoclaved in the same manner as LB medium. Adjusted the pH to 7.0 with 35 % HCl and measured with a 913-pH meter. BHI was then autoclaved for 15 minutes at 121 °C and stored at RT.

2.7.4 M9 Minimal salt (2 x)

Per Kristian Edvardsen kindly provided M9 Minimal salt (2x).

2.7.5 Super Optimal Broth with Catabolite repression (S.O.C.) medium

Liquid medium:

S.O.C. medium is Ready-to-Use by Invitrogen - ThermoFisher Scientific.

2.7.6 Tryptone soya broth (TSB)

Liquid medium:

30.12 g TSB was weighed out, added to a 1000 mL graduated flask, and dissolved in 800 mL dH_2O using a magnet stirrer. The volume was adjusted to 900 mL with dH_2O and autoclaved for 15 minutes at 121 °C. TSB was stored at RT.

2.8 Buffers and Other Solutions

2.8.1 1 M Trizma base hydrochloric acid (Tris-HCl)

The amount of Trizma base (Tris) was weighed out, and 30.285 g was transferred to a 250 mL graduated flask. To dissolve the salt, 150 mL dH_2O was added, and the solution was mixed using a magnet stirrer. 37 % hydrochloric acid (HCl) was then added to the solution until pH was 7.5 or 8, measured with a 913-pH meter. dH_2O was then added until the final volume of 250 mL. The buffer was then sterilized using a 0.20 µm filter. The solution was stored at RT.

2.8.2 0.1 M Sodium acetate (NaAc) and 0.5 M sodium chloride (NaCl)

- 0,82 g NaAc
- 2,92 g NaCl

NaAc and NaCl were weighed out and added to a 100 mL blue cork flask. The powder was then dissolved in 80 mL dH_2O with a magnet stirrer before dH_2O was added to a final volume of 100ml. pH was adjusted to 6.4 with 37 % HCl and measured with a 913-pH meter. The buffer was then sterilized using a 0.20 µm filter. The solution was stored at RT.

2.8.3 2 M Imidazole

Kindly provided by Per Kristian Edvardsen.

2.8.4 5 M NaCl

NaCl was weighed, and 146.1 g was transferred to a 500 mL graduated flask and dissolved in 500 mL dH_2O using a magnet stirrer. The solution was stored at RT.

2.8.5 50 mM Phenylmethylsulphonyl fluoride (PMSF)

- 0,871 g PMSF

- isopropanol

PMSF was weighed, added to a 100 mL graduated flask, and dissolved in 75 mL isopropanol using a magnet stirrer. The volume was then adjusted to 100 mL with isopropanol, and the solution was sterilized using a 0.20 µm filter. The solution was stored at -20 ºC.

2.8.6 Phosphate Solution for TB Medium

0.17 M Potassium phosphate monobasic (KH_2PO_4)

11.568 g KH_2PO_4 was weighed, added to a 500 mL graduated flask, and dissolved in 400 mL dH_2O using a magnet stirrer. The volume was then adjusted to 500 mL with dH_2O. Then the blue cork bottle was autoclaved at 121 °C for 15 minutes. The solution was stored at RT.

0.72 M Potassium phosphate dibasic (K_2HPO_4)

62.70 g K_2HPO_4 was weighed, added to a 500 mL graduated flask, and dissolved in 400 mL dH_2O using a magnet stirrer. The volume was then adjusted to 500 mL with dH_2O. Then the blue cork bottle was autoclaved at 121 °C for 15 minutes. The solution was stored at RT.

2.8.7 PBST

- 49,5 mL PBS
- 500µL of 100 % Triton X-100
- 0.2 µm filter
- Syringe
- Measuring flask

The PBST was made sterile by adding 500 µL of 100 % Triton X-100 to 49,5 mL of PBS in a 50 mL measuring flask. After dissolving the Trixon X-100 by shaking, the solution was made sterile using a 0.2 µm filter and syringe.

2.8.8 Ni-NTA Buffers for Ion Metal Affinity Chromatography (IMAC)

IMAC Binding Buffer:

- 40 mL 5 M NaCl
- 2,5 mL 2 M Imidazole
- 20 mL 1 M Tris-HCl pH 7.5

Imidazole, NaCl, and Tris-HCl were measured and added to a 1000 mL graduated flask. The volume was adjusted to 1000 mL using dH_2O. The final concentration of Imidazole was 5 mM, Tris-HCl pH 7.5 20 mM, and NaCl 200 mM. The solution was then filtered and sterilized through a 0.22 μm vacuum filter and subsequently stored at RT.

IMAC Washing Buffer:

- 10 mL 2M Imidazole
- 20 mL 1M Tris-HCl pH 7.5
- 40 mL 5M NaCl

Imidazole, NaCl, and Tris-HCl were measured and added to a 1000 mL graduated flask. The volume was adjusted to 1000 mL using dH_2O. The final concentration of Imidazole was 20 mM, Tris-HCl pH 7.5 20 mM, and NaCl 200 mM. The solution was then filtered and sterilized through a 0.22 μm vacuum filter and subsequently stored at RT.

IMAC Elution Buffer:

- 125 mL 2 M Imidazole
- 10 mL 1 M Tris-HCl pH 7.5
- 20 mL 5 M NaCl

Imidazole, NaCl, and Tris-HCl were measured and added to a 500 mL graduated flask, and the volume was adjusted to 500 mL using dH_2O. The final concentration of Imidazole was 500 mM, Tris-HCl pH 7.5 20 mM, and NaCl 200 mM. The solution was then filtered and sterilized through a 0.22 μm vacuum filter and subsequently stored at RT.

2.8.9 Size-Exclusion Chromatography (SEC) buffer

- 30 mL 5 M NaCl
- 15 mL 1 M Tris-HCl pH 7.5

NaCl and Tris-HCl were measured and added to a 1000 mL graduated flask, and the volume was adjusted to 1000 mL using dH_2O. The final concentration of Tris-HCl pH 7.5 15 mM, and NaCl 150 mM. The solution was then filtered and sterilized through a 0.22 μm vacuum filter and subsequently stored at RT.

2.8.10 1 M Isopropyl b-D-1-thiogalactopyranoside (IPTG)

IPTG was measured, and 2.383 g was diluted in 10 mL dH_2O in a 15 mL tube. The solution was sterilized using a syringe and 0.20 µm filter. Aliquots were made and stored at -20 °C.

2.8.11 100 mg/mL Lysozyme

Lysozyme was measured, and 1 g was diluted in 10 mL dH_2O in a 15 mL tube. The solution was sterilized using a syringe and 0.20 µm filter. Aliquots were made and stored at -20 °C.

2.8.12 DNS Reagent

DNS reagent was prepared according to Coughlan & Moloney Coughlan and Moloney (1988).

- 1 g DNS
- 30 g of sodium potassium tartrate (Rochelle salt)
- 80 mL of 0.5 M NaOH

DNS and Rochelle salt was added to 80 mL of 0.5 M NaOH in a 0.1 L volumetric flask. The solution was gently heated in a heating bath at 60 °C until dissolved. When fully dissolved, dH_2O was added until a final volume of 0.1 L. The flask was covered in aluminum and placed in a dark room at RT.

2.9 Software and Online Resources

Table 8. gives an overview of the software and online resources used in this study.

***Table 8. Software and Online Resources**. The table shows the software and online resources used in this study, along with the supplier and URL.*

Software/Online Resource	**Supplier**	**URL**
Alphafold2	(Jumper et al., 2021; Varadi et al., 2021)	https://alphafold.ebi.ac.uk
Benchling	Benchling	https://www.benchling.com
CAZy	AFMB	http://www.cazy.org
ExPASy ProtParam Tool	Swiss Institute of Bioinformatics	https://web.expasy.org/protparam/

ExPASy Translate tool	Swiss Institute of Bioinformatics	https://web.expasy.org/translate/
Image Lab™ Software	Bio-Rad	
InterPro	EMBL's European Bioinformatics Institute (EMBL-EBI)	https://www.ebi.ac.uk/interpro/
Jalview	(Waterhouse et al., 2009)	https://www.jalview.org
Ligation calculator		http://www.insilico.uni-duesseldorf.de/Lig_Input.html
NEBcloner	New England Biolabs	https://nebcloner.neb.com/#!/
pblast	NCBI	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins
Pfam	EMBL-EBI	https://pfam.xfam.org
Phyre^2 - Protein Homology/analogY Recognition Engine V 2.0	Structural Bioinformatics Group	http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index
Primer design and other tools	Takara Bio Inc	https://www.takarabio.com/learning-centers/cloning/primer-design-and-other-tools
Pseudomonas Genome DB	(Winsor et al., 2016)	https://www.pseudomonas.com
QuikChange Tm Primer Calculator	Agilent Technologies	https://www.agilent.com/store/primerDesignProgram.jsp
RCSB PDB	(Berman et al., 2000)	https://www.rcsb.org
Reverse complement		https://www.bioinformatics.org/sms/rev_comp.html
Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis	(Szklarczyk et al., 2021)	https://string-db.org
SignalP - 5.0	DTU Health Tech	https://services.healthtech.dtu.dk/service.php?SignalP-5.0

The PyMOL Molecular Graphics System, Version 2.5.2	Schrödinger, LLC	https://pymol.org/2/
Unicorn™ 6.4.1	GE Healthcare Life Sciences	
UniProt	UniProt Consortium	https://www.uniprot.org/uniprot/Q9HYN5

2.10 Antibiotics

2.10.1 Kanamycin

Kanamycin was prepared by dissolving 0.5 g of the antibiotic powder in dH_2O to a concentration of 50 mg/mL. The solution was sterilized through a 0.2 µm filter. Then aliquots were made and stored at -20 °C.

2.10.2 Ampicillin

Ampicillin was prepared by dissolving 1 g of the antibiotic powder in dH_2O to a concentration of 100 mg/mL. The solution was sterilized through a 0.2 µm filter. Then aliquots were made and stored at -20 °C.

3 Methods

An overview of the suppliers for the laboratory equipment and material and the supplier for the chemicals used in this study is found in **Appendix A** (**Table A 1** and **Table A 2**).

3.1 Bioinformatic tools

In **Table 8**, a list of every software- and online- resource are listed with suppliers.

3.1.1 ExPASy Translate tool

ExPASy translate tool translates nucleotide sequence into an amino acid sequence. The nucleotide sequence for *PA1784* was translated into the amino acid sequence with the ExPASy Translate tool.

3.1.2 The Pseudomonas Genome Database

The Pseudomonas Genome Database is a website that provides updates on the PAO1 genome annotation (Winsor et al., 2016). The amino acid sequence for *PA14_41500* was obtained from pseudomonas.com.

3.1.3 SignalP-5.0

SignalP-5.0 was used to analyze the protein sequence to determine if the protein included a signal peptide. The amino acid sequences (**3.1.1**) were pasted into SignalP-5.0, and the program searched for signal protein, TAT signal protein, and Lipoprotein signal protein.

3.1.4 Pairwise sequence alignment (PSA) with BLAST

The well-characterized protein PA1167 was compared with PA14_41500 with a PSA. The amino acid sequences of *PA14_41500* and PA1167 were run and aligned with blastp. The PA1167 sequence was obtained from PDB.

3.1.5 Alphafold2 and PyMOL

Alphafold2 predicts the structure of proteins based on the amino acid sequence (Jumper et al., 2021; Varadi et al., 2021). PyMOL is a molecular visualization system. The 3D structure of PA1167 (PDB ID 1VAV) and the Alphafold2-predicted structure of PA1784 were downloaded and compared in PyMOL.

3.1.6 STRING analysis

STRING analysis was done to see what kind of protein-protein interaction *Pa*Aly7a had. A STRING analysis (Szklarczyk et al., 2021) was done by choosing PA1784 from *P. aeruginosa* on string-db.org. Further, the top 10 interactions with a minimum of medium confidence (0.4) were chosen. Between every protein, each edge was based on confidence, and the thickness of the edge indicated the strength of the data support. The network was a full STRING network that indicated both functional and physical protein associations.

3.2 Gel electrophoresis

3.2.1 DNA agarose gel electrophoresis

DNA agarose gel electrophoresis separates DNA fragments according to size. DNA is negatively charged due to phosphate groups. When a current is applied, the DNA will go through the gel towards the cation. This creates a movement of DNA with a mass to charge (m/z) ratio. The agarose gel is made of agarose, a linear polysaccharide powder containing repeating units of agarobiose and TAE buffer. A network with pores is created by dissolving and heating the agarose in the TAE buffer. By adjusting the amount of agarose, different pore sizes can be made, and different ranges of sizes can be separated. To visualize the DNA fragments on the gel, PeqGREEN is added to the agarose and TAE buffer solution. PeqGREEN is a fluorescent dye that binds to DNA and creates a visible band under UV light. A ladder with the known size is applied to the gel to find the length of the different fragments, and loading dye is added to make the samples more visible, heavier, and easier to apply.

Materials:

- 1 % agarose gel:
    - 0.5 g Agarose powder
    - 50 mL TAE Buffer (1X)
    - 2 µL PeqGreen
    - Loading Buffer: Gel Loading Dye, Purple (6X)
    - Running buffer: TAE Buffer (1X)
    - DNA ladder: Quick-Load® 1kb ladder
- Gel Doc™ EZ Imager system
- Bench top UV Transilluminator
- UV sample tray

- Electrophoresis System, Mini-Sub® Cell GT Horizontal
- 8-Well Comb and 15-Well Comb
- Gel caster
- Power Supply, PowerPac™ Basic

Method:

A 1 % agarose gel was made from 0.5 g agarose powder dissolved in 50 mL 1x TAE buffer in a 250 mL Erlenmeyer flask. With a loose cap, the flask was then placed in a microwave. The solution was boiled several times until the powder dissolved. When dissolved, the flask was cooled under running cold water before 2.5 μL of PeqGREEN was added and gently mixed. The solution was immediately poured into a gel caster with combs. The combs created wells where the samples later were added. After approximately 30-40 minutes, the gel was solid and transported to the Mini-Sub GT cell containing 1X TAE buffer. The ladder and the samples were then mixed with 6X purple loading dye to a final concentration of 1X. In the first chamber, 5 μL of Quick-Load® 1kb ladder was applied, and in the other chambers, 30 μL of the samples were applied. The gel was then run for approximately 45 minutes at 90 V. The gels were then placed on a UV sample tray and placed in the Gel Doc™ EZ Imager system.

3.2.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels separate proteins based on their molecular weight and give an indication of the size of the protein and the purity of the sample. By adding lithium dodecyl sulfate (LDS) sample buffer to the protein sample and then heating it at 70 degrees, the proteins will unfold. LDS will break the non-covalent bonds (except the disulfide bonds) and bind to the protein when heated. The binding between LDS and the protein will create a net negative charge based on the length of the protein. The polyacrylamide gel has a network of crosslinked acrylamide which creates a porous matrix. Samples added to the gel will go through the gel due to an electric current, and due to the porous matrix, the proteins will be separated based on size. Dithiothreitol (DTT) was added prior to heating in order to break the disulfide bonds.

Materials:

- TGS running buffer (1X)
- Protein BenchMark™ Ladder
- mini-PROTEAN® TGX Stain Free™

- Mini-PROTEAN Tetra cell
- PowerPac™ 300
- Gel Doc™ EZ Imager
- ThermoMixer® C
- NuPAGE™ LDS Sample Buffer (4X)
- 90 µM DTT

Method:

15 µL of the protein samples was mixed with 5 µL of sample buffer (NuPAGE™ LDS Sample Buffer (4X)) in a 1.5 mL Eppendorf tube. The tubes were then incubated at 70 °C for 10 minutes on a ThermoMixer® C. While the samples were incubated, the Mini-PROTEAN® TGX Stain-Free™ Precast Gels were placed in a mini-PROTEAN® Tetra cell gel chamber and filled with 1X TGS running buffer. In the first lane, 5-7.5 µL of Protein BenchMark™ Ladder was added. After 10 minutes of incubation, depending on the well size of the gels, either 10 or 15 µL of the samples was applied. The chamber was then connected to a PowerPac™ 300, and the gel was then run at 200 V for 30 minutes. When the samples were at the bottom of the gel, the PowerPac™ was stopped. The gels were then placed on a stain-free sample tray and photographed in the Gel Doc™ EZ Imager system. The sensitivity option (5 minutes imaging) was used in the Image Lab™ software when taking a gel picture.

When adding 90 µM DTT, the samples were heated at 95 °C for 5 minutes.

3.3 Cultivation of bacteria

In this study, two different *E. coli* were used, TOP10 and BL21. TOP10 was used to mass-produce the plasmid pNIC-CH+*PA14_41500*, while BL21 was used to express *Pa*Aly7a in high quality and quantity. Both strains share numerous properties, and therefore they were incubated in the same manner. In addition, were *P. aeruginosa* strains PAO1, PAO1 ΔWspf, PAO1 ΔWspf ΔPsl, PAO1 ΔWspf ΔPel, and mucoid strain PAU2 used.

3.3.1 Overnight culture

Materials:

- LB agar plate with 50 µg/mL kanamycin (TOP10/BL21 with pNIC-CH or pET)
- LB agar plate (*P. aeruginosa* strains)

- LB agar plate with 100 μg/mL ampicillin (TOP10/BL21 with pBAD)
- White loops
- Bacterial glycerol stock
- 5 mL LB liquid medium
- 5 μL kanamycin (50 mg/mL) (TOP10/BL21 with pNIC-CH or pET)
- 5 μL ampicillin (100 mg/mL) (TOP10/BL21 with pBAD)
- 15 mL tube
- New Brunswick™ Innova® 44

Method:

A white loop was used to spread the bacteria from a glycerol stock to an LB agar plate. TOP10 or BL21 with pBAD was spread on LB agar plate with 100 μg/mL ampicillin, TOP10 or BL21 with pET or pNIC-CH was spread on LB agar plate with 50 μg/mL kanamycin, and *P. aeruginosa* strains were spread on LB agar plates without antibiotics.

For cultivation with *E. coli*, the plate was incubated at 37 °C for 16-20 hours. One colony was picked with a white loop from the plate and put in a 15 mL tube with a 5 mL LB medium. Kanamycin or ampicillin was added until a final concentration of 50 μg/mL or 100 μg/ml, respectively. The tube was then incubated in New Brunswick™ Innova® 44 at 37 °C with shaking (220 rpm) for another 16-20 hours. The cap on the tube was a bit loose.

P. aeruginosa strains were cultured without antibiotics, and the mucoid strain (PAU2) was first incubated on an LB agar plate for 3 days at 37 °C. One colony was picked with a white loop from the plate and put in a 15 mL tube with a 5 mL LB medium. The tube was then incubated in New Brunswick™ Innova® 44 at 37 °C with shaking (220 rpm) for 16-20 hours with a bit loose cap on the tube. PAO1 strains were incubated as the *E. coli* strains.

3.3.2 Long-Term Storage of Bacteria – Glycerol Stocks

Materials:

- Sterile 85 % glycerol (which earlier has been autoclaved)
- 2 mL screw cap microtube
- Overnight culture, as described in section **3.3.1**

Method:

In a sterile cabinet, 130 µL of sterile 85 % glycerol was mixed with 830 µL overnight culture of the bacterial strain in a 2 mL screw cap microtube. The tube was then adequately marked and stored at -80 °C.

3.4 Cloning of *PA14_41500* into pNIC-CH carrying a His-Tag

The plasmid pNIC-CH was a kind gift from Opher Gileadi, and the supplier is listed in **Table 1**, the primer used is described in **Table 2**, and the supplier of the kit is listed in **Table 5**.

3.4.1 Overnight culture of pNIC-CH from TOP10

The plasmid pNIC-CH was stored in TOP10. Therefore, these bacteria were obtained and then cultivated as an overnight culture as described in **3.3.1.**

3.4.2 Isolation of pNIC-CH from TOP10

The plasmid was isolated from an overnight culture made of TOP10 with pNIC-CH (**3.3.1**).

Materials:

- Overnight culture (**3.3.1**)
- Allegra X-30R Benchtop centrifuge
- Nucleospin® Plasmid DNA purification kit
- dH_2O
- NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer

Method:

The overnight culture was centrifuged in an Allegra X-30R Benchtop centrifuge for 10 minutes at 4 °C at 4255 g. The protocol Plasmid (NoLid) was used from the Nucleospin® Plasmid DNA purification kit. Instead of an elution buffer, dH_2O was added. NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer measured the concentration and the purity of the sample.

3.4.3 Double digestion of pNIC-CH

To clone the gene *PA14_41500* into pNIC-CH, the plasmid and the gene needed to be treated with the same restriction enzymes. For this purpose, the restriction enzymes AflII and PstIHF were chosen.

Materials:

- NEBcloner
- CIP
- 0.2 mL PCR tubes

Table 9. Double digestion pNIC-CH*. The amount of each reagent for double cutting and negative control is shown.*

	Samples		
Reagents	**Double cutting**	**Negative control**	**Negative control**
pNIC-CH	1 µg	1 µg	1 µg
AflII	1.0 µL	1.0 µL	-
PstIHF	1.0 µL	-	1.0 µL
10X buffer	5 µL	5 µL	5 µL
UltraPure™ DNase/RNase-Free Distilled Water	up to 50 µL	up to 50 µL	up to 50 µL

Method:

NEBcloner was used to find the correct NEBuffer for both restriction enzymes. In PCR tubes, the components of double digestion were added (**Table 9**). In this case, there were two samples and two controls. All the samples were incubated at 37 °C for 2 hours, but after 1 hour and 40 minutes, 1 µL of CIP was added. After 2 hours, the samples were heat-inactivated at 65 °C for 25 minutes.

3.4.4 Gel excision and purification

The double digestion of pNIC-CH (**3.4.3**) was controlled with 1 % agarose gel, as described in section **3.2.1**.

Materials:

- Scalpel
- 1.5 mL Eppendorf tubes
- Scale, Entris 3202I-1S
- dH_2O
- NucleoSpin® Gel and PCR Clean-up
- NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer

The bands were visualized and verified by placing the gel on a Benchtop UV Transilluminator. After identifying the double digested plasmid from section **3.4.3** on the gel, the plasmid was cut out of the gel using a scalpel. Next, the gel piece was weighted on Scale, Entris 3202I-1S, and then cleaned by NucleoSpin® Gel and PCR Clean-up protocol DNA extraction from agarose gels. The cleaned vector was then measured by NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer to find the concentration and the purity of the sample.

3.4.5 Insert amplification

PA14_41500 was amplified by PCR amplification. Per Kristian Edvardsen kindly provided the primers used.

Materials:

- 0.2 mL PCR tubes
- SimpliAmp™ Thermal Cycler
- NucleoSpin® Gel and PCR Clean-up
- NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer
- Q5 master mix
- Alg_Lya_*PA14_41500*_For_AflII 10 mM
- Alg_Lya_*PA14_41500*_Rev_PstI 10 mM
- UltraPure™ DNase/RNase-Free Distilled Water
- pUC19+*PA14_41500* 5 ng/µL

Method:

Primer Alg_Lya_*PA14_41500*_For_AflII and Alg_Lya_*PA14_41500*_Rev_PstI listed in **Table 2** were used to amplify *PA14_41500* in vector pUC19.

Table 10. Insert amplification. *The table shows PCR reagents for insert amplification of PA14_41500 with a reaction volume of 1 reaction and X reactions.*

Reagents	**1 reaction**	**X reactions**
Q5 master mix	25 µL	(25) X µL
Alg_Lya_*PA14_41500*_For_AflII (10 mM)	2.0 µL	(2.0) X µL
Alg_Lya_*PA14_41500*_Rev_PstI (10 mM)	2.0 µL	(2.0) X µL
UltraPure™ DNase/RNase-Free Distilled Water	19 µL	(19) X µL
pUC19 *PA14_41500* 5 ng/µL	2.0 µL	(2.0) X µL

Table 11. PCR conditions. *The table shows the PCR conditions for amplification of PA14_41500 from pUC19 and the temperature and duration.*

PCR conditions (Temperature and duration)	**Cycles**
98 °C (5 minutes)	1 x
98 °C (10 seconds)	35 x
Sample 1+2 at 63 °C (30 seconds)	
Sample 3+4 at 66 °C (30 seconds)	
Sample 5+6 at 68 °C (30 seconds)	
Neg control at 66 °C (30 seconds)	
72 °C (90 seconds)	
72 °C (5 minutes)	1 x

The components in **Table 10** were added to six different 0.2 mL PCR tubes on ice. In addition, the negative control was made with UltraPure™ DNase/RNase-Free Distilled Water instead of pUC19+*PA14_41500*. The samples were annealed at three different temperatures since the optimal temperature for the primers was unknown (**Table 11**). The amplification was verified by analyzing the samples with a 1 % agarose gel as described in section **3.2.1**. After verifying that the PCR product included only *PA14_41500*, the PCR product was cleaned with the kit NucleoSpin® Gel and PCR Clean-up protocol PCR clean-up. NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer was then used to measure the concentration of the gene and the purity of the clean process.

3.4.6 In-Fusion reaction

pNIC-CH linearized plasmid kindly provided by Hibaq Ahmed Farah.

A ligation calculator was used to find the optimal infusion cloning under standard conditions.

Materials:

- Ligation calculator
- In-Fusion® HD Cloning kit
- dH_2O
- pNIC-CH linearized plasmid, section **3.4.3**
- Purified PCR fragment (*PA14_41500*), section **3.4.5**
- ThermoMixer® C

Method:

The ratio in the infusion calculation was set to 2:1 of insert and vector. In Eppendorf tubes placed on ice, cloning reaction, negative control, and positive control were made with the reagents listed in **Table 12**.

Table 12. In-Fusion of PA14_41500 in linearized pNIC-CH*. The table shows the components and the amount to make a cloning reaction, a positive control, and negative control. Based on the In-Fusion® HD Cloning kit.*

	Samples		
Reagents	**Cloning reaction**	**Negative control**	**Positive control**
Purified PCR fragment (*PA14_41500* ca 696 bp) 26.4 ng	2.64 µL	-	2 µL of control insert (2000bp)
Linearized pNIC-CH (5279 bp) 100 ng	6 µL	1 µL	1 µLµL control vector (PUC19)
5X In-Fusion HD Enzyme Premix	2 µL	2 µL	2 µL
dH_2O		7 µL	5 µL

The infusion reactions were incubated for 60 minutes at 50 °C and then placed on ice. If the infusion reactions were not used the same day, the samples were stored at -20 °C until the next day.

3.4.7 Transformation of pNIC-CH+*PA14_41500* into TOP10

From the In-Fusion reaction (**3.4.6**), pNIC-CH+*PA14_41500*, positive control, and negative control were used in the transformation of TOP10.

Materials:

- pNIC+*PA14_41500* (**3.4.6**)
- positive control and negative control from In-Fusion reaction (**3.4.6**)
- Microcentrifuge, 5418 R
- TOP10
- 13 mL round bottom tube
- SBB Aqua 5 plus
- SOC media
- LB agar plates with kanamycin 50 μg/mL
- LB agar plate with ampicillin 100 μg/mL
- T-shaped cell spreader

Method:

The vial containing pNIC+*PA14_41500* was briefly centrifuged and then placed on ice, along with the rest of the components. From the infusion reaction (**3.4.6**), 5 μL of pNIC+*PA14_41500* was mixed with 50 μL of TOP10 in a 13 mL round bottom tube. Positive and negative transformants were made using 5 μL of the positive and the negative control from the In-Fusion reaction. Both the positive and the negative control were mixed with 50 μL of TOP10. All the tubes were mixed by flicking before the tubes were incubated on ice for 30 minutes. Each sample was then heat shocked by placing the tube in a 42 °C water bath for approximately 40-45 seconds and then back on the ice for 2 minutes. After 2 minutes, 200 μL of prewarmed SOC media was added to the tubes, and the samples were incubated at 37 °C and 230 rpm for 1 hour. After this hour, aliquots were made on preheated LB agar plates with kanamycin 50 μg/mL. From the bacterial solution containing pNIC+*PA14_41500*; 10, 50, and 100 μL were spread on separate plates using a T-shaped cell spreader. The positive control was made by adding 100 μL of the bacteria solution to an LB agar plate with 100 μg/mL ampicillin. At the same time, the negative control was made by adding 100 μL bacterial solution to an LB agar plate containing 50 μg/mL kanamycin. All the plates were incubated at 37 °C overnight.

3.4.8 Colony PCR of TOP10 with pNIC-CH+*PA14_41500*

The colonies growing on the LB plates after transformation (**3.4.7**) were run with colony PCR and the primers for *PA14_41500*. Colony PCR identifies transformant cells with pNIC-CH+*PA14_41500*.

Per Kristian Edvardsen kindly provided the primers used.

Materials:

- LB-agar plate with 50 μg/mL kanamycin
- dH_2O
- 1.5 mL Eppendorf tubes
- SimpliAmp™ Thermal Cycler
- 0.2 μL PCR tubes
- Redtaq master mix
- pNIC-For
- pNIC-Rev

Method:

From the LB agar plates with TOP10 transformants (**3.4.7**), every growing colony was dissolved in 20 μL dH_2O in Eppendorf tubes and streaked out on a new LB-agar plate with 50 μg/mL kanamycin. The plates with positive and negative control were visually controlled.

Table 13. Colony PCR Mastermix*. The table shows PCR reagents for colony PCR with the volume of 1 reaction and X reactions.*

Reagents	**1 reaction**	**X reactions**
Redtaq master mix	12.5 μL	(12.5) X μL
pNIC-For (10 μM)	1.0 μL	(1.0) X μL
pNIC-Rev (10 μM)	1.0 μL	(1.0) X μL
UltraPure™ DNase/RNase-Free Distilled Water	8.5 μL	(8.5) X μL
Dissolved CFU (5 ng/μL)	2.0 μL	(2.0) X μL

Primer pNIC-For and pNIC-Rev were used to amplify pNIC-CH+*PA14_41500*. The different reagents listed in **Table 13** were added to a 0.2 mL PCR tube, and then the PCR cycle was run as described in **Table 14**. All the components were placed on ice during the experiment.

***Table 14. PCR conditions**. The table shows the temperature and duration of colony PCR.*

PCR conditions (Temperature and duration)	**Cycles**
94 °C (10 minutes)	1 x
94 °C (30 seconds)	35 x
58 °C (30 seconds)	
72 °C (150 seconds)	
72 °C (5 minutes)	1 x

The PCR samples were then verified by analyzing the samples on a 1 % agarose gel as described in section **3.2.1.** Due to the Redtaq master mix, loading dye was not added to the samples.

An overnight culture was made from the colonies spread on LB agar plates with kanamycin 50 μg/mL) as described in section **3.3.1.** From this overnight culture, a glycerol stock was made as described in **3.3.2,** and with the rest of the overnight culture, pNIC-CH+*PA14_41500* was purified and measured as outlined in **3.4.2.**

3.4.9 Sequencing

To check if errors were made during PCR in **3.4.5,** the plasmid was isolated and sequenced using the LightRun service from Eurofins Genomics.

Materials:

- Primer pNIC-For (10 μM)
- Primer pNIC-Rev (10 μM)
- pNIC-CH+*PA14_41500*
- 1.5 mL Eppendorf tubes
- Jalview
- Reverse complement

Method:

Eppendorf tubes were marked with individual barcodes, and 5 µL of a DNA sample with a concentration between 50-100 ng/µL was added. In addition to the sample was 5 µL of a 5 µM primer added. Two samples were made; the forward primer was added to one tube and the reverse primer in the second tube.

When the results from the sequencing came back, separate word files were made for forward FASTA files and reverse FASTA files. The reverse files were then converted into reverse complement in the bioinformatic tool Reverse complement. These sequences were then pasted into Jalview, and the original FASTA file of *PA14_41500* was added and set as a reference. Then, a multiple sequence alignment (MSA) was made using the MUSCLE algorithm (default settings), and the percent identity was marked with colors. Finally, the chromatograms from the sequencing were studied and compared to the original *PA14_41500* file.

3.4.10 Transformation of pNIC-CH+*PA14_41500* into BL21

After confirming that pNIC-CH+*PA14_41500* had been correctly transformed into TOP10 and no mutations had occurred in the gene (**3.4.9**), pNIC-CH+*PA14_41500* was further transformed into BL21 as described in **3.4.7**.

3.4.11 Colony PCR of BL21 with pNIC-CH+*PA14_41500*

After transforming the plasmid into BL21, the transformation was verified with colony PCR.

Per Kristian Edvardsen kindly provided the primers used.

Materials:

- 0.2 mL PCR tubes
- SimpliAmp™ Thermal Cycler
- LB-agar plate with 50 µg/mL kanamycin
- dH_2O
- 1.5 mL Eppendorf tubes
- NucleoSpin® Gel and PCR Clean-up
- NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer

***Table 15. Colony PCR Master mix**. The table shows PCR reagents for colony PCR with a reaction volume of 1 reaction and X reactions. These reagents are for BL21.*

Reagents	**1 reaction**	**X reactions**
Q5 master mix	25 µL	(25) X µL
pNIC-For (10 µM)	2.0 µL	(2.0) X µL
pNIC-Rev (10 µM)	2.0 µL	(2.0) X µL
UltraPure™ DNase/RNase-Free Distilled Water	19 µL	(19) X µL
Dissolved CFU (5 ng/µL)	2.0 µL	(2.0) X µL

Method:

From the **Transformation of pNIC-CH+PA14_*41500* into BL21,** each growing colony on the LB agar plates was dissolved in 20 µL dH_2O in Eppendorf tubes. The colonies were then streaked out on a new LB-agar plate with 50 µg/mL kanamycin. The negative and positive control was virtually controlled.

Primer pNIC-For and pNIC-Rev were used to amplify pNIC-CH+*PA14_41500*. The different reagents listed in **Table 15** were added to a 0.2 mL PCR tube, and then the PCR cycle was run as described in **Table 16**. All the components were placed on ice during the experiment.

***Table 16. pNIC-CH+PA14_41500 colony PCR conditions.** The table shows the PCR conditions for colony PCR of BL21 with pNIC-CH+PA14_41500 and the temperature and duration for this amplification.*

PCR conditions (Temperature and duration)	**Cycles**
98 °C (10 minutes)	1 x
98 °C (10 seconds)	35 x
63 °C (30 seconds)	
72 °C (90 seconds)	
72 °C (5 minutes)	1 x

The PCR samples were then verified by analyzing the samples on a 1 % agarose gel as described in section **3.2.1.** After verifying that the PCR product included only *PA14_41500*, the PCR product was cleaned with the kit NucleoSpin® Gel and PCR Clean-up protocol PCR clean-up. NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer was then used to measure

the concentration of the gene and the purity of the clean process. The cleaned PCR products were then sent to sequencing and analyzed as described in section **3.4.9**.

An overnight culture was made from the LB agar plates with kanamycin 50 µg/mL and dissolved CFU as described in section **3.3.1**. A glycerol stock was made from the overnight culture as described in **3.3.2**.

3.5 Cloning of *PA14_41500* into pET carrying a His-Tag

To test if other plasmids could produce more *Pa*Aly7a, *PA14_41500* was cloned into pET and pBAD (**3.6**). The suppliers for the plasmids are listed in **Table 1**, the primer used is described in **Table 2**, and the kit supplier is listed in **Table 5**.

3.5.1 Overnight culture of pET from TOP10 isolation of the plasmid

An overnight culture of *E. coli* with pET was made as described in **3.3.1**. The plasmid was then isolated as written in **3.4.2**.

3.5.2 Double digestion of pET and Gel excision and purification

Double digestion was done as described in section **3.4.3** with other restriction enzymes and buffer (**Table 17**).

Materials:

- NcoI
- XhoI
- 10X NEBuffer r3.1

Table 17. Double digestion of pET*. The amount of each component for double cutting and negative control is shown.*

	Samples		
Reagents	**Double cutting**	**Negative control**	**Negative control**
pET	1µg (10.1 µL)	1 µg	1 µg
NcoI	1.0 µL	1.0 µL	-
XhoI	1.0 µL	-	1.0 µL
10X NEBuffer r3.1	5 µL (1X)	5 µL	5 µL

UltraPure™ DNase/RNase-Free Distilled Water	up to 50 µL	up to 50 µL	up to 50 µL

Method:

After 2 hours and 40 minutes of incubation at 37 °C, the samples were heat-inactivated at 80 °C for 25 minutes. Afterward, the samples were analyzed with 1 % agarose gel and purified (**3.4.4**).

3.5.3 Insert amplification

To later clone *PA14_41500* into pET, both *PA14_41500* and pET needed the same ends. Therefore, *PA14_41500* was amplified with primers that included ends that complimented the ends of pET. The procedure was based on section **3.4.5** with reagents and PCR conditions deviations.

Materials:

- *PA14_41500* (10 ng/µL)
- pET_28a(+)_TEV_conf_F
- pET_28a(+)_TEV_conf_R

Table 18. PCR reagents for insert amplification. *The table shows PCR reagents for amplification of PA14_41500 from pUC19 with reaction volume of 1 reaction and X reactions.*

Reagents	**1 reaction**	**X reactions**
Q5 master mix	25 µL	(25) X µL
pET_28a(+)_TEV_conf_F	2.0 µL	(2.0) X µL
pET_28a(+)_TEV_conf_R	2.0 µL	(2.0) X µL
UltraPure™ DNase/RNase-Free Distilled Water	19 µL	(19) X µL
PA14_41500 (5 ng/µL)	2.0 µL	(2.0) X µL

Method:

A PCR mix was set up as described in **Table 18**, and the PCR was run in the terms stated in **Table 19**.

Table 19. PCR conditions for insert amplification. *The table shows the PCR conditions for amplification of PA14_41500 and the temperature and duration for this amplification.*

PCR conditions (Temperature/duration)	**Cycles**
98 °C (5 minutes)	1 x
98 °C (10 seconds)	35 x
67 °C (30 seconds)	
72 °C (90 seconds)	
72 °C (5 minutes)	1 x

The PCR product was then analyzed on a 1 % agarose gel as described in section **3.2.1** and purified by NucleoSpin® Gel and PCR Clean-up as described in **3.4.4**.

3.5.4 In-Fusion reaction

The In-Fusion reaction was carried out as described in section **3.4.6**, with different plasmid and amplified inserts. From insert amplification (**3.5.3**), 2 µL of *PA14_41500* was mixed with 5.2 µL of linearized pET (**3.5.2**). The volume of the other reagents was regulated based on the insert gene and plasmid. A second In-Fusion reaction was carried out with a ratio: of 4:1 of insert and vector.

3.5.5 Transformation, Colony PCR of TOP10 pET+*PA14_41500*, and sequencing

With pET+*PA14_41500* (**3.5.4)**, the transformation of TOP10 was done as explained in section **3.4.7**. After incubation, the colonies were amplified in colony PCR to verify the transformation. The procedure is explained in **3.4.8**. However, the primers and the last elongation step in the PCR procedure deviated since the elongation step lasted 70 seconds, not 150 seconds, as written in **Table 14**.

Methods:

The primers used in colony PCR were pET_28a(+)_TEV_conf_F and pET_28a(+)_TEV_conf_R. After colony PCR, gel electrophoresis, making glycerol stocks, and purifying the plasmids were described in **3.4.8**. Finally, the purified plasmids from different colonies were sent to sequencing. The procedure is described in **3.4.9**.

3.5.6 Transformation of pET+*PA14_41500* into BL21, Colony PCR, and Sequencing

The pET+*PA14_41500* was later transformed into BL21 as described in **3.4.7**, then a colony PCR plus sequencing was done as described in **3.4.8** and **3.4.9** with the primers pET_28a(+)_TEV_conf_F and pET_28a(+)_TEV_conf_R. The last elongation step lasted 70 seconds, not 150 seconds, as written in **Table 14**.

3.6 Cloning of *PA14_41500* into pBAD carrying a His-Tag

The suppliers for the plasmids are listed in **Table 1**, the primer used is described in **Table 2**, and the kit supplier is listed in **Table 5**.

3.6.1 Overnight culture of pBAD from TOP10 and isolation of the plasmid

Materials:

- LB agar plate with 100 µg/mL Ampicillin
- 100 mg/mL Ampicillin

Method:

An overnight culture of *E. coli* with pBAD was made as described in **3.3.1**. The plasmid was then isolated as written in **3.4.2**.

3.6.2 Double digestion of pBAD and Gel excision and purification

Double digestion was done as described in section **3.4.3** with other restriction enzymes and buffer (**Table 20**).

Materials:

- EcoRI
- BglII
- 10X NEBuffer r3.1

Table 20. Double digestion of pBAD*. The amount of each component for double cutting and negative control is shown.*

	Samples		
Reagents	**Double cutting**	**Negative control**	**Negative control**
pBAD	1µg (6.5 µL)	1 µg	1 µg

BglII	1.0 µL	1.0 µL	-
EcoRI	1.0 µL	-	1.0 µL
10X NEBuffer r3.1	5 µL (1X)	5 µL	5 µL
UltraPure™ DNase/RNase-Free Distilled Water	up to 50 µL	up to 50 µL	up to 50 µL

Method:

After 2 hours and 40 minutes of incubation at 37 °C, the samples were heat-inactivated at 65 °C for 25 minutes. Afterward, the samples were analyzed with 1 % agarose gel and purified (**3.4.4**).

3.6.3 Insert amplification

As described in **3.5.3**, the procedure was done with the primers pBAD_conf_F and pBAD_conf_R. *PA14_41500* was amplified as described in **Table 18** and **Table 19** and purified using the kit NucleoSpin® Gel and PCR Clean-up as described in **3.4.4**.

3.6.4 In-Fusion reaction

The In-Fusion reaction was carried out as described in section **3.4.6**, with different plasmid and amplified inserts. From insert amplification (**3.6.3**), 2 µL of *PA14_41500* was mixed with 4.5 µL of linearized pBAD (**3.6.2**). The volume of the other reagents was regulated based on the insert gene and plasmid.

3.6.5 Transformation of pBAD+*PA14_41500* into TOP10 and colony PCR

The transformation of pBAD+*PA14_41500* (**3.6.4**) into TOP10 was done as explained in section **3.4.7**. After incubation, the colonies were amplified in colony PCR to see if the transformation worked. The procedure was carried out as described in **3.4.8** with other primers and a shorter elongation step. The primers used were pBAD_conf_F and pBAD_conf_R, and the last elongation step in PCR lasted 70 seconds, not 150 seconds as written in **Table 14**. For pBAD+*PA14_41500*, the annealing step was at 62 °C.

3.6.6 Control PCR

To double-check if the transformation worked with pBAD+*PA14_41500*, both colony PCR and amplification PCR proceeded a second time. The same setup as insert amplification (**3.6.3**) and

colony PCR (**3.6.5**). Both PCR products were then analyzed for comparison with 1 % agarose gel as described in **3.2.1**.

3.6.7 Sequencing

After colony PCR, gel electrophoresis, making glycerol stocks, and purifying the plasmids as further described in **3.4.8**, the purified plasmids from different colonies were sent to sequencing. The procedure is described in **3.4.9**. A deviation from the procedure was the plasmids. When pBAD+*PA14_41500* was sequenced, the primers pBAD_conf_F and pBAD_conf_R were used.

3.6.8 Transformation of BL21, Colony PCR, and Sequencing

The pBAD+*PA14_41500* was later transformed into BL21 as described in **3.4.7**, then a colony PCR plus sequencing was done as described in **3.4.8** and **3.4.9** with the primers pBAD_conf_F and pBAD_conf_R. The last elongation step lasted 70 seconds during colony PCR, not 150 seconds, as written in **Table 14**. For pBAD+*PA14_41500*, the annealing step was at 62 °C.

3.7 Cultivation of *E. coli*

3.7.1 Small-scale cultivation of bacteria in Erlenmeyer flask

Materials:

- Overnight culture (**3.3.1**)
- 50 mg/mL Kanamycin
- Growing medium: LB/TB/BHI/M9/TSB
- Erlenmeyer flask 250 mL
- New Brunswick™ Innova® 44

Method:

An overnight culture was made as described in section **3.3.1**.

30 mL from a sterile medium was inoculated with 1 mL overnight culture in a 250 mL Erlenmeyer flask. The general rule was followed to optimize bacterial growth during cultivation: a maximum of 25 % of the total volume in an Erlenmeyer flask was filled with medium. For example, when cultivating a 30 mL medium, the smallest Erlenmeyer flask was 120 mL (30 mL • 4 = 120 mL). Therefore, a 250 mL Erlenmeyer flask could be used. Kanamycin was then added to the flask to a final concentration of 50 µg/mL. The flask was

then covered in aluminum foil and placed on an adhesive mat in the New Brunswick™ Innova® 44 and grown at 37 °C with shaking (220 rpm) for 16-20 hours.

The small-scale culture was then used to express *Pa*Aly7a in small-scale expression (**3.8.1**).

3.7.2 Large-scale cultivation of bacteria in Erlenmeyer flask

Materials:

- Overnight culture (**3.3.1**)
- 50 mL falcon tube
- 50 mg/mL Kanamycin
- 1.1X TB medium (**2.7.2**)
- K_2HPO_4 **(2.8.6**)
- KH_2PO_4 **(2.8.6**)
- Erlenmeyer flask 2-5 L
- Antifoam 204
- New Brunswick™ Innova® 44

Method:

TB medium was prepared by mixing 50 mL of K_2HPO_4 and 50 mL of KH_2PO_4 to 900 mL 1.1X TB medium. The final concentration was 1X TB medium. An overnight culture was made as described in **3.3.1,** with 20 mL LB medium instead of 5 mL. The overnight culture was incubated in a 50 mL flask. From the overnight culture, 15 mL was added to 0.5 L 1X TB medium in a sterile 2 L plastic Erlenmeyer flask, following the general rule (**3.7.1**). Further, 150 µL of Antifoam 204 and kanamycin (50 mg/mL), with a final concentration of 50 µg/mL, were added to the medium. The bacteria were then incubated at 37 °C with shaking (220 rpm) for 16-20 hours in New Brunswick™ Innova® 44.

3.8 Expressing *Pa*Aly7a

3.8.1 Small-scale expression of *Pa*Aly7a in *E. coli*

First, a small-scale expression of *Pa*Aly7a was set up with different mediums, temperatures, and concentrations of IPTG to optimize the expression.

BL21 with pNIC and BL21 with pET have a system for controlling gene expression. The system includes the gene *lacI*, which codes for the repressor LacI, and *lacO*, the operator sequence. When LacI is expressed, the protein will bind to the operator, the *lacO*, and prevent transcription by physically blocking the initiation of the transcription. *lacO* is upstream for the gene coding for T7 RNA polymerase, and the T7 RNA polymerase is necessary for transcribing the *Pa*Aly7a on the vector. However, IPTG can bind to the LacI and prevent this protein from binding to *lacO*. So, when adding IPTG, the *lacO* operator will not be blocked, and the T7 RNA polymerase can be transcribed. The T7 RNA polymerase can then transcribe the gene *PA14_41500* (Brown et al., 1987; Jacob & Monod, 1961). The bacteria cannot degrade IPTG, so the transcription of *Pa*Aly7a will continue.

For pBAD+*PA14_41500*, the inducer was arabinose.

Materials:

- Small-scale cultivation of *E. coli* (**3.7.1)**
- 1 M IPTG (**2.8.10**)
- Arabinose
- PBST (**2.8.7**)
- 25x protease inhibitor
- dH_2O
- 250 mL Erlenmeyer flask
- 1.5 mL Eppendorf tubes
- Sonics Vibra-Cell™ Ultrasonic Processor, VC750
- Ultrospec 10
- Glass tubes
- Microcentrifuge, 5418 R
- New Brunswick™ Innova® 44

Method:

Small-scale cultivation of the transformant cell was made as described in **3.7.1.** First, the bacteria were grown at 37 °C with shaking (220 rpm) until an OD_{600} of 0.6-0.8 was reached. When the desired OD was reached, 1 mL culture was collected and placed on ice. This sample was harvested by centrifugation later. Further, 6 mL of the bacteria culture was transferred to eight glass tubes. To half of the tubes, IPTG was added with a final concentration of 0.2 mM,

and in the other half, IPTG was added with a final concentration of 1.0 mM. After adding IPTG, 1 mL from each tube was collected and placed on ice. Further, half of the tubes were incubated at 20 °C with shaking (220 rpm) overnight, and the other half was incubated at 37 °C for 3 hours with shaking (220 rpm) in New Brunswick™ Innova® 44. After 3 hours, new samples were collected from the tubes at 37 °C, and the tubes were placed back for incubation overnight. The next day all the cells were collected by centrifugation.

Harvesting the cells and separation of proteins

The collected samples were centrifuged on Microcentrifuge, 5418 R, for 10 minutes at 4 °C with 16900 g. Afterward, the supernatants were discarded, and the pellets were resuspended in PBST (**2.8.7**) and 1X protease inhibitor (PBSTp). PBSTp was made by adding 4 µL of a 1X protease inhibitor to 196 µL of sterile PBST. The 25X protease inhibitor was made by dissolving a tablet in 2 mL dH_2O. Until analysis, the samples were kept at -20 °C. The samples were then sonicated using Sonics Vibra-Cell™ Ultrasonic Processor VC750 five times while on ice (5 seconds off and 5 seconds on, 27 % amplitude). The samples were then centrifuged for 10 minutes at 4 °C with 16900 g. The supernatant from each sample was then analyzed with SDS-PAGE gel as described in section (**3.2.2**).

From the small-scale expression of *Pa*Aly7a in TSB and BHI, as an addition, 1 mL of each sample was collected but not sonicated. These samples were mixed with 2 µL of 85 % glycerol, 5 µL of LDS sample buffer, and 15 µL of the samples before they were heated and analyzed with SDS-PAGE gel as described in section (**3.2.2**). Glycerol was added to make the samples heavier and easier to apply to the gel. These samples are called total protein samples.

3.8.2 Large-scale expression of *Pa*Aly7a in *E. coli*

Based on small-scale expression (**3.8.1**), the transformant was further cultivated in the medium, the concentration, and the temperature, which expressed the most *Pa*Aly7a.

Materials:

- Large-scale cultivation of *E. coli* (**3.7.2)**
- 1 M IPTG (**2.8.10**)
- Microcentrifuge, 5418 R
- PBST (**2.8.7**)

- protease inhibitor
- 250 mL Erlenmeyer flask
- 1.5 mL Eppendorf tubes
- Sonics Vibra-Cell™ Ultrasonic Processor, VC750
- Ultrospec 10
- 1 L centrifugal flask
- Avanti J-26S XP centrifuge - JLA 8.1 rotor
- IMAC binding buffer **(2.8.8**)
- Complete™ Mini EDTA free protease inhibitors
- DNase I
- 100 mg/mL lysozyme
- 50 mL falcon tube
- F21-8 X 50Y rotor
- Sorvall LYNX 6000
- 0.22 μm filter
- New Brunswick™ Innova® 44

Method:

Small-scale cultivation of the transformant cell was made as described in **3.7.2**. The bacteria were then incubated at 37 ℃ with shaking (220 rpm) until they reached an OD_{600} of 0.6-0.8. When the correct OD was reached, 1 mL of the culture was collected and stored on ice. This sample was used as a negative control since *Pa*Aly7a was non-induced. After this, 1 M IPTG was added to the bacteria with a final concentration of 1.0 mM, and then 1 mL of the culture was collected. Further on, the Erlenmeyer flask was incubated at 20 ℃ with shaking (220 rpm) overnight in New Brunswick™ Innova® 44. Both samples collected were centrifuged at 4 ℃ at 16900 g, and the supernatant was removed. The pellet was resuspended in 200 μL PBSTp and stored at -20 ℃ overnight. Both collected tubes with PBSTp were sonicated five times with 5 seconds on and 5 seconds off at 27 % amplitude on Sonics Vibra-Cell™ Ultrasonic Processor, VC750. After sonication, both samples were centrifuged at 4 ℃ for 10 minutes at 16900 g.

Harvesting the bacteria cells and making the cytoplasmic protein extract

The cells were harvested by centrifuging the overnight culture in a 1 L centrifugal flasks in the JLA 8.1 rotor for 15 minutes at 7500 rpm at 4 ℃ in the Avanti J25 series centrifuge. The supernatant was then discarded, and the pellet was resuspended in 30 mL ice-cold IMAC

binding buffer **(2.8.8**) supplemented with Complete™ Mini EDTA free protease inhibitors with a final concentration of 1 tablet/10 mL. The sample was then transferred to a 50 mL falcon tube, and 3 µL of DNase I and 30 µL of 100 mg/mL lysozyme were added. Afterward, the solution was incubated on ice for 30 minutes, and every 10 minutes, the sample was shaken. Then the sample was lysed by sonication while still on ice. During sonication, a maximum of 30 mL of the sample in a 50 mL falcon tube could be lysed. The sonicated lasted for 10 minutes with 30 % amplitude and 5 seconds off and 5 seconds on. The cell lysate was then centrifuged with the F21-8 X 50Y rotor at 20 000 rpm for 15 minutes at 4 °C to remove cell debris in Sorvall LYNX 6000. To fill the flask, dH_2O was added. After centrifugation, the supernatant was filtered through a 0.22 µm filter to remove cell debris and DNA.

3.9 Purification methods

3.9.1 IMAC

From the large-scale expression, *Pa*Aly7a was purified with Äkta Start and HisTrap HP 5 mL column. This column contained Ni Sepharose resin. The gene *PA14_41500*, coding for *Pa*Aly7a, was added a His-tag, an area in the sequence coding for six histidine residues. When *Pa*Aly7a was added to the column, this His-tag will bind to the Ni^{2+} in the Ni Sepharose resin due to the histidine imidazole ring (Bornhorst & Falke, 2000).

To purify the protein, IMAC binding buffer, IMAC washing buffer, and IMAC elution buffer were used **(2.8.8).** These buffers contained different concentrations of free imidazole. Imidazole has a similar structure as histidine and can compete with the protein for the binding site on the column. However, the protein has at least six histidine residues and will outcompetes the free imidazole. Nevertheless, the protein will be eluted when a high concentration of free imidazole is added.

The binding buffer had a concentration of 5 mM imidazole and was used to equilibrate the column. Non-specific binding to the column was washed away with the washing buffer with 20 mM imidazole. Only the protein with a His-Tag should be bound to the column after the washing step. However, some substances can still be bound. The elution buffer contains 500 mM imidazole, and due to this concentration, the free imidazole outcompetes the protein forcing it to elute (Bornhorst & Falke, 2000).

The flow-through, washing and eluted samples were collected and analyzed on an SDS-PAGE gel. This was a precaution since the protein may not fold or bind to the column as expected.

Materials:

- Äkta Start/Äkta Pure
- HisTrap HP 5 mL column
- 50 mL falcon tube
- dH_2O
- IMAC binding buffer, IMAC washing buffer, and IMAC elution buffer **(2.8.8**)
- 20 % ethanol

Method:

*Pa*Aly7a was purified on Äkta Pure with the column HisTrap HP 5 mL. The column can be destroyed with a pressure higher than 0.4 mPa, so a constant flow rate of 2 mL/minutes was used.

Before the purification started, all the tubing was flushed with 5 column volumes (CVs) of dH_2O. The system was flushed with water to prevent buffer interactions with ethanol. After washing the system, the column was equilibrated with 5 CVs of IMAC binding buffer until the UV stabilized. When the UV was stable, the UV detector was set to zero. The protein extract from large-scale (**3.8.2**) was then loaded to the column with the sample tube, and the flow-through was then collected in a 50 mL falcon tube. When all the sample was applied, the sample tube was flushed with IMAC binding buffer until the UV signal started to decrease. With the buffer tube, the column was washed with IMAC washing buffer. The wash fraction was collected, and the buffer was run until the UV signal stabilized. IMAC elution buffer was then pumped into the system, and when a peak was detected, the eluted sample was collected. Afterward, the tubes and the column were washed with 5 CVs of dH_2O, before they were stored in 20 % ethanol. Each sample was then analyzed with SDS-page gel as described in section (**3.2.2**). In one SDS-PAGE analysis, a sample from the lysate was also included.

3.9.2 SEC

The chromatographic method SEC was used to remove unwanted proteins from the protein samples purified on IMAC. Before the protein could be purified, the sample had to be concentrated down to 1 mM, and buffer had to be exchanged (**3.10**).

Barth and Boyes (1992) wrote a description of SEC, "SEC is an entropically controlled separation technique that depends on the relative size or hydrodynamic volume of a macromolecule with respect to the average pore size of the packing." This definition describes how macromolecules are separated in the SEC column. In contrast to the IMAC purification method, only one buffer is used in SEC. This is because SEC purification is based on the size or the hydrodynamic volume of the protein. A protein with large size will use more time through the column than a small molecule, and since the protein often has a known size, it can be purified and separated using this method.

Materials:

- Purified *Pa*Aly7a (**3.9.1**)
- SEC buffer (**2.8.9**)
- SEC column storage buffer (20 % ethanol + 0.2 M Sodium acetate ($NaCH_3COO$) and 20 % ethanol)
- dH_2O
- 20 % ethanol
- 10 mL syringe
- 1 mL syringe
- Eppendorf tubes, 2 mL
- HiLoad 16/600 superdex 75 pg column (3 – 70 kDa)
- Äkta purifier system
- 0.7 mm injection needle

Method:

This protocol used the HiLoad 16/600 superdex 75 pg column on the Äkta purifier system. Before starting, the column and the system were flushed with dH_2O to remove the storage buffer in the system. After the cleaning step, the column was equilibrated with one CV of SEC buffer. The column was equilibrated when the conductivity signal had increased and stabilized. Afterward, the sample loop was washed with SEC buffer. This was done by first cleaning the

needle in boiling water, then a 10 mL syringe was filled with SEC buffer, before the needle was placed on the syringe. Before the buffer was injected, the flow rate was adjusted to 0.1 mL/minute. Afterward, the needle was carefully injected into the injector value, and then the buffer was ejected slowly. Next, the protein sample was injected with a 1 mL syringe in the same manner. After applying the protein sample, the flowrate was slowly adjusted to 1 mL/minute.

Forty minutes after applying the protein sample, fractionation was initiated, and the fraction volume was set to 1 mL. Fractions were collected in 2 mL Eppendorf tubes. After approximately 50 and 70 minutes, the proteins of interest were eluted. This was shown as an elevated peak of absorbance. The fractions of interest were analyzed with SDS-PAGE, and the purity was decided as described in section **3.2.2**. Based on the SDS-PAGE, pure protein fractions were concentrated as written in section **3.10** before measuring the concertation as described in section **3.11.1**.

The two samples eluted at 50 and 70 minutes are further described as *Pa*Aly7a-P1 and *Pa*Aly7a-P2, respectively.

3.10 Protein concentration and buffer exchange

The eluted proteins from IMAC purification and SEC purification (**3.9.1**) (**3.9.2**) were concentrated by centrifuging the sample in Vivaspin 20 centrifugal concentrator 3 000 MWCO. The protein samples from IMAC purification contained a high imidazole concentration. Therefore, by diluting the samples in a low concentrated buffer and then centrifuging the samples repeatedly, the high imidazole concentration would be diluted, and the sample concentrated.

Materials:

- Centrifuge, Allegra X-30R
- 50 mM Tris-HCl pH 7.5
- Vivaspin 20 centrifugal concentrators 3 000 MWCO
- Protein samples from IMAC (**3.9.1**) and SEC (**3.9.2**)

Method:

The sample was centrifuged in a Vivaspin 20 centrifugal concentrators 3 000 MWCO at 4255 g at 4 °C until the sample had a volume of 1.5 mL. To change the buffer, 50 mM Tris-HCl pH 7.5 was added to the tube until a final volume of 15 mL. The sample was again centrifuged at 4700 rpm at 4 °C until the volume was 1.5 mL. These steps were repeated until the protein sample had an imidazole concentration of approximately 1.33 mM.

3.11 Protein concentration determination

3.11.1 Absorbance at 280 nm

By measuring the absorbance at 280 nm, the concentration of a protein sample can be estimated based on the absorbance and the hypothetical extinction coefficient value for the amino acid sequence. The extinction coefficient was found by pasting the amino acid sequence into ExPASy´s ProtParam Tool. Two of the amino acids: tryptophan and tyrosine, absorb UV light at 280 nm, and this absorbance is proportional to the concentration of tryptophan and tyrosine in the protein. The extinction coefficient reflects the amount of tryptophan and tyrosine in the protein in proportion to the other amino acids (Pace et al., 1995). Therefore, the protein concentration can be estimated by measuring the absorbance at 280 nm (Anthis & Clore, 2013).

Materials:

- µCuvette G1.0
- dH_2O
- Lens cleaning tissue
- Protein sample
- Protein buffer - 50mM Tris-HCl buffer
- D30 BioPhotometer(R)

Method:

To measure the protein concentration a µCuvette and a D30 BioPhotometer were used. First, the µCuvette was cleaned with dH2O and lens tissue paper, this was an essential step between every measuring. Then, to get a correct measurement absorbance at 280 nm, the BioPhotometer was blanked with 2 µL 50 mM Tris-HCl buffer (**3.10**). Afterward, 2 µL of protein sample was measured at 280 nm. The absorbance value was used with the formula below to find the protein

concentration. Both the extinction coefficient and the molecular weight were found by ExPASy´s ProtParam Tool.

$$Consentration\ mg/ml = \frac{A_{280}}{extinction\ coefficient \times path\ length} \times molecular\ weight$$

3.12 Protein melting point analysis

Thermal shift analysis was used to find the protein melting temperature (T_m). Both samples eluted from SEC purification were analyzed (**3.9.2**). The samples were slowly heated up from 25 ºC to 100 ºC. Subsequently, graphs were made based on the differentiated fluorescence data. The protein melting point (T_m) is defined as the temperature where the protein denatures. On the graph representing the derivative data, the T_m is the temperature of the lowest value belove zero (Huynh & Partch, 2015).

Materials:

- Eppendorf tube 1.5 mL
- *Pa*Aly7a-P1 and *Pa*Aly7a-P2
- SYPRO Orange dye (8x)
- Tris-HCl pH 7.5 50 mM
- qPCR StepOnePlus™
- Plate centrifuge, Plate spin II
- MicroAmp™ optical 96-well reaction plate
- MicroAmp™ Adhesive optical covers
- dH_2O

Methods:

Step One Plus system with optical 96-well plates was used for protein melting point analysis. From the manufacturer, a recommendation of four parallels per sample was given. This was for both the protein samples and the control sample. The dye used in this analysis was sensitive to light. Therefore, the plate and the SYPRO Orange dye were always covered with aluminum foil. In addition, the 96-well plate was always placed on ice during preparation to avoid protein degradation and contamination.

***Table 21. Protein thermal shift reaction**. An overview of the components and the volume needed for Protein thermal shift reaction.*

Reagent	**Protein sample**	**Negative control**
SYPRO orange dye	12.5 µL	12.5 µL
*Pa*Aly7a -P1/P2	10 µL	-
Tris-HCl pH 7.5 50 mM	25 µL	25 µL
dH_2O	Up till 100 µL	Up till 100 µL

The samples were mixed in separate 1.5 mL Eppendorf tubes as described in **Table 21**, and from the Eppendorf tubes, four parallels were added to the 96-well plate with 20 µL in each well. After adding all the samples to the 96-well plate, the plate was sealed with optical adhesive film. Then the plate was centrifuged using a plate centrifuge at 1000 g for 1 minute. The plate was then directly moved to the qPCR instrument. As recommended, the plate was first incubated for 2 minutes at 25 °C. Then the temperature was elevated from 25 °C to 97 °C over 50 minutes before being kept at 97 °C for 2 minutes. During the ramping period, measurements were conducted continuously.

3.13 Production of alginate from mucoid *P. aeruginosa*

The mucoid *P. aeruginosa* strain PAU2 overproduces alginate. Therefore, by isolating alginate produced by this strain, O acetylated alginate could be used as a substrate. This method is based on the protocol "Isolation and Assay of *Pseudomonas aeruginosa* Alginate" by May and Chakrabarty (1994). Per Kristian Edvardsen kindly provided *Pseudomonas* isolation agar (PIA) plates containing 1 % glycerol with PAU2 spread.

Materials:

- *P. aeruginosa* PAU2 (**Table 7**)
- Saline (0.9 % NaCl)
- Falcon tube with a grey lid
- Heraeus Multifuge X1R centrifuge
- QBD2 hot block
- 95 % ethanol
- Stirring plate
- Stirring magnets

- Eppendorf tube
- Absolut ethanol
- Vacufuge plus
- dH_2O

Method:

Cell material was washed from PIA with 10 mL saline. The solution was then collected and added to a Falcon tube with a grey lid. The sample was centrifuged for 30 minutes at 13700 g, and then the supernatant was transferred to a new tube stored on ice. Next, the pellet was washed with 1 mL saline and centrifuged again. By doing so, as much alginate as possible was collected from the cell material. From the second centrifugation, the supernatant was added to the first collected supernatant on ice. This sample was then centrifuged again, as described earlier. After centrifugation, the supernatant was transferred to a new tube and heated at 100 °C for 5 minutes. The pellet was stored on ice to find the dry weight. Three times the sample volume of the boiled supernatant was added as ice-cold 95 % ethanol. The ethanol was added while the boiled supernatant was stirred to precipitate the alginate. Alternatively, sodium acetate (1 % final concentration) could be added to the boiled supernatant to aid ethanol precipitation. The sample was then centrifuged at 13700 g for 15 minutes at 4 °C, and the supernatant precipitated. Next, the pellet was washed with 3 mL of ice-cold 95 % ethanol and then centrifuged at 13700 g for 15 minutes at 4 °C. This step was repeated once, and then the pellet was washed with 1 mL ice-cold absolute ethanol. After centrifugation, the supernatant was removed as described earlier. In the last step, the pellet was dried under vacuum for approximately 1.5 hours at 30 °C before being resuspended in dH_2O (or in an appropriate buffer). The amount is based on the dry weight, 1 mg dry weight per mL dH_2O.

3.14 Activity assay

Activity assays were proceeded to analyze the activity of *Pa*Aly7a and AMOR_PL7A with different substrates, conditions, and reactions. AMOR_PL7A has been included as a positive control in every activity assay since this enzyme can degrade sodium alginate (Vuoristo et al., 2019).

Materials:

- AMOR_PL7A

- 100 mM NaAc + 500 mM NaCl pH 6.6, and pH 7.5
- UltraPure™ DNase/RNase-Free Distilled Water
- 100 mM Tris-HCl buffer pH 7.5
- *Pa*Aly7a-P1/P2 (**3.9.2**), or *Pa*Aly7a from IMAC purification (**3.9.1**)
- ThermoMixer® C
- 1.5 mL Eppendorf tubes
- Substrates
    - Sodium alginate (**2.5.1**)
    - Alginate from *P. aeruginosa* (**3.13**)
    - DNA (**3.14.6** and **3.6.3**)

Methods:

Sodium alginate (**2.8.1**) and alginate isolated from *P. aeruginosa* (**3.15**) were tested as substrates with *Pa*Aly7a and AMOR_PL7A. The procedure for AMOR_PL7A is described in **Table 23**, and the procedure for *Pa*Aly7a is shown in **Table 22**. The negative control contained the same reagents as listed in **Table 22** with dH_2O instead of enzyme. DNA was also tested as a substrate, but only on *Pa*Aly7a (**3.16.5**).

Table 22. **Pa*Aly7a activity assay experiment conditions.*** *The table contains the reagents, the end concentration, and the volume for activity assays with* Pa*Aly7a. The volume of* Pa*Aly7a differs based on the different protein samples.*

Reagents	**End concentration**	**Volume**
*Pa*Aly7a (161.2 mM)	5 mM	15.2 µL
Sodium alginate (5 %)	0.5 %	49 µL
Tris-HCl pH 7.5 (100 mM)	50mM Tris-HCl pH 7.5	245 µL
dH_2O		Up to 490 µL

Table 23. ***AMOR_PL7A activity assay experiment conditions.*** *The table contains the reagents, the end concentration, and the volume for activity assays with AMOR_PL7A.*

Reagents	**End concentration**	**Volume**
AMOR_PL7A (5.227 µM)	80 nM	7.5 µL
Sodium alginate (5 %)	0.5 %	49 µL
100 mM NaAc + 500 mM NaCl pH 6.6	50 mM NaAc + 250 mM NaCl pH 6.6	245 µL

dH_2O		Up to 490 µL

A master mix with all the reagents without the enzymes was made (**Table 22** and **Table 23**) based on the number of samples tested. The master mix was distributed to Eppendorf tubes, and the reaction started when the enzyme was added. In addition to the negative control without enzyme, a second negative control with unfunctional protein was made. This control contained the same as the sample, but before incubation, the second negative control was boiled for 15 minutes at 100 °C.

Samples containing AMOR_PL7 or NaAc were incubated at 65 °C with shaking (700 rpm), and samples containing *Pa*Aly7a were incubated at 23 °C with 700 rpm agitation, both on ThermoMixer® C. When the Varioskan LUX was used, all the samples were incubated on a Micro test plate, 96-well, at 37 °C. Before each reading, the plate was agitated (420 rpm) for 10 seconds. These samples were later analyzed with DNS (**3.14.1)**, the absorbance of double bonds at 235 nm (**3.14.2)**, agarose assay (**3.14.6**), and MALDI-TOF MS (**3.14.3**).

3.14.1 DNS reducing end assay

The DNS method is used to quantify sugars with reducing ends. DNS will be reduced to 3-amino-5-nitrosalicylic acid (ANS) in a redox-reaction with aldehyde groups on the sugar, and the aldehyde group will simultaneously be oxidized to a carboxyl group. ANS has an orange color that can be measured by absorbance at 540 nm (Saqib & Whitney, 2011). When alginate is cleaved by ß-elimination, a reducing end is created. This method was therefore used to analyze if *Pa*Aly7a could degrade alginate by ß-elimination. Since glucose has many reducing ends, this sugar was used as a positive control in this experiment.

Materials:

- *Pa*Aly7a samples and positive and negative controls (**3.14**)
- 1 % sodium alginate, 0.5 % sodium alginate
- 50 mM Tris-HCl
- 1 mM glucose
- Water bath 100 °C
- Microcentrifuge 5418 R
- DNS solution (**2.8.12**)

- Varioskan LUX
- Micro test plate, 96-well,

Method:

As described in **3.14**, a setup was made with three parallels of the positive control (**Table 23**) and three parallels of *Pa*Aly7a (**Table 22**). In addition, a 0.3 mM glucose sample was made, and negative controls with sodium alginate and Tris-HCl or NaAc. In this experiment, each sample was made with a total volume of 490 µL and then incubated. Samples containing AMOR_PL7 or NaAc were incubated at 65 °C with 700 rpm agitation, while samples containing *Pa*Aly7a were incubated at 23 °C with 700 rpm agitation, both on ThermoMixer® C. Seventy µL sample was collected from this tube after 0-, 30-, 60-, 120-, 180-, and 240-minutes and added to a new 1.5 µL Eppendorf tube. After collecting the samples, the tubes were subsequently boiled for 5 minutes to stop the reaction and then centrifuged for 10 minutes at 16900 g at 4 °C. After every sample was collected, boiled, and centrifuged, 60 µL of the sample was added to a 96-well plate. Then 120 µL of DNS was mixed in, and the plate was heated on a ThermoMixer® C at 100 °C for 15 minutes with a lid. The plate was then transferred to a Varioskan LUX, and absorbance at 540 nm was measured.

A standard curve for glucose was also made to quantify the measurements. The standard curve consisted of the glucose concentrations: 7.81 µM, 15.63 µM, 31.25 µM, 62.50 µM, 125 µM, 250 µM, 500 µM, and 1000 µM mixed with 50 mM Tris-HCl pH 7.5. 70 µL of each sample were mixed with 140 µL DNS in a 96-wells plate. Then the plate was heated for 15 minutes at 100 °C with a lid on a ThermoMixer® C. The absorbance was measured using a Varioskan LUX at A_{540}.

This experiment was also tested with 1 % alginate, a 1:1 ratio of sample and DNS, incubation at 23 °C, 37 °C, and 60 °C. In addition, the lysate of *Pa*Aly7a was incubated with sodium alginate to test the isolation process. In this experiment, 19.5 µL of the lysate (pNIC-CH+*PA14_41500* incubated overnight in TB medium at 20 °C) was incubated with Tris-HCl and sodium alginate until a final concentration of respectively 50 mM and 1 %. The lysate sample was incubated at 23 °C for 24 hours before reading.

3.14.2 Monitoring A_{235} for generation of double bonds

When alginate is cleaved by ß-elimination reaction, the 1,4-glycosidic bond is broken, and a double bond between carbon 4 and 5 on DEH, and a reducing end is created. This double bond can be measured by analyzing the absorption at 235 nm. As a positive control for the double bond, imidazole was used.

Materials:

- 2 M Imidazole
- $CaCl_2$
- *Pa*Aly7a samples and positive and negative controls (**3.14**)
- 1 % sodium alginate, 0.5 % sodium alginate
- Alginate purified from *P. aeruginosa* (**3.13**)
- 96-wells plate
- Varioskan LUX

Method:

The setup for this activity assay is described in section **3.14**, with three parallels of the protein samples and one of each negative control. These samples were measured using a time loop over 5 hours, with readings every half hour. The plate was incubated at 37 °C with shaking (420 rpm) for 10 seconds before each reading. Both sodium alginate and alginate purified from *P. aeruginosa* were used, and concentrated protein from IMAC (**3.9.1**) and both fractions from the SEC isolation (**3.9.2**). In addition to Bis-tris HCl pH 6.5 and Tris-HCl pH 8.5. These buffers were kindly provided by Hibaq Ahmed Farah.

$CaCl_2$ was added to *Pa*Aly7a-P1 and -P2 to test if the enzyme required Ca^{2+} ions for optimal activity (**3.9.2)**. The samples had a final concentration of 50 mM $CaCl_2$ and 5 mM *Pa*Aly7a. *Pa*Aly7a-P1 and -P2 with $CaCl_2$ were then incubated with sodium alginate and alginate from *P. aeruginosa* as described in section **3.15.** These samples were tested using a time loop over 5 hours, measuring every half hour on Varioskan LUX. The plate was incubated at 37 °C with shaking (420 rpm) for 10 seconds before each measurement. In addition, AMOR_PL7A and negative controls were used. Neither of these was added $CaCl_2$.

3.14.3 Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS)

MALDI-TOF MS shoots a laser beam at macromolecules embedded in a matrix, creating ions detectable by the instrument (Karas & Hillenkamp, 1988). The matrix is mixed with the sample before it is added to a metal plate. When added, the matrix will evaporate and crystallize, and since the sample is mixed with the matrix, the sample will co-crystallize (Singhal et al., 2015). The plate is then placed in the MALDI-TOF MS machine, and the machine will then create a vacuum and an electric-field-free flight tube (Steen & Mann, 2004). After this step, a laser will shoot at the sample, often with a UV laser, and the matrix will absorb this energy and desorb and create singly protonated ions in positive mode. It is possible to choose a negative mode on MALDI-TOF MS as well. During negative mode, the ions will have a negative charge after ionization. These ions will then go from the sample to a detector through an electric field generator. This electric field generator will cause a union kinetic energy for the ions passing. Since the tube is under vacuum, the time the ions use to hit the generator is proportional to the m/z ratio (Steen & Mann, 2004). Based on this m/z ratio, TOF is set, and this information is used to create a mass list for identification or comparison (Singhal et al., 2015).

Materials:

- UltraFlextreme MALDI-TOF-MS
- MTP 384 ground steel BC MALDI-TOF target plate
- MTP Target Fame III
- 2,5 Dihydroxybenzoic acid (DHB)
- PCR tube
- dH_2O
- Hairdryer
- Standard: 1-6 Chitooligosaccharides kindly provided by Ole Golten
- *Pa*Aly7a samples and positive and negative controls (**3.14**)

Method:

Preparations of samples were done as described in **3.14**. However, MALDI-TOF MS is more effective with low concentrations of the sample. Therefore, the samples from incubation were first frozen for 15 minutes to stop the reaction and then diluted 100 times. Then 1 μL of the sample was mixed with 2 μL DHB in the cap of a PCR tube, and from this cap, 1 μL was added

to the MTP 384 ground steel BC MALDI-TOF target plate. The samples were carefully dried and properly crystallized by a hairdryer. When the samples were dried, the plate was placed on the MTP Target Fame III and further placed in the UltraFlextreme MALDI-TOF-MS.

Two different methods were used, positive mode and negative mode. For each method, calibration with a known standard was recommended. No calibration was done in negative mode. For positive mode, 1-6 chitooligosaccharides were used as a standard. The standard was analyzed with 1000 shots fired at an intensity of 60 %. While measuring the samples, 1000 – 2000 shots were fired with 60-85 % intensity. One spectrum was created by 1000 – 2000 shots, and each shot with low background noise was saved. Together approximately 6-12 spectrums made up the resulting spectra for that sample. This spectrum was then saved and opened in the analysis software, and the peak masses were automatically found.

Different approaches were also tested; the samples were filtered before being mixed with the matrix, 0.3 M acetic acid was added until the sample had a pH less than 3, and when mixing the sample with the matrix, a 1:1 dilution was tested. In addition, a different concentration of sodium alginate (1%) and alginate purified from *P. aeruginosa* were also attempted.

3.14.4 Formation of halos on biofilm

PAO1, PAO1 ΔWspf, PAO1 ΔWspf ΔPsl, PAO1 ΔWspf ΔPel, and mucoid strain PAU2 can produce biofilm. PAO1 ΔWspf can make a biofilm with both the exopolysaccharides Psl and Pel. PAO1 ΔWspf ΔPsl overproduces the extracellular polysaccharide Pel due to a mutation in *Psl.* For PAO1 ΔWspf ΔPel, the bacteria overproduced a biofilm with Psl. Neither of these mutants had an overproduction of alginate. Therefore, a fifth bacteria strain was included, PAU2. By using all these different strains, multiple exopolysaccharides were tested with *Pa*Aly7a. If the enzyme degraded or prevented biofilm formation, a halo was visible in the area where the enzyme was applied. Suppliers of the bacterial strains are listed in **Table 7**.

Materials:

- Overnight culture of PAO1, PAO1 ΔWspf, PAO1 ΔWspf ΔPsl, PAO1 ΔWspf ΔPel, mucoid strain PAU2 (**3.3.1)**
- Ultrospec 10
- Cuvette plastic

- LB liquid medium
- L-shaped loop
- *Pa*Aly7a from IMAC (**3.9.1**) and SEC (**3.9.2**)
- AMOR_PL7A
- Tris-HCl buffer 50 mM and 100 mM pH 7.5
- 100 mM NaAc + 500 mM NaCl pH 6.6
- Sterile filter pads

Method:

Overnight cultures with PAO1, PAO1 ΔWspf, PAO1 ΔWspf ΔPsl, PAO1 ΔWspf ΔPel, and mucoid strain PAU2 were first made. The protocol is described in section **3.3.1.** The overnight cultures were diluted to an OD_{600} of 0.01 in LB medium. Before the overnight culture was added to the plates, the plate was marked and separated into four similar parts on the back with a marker. Then, 1 mL of each diluted overnight culture was spread using an L-shaped loop on separate LB agar plates. The excess culture was removed, and before the bacterial solution was solidified, 20 µL of protein samples were added. To one plate, three different bacterial solutions were added; 20 µM, 10 µM, and 5 µM *Pa*Aly7a (**3.9.1**). In addition, a negative control consisting of 50 mM Tris-HCl buffer was also added to the plate. Each of the samples was added in the middle of their part. To LB agar plate with PAU2, 20 µL of *Pa*Aly7a-P2 and AMOR_PL7A were added to sterile filter pads. *Pa*Aly7a-P2 (**3.9.2**) was mixed with 100 mM Tris-HCl to a final concentration of 35.9 µM protein and 50 mM buffer. AMOR_PL7A was mixed with 100 mM NaAc + 500 mM NaCl to a final concentration of 144.4 µM protein and 50 mM NaAc + 250 mM NaCl. The negative controls consisted of only buffers. Before the plates were incubated overnight at 37 °C, each plate was dried on the LAF bench. After incubation, the haloes were measured.

3.14.5 Biofilm degradation assays

The same strains as in **3.14.4** were used and each strain was cultivated in a Nunc® MicroWell™ 96-well Polystyrene Plate for biofilm formation. After the formation, the enzyme was added to the biofilm to analyze the activity. An enzyme capable of degrading the biofilm would loosen the biofilm. Therefore, during the washing step, the biofilm would be removed. The degradation was detected by coloring the biofilm and measuring the absorbance at 595 nm. The intensity of the absorbance gives an indication of the degradation.

Materials:

- Overnight culture of PAO1, PAO1 ΔWspf, PAO1 ΔWspf ΔPsl, PAO1 ΔWspf ΔPel, mucoid strain PAU2 (**3.3.1)**
- Varioskan LUX
- Nunc® MicroWell™ 96-well Polystyrene Plate
- LB medium
- bleach
- container
- saline
- *Pa*Aly7a-P2 (**3.9.2**)
- AMOR_PL7A
- 100 mM NaAc + 500 mM NaCl pH 6.6
- 100 mM Tris-HCl buffer pH 7.5
- 0.1 % (wt/vol) Crystal Violet (CV) solution kindly provided by Per Kristian Edvardsen
- orbital shaker
- 70 % ethanol

Method:

Overnight cultures of PAO1, PAO1 ΔWspF, PAO1 ΔWspF ΔPsl, PAO1 ΔWspF ΔPel, and PAU2 were made without antibiotics (**3.3.1**). The overnight cultures were diluted to an OD_{600} of 0.1 in LB medium. To a 96-Well Polystyrene Microwell Plate, 100 µL of each culture was added to 5 wells x 2. In 5 wells x 2, only LB medium was added as a negative control. The plate was incubated with a lid under static conditions for 16–20 h at 37 °C. After incubation, the OD_{600} was measured. This measurement was a checkpoint to see if there had been bacterial growth on the plate. The medium was then discarded into a container with bleach, and the wells were rinsed three times with 150 µL of saline. Again, the wells were emptied, and the plate dried upside-down on dry paper towels for 5 minutes. The OD_{600} was measured before AMOR_PL7 mixed with 100 mM NaAc + 500 mM NaCl pH 6.6 to a final concentration of 80 nM or *Pa*Aly7a-P2 (**3.9.2**) with 100 mM Tris-HCl buffer pH 7.5 to a final concentration of 5 µM, was added to half of the wells (5 wells x1). The plate was incubated for 4 hours at 37°C. After 4 hours, the plate was again rinsed with saline and dried as described above. When dried, 120 µL of 0.1 % (wt/vol) Crystal Violet solution was added to each of the wells, and the plate was placed on an orbital shaker rotating at low speed for 30 minutes at RT, allowing the biofilm

to be stained. The Crystal Violet solution was then discarded, and the biofilm was rinsed three times with 150 µL of saline before the plate was dried. To de-stain the Crystal Violet, 120 µL of 70 % ethanol was added to the wells before the plate was incubated at RT for 30 minutes with shaking. From the wells, the ethanol-solubilized Crystal Violet was transferred to a fresh 96-Well Polystyrene Microwell Plate, and the absorbance was measured at A_{595}.

3.14.6 DNA-degradation assay

Based on the STRING analysis (**3.1.6**), an assay with DNA as substrate was tested with *Pa*Aly7a. Both DNA extracted from *P. aeruginosa* PAO1 and plasmid DNA from pBAD amplification (**3.6.3**) were used. The kit supplier is listed in **Table 5**.

DNA isolation

Materials:

- Overnight culture of PAO1
- Kit Genomic DNA from Microorganisms
- FAST PREP
- UltraPure™ DNase/RNase-Free Distilled Water
- Microcentrifuge, 5418 R
- NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer

Method:

An overnight culture of PAO1 was made without antibiotics as described in **3.3.1**. From this overnight culture, 4 mL was separated into four different tubes. By using the kit "Genomic DNA from Microorganisms" and the protocol for "gram-positive and gram-negative bacteria", DNA from PAO1 was isolated. In the agitate step, FAST PREP was used. The sample was run at 6.5 m/s for 30 seconds on the FAST PREP three times, and between each interval, the samples were placed on ice for 5 minutes. After agitation, the samples were centrifuged for 30 seconds at 11 000 g on the Microcentrifuge, 5418 R. The protocol was followed until elution, where the Elution Buffer BE was replaced with UltraPure™ DNase/RNase-Free Distilled Water. After the DNA was isolated, the sample was measured on NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer.

*Pa*Aly7a degrading DNA

Materials:

- Isolated DNA from PAO1
- Restriction enzymes: EcoRI, XhoI, AflII
- Restriction enzymes buffer: 10X NEBuffer r3.1, 10X NEBuffer 2.1
- pBAD+*PA14_41500* 394.1 ng/µL (**3.6.3**)
- UltraPure™ DNase/RNase-Free Distilled Water
- Eppendorf tubes
- ThermoMixer® C
- *Pa*Aly7a-P1 and -P2 and AMOR_PL7A samples as described in **3.14**
- DNA agarose gel (**3.2.1**)

Method:

The following samples were prepared with the DNA isolated from PAO1: restriction enzymes, AMOR_PL7A, *Pa*Aly7a-P1, *Pa*Aly7a-P2, negative control with Tris-HCl buffer, and negative control with NaAc buffer. Each sample contained 1 050 ng DNA (30 µL). The sample with restriction enzymes contained 2 µL EcoRI, 2 µL XhoI, 10 µL 10X NEBuffer r3.1, and 56 µL UltraPure™ DNase/RNase-Free Distilled Water. The remaining samples were prepared as described in **3.14**, with DNA isolated from *P. aeruginosa* PAO1 instead of alginate. All the samples were incubated for 20 hours at 37 °C with shaking (500 rpm) and then analyzed on a 1 % agarose gel.

With pBAD+*PA14_41500* 394.1 ng/µL three samples were made, one with *Pa*Aly7a-P2, one negative control, and one positive control with restriction enzymes. The samples were prepared as described in **3.14** with 2.6 ng/µL plasmid instead of alginate and 35.9 µM *Pa*Aly7a in the protein samples. In the positive control, 2 µL AflII was mixed with 2 µL EcoRI, 10 µL 10 X NEBuffer 2.1, dH_2O up to 100 µL, and 11.8 ng/µL plasmid. The sample with *Pa*Aly7a was made with a final volume of 450 µL and placed on incubation at 37 °C with shaking (500 rpm). Samples of 50 µL were collected from this tube after 5 minutes, 60 minutes, 3 hours, and 21 hours incubation. For each time point, two samples were collected. One was boiled at 100 °C for 5 minutes and then placed in the freezer, while the other was directly placed in the freezer. The positive and negative control were incubated for 21 hours, and two samples from each

control were collected as previously described. Each of these samples was analyzed on a 1 % agarose gel with a 1 kb ladder.

3.15 Proteomics

3.15.1 Reduction/denaturation and electrophoresis of the protein sample

After purification on IMAC (**3.9.1**), the elution sample showed multiple bands on the SDS-PAGE gel. Two strong bands were visible, one at 25 kDa and one at 70 kDa. Both were analyzed by proteomics to verify that *Pa*Aly7a was at approximately 25 kDa.

Materials:

- Protein sample
- SDS-PAGE (**3.2.2**)
- Glows
- Microwave safe plastic container
- Coomassie staining
- LDS sample buffer (4x)
- dH_2O

Method:

The protein sample was analyzed with SDS-PAGE gel as described in section **3.2.2.** However, in this step, 25 µL of the protein sample was mixed with 8.33 µL of LDS sample buffer (4X) and heated on a ThermoMixer® C at 70 °C for 5 minutes. When the gel was finished, it was rinsed in dH_2O and placed in a clean plastic container filled with Coomassie staining. Glows were used when the gel was touched.

Coomassie staining and de-staining

Materials:

- Microwave
- De-staining solution kindly provided by Ole Golten
- Rotating table
- Benchtop UV Transilluminator
- Scalpel
- Eppendorf Protein LoBind tubes

Method:

The container with the gel and Coomassie staining was transferred to a microwave and ran at full power for 30 seconds without the solution boiling. After 30 seconds, the container was shaken carefully and then heated again for 30 seconds. The staining solution was removed, the de-staining solution was added, and the same heating procedure was repeated. After heating the gel for 2 x 30 seconds, the box was placed on a rotating table at 120 for 10 minutes at RT. Like the staining solution, the de-staining solution was poured out, and a new de-staining solution was added. Again, the container was heated 2 x 30 seconds before it was rotated on a rotating table. The gel was then placed on a clean Benchtop UV Transilluminator, and the bands of interest were cut out in 1x1 mm cubes with a scalpel. Each band was placed in an Eppendorf Protein LoBind tube.

In-gel reduction, alkylation, and digestion

Materials:

- dH_2O
- Microcentrifuge, Heraeus™ Pico™ 21
- ThermoMixer® C
- 100% ACN
- 1M Ambic
- 1M DTT
- IAA
- 10 % TFA
- Bransonic® Ultrasonic Cleaner, Branson 3510
- 500 ng/µL Trypsin
- pH paper
- STAGE tips
- Methanol
- Vacufuge plus
- NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer
- AS vials

Method:

The samples were centrifuged in Microcentrifuge, Heraeus™ Pico™ 21.

The tubes with a gel band from SDS-PAGE were added 200 µL of dH_2O and placed in a ThermoMixer® C at 20 °C for 15 minutes. After the incubation, the liquid was removed, and 200 µL of 50 % ACN and 25 mM AmBic were added. The incubation was repeated, and again the liquid was removed. For a second time, 200 µL of 50 % ACN and 25 mM AmBic was added, incubated, and removed. Then 100 µL of 100 % ACN was added, and the sample was incubated at 20 °C for 5 minutes. The liquid was removed, and the sample was air-dried for 1-2 minutes. Afterward, the gel pieces were reduced by adding 50 µL of DTT solution, consisting of 10 µL 1M DTT (frozen), 100 µL 1M AmBic (frozen), and 900 µL dH_2O, before incubating at 56 °C for 30 minutes. The samples were cooled down at RT, and the liquid was removed. 50 µL of IAA solution, consisting of 10 mg IAA, 100 µL 1M AmBic (frozen), and 900 µL dH_2O, was added to the sample tube. The samples were placed in the dark at RT for incubation for 30 minutes. Then, the IAA solution was removed, 100 µL of 100% ACN was added, and the sample was incubated at 20 °C for 5 minutes. The liquid was removed, and the sample was air-dried for 1-2 minutes. When air-dried, 30 µL of 10 ng/µL Trypsin solution was added. The Trypsin solution consisted of 5 µL 500 ng/µL Trypsin (-80 °C) and 245 µL Trypsin buffer (25 µL 1M AmBic (frozen), 875 µL dH_2O, and 100 µL 100% ACN). The sample was incubated on ice for 30 minutes. After incubation, Trypsin solution was added to cover the gel pieces, if necessary. The samples were then incubated at 37 °C overnight. The next day the samples were cooled down, and 40 µL 1% TFA was added before the samples were sonicated in a Bransonic® Ultrasonic Cleaner, Branson 3510, for 15 minutes.

While the samples were sonicated, the STAGE tips were prepared. By using an adaptor, the tip was first mounted in a retainer tube, an Eppendorf tube with the lid cut off. Then 50 µL of methanol was added above the column to activate it. Afterward, the Eppendorf tube with the tip was centrifuged for 5 minutes at 2500 g, and the flow-through was discarded. If all the liquid had not passed through the column, the tip was centrifuged again. The next step was to equilibrate the column by adding 100 µL of 0.1% TFA, centrifuge the tip for 5 minutes at 2500 g, and discard the flow-through. After this step, the tips were prepared for the samples.

After sonication, the samples were centrifuged briefly, and 70 µL of peptide extraction solution, consisting of 100 µL 10% TFA and 1.9 mL dH_2O, was added. The solutions were mixed well, and 0.5 µL of the sample was added to a pH paper to ensure that the sample was acidic. If the samples were not acidic, 0.5 µL of 10% TFA would have been added. In the Bransonic®

Ultrasonic Cleaner, Branson 3510, the samples were sonicated for 10 minutes before centrifuged for 5 minutes at 16000 g at RT. The supernatants were then transferred to the prepared STAGE tips, and the tips were centrifuged for 5 minutes at 2500 g. After centrifugation, the flow-throughs were discarded, 100 µL of 0.1% TFA was added to the column, and the tips were again centrifuged as earlier. Afterward, the tips were transferred to new clean 1.5 mL Eppendorf tubes, and 50 µL of ACN/TFA elution solution, consisting of 800 µL 100% ACN and 200 µL 0.5% TFA, was added. The tips were centrifuged again, removed from the Eppendorf tube and stored in an empty pipette box, while the tubes were capped. Next, the samples were evaporated to dryness in a Vacufuge plus at 30 °C for approximately 1.5 hours. After this, the samples were sonicated for 10 minutes after being redissolved in 10 µL LC-MS loading solution, consisting of 200 µL 100% ACN, 50 µL 10% TFA, and 9.75 mL dH_2O. The peptide concentrations were then measured using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer at A_{205}, method 31, and from each sample, 6 µL were transferred to AS vials. No bubbles could be at the bottom of the AS vial.

The peptide analysis was performed by Morten Skaugen using timsTOF Pro, and Per Kristian Edvardsen examined the results.

3.15.2 STrap protocol

After the agarose assay (**3.8.1**), *Pa*Aly7a-P2 was analyzed by proteomics to see if a DNase was present.

Materials:

- dH_2O
- ThermoMixer® C
- SDS
- 1M Tris-HCl pH 8 and pH 7
- 100% ACN
- 1M DTT
- IAA
- 85% phosphoric acid (PA)
- 1 M ABC
- 10 % TFA

- Bransonic® Ultrasonic Cleaner, Branson 3510
- 500 ng/µL Trypsin
- STrap tip
- Parafilm
- Methanol
- Vacufuge plus
- NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer
- AS vials
- *Pa*Aly7a-P2 from SEC (**3.9.2**)
- Microcentrifuge, Heraeus™ Pico™ 21

Method:

The sample was centrifuged in Microcentrifuge, Heraeus™ Pico™ 21. To the sample, SDS was added to a final concentration of 5 %, Tris-HCl (pH 8) to a final concentration of 50 mM, and DTT to a final concentration of 10 mM. Then the sample was heated at 70 °C for 10 minutes before it was cooled to RT. After the sample was cooled, 500 mM IAA solution, consisting of 9.25 mg IAA and 100 µL dH_2O, was added with 0.1 times the sample volume. The sample was mixed by pipetting up and down a few times and incubated for 20 minutes in the dark. After incubation, the PA solution was added with 0.1 times the sample volume and mixed by pipetting. The PA solution consisted of 14.1 µL 85 % PA and 86 µL dH_2O. Then the STrap tip was prepared by mounting the tip in a retainer tube, an Eppendorf tube with the lid cut off, using an adaptor. Before the sample was added, 170 µL[1] of Strapping solution, which consisted of 90 % methanol + 50 mM Tris-HCl pH 7, was added. Then carefully, the sample was added to the top third of the Strapping solution. The tip was centrifuged for 10 minutes at 2500 g[2] with the label of the tip facing outwards. For each centrifugation, the tube/the tip was rotated 180°, and the flow-through was discarded by no other notice. After centrifugation, 50 µL of Strapping solution was added, and the sample was centrifuged again. Then 70 µL of ABC solution was added, and the sample was centrifuged. ABC solution consisted of 200 µL 1 M ABC, 100 µL 100 % ACN, and 1.7 mL dH_2O. After this step, the tip was transferred to a clean, labeled 1.5

[1] *This volume assumed a total sample volume of 20-30 µL. If the sample volume was larger than this, the trapping had to be performed in two or more operations since the ratio between the sample and the Strapping solution should be between 1:6 and 1:8.*

[2] *If the solution did not go through after the first centrifugation, the speed could have been elevated to 4000 g after rotating the sample 180°. A maximum of 50-60 µg of protein could be added to prevent clogging of the column.*

mL Eppendorf tube, and 15 µL of Trypsin solution, consisting of 20 ng 500 ng/µL Trypsin (-80 °C) and 500 µL ABC solution, and 15 µL of ABC solution were added. The tip was centrifuged for 1 minute at 1000 g, leaving a few mm of liquid above the stacked filters. The top of the tip was covered with parafilm before the tip was incubated for 60 minutes at 47 °C in a ThermoMixer® C. After incubation, the tip was centrifuged for 2 minutes at 4000 g, and 50 µL of 0.5 % TFA was added to the flow-through. The flow-through and TFA mix was transferred back to the tip and centrifuged for 5 minutes at 2500 g. Then 100 µL of 0.1 % TFA was added to the tip and centrifuged for 5 minutes at 2500 g.

The tip was transferred to a new clean 1.5 mL Eppendorf tube, and 50 µL of ACN/TFA elution solution was added. ACN/TFA consisted of 800 µL 100 % ACN and 200 µL 0.5 % TFA. The tip was centrifuged for 10 seconds at 1000 g and then incubated for 10 minutes at RT without discarding the flow-through. After incubation, the tip was centrifuged again for 10 minutes at 1000 g. After the last centrifugation, the tip was removed from the Eppendorf tube and stored in an empty pipette box while the flow-through was kept. The sample was evaporated to dryness in a Vacufuge plus at 30 °C for approximately 1.5 hours. After this, the sample was sonicated for 10 minutes in Bransonic® Ultrasonic Cleaner, Branson 3510, after being redissolved in 10 µL LC-MS loading solution, consisting of 200 µL 100 % ACN, 50 µL 10 % TFA, and 9.75 mL dH_2O. The peptide concentration was measured using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer at A_{205}, method 31. Then, 6 µL of the peptide sample was transferred to AS vials. While adding the sample to AS vials, no bubbles could be at the bottom.

The peptide analysis was performed by Morten Skaugen using an Orbitrap LC-MS system, and Per Kristian Edvardsen examined the results.

4 Results

P. aeruginosa is known for being an opportunistic pathogen, and most of the strains can create an infection in humans. In the lungs of patients with CF, such an infection can develop into a chronic and deadly infection. These chronic infections often consist of mucoid strains of *P. aeruginosa*, which are strains that can overproduce the exopolysaccharide alginate. Mucoid strains are known for being pathogenic, and these strains are often more difficult to treat. It is therefore of fundamental and applied interest to understand the function of alginate lyases as they may reduce the alginate protecting the mucoid bacteria, e.g., by applying the enzyme to the bacterial biofilm or by interfering with alginate synthesis. Many of the alginate-related enzymes of *P. aeruginosa* have been thoroughly characterized, for instance, AlgL. AlgL has an important function in relation to the synthesis of alginate in *P. aeruginosa*. However, another putative *P. aeruginosa* alginate lyase, encoded by the *PA14_41500* gene in the PA14 strain genome, is hitherto slightly described in the literature. The goal of the present project is to determine the properties of the *PA14_41500* protein product and investigate its putative activity toward alginate.

4.1 Bioinformatic analysis of *PA14_41500*

The nucleotide sequences encoded to PA14_41500 from the *P. aeruginosa* strain PA14 and PA1784 from the *P. aeruginosa* strain PAO1 were obtained from www.pseudomonas.com and translated with the ExPASy Translate tool to the amino acid sequence (**3.1.1**). Each amino acid sequence was used in the subsequent analyses (**3.1**). Firstly, the sequence of protein *PA14_41500* was manually inspected for the conserved sequence motifs of family PL7 alginate lyases (**1.4.2**). Indeed, these sequence motifs were identified (**Figure 9**), supporting the hypothesis that the protein is an active alginate lyase belonging to the PL7 family. Some of the amino acids differed from the conserved motif, ML in the first sequence and Q in the last. Based on this finding, the *PA14_41500* protein was renamed *Pa*Aly7a. "*Pa"* is short for *P. aeruginosa*, "Aly" is short for alginate lyase, "7" is short for PL7, and "a" since it is the first protein the name *Pa*Aly7.

MIDLSTWNLTIPTEPTPQVITTQQLNNGYQSRYFQQGNDGGLVFWVPIDGSHTEDSTY
PRTELRETQADGSLDNWYYWQADNELRVTMSVEKVPSKNKVIIGQIHSKSPQTDDG
EPLVKLQYYYKPEEGLGRVEALVRNHPDDSYSRNVSILEQVRLGERFNYSLRVTSSG
ELAVRARSQDGEEDAYYQTLGSAWNDQLLYFKAGAYVNDNAGDSGEGSRVTIYHL
NTAHR

Figure 9. The amino acid sequence of* Pa*Aly7a from* P. aeruginosa *PA14. *The three conserved sequence motifs characteristic of family PL7 alginate lyases are highlighted in purple.*

Since alginate lyases are responsible for the depolymerization of extracellular alginate secreted out of the bacterial cell, one may assume that the protein has a signal peptide. The sequence was analyzed by SignalP-5.0 for the identification of either a TAT signal peptide or a Lipoprotein signal peptide (**3.1.3**). No signal peptide was detected by this analysis (**Figure 10**), indicating an intracellular location of the protein or secretion by a secretion mechanism not identified by SignalP-5.0.

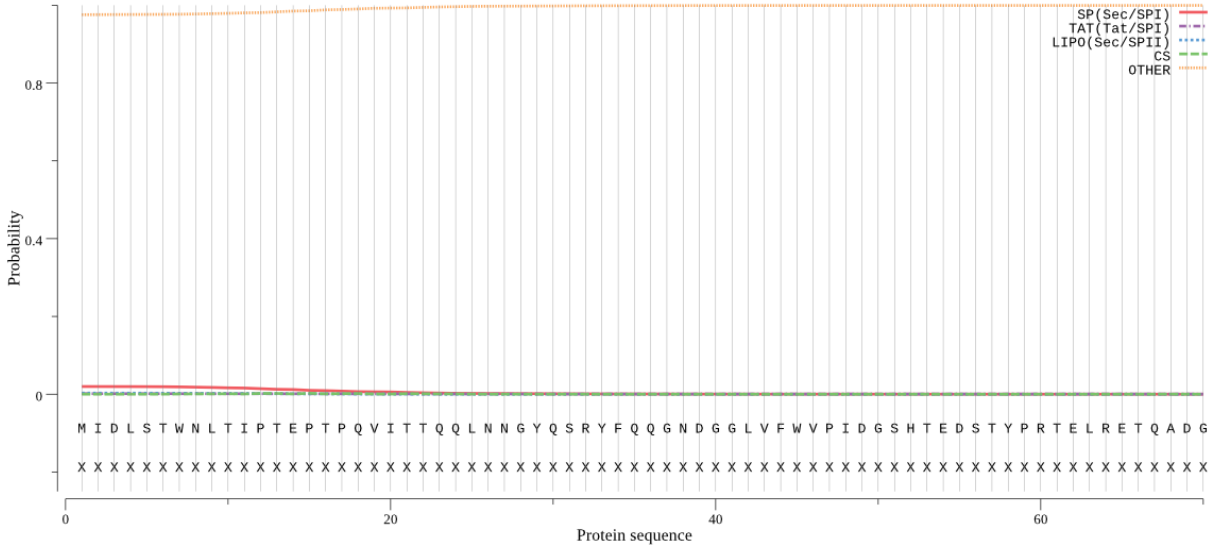

Figure 10. Signal peptide prediction by SignalP-5.0*. This graph shows the predicted signal peptide and the placement on PaAly7a. SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) identified signal peptides secreted based on the amino acid sequence. These signal peptides can be either transported by the SEC translocon or the Tat translocon (Sec/Tat) and cleaved by signal peptidase I (SPI) or by signal peptidase II (SPII). The figure includes the protein sequence marked with OTHER as a yellow line. SP (Sec/SPI) is marked with a red line, and the Cleaving Site (CS) is marked with a green line.*

To identify similarities and conserved sequence motifs in other enzymes in the PL7 family, a figure with MSA is shown (**Figure 11**). The MSA shows that the conserved sequence motifs R*ELR*ML, VIIQ(I/V)H, and YFKAG*Y*Q are present in most of the proteins from PL7 (**Figure 11**) (Wong et al., 2000). The conserved regions are marked in the figure with a red box. The *P. aeruginosa* alginate lyase marked P. aeruginosa_PAO1 (PDB:1VAV) in **Figure 11** is the sequence of PA1167.

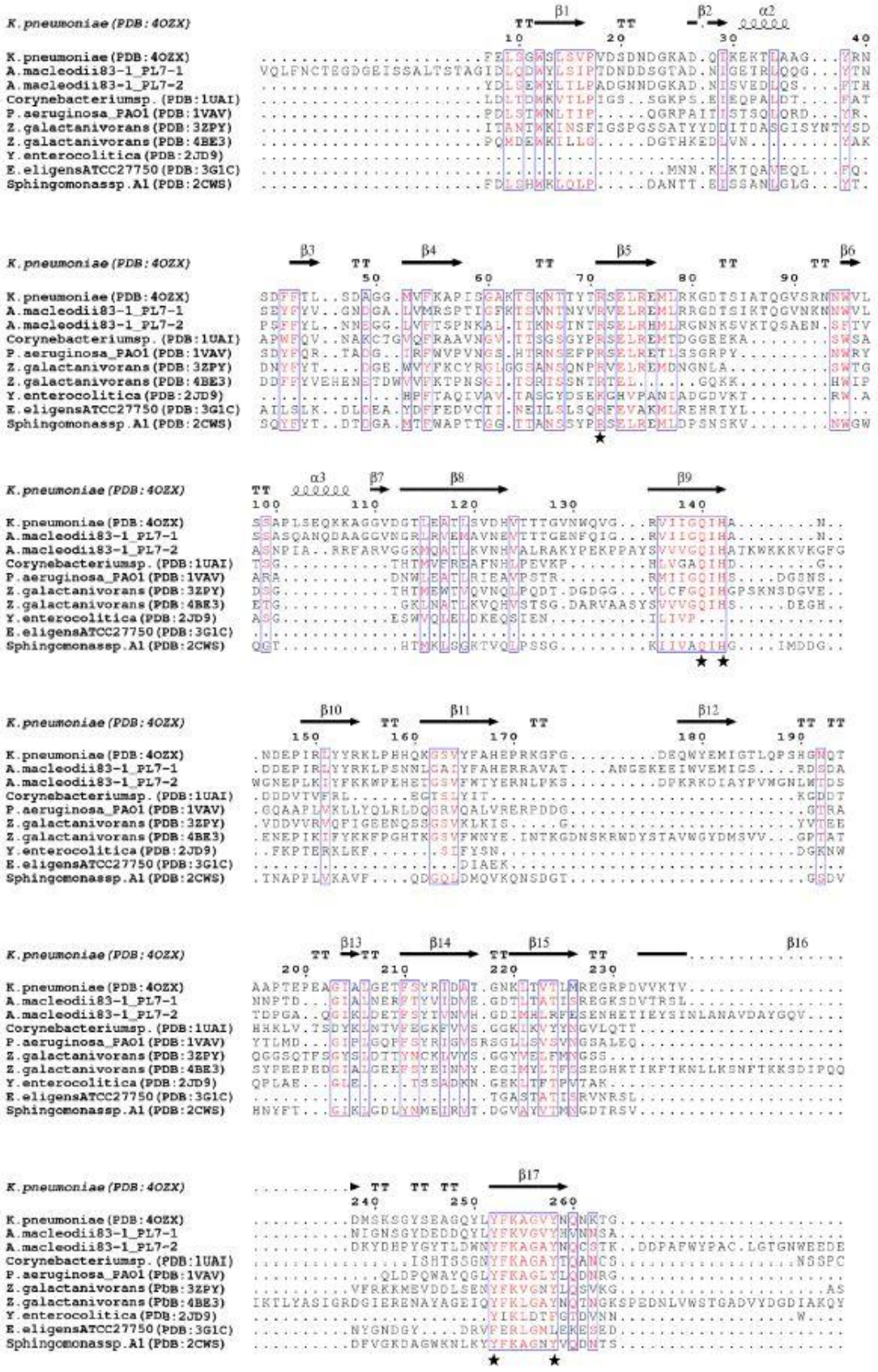

Figure 11. MSA of alginate lyases in PL7*. The figure shows an MSA of ten different alginate lyases from PL7 in addition to a predicted secondary structure of the crystallized PL7 from* Klebsiella pneumoniae *(PDB ID 4OZX). Conserved residues are marked with a red box. The figure is obtained from (Gerlach, 2015).*

To obtain more functional information about *Pa*Aly7a, the amino acid sequence was compared in a PSA with PA1167, which is the only well-characterized subgroup 1 PL7 enzyme with a known 3D structure (**3.1.4**).

```
Query: PA14_41500 Query ID: lcl|Query_13191 Length: 231


>1VAV_1|Chains A, B|Alginate lyase PA1167|Pseudomonas aeruginosa (208964)
Sequence ID: Query_13193 Length: 222
Range 1: 2 to 221

Score:192 bits(488), Expect:2e-66,
Method:Compositional matrix adjust.,
Identities:107/228(47%), Positives:141/228(61%), Gaps:8/228(3%)

Query  3    DLSTWNLTIPTEPTPQVITTQQLNNGYQSRYFQQGNDGGLVFWVPIDGSHTEDSTYPRTE  62
            DLSTWNLTIP       I+T QL   Y+S YFQ+  DG + FWVP++GSHT +S +PR+E
Sbjct  2    DLSTWNLTIPQGRPAITISTSQLQRDYRSDYFQRTADG-IRFWVPVNGSHTRNSEFPRSE  60

Query  63   LRETQADGSLDNWYYWQADNELRVTMSVEKVPSKNKVIIGQIHSKSPQTDDGEPLVKLQY  122
            LRET + G   NW Y +ADN L  T+ +E VPS  ++IIGQIHS    +    PLVKL Y
Sbjct  61   LRETLSSGRPYNWRYARADNWLEATLRIEAVPSTRRMIIGQIHSDGSNSGQAAPLVKLLY  120

Query  123  YYKPEEGLGRVEALVRNHPDDSYSRNVSILEQVRLGERFNYSLRVTSSGELAVRARSQDG  182
              + ++  GRV+ALVR  PDD  +R  ++++ + LG+ F+Y + V+ SG L+V   S +G
Sbjct  121  QLRLDQ--GRVQALVRERPDDGGTRAYTLMDGIPLGQPFSYRIGVSRSGLLSV---SVNG  175

Query  183  EEDAYYQTLGSAWNDQLLYFKAGAYVNDNAGDSGEGSRVTIYHLNTAH  230
               A  Q L   W  Q LYFKAG Y+ DN G S EG R T   L  +H
Sbjct  176  S--ALEQQLDPQWAYQGLYFKAGLYLQDNRGPSSEGGRATFSELRVSH  221
```

Figure 12. Pairwise alignment of PaAly7a and PA1167. *PaAly7a (*PA14_41500*) was listed as the query and compared to PA1167, which was listed as the Sbjct. The conserved motifs were marked with a black box.*

The conserved PL7 sequence motifs are shared between the two proteins, apart from some amino acids. As described in **1.4.3.2**, PA1167 contains the conserved regions RSELR, QIH, and YFKAG*Y*Q. *Pa*Aly7a lacks the last Q (glutamine), this amino acid is substituted for N (asparagine), and *Pa*Aly7a has RTELR instead of RSELR. In addition, PA1167 has substituted valine against methionine (**Figure 12**). Also, it seems that *PA14_41500* has four small insertions compared to PA1167 (**Figure 12**).

To determine the potential significance of these insertions and to identify other putative structural differences between PA1167 and PA1784, the 3D structure of PA1167 (PDB ID 1VAV) and the Alphafold2-predicted structure of PA1784 were compared (**Figure 13**) (**3.1.5**) (Jumper et al., 2021; Varadi et al., 2021). The structure of PA14_41500 is not yet estimated, so the structure of PA1784 was used.

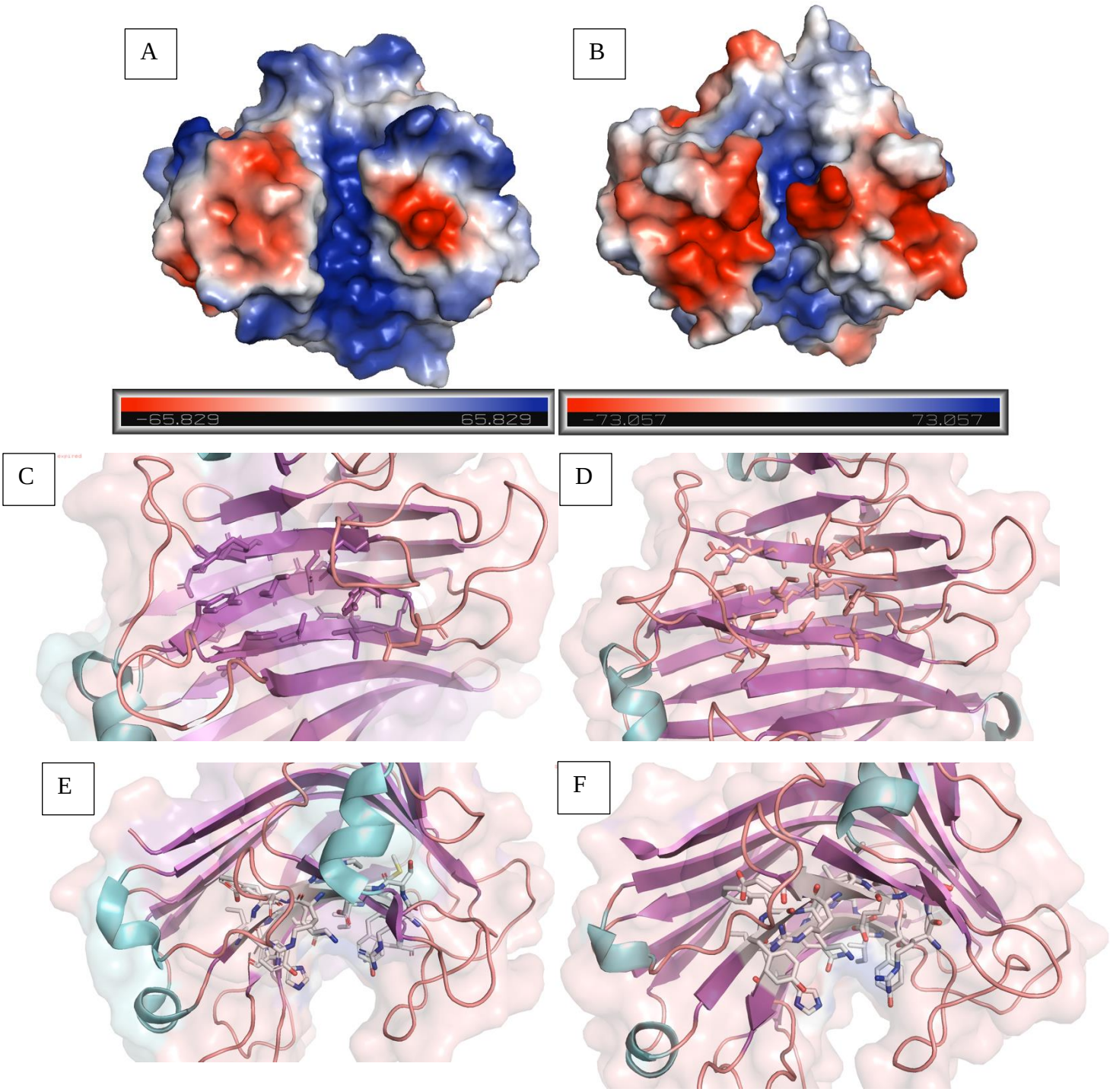

Figure 13. The predicted 3D structure of PA1167 and PA1784. *Structure of PA1167 (A, C, E) and PA1784 (B, D, F) with the active site as the point of view. Panels A and B show the predicted surface structure with charge. The blue color indicates positive charged areas, and the red color indicates negative charged areas. Panels C and D show the active site of PA1167 and PA1784, respectively. The conserved amino acids marked in* ***Figure 12*** *are shown as sticks and colored based on the secondary structure; pink represents loop, blue represents α-helix, and purple represents β-blocks. Panels E and F show the entire predicted protein of PA1167 and PA1784 with the surface 80 % transparent, respectively. The conserved amino acids are colored based on the atom in panels E, and F. Carbon and hydrogen are colored grey, nitrogen is colored blue, oxygen is colored dark purple, and sulfur is colored yellow.*

As already observed by the sequence alignments, the conserved sequence motifs marked in **Figure 12** were present in almost identical conformations in the active sites (**Figure 13**, panels **C, D, E**, & **F**). In addition, both panels showed an active site with a positive charge (**Figure 13**, panels **A** & **B**). On the other hand, the putative substrate-binding cleft of PA1784 seemed more closed, and the amino acids around the active site were more negatively charged compared to PA1167 (**Figure 13**, panels **A** & **B**). The conserved sequence motifs colored by secondary structure show that the amino acids in the active site to *Pa*Aly7a are loops (pink) (**Figure 13D)** while the amino acids in the active site to PA1167 are β-helixes (**Figure 13C**).

Finally, to obtain more insight into the potential function of the protein, PA1784 was explored by STRING analysis (**3.1.6**). The STRING analysis gives an overview of proteins that are functionally or physically associated with the protein of interest, and it gives a prediction of protein-protein interaction (Szklarczyk et al., 2021). This analysis places the chosen protein in a network of proteins with edges between them. Each edge has a specific thickness based on the strength of data support. This means that a thick edge has a higher possibility that the proteins have a physical or functional association than a thin edge, based on data.

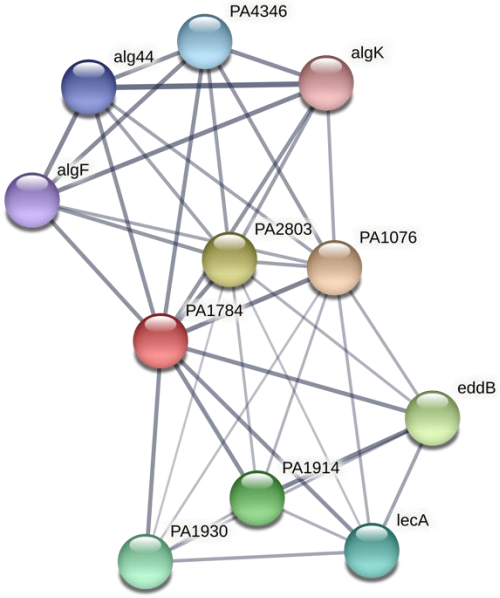

Figure 14. STRING analysis*. The PA1784 is marked in the figure with a red circle. This figure is made of the top 10 interactions with minimum medium confidence (0.4). The network is a full STRING network which indicates both functional and physical protein associations. Between every protein, each edge is based on confidence, and the thickness of the line indicates the strength of the data support.*

Based on the STRING analysis (**Figure 14),** PA1784 has the strongest confidence connection to PA1076, PA2803, and eddB. eddB is an extracellular DNA degradation protein, while

PA1076 and PA2803 have unknown functions. Further, PA1784 is also associated with AlgF, AlgK, and Alg44 which are proteins essential in the alginate synthesis complex: Alg44 is a mannuronic synthase, AlgF is involved in the acetylation of alginate, and AlgK is thought to have a function as a regulator of the alginate degrading protein AlgL.

4.2 Cloning of *PA14_41500*

Since the bioinformatic analysis of PA14_41500 indicated a functional alginate lyase, the gene representing *Pa*Aly7a from the *P. aeruginosa* PA14 strain was cloned into the expression vector pNIC-CH.

4.2.1 Cloning of *PA14_41500* into pNIC-CH

To prepare the pNIC-CH expression vector for insertion of the *PA14_41500* gene, the vector was digested with the AflII and PstIHF restriction enzymes and analyzed by agarose gel electrophoresis (**3.4.3**). The linearized vector showed a size of 5279 bp, and the controls with only one restriction enzyme showed only one band, indicating successful restriction digestion (**Figure 15a**). Subsequently, the *PA14_41500* gene was amplified by PCR using the primers indicated in **Table 2** (**3.4.5**). The PCR product was analyzed by agarose gel electrophoresis that showed a band with the expected size of 696 bp (**Figure 15b**).

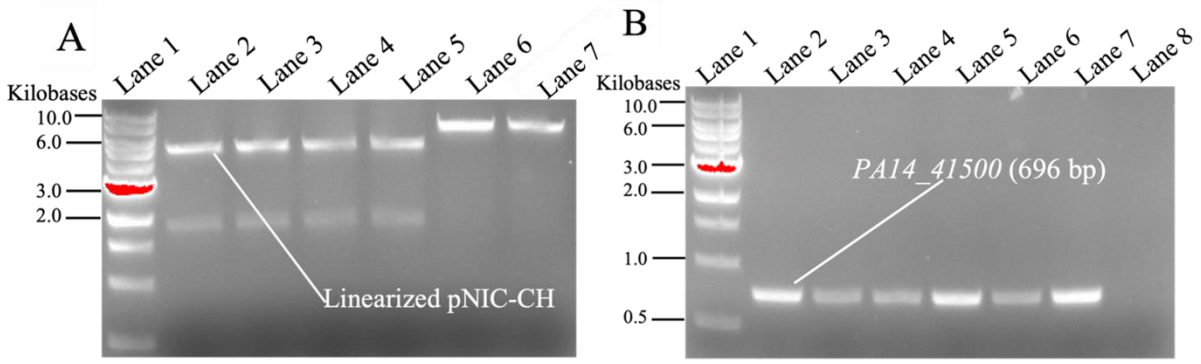

***Figure 15. Agarose gel analysis of PCR products from the linearization of pNIC-CH and amplification of* PA14_41500.** *(A) Linearization of pNIC-CH using AflII and PstIHF. The lanes show the following samples: 1: QuickLoad® 1kb ladder, 2-5: double digestion, 6,7: negative controls with one restriction enzyme each. (B) The amplification of* PA14_41500. *The lanes show the following samples: 1: QuickLoad® 1kb ladder, 2,3:* PA14_41500 *annealed at 63 ºC, 4,5:* PA14_41500 *annealed at 66 ºC, 6,7:* PA14_41500 *annealed at 68 ºC, 8: the negative control without DNA.*

The PCR product representing the *PA14_41500* gene was inserted into the linearized pNIC-CH plasmid by an In-Fusion ligation (**3.4.6**), transformed into TOP10 cells that were spread on an LB agar plate supplemented with kanamycin (**3.4.7**). After overnight incubation of the

transformed cells, colony PCR was used to identify transformants carrying the complete construct (**3.4.8**). The expected PCR product of approximately 750 bp was marked with a blue arrow and observed for all transformants, indicating a successful insertion of the *PA14_41500* gene (**Figure 16**).

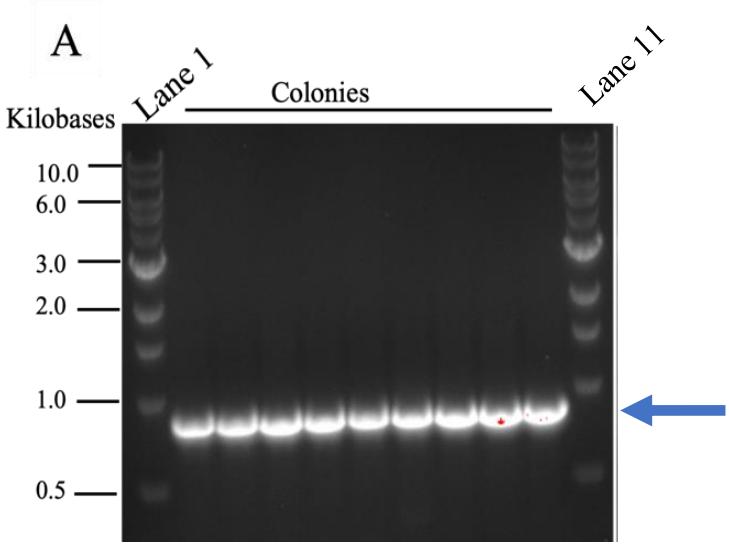

Figure 16. Colony PCR of TOP10 with pNIC-CH*+PA14_41500 *transformant. *The gel image shows the colony PCR of every TOP10 colony after transformation. Lanes 1 and 11 contain QuickLoad® 1kb ladder.*

The colonies carrying the complete plasmid were used to inoculate fresh LB medium to make cultures for -80 ºC glycerol stocks. All plasmids were verified by sequencing and subsequently transformed into *E. coli* BL21 cells for expression of the *Pa*Aly7a (**3.4.9**, **3.4.10**).

4.2.2 Cloning of *PA14_41500* into pBAD and pET

The expression of *Pa*Aly7a with pNIC-CH+*PA14_41500* was less than expected (**Appendix C**). *PA14_41500* were therefore cloned into the plasmids pET and pBAD (**3.5**) (**3.6**). pET and pBAD were linearized (**3.5.2**) (**3.6.2**), and the linearized vector of pBAD showed a size of 3992 bp, and by using a Benchtop UV Transilluminator, the linearized vector of pET showed a size of 5231 bp with an additional band at 141 bp (**Figure 17**). The controls were only digested with one restriction enzyme, and therefore only one band was visible. This indicated successful restriction digestion (**Figure 17**). Subsequently, the *PA14_41500* was amplified with primers (**Table 2**) compatible with the cutting site on pET and pBAD (**3.5.3**) (**3.6.3**). The insert amplification of *PA14_41500* showed a band at approximately 750 bp marked with a blue arrow in the figure (**Figure 18**).

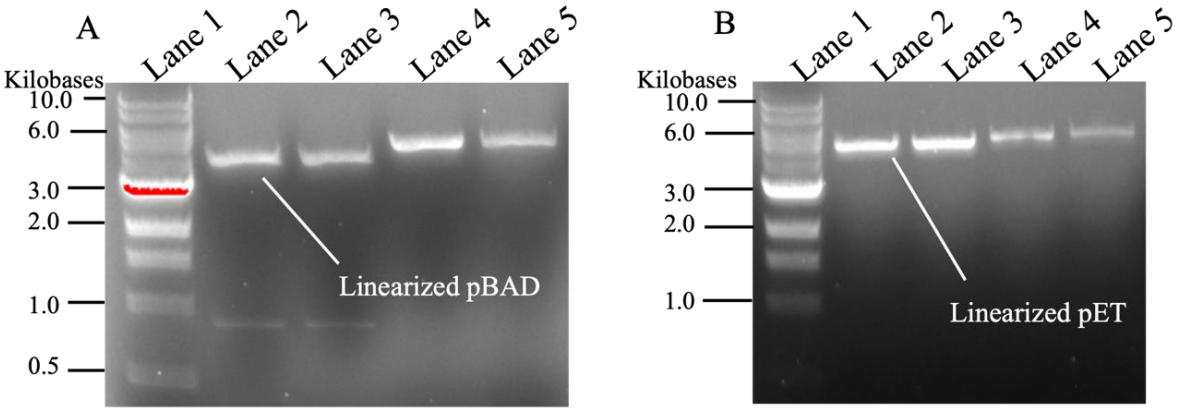

Figure 17. Agarose gel analysis of PCR products from the linearization of pBAD and pET*. (A) Linearization of pBAD using EcoRI and BglII. The lanes show the following samples: 1: QuickLoad® 1kb ladder, 2, 3: pBAD with EcoRI and BglII, 4, 5: negative controls with one restriction enzyme each. (B) Linearization of pET using NcoI and XhoI. The lanes show the following samples: 1: QuickLoad® 1kb ladder, 2, 3: pET with NcoI and XhoI, 4, 5: negative controls with one restriction enzyme each.*

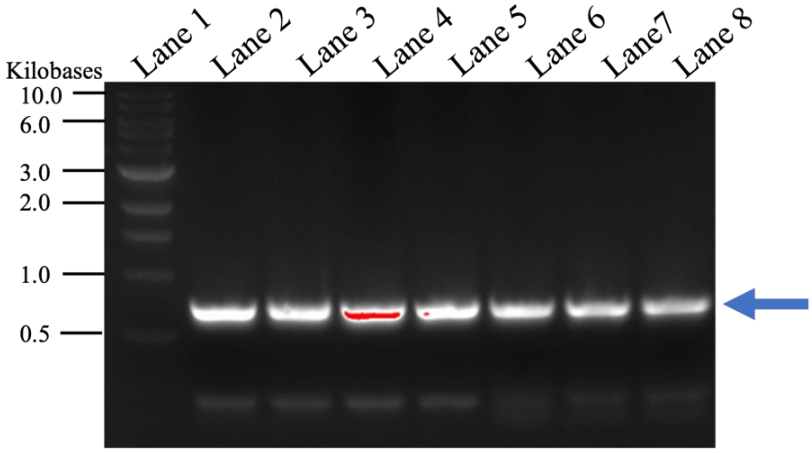

***Figure 18. Agarose gel analysis of PCR amplification of* PA14_41500.** *The PCR product resulting from amplification of* PA14_41500 *with primers compatible with the pBAD and pET vectors are shown in lanes 2-5 and lanes 6-8, respectively. Lane 1: QuickLoad® 1kb ladder.*

The PCR products representing the *PA14_41500* gene were inserted into the linearized pET and pBAD plasmids by an In-Fusion ligation (**3.5.4**) (**3.6.4**) and transformed into TOP10 cells that were plated on LB agar plate supplemented with kanamycin or ampicillin (**3.5.5**) (**3.6.5**). After overnight incubation of the transformed cells, colony PCR was used to identify transformants carrying the complete construct (**3.5.5**) (**3.6.5**).

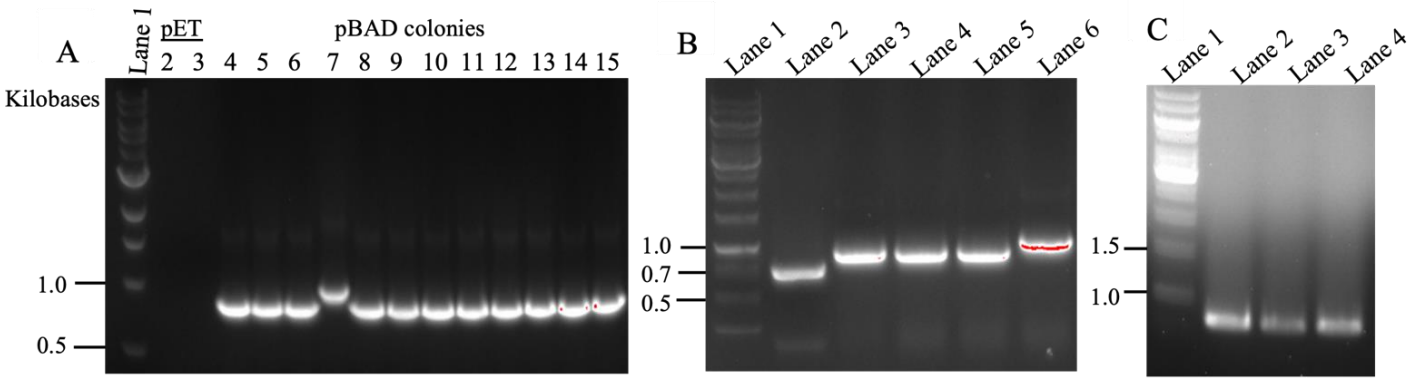

Figure 19. Colony PCR of TOP10 and control PCR of pBAD*+PA14_41500.** *(A) Colony PCR of pET*+PA14_41500 *and pBAD*+PA14_41500. *The lanes show the following samples: 1: QuickLoad® 1kb ladder, 2, 3: Colonies transformed with pET*+PA14_41500, *4-15: colonies transformed with pBAD*+PA14_41500. ***(*B) Second colony PCR of pBAD*+PA14_41500 *with colonies from lanes 3-6 in gel A. The lanes show the following samples: 1: 1 kb GeneRuler ladder, 2: positive control of* Pa*Aly7a analyzed with amplification PCR, 3-5: a new colony PCR of colonies 1, 2, and 3, 6: colony four from gel A. (C) The lanes show the following samples: 1: 1 kb GeneRuler ladder, 2-4: a new colony PCR of pET*+PA14_41500.

The two colonies from transformation with pET+*PA14_41500* did not give a visible band in lanes 2 and 3 (**Figure 19A**). A new In-Fusion reaction with an insert: vector ratio of 4:1 was therefore carried out, resulting in successful insertion (**Figure 19C**).

Twelve of the 34 colonies from the transformation of pBAD+*PA14_41500* into TOP10 were amplified by colony PCR. All 12 colonies showed a PCR product of approximately 750 bp (**Figure 19A)**. However, the PCR product in lane 7 had more base pairs than the rest, so a second colony PCR of the colonies in lanes 4-7 was run. *PA14_41500* was amplified by PCR and added as a positive control (**3.6.6**). The expected value of the colony PCR was approximately 750 bp, and the samples in lanes 3-5 showed a band at that size (**Figure 19B)**. This confirmed that the samples in lanes 4-6 and 8-15 were of the correct size (**Figure 19A)**.

The colonies carrying the complete plasmid were used to inoculate fresh LB medium to make cultures for -80 ºC glycerol stocks. All plasmids were verified by sequencing and subsequently transformed into *E. coli* BL21 cells for expression of the alginate lyase (**3.5.6) (3.6.7**, **3.6.8**).

4.3 Expression, purification, and validation of *Pa*Aly7a

The successful cloning of *PA14_41500* into pNIC-CH, pET, and pBAD vectors enabled the expression of *Pa*Aly7a in *E. coli* BL21 cells. In short, the first step was to optimize the protein

production (**3.7.1**, **3.8.1**), followed by the cultivation of 0.5-2 L cultures to produce a sufficient amount of protein for purification (**3.7.2**).

Primarily the expression and purification of *Pa*Aly7a proceeded with the vector pNIC-CH+*PA14_41500*. A substantial amount of work was put into the optimization of *Pa*Aly7a production and purification from BL21 with pNIC-CH+*PA14_41500*. However, due to a low expression of *Pa*Aly7a with pNIC-CH+*PA_41500* and the readability of this thesis, the results from the expression and purification of *Pa*Aly7a from BL21 with pNIC-CH+*PA_41500* are placed in **Appendix C**.

Based on analysis by ProtParam *Pa*Aly7a with a 6x His-tag is expected to have a mass of approximately 27 kDa (Gasteiger et al., 2005). The band at approximately 27 kDa was therefore marked with a red box in **4.3.1**, **4.3.2, Appendix B,** and **Appendix C**.

4.3.1 Optimization of culture conditions for protein production

To find the optimized conditions for *Pa*Aly7a production, different mediums, temperatures, and inducer concentrations were tested on small-scale production of *Pa*Aly7a. BL21 with pNIC-CH+*PA14_41500*, pET+*PA14_41500*, and pBAD+*PA14_41500* were incubated in LB, TB, M9, BHI, and TSB medium at 20 and 37 ºC and with two concentrations of inducer (**3.7.1**, **3.8.1**). The results with pBAD+*PA14_41500* are shown in **Appendix B** - **Figure B 1**, pET+*PA14_41500* incubated in LB, TSB, and BHI are shown in **Appendix B** - **Figure B 2**, **Figure B 3**, and **Figure B 4**, and the results with pNIC-CH+*PA14_41500* are shown in **Appendix C** - **Figure C 1** and **Figure C 2**. A summary of the results from the optimization is found in **Table 24**.

*Pa*Aly7a at approximately 27 kDa was more visible after incubation at 20 ºC than after incubation at 37 ºC. For each sample incubated, no difference between 0.2 mM and 1 mM IPTG was visible (**Figure 20**).

The expression of *Pa*Aly7a was weaker than expected. Therefore, total protein samples were analyzed on the SDS-PAGE gel in addition to the lysate to determine if the protein could be present in the insoluble fraction (**Figure 21**).

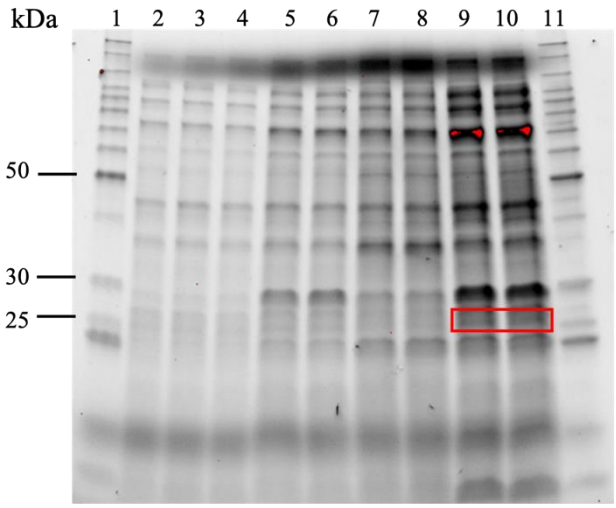

***Figure 20*. *Small-scale expression trials for TB medium*.** *BL21 with pET*+PA14_41500 *lysate was incubated in TB medium. The lanes show the following samples: 1, 11: the protein ladder Benchmark, 2: cell lysate without IPTG, 3: 0 hours incubation with 1 mM IPTG, 4: 0 hours incubation with 0.2 mM IPTG, 5: 3 hours incubation at 37 °C with 1 mM IPTG, 6: 3 hours incubation at 37 °C with 0.2 mM IPTG, 7: incubated at 37 °C overnight with 1 mM IPTG, 8: incubated at 37 °C overnight with 0.2 mM IPTG, 9: incubated overnight at 20 °C with 1 mM IPTG, 10: incubated overnight at 20 °C with 0.2 mM IPTG.*

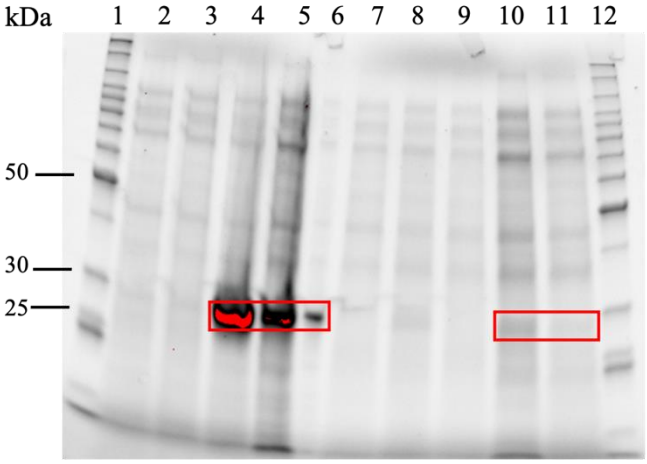

Figure 21*. *Small-scale expression trials for BHI medium. *BL21 with pET*+PA14_41500 *incubated in BHI medium with 0.2 mM IPTG was incubated at 20 or 37 ºC. In lanes 2-6, the total protein samples were added, while in lanes 7-11, lysate samples were added. The lanes show the following samples: 1, 12: the protein ladder Benchmark, 2, 7: non-induced culture, 3, 9: 0 hours incubation with IPTG, 4, 8: 3 hours incubation at 37 °C with IPTG, 5, 10: incubated at 20 °C overnight with IPTG, 6, 11: incubated at 37 °C overnight with IPTG*

The cell lysate incubated overnight gave low-intensity bands at 27 kDa, while the total protein samples gave a very strong band. Compared to **Figure 20**, the total protein samples gave a much stronger signal than the cell lysate. This could indicate that the protein had formed inclusions bodies (insoluble protein aggregates) (**Figure 21**). The sample incubated at 20 ºC overnight was not added to the gel because of the viscosity of the sample. A second SDS-PAGE gel was therefore made with only the total protein samples (**Appendix B - Figure B 5)**.

Even though a band at approximately 27 kDa was seen in the non-induced sample (**Figure 20**). The optimization experiments showed that pET+*PA14_41500* cultivated in TB medium overnight at 20 ºC with 1 and 0.2 mM IPTG represented the optimal conditions for protein production as these conditions gave substantially more protein than all other conditions (**Table 24**). Thus, pET+*PA14_41500* was used for further experiments.

Table 24. Optimization of protein expression. *Production of* Pa*Aly7a cloned into the three specified vectors was evaluated semi-quantitatively by visual inspection, +++ indicating high production, ++ indicating medium production, + indicating low production, and – indicating no production. In the case of inclusion bodies, these are indicated by "IC". The result is a summary of* ***Figure 20***, ***Figure 21***, ***Appendix B,*** *and* ***Appendix C.***

Vector/condition	**LB**	**TB**	**M9**	**BHI**	**TSB**
pNIC-CH+*PA14_41500*	-	+	-	-	NA
pBAD+*PA14_41500*	-	-	NA	NA	NA
pET+*PA14_41500*	++	+++	NA	- IC	- IC

4.3.2 *Pa*Aly7a purified with IMAC

Based on the optimization (**4.3.1**), pET+*PA14_41500* and pNIC-CH+*PA14_41500* were cultivated in TB medium at 20 ºC overnight with 1 mM IPTG to produce *Pa*Aly7a (**3.7.2, 3.8.2**). The cell lysate was purified with IMAC based on protocol **3.9.1**.

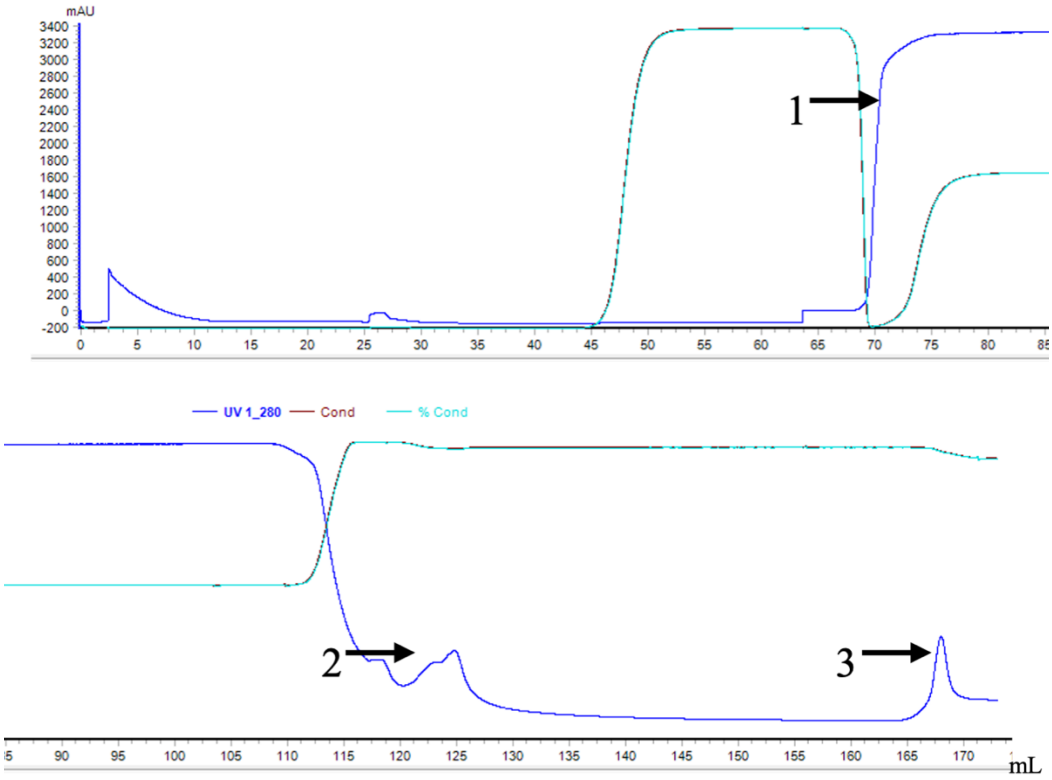

Figure 22. IMAC purification of* Pa*Aly7a. *The chromatograms represent the purification of* Pa*Aly7a bound to a 5 mL HisTrap™ column. The y-axis shows the intensity of the absorbance measured by* A_{280} *in mAU, and the x-axis shows the mL added to the column. The eluted proteins were detected by monitoring* A_{280}*, shown by the dark blue curve. The light blue and brown curves represent the conductivity of the solution within the column. The conductivity measures the imidazole added in the different buffers added to the system. The chromatogram shows three major peaks: the flow-through (non-bound protein) peak is indicated with the number 1, the wash peak is indicated with the number 2, and the peak representing the target protein is indicated with the number 3. The chromatogram was generated using the Unicorn™ 6.4.1 Software.*

The chromatogram of the purification shows a peak at 800 mAU (167 mL) during elution (**Figure 22**), and the analysis of the wash sample and the elution sample showed that *Pa*Aly7a had bound to the column. However, the analysis also showed that the purified sample also contained numerous contaminants (**Figure 23**). ChiC expressed from BL21 with pNIC-CH+ChiC was purified alongside *Pa*Aly7a from BL21 with pET+*PA14_41500* as a control to verify the functionality of the production and the purifying procedure (**Figure 23B**).

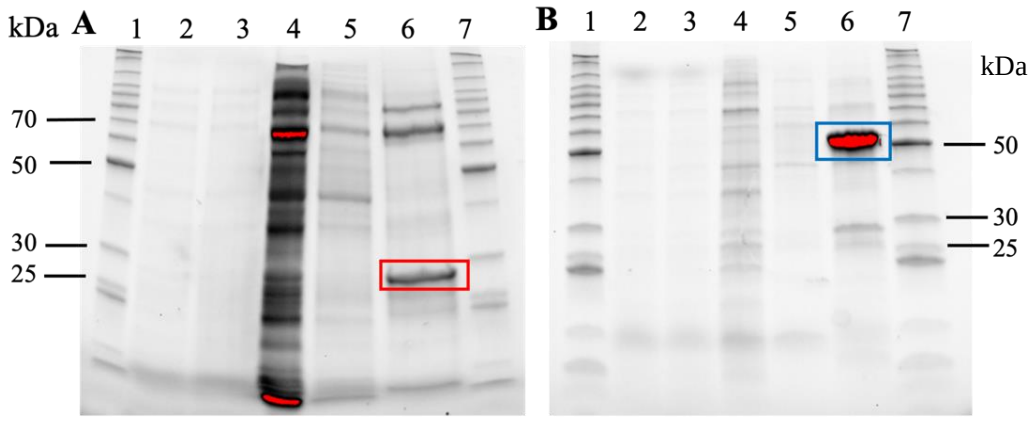

Figure 23. SDS-PAGE analysis of* Pa*Aly7a purification. *The SDS-PAGE gels show analysis of expression and purification of* Pa*Aly7a from* E. coli *BL21 containing pET*+PA14_41500 *(A). The second gel (B) shows a control experiment; expression and purification of ChiC from* E. coli *BL21 containing pNIC-CH+ChiC. In all panels, the following lanes show samples: 1, 7: BenchMark™ protein ladder, 2: cell extract from a culture without IPTG, 3: the sample 0 hours with 1 mM IPTG, 4: the flow-through, 5: the wash fraction, 6: the eluted fraction.*

*Pa*Aly7a has a theoretical value of 27 kDa, and the red box shows the molecular weight where the expected protein was found in the elution sample. A band at 27 kDa was also visible in the flow-through, and a very weak band was also visible in the non-induced sample (**Figure 23a**). However, in the flow-through, other proteins with different molecular weights contaminated *Pa*Aly7a.

Per Kristian Edvardsen kindly provided the BL21 pNIC-CH+ChiC with the expected molecular weight of 53 kDa, marked with a blue box in **Figure 23B**. This protein was used as a control to verify that the purification procedure was done correctly. ChiC was successfully expressed and purified on IMAC with a strong signal and few contaminants (**Figure 23B**).

The samples analyzed by SDS-PAGE (**Appendix C - Figure C 3**) were treated with LDS and heated at 70 ºC for 10 minutes. To try different denaturation approaches, cell lysate from BL21 pNIC-CH+*PA14_41500* cultivated in TB medium purified on IMAC and culture samples were added LDS and 90 µM DTT, boiled at 95 ºC for 5 minutes before they were analyzed on the SDS-PAGE gel. These conditions were used to determine whether milder conditions could yield non-complete dissolving of the protein, e.g., that the band at 70 kDa could be a *Pa*Aly7a dimer (**Appendix C - Figure C 3**). However, these conditions did not change the results (**Appendix C - Figure C 4.**).

Since a band at 27 kDa was visible from the flow-through samples (**Figure 23A**), a new hypothesis was tested. The flow-through, from BL21 with pNIC-CH+*PA14_41500* purified on IMAC, was added sodium alginate to a final concentration of 0.1 % to test if *Pa*Aly7a needed to form an interaction with alginate to bind to the column. However, this approach did not change the results (**Appendix C - Figure C 5.**, - **Figure C 3**).

4.3.3 Verification of *Pa*Aly7a by proteomics

First, a proteomics analysis was proceeded to verify that the band on the SDS-PAGE was *Pa*Aly7a (**Figure 23A**). After large-scale expression and purification on IMAC, the analysis of the SDS-PAGE gel showed a band at approximately 27 kDa. However, compared to the ChiC purification, this band was very weak, and there were numerous contaminants in the elution sample. In addition, another band at the gel at approximately 70 kDa had a stronger signal than the band at 27 kDa. Both bands were therefore tested since the kDa size was theoretical, the protein may be folding differently than expected, and the protein may have been a dimer. Each band was therefore cut out of the SDS-PAGE gel and treated with protocol **3.15.1**. The peptide analysis was performed by Morten Skaugen using the timsTOF Pro system, and the results were examined by Per Kristian Edvardsen. The result from the proteomics showed that there were 35 times as much *Pa*Aly7a present in the gel piece at 27 kDa compared to the gel piece at 70 kDa (data not shown).

4.3.4 *Pa*Aly7a purified with SEC

BL21 pET+*PA14_41500* cultivated in TB medium was first purified on IMAC (**3.9.1**) (**4.3.2**). Due to the low purity of *Pa*Aly7a obtained from IMAC purification (**Figure 23A**), the protein was further purified by SEC (**3.9.2**). Before the sample was added to the SEC column, the protein sample was concentrated (**3.10**), and the protein concentration was measured by A_{280} (**3.11.1**).

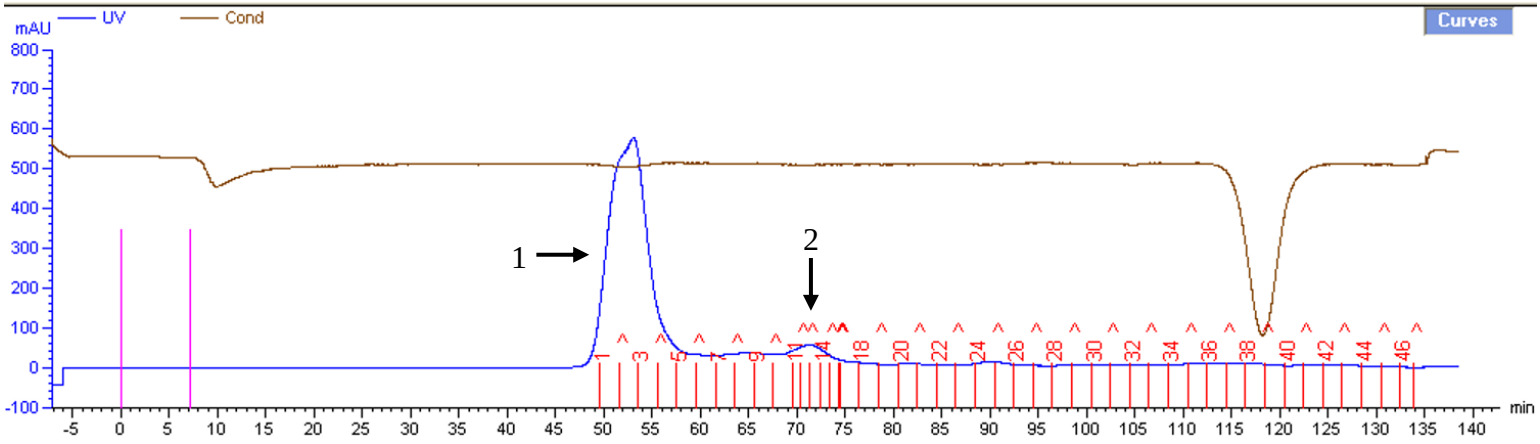

Figure 24. SEC purification of* Pa*Aly7a. *The chromatograms represent the purification of* Pa*Aly7a. The y-axis shows the intensity of the absorbance measured by* A_{280} *in mAU, and the x-axis shows the time in minutes (min) after 1 mL of the sample was added. The eluted proteins were detected by monitoring* A_{280}*, shown by the dark blue curve. The brown curve represents the conductivity of the solution within the column. The conductivity measures the salt concentration added to the system. The chromatogram shows two peaks: the first peak is indicated with the number 1, and the second peak is indicated with the number 2.*

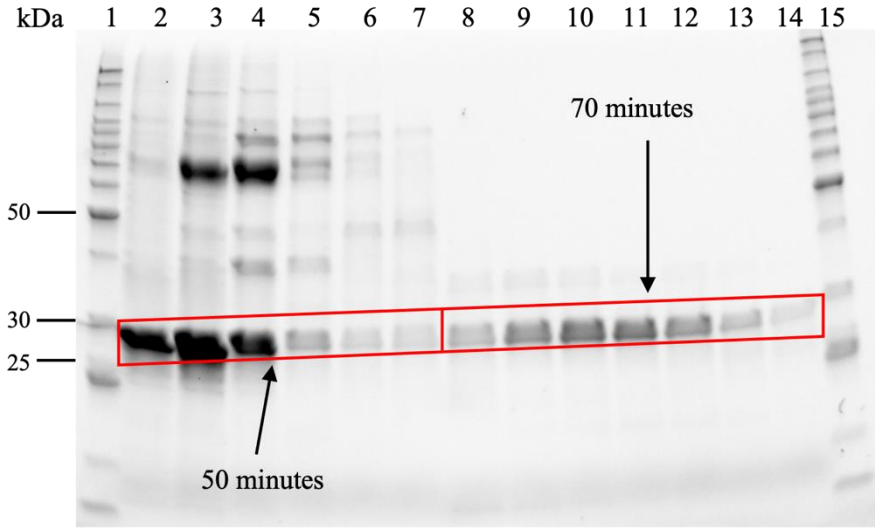

Figure 25. SDS-PAGE analysis of SEC purification of* Pa*Aly7a samples purified on IMAC. *The gel shows SEC elution fractions of* Pa*Aly7a from BL21 with pET+*PA14_41500 *in TB medium earlier purified on IMAC. Fractions in lanes 2-7 were eluted after 50 minutes, while fractions in lanes 8-14 were eluted after 70 minutes (**Figure 24**). Lanes 1 and 15 show the BenchMark™ protein ladder.*

The protein applied to the column eluted in two main peaks, the major peak eluting at approximately 50 minutes and a smaller peak eluting at 70 minutes (**Figure 24**). Six 1 mL fractions from the first peak and seven fractions from the last peak were collected and analyzed by SDS-PAGE (**Figure 25**). Interestingly, both peaks showed the 27 kDa protein believed to be *Pa*Aly7a, indicating that the protein may exist as a multimer or as soluble aggregates. Based on the purity of the samples, fraction 1 in lane 2 from peak 1 was kept (further named *Pa*Aly7a-

P1), in addition to fractions 10-16 in lanes 8-14 from peak 2 (further named *Pa*Aly7a-P2) (**Figure 25**). These samples were later used on protein melting point analysis (**4.4**) and activity assays (**4.5**).

4.4 Protein melting point analysis

The pooled and concentrated fractions of pure *Pa*Aly7a obtained by SEC purification, *Pa*Aly7a-P1 (*Pa*Aly7a peak 1) and *Pa*Aly7a-P2 (*Pa*Aly7a peak 2) (**4.3.4**), were analyzed by protein melting point analysis (**3.12**) with four replicates to determine the integrity of the purified protein. This test was proceeded to see if the eluted protein was a multimer or as soluble aggregates.

For *Pa*Aly7a-P1, the spectrum from protein melting point analysis did not show a protein melting point (**Figure 26a)**, which might indicate a misfolded protein. The spectrum for *Pa*Aly7a-P2 showed a melting curve similar to what is expected for a folded protein. Based on the curve, T_m was determined to be approximately 51 ºC (**Figure 26b)**. The negative control without protein did not show a protein melting point (**Figure 26c)**.

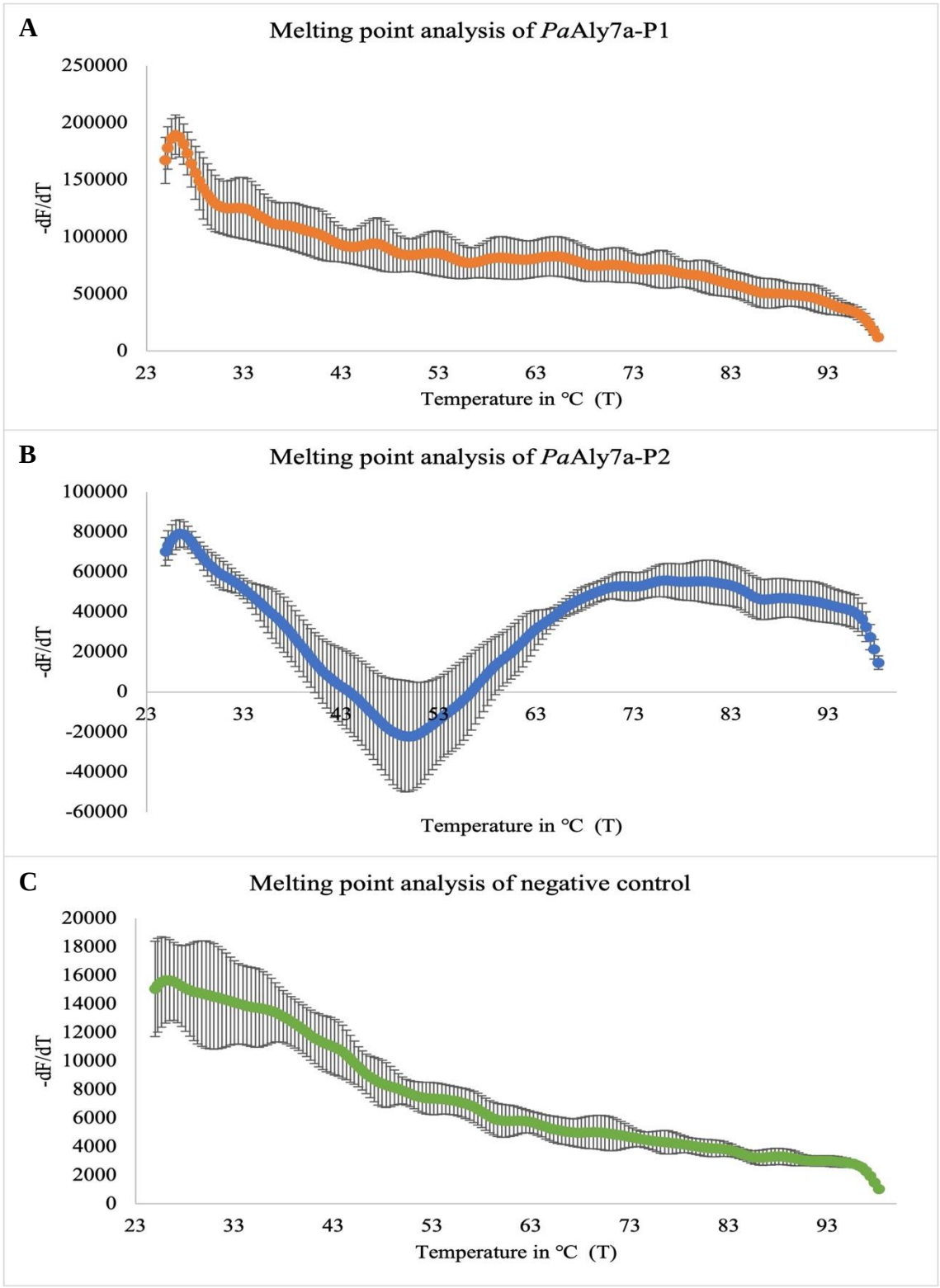

Figure 26. Protein melting point analysis*. Two samples from SEC purification were concentrated and analyzed by protein melting point analysis. The samples were added orange dye and slowly heated from 25 to 100 ºC. The figure is shown with three spectra, A, B, and C. (A) is of PaAly7a-P1 marked in orange, (B) is of PaAly7a-P2 marked in blue, and (C) is the negative control without protein added marked in green. The y-axis shows the first derivative of fluorescence (F) emission as a function of temperature (T), whilst the x-axis shows the temperature (T). Four replicates were used for each sample, and the average was calculated with standard deviation.*

4.5 Activity assay

Protein samples purified from both BL21 with pNIC-CH+*PA14_41500* and BL21 with pET+*PA14_41500* on IMAC (**4.3.2**) in addition to *Pa*Aly7a-P1 and *Pa*Aly7a-P2 (**4.3.4**) were tested for *Pa*Aly7a activity using a variety of activity assays (results from pNIC-CH+*PA14_41500* is not shown). In addition, the characterized PL7 alginate lyase AMOR_PL7A was also included in most of the assays as a positive control. The assays used for determining activity towards sodium alginate and alginate purified from *P. aeruginosa* (**3.13**) were DNS assay for reducing end generation (**3.14.1**), monitoring A_{235} for generation of double bonds (**3.14.2**), and MALDI-TOF MS for detection of reaction products (**3.14.3**). Furthermore, the potential activity of the protein was investigated in biofilm degradation assays (**3.14.5**) and agar plate biofilm degradation assays (**3.14.4**). Finally, a DNA-degradation assay was used to probe the putative role of *Pa*Aly7a in DNA depolymerization (**3.14.6**).

4.5.1 DNS reducing end assay

Purified *Pa*Aly7a was incubated with sodium alginate and alginate from *P. aeruginosa* (**3.13**) and analyzed with the DNS reducing end assay (**3.14.1**). To verify the process, a sample of glucose with a concentration of 0.3 mM was included to visualize potential drift in the analysis. The glucose concentration was used based on the result from a standard curve (**Figure 28**). In addition to a glucose sample, AMOR_PL7A was used as a positive control, and negative controls for each protein sample were used (**3.14**).

The *Pa*Aly7a did not show activity when measuring the reducing ends with DNS. However, AMOR_PL7A did have an increasing absorbance measured at A_{540}. All other samples had no change in their absorbance at approximately 0.1 A_{540} (**Figure 27**).

The standard curve for glucose was linear, with an R^2 value of 0.9625 (**Figure 28**). Based on the standard curve, a 0.3 μM glucose sample will give an absorption of approximately 0.12 measured at A_{540}.

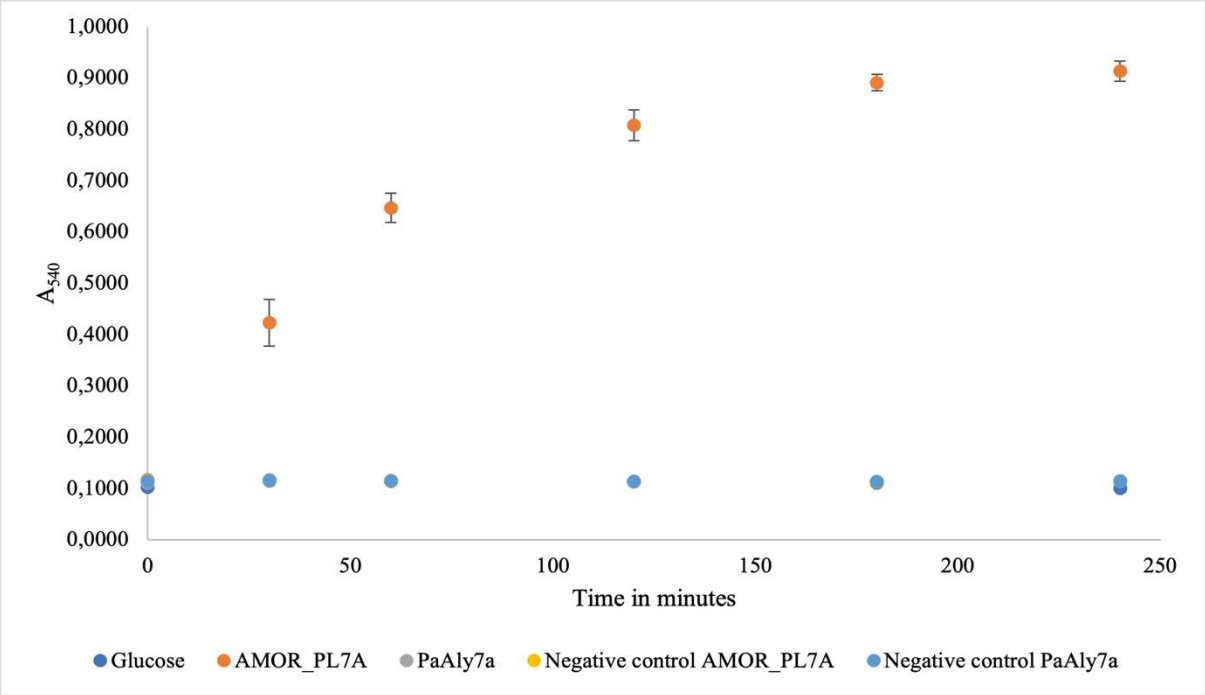

Figure 27. DNS reducing end assay measuring the degradation of sodium alginate*. The figure shows the absorption measured at A_{540} of 0.3 mM glucose (dark blue), AMOR_PL7A (orange), and* Pa*Aly7a from pET*+PA14_41500 *purified on IMAC (grey), negative control for* Pa*Aly7a (light blue, and negative AMOR_PL7A (yellow) with sodium alginate measured over 240 minutes. The negative controls were not added protein. All the data points overlap except for AMOR_PL7A. Therefore, the only samples visible are AMOR_PL7A and negative control for* Pa*Aly7a. For* Pa*Aly7a and AMOR_PL7A, three replicates were used, and the average was calculated with standard deviation.*

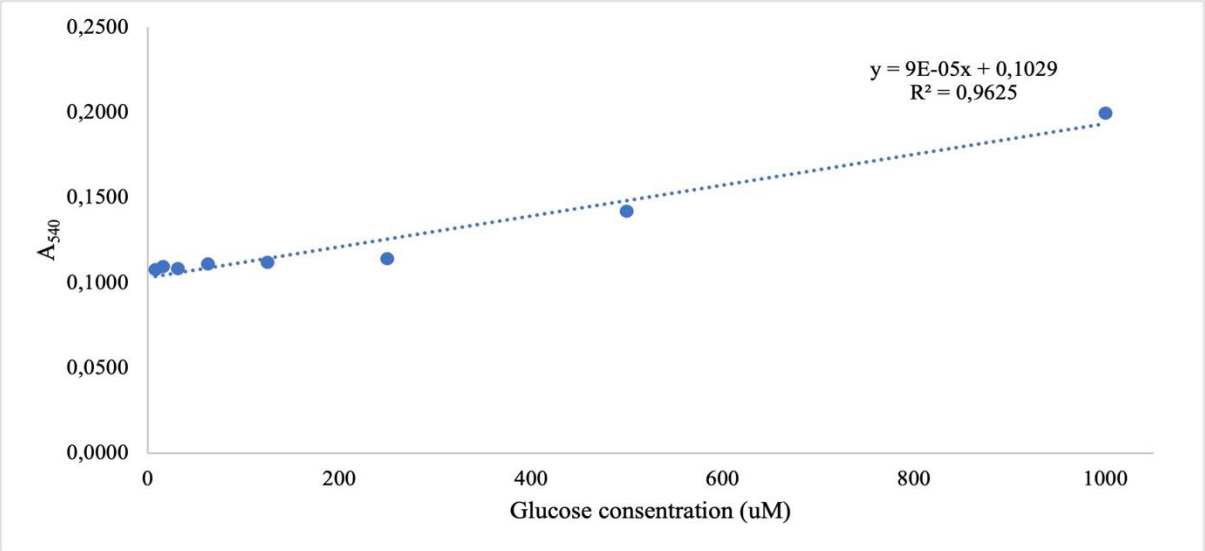

Figure 28. Glucose standard curve*. The graph shows glucose concentrations from 7.8 µM to 1000 µM measured at A_{540}. Linear regression is also shown.*

4.5.2 Monitoring A_{235} for generation of double bonds

The DNS reducing end assay showed no activity for *Pa*Aly7a measured at A_{540} (**4.5.1**). Therefore, another activity assay was tested. By monitoring A_{235} over time, the number of carbon-carbon double bonds potentially produced by *Pa*Aly7a was measured (**3.14.2**). The *Pa*Aly7a used for this assay was purified from BL21 with pET+*PA14_41500* on IMAC. To verify that the assay conditions did not cause drift in the analysis, imidazole at 2 M concentration was also added to the 96-well plate. Imidazole was used since it has double bonds that absorb light at 235 nm.

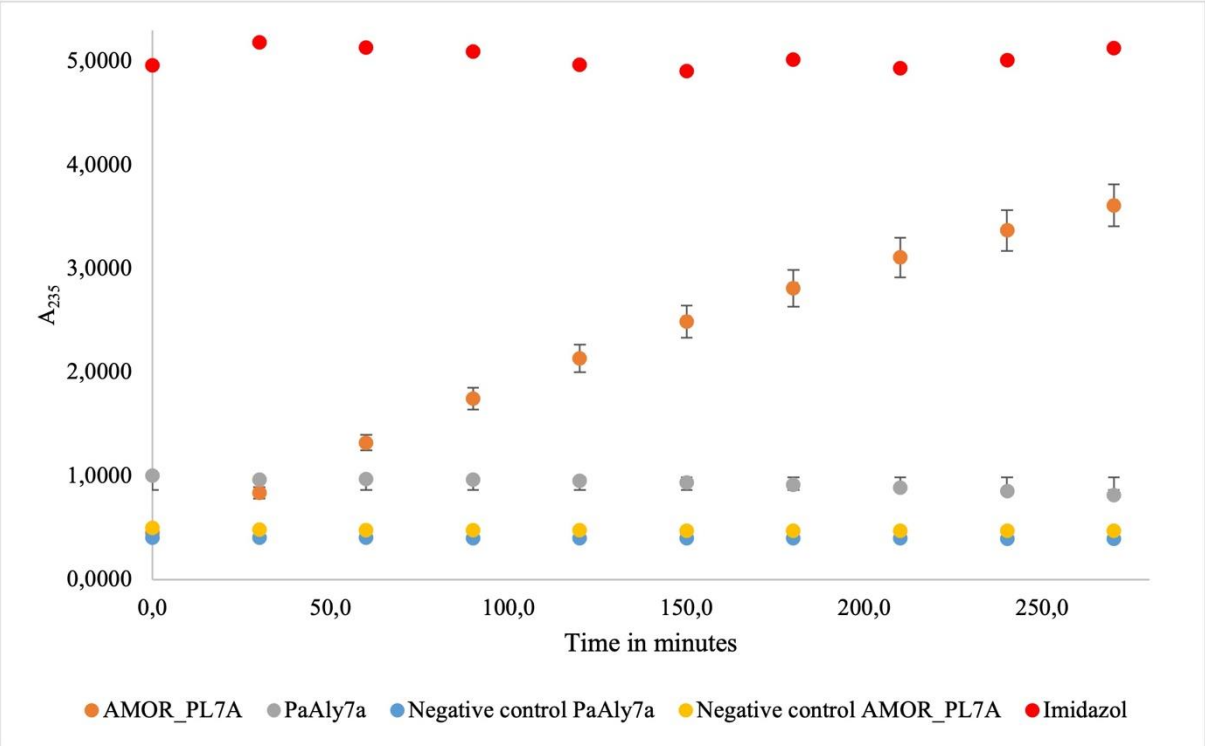

Figure 29. Monitoring A_{235} for generation of double bonds. *The figure shows the absorption at A_{235} of AMOR_PL7A (orange),* Pa*Aly7a from pET+*PA14_41500 *purified on IMAC (grey), and negative control for* Pa*Aly7a (light blue) and AMOR_PL7A (yellow) with sodium alginate measured over 240 minutes with reading every 30 minutes. The negative controls were not added protein, and imidazole (red) was measured as a positive control. For* Pa*Aly7a and AMOR_PL7A, three replicates were used, and the average was calculated with standard deviation.*

The positive control AMOR_PL7A with sodium alginate showed increasing absorbance over time (**Figure 29**), while the other samples showed no change. Imidazole showed absorbance at approximately 4.8, while *Pa*Aly7a and both the negative controls had an absorbance at approximately 0.1-1 measured at A_{235} (**Figure 29**).

Since the *Pa*Aly7a did not show activity against sodium alginate, alginate partly purified from a mucoid *P. aeruginosa* strain was tested in a qualitative assay using the same concentrations and the same conditions as for sodium alginate, except the alginate concentration was unknown (a crude purification product was used, see methods section **3.13** for details). No degradation products could be observed for either of the alginate lyases towards the *P. aeruginosa* alginate (results not shown).

To test if the activity of *Pa*Aly7a was determined by pH, Bis-tris HCl pH 6.5 and Tris-HCl pH 8.5 were tested (**3.14.2**). Neither of these samples showed an improvement in activity (results not shown). Since some of the protein was eluted in the flow-through during IMAC purification, the lysate was also tested. The lysate was so cloudy that the absorption was out of the measuring range (results not shown). $CaCl_2$ was then added to *Pa*Aly7a since some marine alginate lyases are dependent on divalent cations to bind to substrates (Thomas et al., 2013). *Pa*Aly7a-P1 and *Pa*Aly7a-P2 (**4.3.4**) were therefore mixed with $CaCl_2$ and then incubated in sodium alginate or alginate from *P. aeruginosa.* $CaCl_2$ was neither added to the negative control nor the positive control.

By adding $CaCl_2$, the activity of *Pa*Aly7a was unchanged (**Figure 30**), neither with the sodium alginate nor with the alginate from *P. aeruginosa*. All the samples except the AMOR_PL7A with sodium alginate showed a steady flat absorbance, which indicated no activity.

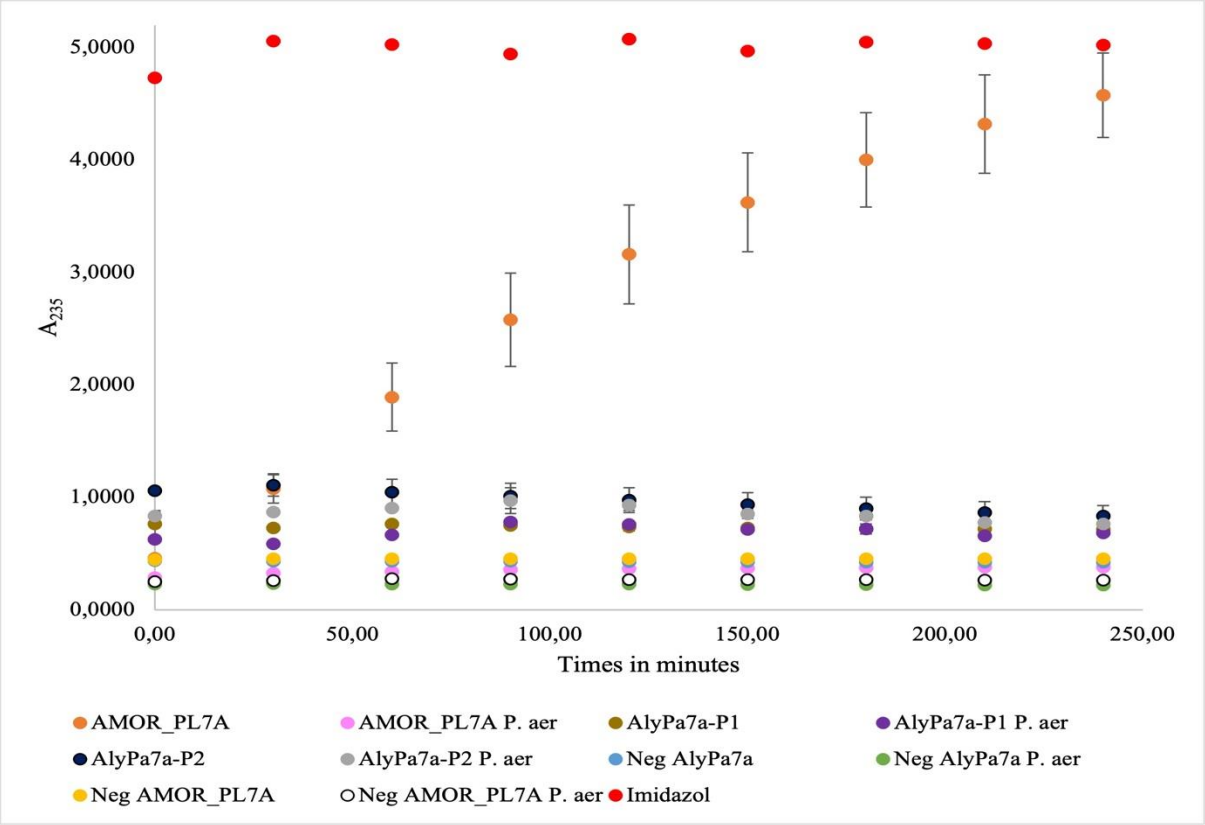

Figure 30. Monitoring A_{235} for generation of double bonds with* Pa*Aly7a added $CaCl_2$. *The figure shows the absorption at A_{235} over 240 minutes with a reading every 30 minutes.* Pa*Aly7a-P1 (F1) and* Pa*Aly7a-P2 (F10-16) were added $CaCl_2$ and then incubated with sodium alginate or alginate from* P. aeruginosa*. All samples incubated with alginate purified from* P. aeruginosa *are named P. aer, and negative controls are named neg. Each sample is marked with a color: AMOR_PL7A (orange), AMOR_PL7A P. aer (pink), F1 (brown), F1 P. aer (purple), F10-16 (black), F10-16 P. aer (green), neg* Pa*Aly7a (light blue), neg* Pa*Aly7a P. aer (grey), neg AMOR_PL7A (yellow), neg AMOR_PL7A P. aer (white), and imidazole (red). AMOR_PL7A and negative control was not added $CaCl_2$. Two replicates were used, and the average was calculated with standard deviation, except for negative controls and imidazole. The negative controls were not added protein.*

4.5.3 MALDI-TOF MS detection of reaction products

To investigate if *Pa*Aly7a was able to degrade alginate into alginate oligosaccharides, samples with sodium alginate or alginate from *P. aeruginosa* were analyzed using MALDI-TOF MS (**3.14**). For this analysis, *Pa*Aly7a purified on IMAC, and AMOR_PL7A were used. For each sample, negative control without protein was included. In addition, a duplicate of each sample was made and boiled before incubation.

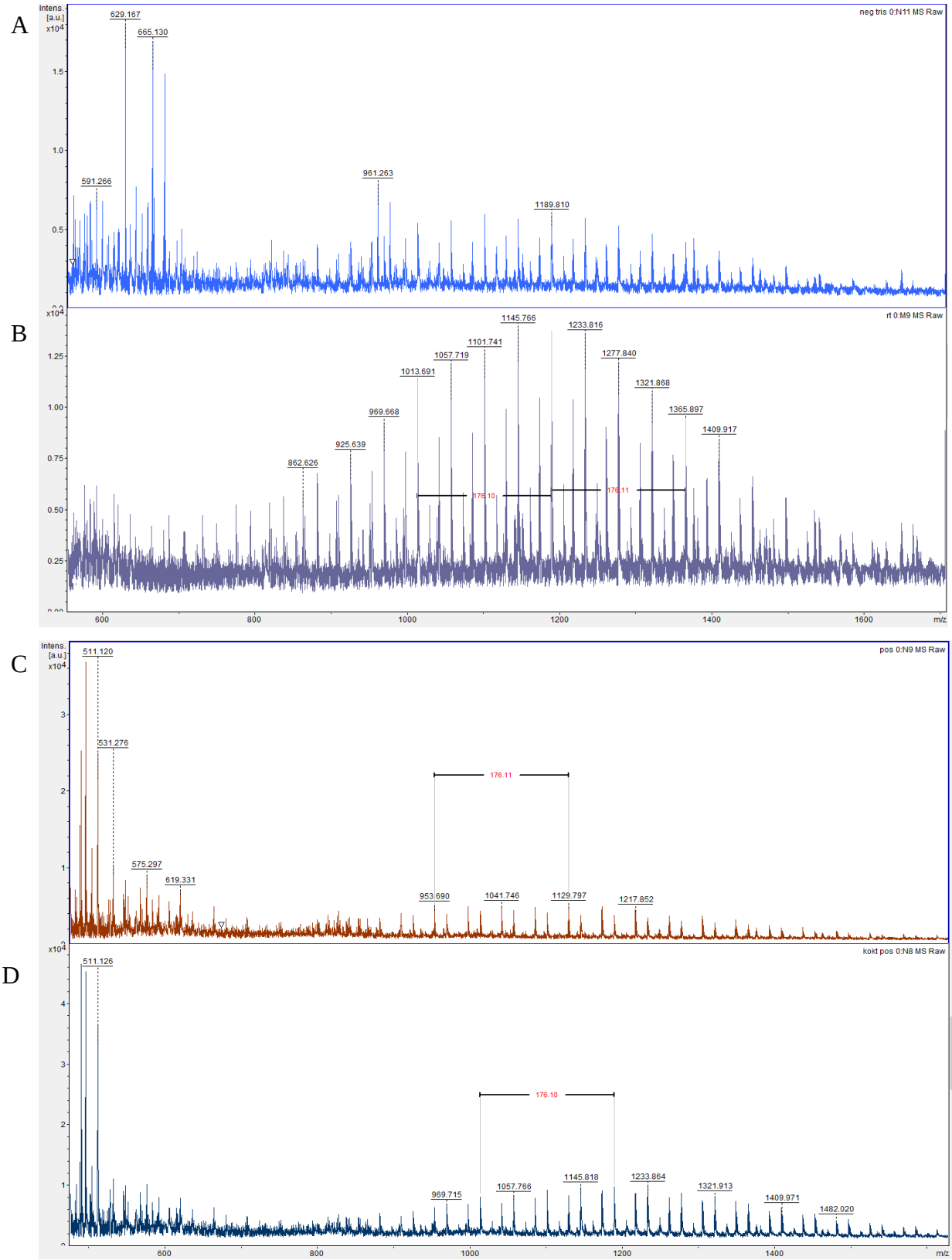

Figure 31. MALDI-TOF MS spectra analyzed with positive mode. *The spectra show: (A) the negative control for* Pa*Aly7a containing buffer and sodium alginate (in blue), (B)* Pa*Aly7a incubated at RT (in purple), (C) AMOR_PL7A (in brown), and (D) boiled AMOR_PL7A (in dark blue), analyzed with positive mode on MALDI-TOF MS. The x-axis is the m/z, and the y-axis is the intensity measured in absorbance units (a. u.).*

After comparing the boiled samples, and the negative control, against not-boiled samples, no significant differences were found, and the spectra looked similar (**Figure 31**). Peaks with a size difference of 176 were visible in **Figure 31B**, and this size could have represented a monosaccharide of alginate, a DEH. However, this spectrum was also visible in samples without protein (**Figure 31A**) and in the sample with boiled protein (**Figure 31D**). Therefore, no activity of protein could be detected using MALDI-TOF MS under these conditions. The samples were therefore analyzed with negative mode on MALDI-TOF MS. However, this method did not give a sufficient difference between the negative control and AMOR_PL7A with sodium alginate (**Figure 32**).

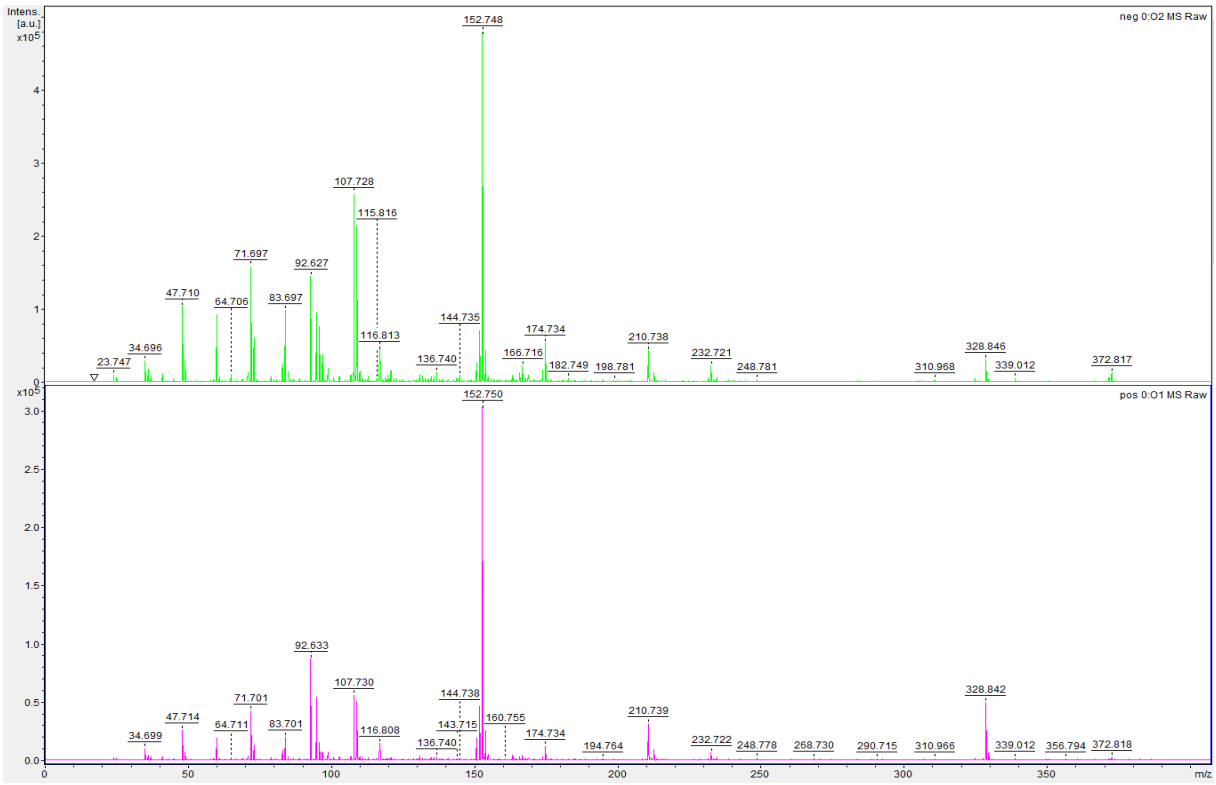

Figure 32. MALDI-TOF MS spectra analyzed with negative mode of AMOR_PL7A and negative control. *The spectra show: (A) the negative control (in green) and (B) the positive control (in pink) analyzed with negative mode on MALDI-TOF MS. Positive control consisted of AMOR_PL7A incubated in buffer and sodium alginate, and the negative control consisted of buffer and sodium alginate. The x-axis is the m/z, and the y-axis is the intensity measured in absorbance units (a. u.).*

4.5.4 Agar plate biofilm degradation assays

In the activity assays, DNS and A_{235} were *Pa*Aly7a and AMOR_PL7A only tested against alginate solutions, and *Pa*Aly7a showed no activity against sodium alginate from brown algae or a crude alginate preparation from *P. aeruginosa* (**3.13**). Therefore, other sources of polysaccharides in *P. aeruginosa* biofilm were tested. The *P. aeruginosa* PAO1 strain can

create a biofilm with the exopolysaccharide's alginate, Pel, and Psl. PAO1 ΔWspf overproduces the exopolysaccharide Pel and Psl, while PAO1 ΔWspf ΔPsl will overproduce Pel, and PAO1 ΔWspf ΔPel will overproduce Psl. The mucoid strain PAU2 will overproduce alginate. These different strains can produce five different kinds of biofilms and were of interest to further investigate the enzyme activity of *Pa*Aly7a (**3.14.4**).

Diluted overnight cultures of PAO1, PAO1 ΔWspf, PAO1 ΔWspf ΔPsl, and PAO1 ΔWspf ΔPel were added to LB agar plates without antibiotics (**3.14.4**). The hypothesis was that the protein could be able to degrade the biofilm or prevent biofilm formation, causing a clear zone without biofilm formation. Both *Pa*Aly7a purified on IMAC (**3.9.1**), *Pa*Aly7a-P1, and *Pa*Aly7a-P2 (**3.9.2**) were tested. Neither of these showed any haloes (results not shown). After testing the different PAO1 strains, the mucoid strain PAU2 was tested with AMOR_PL7A and *Pa*Aly7a-P2.

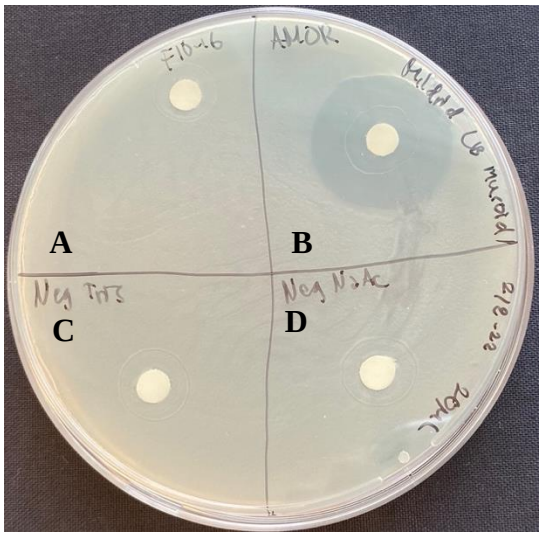

***Figure 33*. Formation of haloes on biofilm.** *In this figure, PAU2 was spread on an LB agar plate with four different samples added. The figure shows the following samples: (A): PaAly7a-P2, (B): AMOR_PL7A, (C): negative control with Tris-HCl buffer, (D): negative control with NaAc buffer.*

Neither of the negative controls nor the *Pa*Aly7a-P2 added to the LB agar plate with PAU2 showed a clear zone (**Figure 33**). AMOR_PL7A did create a clear halo of approximately 2.5 cm. In **Figure 33**, it is possible to see a ring where the protein was added, but the halo extends past this ring.

4.5.5 Biofilm degradation assays

The agar plate degrading assay was a crude assay, and therefore a second biofilm assay was tested. PAO1 and its mutants were used to create four different biofilms in a 96-wells plate. Since the *Pa*Aly7a-P1 and *Pa*Aly7a-P2 did not show activity against PAO1 and the mutants, AMOR_PL7A were tested. The hypothesis was that when adding AMOR_PL7A to the biofilm, the biofilm could be degraded if the enzyme had activity towards biofilm polysaccharides other than alginate. A degraded biofilm would be washed away, so when adding color, the wells with little or no biofilm would have a small color change compared to the wells with plenty of biofilms. To verify this experiment, negative controls were used. For each sample, two wells were created, one was added AMOR_PL7A, and the other was added dH_2O (**3.14.5**).

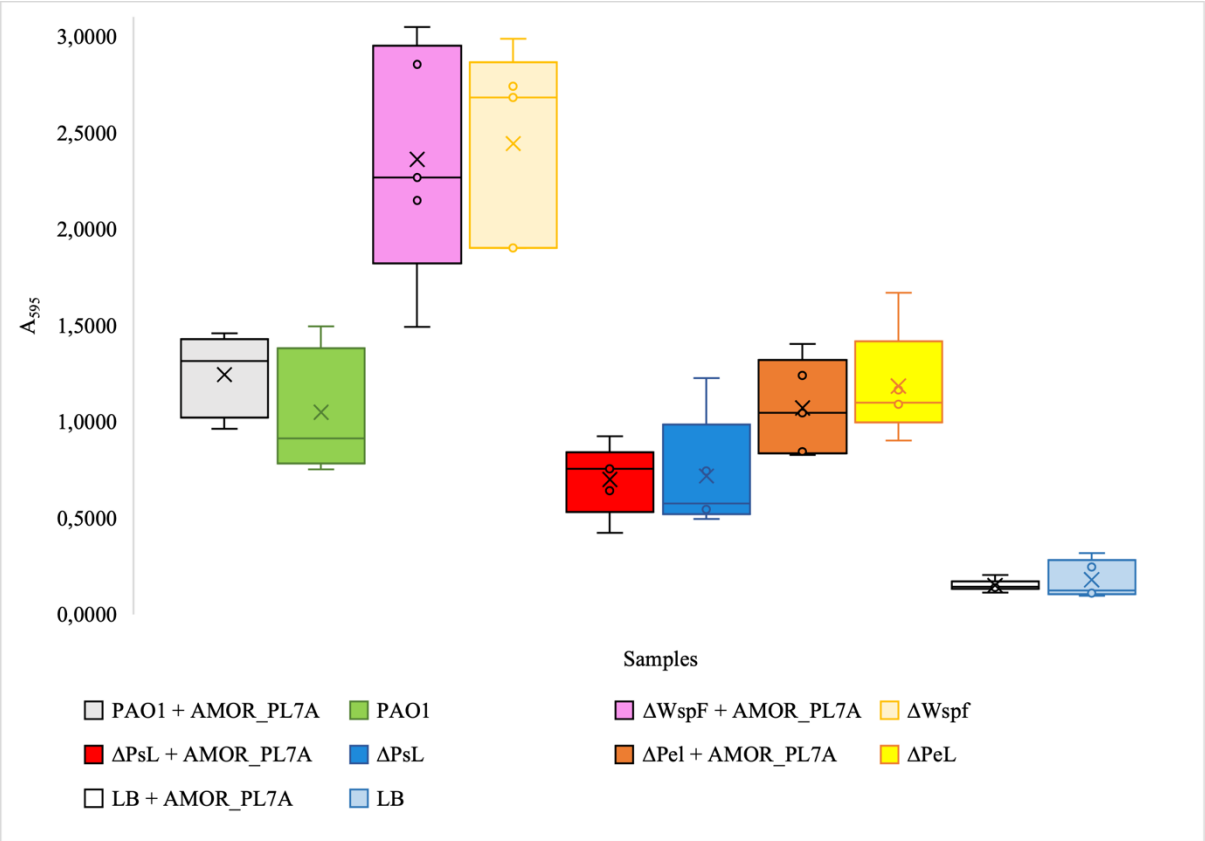

***Figure 34*. Microtiter biofilm assay with AMOR_PL7A.** *This box diagram shows the microtiter biofilm assay for PAO1, PAO1 ΔWspf (ΔWspf), PAO1 ΔWspf ΔPsl (ΔPsl), and PAO1 ΔWspf ΔPel (ΔPel), with and without adding AMOR_PL7A. The LB samples, with and without AMOR_PL7A, consisted only of LB medium. Samples with a black figure outline were added enzyme. There were five replicates per sample, and the average score for each sample is marked with a cross. The samples were measured at* A_{595}.

After measuring the samples at A_{595}, the difference between the samples with AMOR_PL7A and the samples with dH_2O was marginal (**Figure 34**). The average absorbance for the samples

with protein and the samples with dH_2O was approximately the same, despite the considerable variation between some of the replicates.

4.5.6 DNA-degradation assay

*Pa*Aly7a did not show any activity with alginate or biofilm. The results from the STRING analysis were therefore investigated further, and due to a link between *Pa*Aly7a and a DNase, a new activity assay was tested with DNA as the substrate (**Figure 14**). eDNA is an important part of the biofilm structure, and the eDNA is released from *P. aeruginosa* through cell lysis (Allesen-Holm et al., 2006). Since the protein *Pa*Aly7a is a part of the *P. aeruginosa* genome, DNA from *P. aeruginosa* was used. This was done by isolating genomic DNA from overnight cultures of PAO1 (**3.14.6**). This procedure took some time and did not create enough DNA, so pBAD+*PA14_41500* from earlier cloning was also used as substrate (**3.6.4**). *Pa*Aly7a-P1, *Pa*Aly7a-P2, negative controls, positive control with restriction enzymes, and AMOR_PL7A were then incubated with DNA overnight (**Figure 35A**). All these samples were analyzed on a 1 % agarose gel.

The results from the gel showed a possible degrading of DNA from PAO1 by *Pa*Aly7a-P2 (**Figure 35A**). In the figure, *Pa*Aly7a-P2 caused a smear with a lower intensity at a lower size range than the other protein sample and the negative control. The positive control with restriction enzymes shows quite similar results as *Pa*Aly7a-P2. These results may be caused by binding between the protein and the DNA, degradation of DNA by the protein, or contamination. Fractions 10-16, in addition to negative control and positive control with restriction enzymes, were therefore tested again with pBAD+*PA14_41500*. Fractions 10-16 were incubated overnight, and multiple samples were collected at different time points. To check if the protein was only binding to the DNA, one sample from each time point was boiled before it was placed in the freezer. Every sample was analyzed on a 1 % agarose gel.

The DNA incubated with *Pa*Aly7a-P2 was less and less visible over time, while the DNA in both the negative sample and the positive sample was present (**Figure 35b**). All samples contained the same amount of DNA, and the last sample of *Pa*Aly7a-P2 with DNA was incubated for the same duration as the controls. To verify that the protein sample did not include a contaminating DNase that could be responsible for the DNase activity observed, the protein sample was analyzed by proteomics (**3.15.2**).

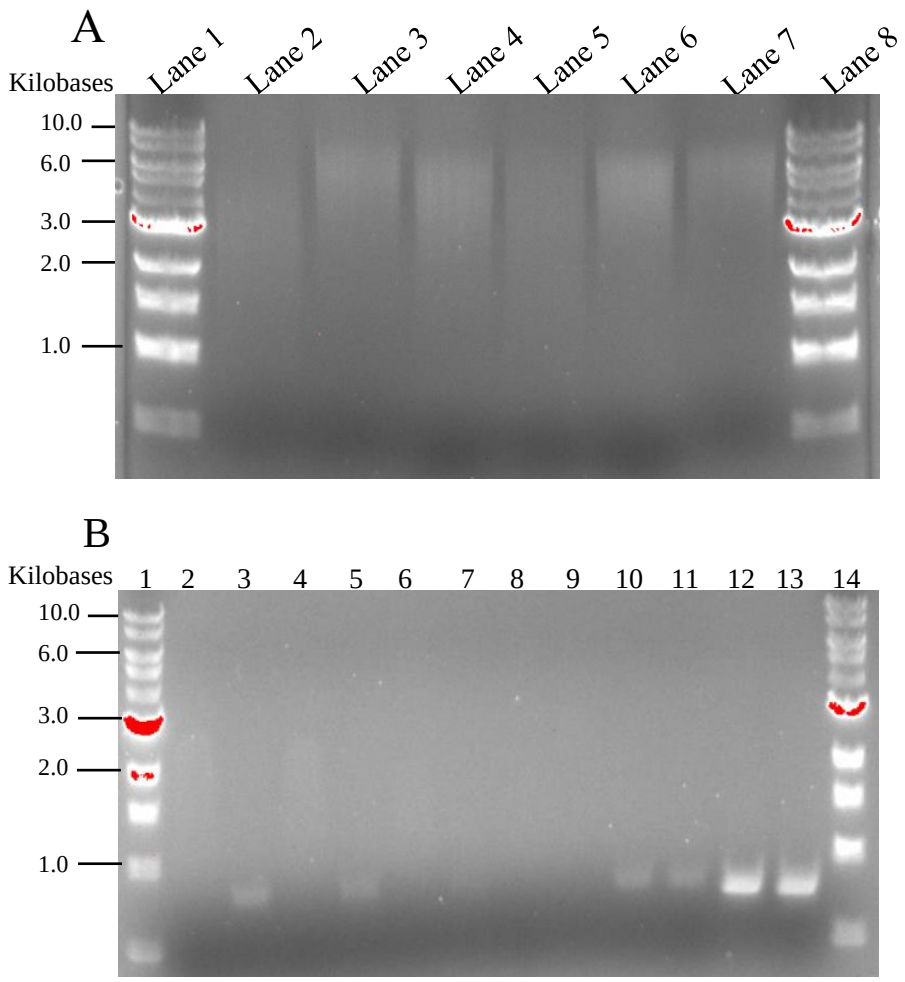

Figure 35. DNA degradation assay on an agarose gel. *These figures show samples of PaAly7a incubated with DNA and controls. (A) PaAly7a from SEC was incubated with 35 ng/µL DNA from PAO1. The lanes show the following samples: 2: DNA with restriction enzymes EcoRI and XhoI, 3: AMOR_PL7A with DNA, 4: PaAly7a-P1 incubated with DNA, 5: PaAly7a-P2 incubated with DNA, 6: negative control with Tris-HCl and DNA, 7: negative control with NaAc and DNA, 1 and 8: QuickLoad® 1kb ladder. (B) This figure shows PaAly7a-P2 incubated with pBAD+PA14_41500. Lanes 3, 5, 7, 9, 11, and 13 were the same samples as the ones added to 2, 4, 6, 8, 10, and 12; only the odd samples were boiled for 5 minutes after collection. The lanes show the following samples: 2, 3: PaAly7a-P2 incubated with pBAD+PA14_41500 for 5 minutes, 4, 5: PaAly7a-P2 incubated with pBAD+PA14_41500 for 60 minutes, 6, 7: PaAly7a-P2 incubated with pBAD+PA14_41500 for 3 hours, 8, 9: PaAly7a-P2 incubated with pBAD+PA14_41500 overnight, 10, 11: the negative control with Tris-HCl and pBAD+PA14_41500, 12, 13: pBAD+PA14_41500 added AflII and EcoRI, 1 and 14: QuickLoad® 1kb ladder.*

4.6 Proteomics analysis of *Pa*Aly7a-P2

In the proteomics analysis of *Pa*Aly7a-P2, the goal was to investigate whether an enzyme capable of DNA degradation was present or not. In the agarose assay (**4.5.6**), the DNA was degraded over time, and the hypothesis was that *Pa*Aly7a-P2 was able to degrade DNA. *Pa*Aly7a-P2 were therefore prepared with the Strap protocol (**3.15.2**) and sent for analysis on

the Orbitrap. The peptide analysis was performed by Morten Skaugen, and the results were examined by Per Kristian Edvardsen.

Table 25. ***Proteomics of* Pa*Aly7a-P2***. *The table shows the result from Orbitrap with UniProt ID, gene name, protein, sequence coverage, and intensity for the top 10 proteins identified in the protein sample PaAly7a-P2.*

UniProt ID	**Gene name**	**Protein**	**Sequence coverage (%)**	**Intensity** *Pa*Aly7a
A0A0H2Z9V3	*PA14_41500*	Putative lyase	100	39,466
A0A140NG17	ECBD_3624	Transcriptional regulator, AraC family	63	37,344
A0A140NB62	ECBD_1819	rRNA (Guanine-N(1)-)-methyltransferase	79,9	37,075
A0A140NE13	fur	Ferric uptake regulation protein	100	36,894
A0A140N6V1	ECBD_0400	Peptidyl-prolyl cis-trans isomerase	50,5	36,575
A0A140ND09	ECBD_2498	Histidine triad (HIT) protein	97,5	36,257
A0A140NA89	nei	Endonuclease 8	84,4	35,872
A0A140N7Y4	ECBD_1474	Pseudouridine synthase	90,9	35,859
A0A140N421	rlmJ	Ribosomal RNA large subunit methyltransferase J	90,7	35,643
A0A140N6H0	mutM	Formamidopyrimidine-DNA glycosylase	91,1	35,596

Among the top 10 identified proteins, Endonuclease 8 was found (**Table 25**), indicating that the DNA degradation observed for purified *Pa*Aly7a-P2 was likely the result of a contaminating exonuclease.

5 Discussion

This thesis aimed to investigate the activity of the putative family PL7 alginate lyase *Pa*Aly7a from *P. aeruginosa*. Initial sequence analysis showed that the *Pa*Aly7a sequence contained the conserved regions diagnostic for the activity of PL7 enzymes (**Figure 9**). Nevertheless, no activity towards alginate, neither with pure alginate from brown algae nor crude bacterial alginate isolated from *P. aeruginosa*, could be observed for the purified protein. Based on the results presented in this thesis, there are three alternative explanations to why activity towards alginate was not detected – the protein was not appropriately folded, the protein is active towards an alginate variant that was not tested, or the protein is active towards a substrate different from alginate. All these scenarios will be discussed below.

5.1 Protein folding

It is usually taken for granted that proteins that appear soluble after expression and purification are correctly folded, thus showing the expected activity. This is usually a correct hypothesis, but it may not be accurate in some instances. For example, hNK_1R showed no activity in a ligand-binding assay even though the protein was soluble Bane et al. (2007). The solubility of the protein can therefore not determine if a protein is active or not. Toledo 2010 suggests that soluble aggregates are just in a preface before becoming insoluble. Analysis of SDS-PAGE gel and purification on IMAC and SEC indicated that *Pa*Aly7a was soluble, but neither activity assay showed any signs of activity.

The protein expression experiments caused multiple difficulties, and different solutions were tested to obtain pure *Pa*Aly7a. Three different constructs were used to express *Pa*Aly7a: pNIC-CH+*PA14_41500*, pBAD+*PA14_41500*, and pET+*PA14_41500*. pBAD gave a deficient protein expression, but neither the pNIC-CH+*PA14_41500* nor pET+*PA14_41500* expressed a significant amount of protein compared to the control protein production ChiC (**Figure 23**) (**Table 24**). The eluted product of *Pa*Aly7a from IMAC purification included several proteins in addition to the *Pa*Aly7a, which indicated an impure product (**Figure 23**). Different methods were tested to purify further and obtain more protein, but the changes did not make any difference (**Appendix C**). Due to the low expression, total protein samples were analyzed with SDS-PAGE, and inclusion bodies were identified. Inclusion bodies can be formed in *E. coli* due to the forced mass production of one protein. This mass production causes stress for the bacteria, which will translate the amino acid sequence faster than the protein manages to fold.

Therefore, the polypeptide will incorrectly fold and form aggregates of inactive protein. Other reasons behind this aggregation are the lack of chaperones in *E. coli*, high concentration of inducer, a too strong promoter, and too high temperature during incubation (Singh et al., 2015). The inclusion bodies had a much stronger signal than the lysate samples, which indicated that most of the protein formed inclusion bodies (**Figure 21**). By finding a solution for refolding the inclusion bodies or preventing the formation, the yield of *Pa*Aly7a could be much higher (Singh et al., 2015).

Furthermore, two peaks were detected when *Pa*Aly7a was further purified by SEC. Based on the properties of SEC, two different peaks indicate that the proteins eluted are of two different sizes (**Figure 24**). Nevertheless, the SDS-PAGE gel analysis showed that both proteins had a molecular weight of 27 kDa (**Figure 25**). There are two possible explanations for this, either that *Pa*Aly7a formed a multimer with non-covalent bindings or that it formed soluble aggregates. Aggregation can be caused by numerous stresses and other exterior factors, such as pH and temperature (den Engelsman et al., 2011). Ryan et al. (2012) showed that aggregated protein would be eluted earlier than native proteins during purification with SEC. Since the protein had been purified and analyzed with SEC, the protein was apparently soluble. According to den Engelsman et al. (2011), proteins can form aggregates during purification on SEC, and as previously mentioned, proteins can form soluble aggregates as a pre-step before forming insoluble aggregates (Toledo-Rubio et al., 2010). Since SDS can only disturb non-covalent bindings, the first peak had to be a multimer or an aggregate with non-covalent binding (den Engelsman et al., 2011). Both peaks were therefore analyzed further with melting point analysis and activity assays.

Based on the melting point analysis of the two peaks from the SEC purification, *Pa*Aly7a-P2 showed a promising protein melting point curve and could therefore be considered a folded protein, in contrast to *Pa*Aly7a-P1 (**Figure 26**). This hypothesis is supported by Crowther et al. (2010), who presented evidence that melting curves could be used to indicate whether a protein is adequately folded or not. However, no positive control was included during the protein melting point analysis. Complete verification of a positive protein melting curve could therefore not be done. Therefore, kindly provided by Ole Golten (Golten, 2020), **Figure 36** is included as a positive control. The figure shows a protein melting point analysis of $rCbpD_{EC}$ M1 with an apparent T_m (**Figure 36**). By comparing the $rCbpD_{EC}$ M1 graph (**Figure 36**) and the negative control (**Figure 26C**) with *Pa*Aly7a-P2 (**Figure 26B**), the graph representing *Pa*Aly7a-P2 is

most similar to the rCbpD$_{EC}$ M1 graph (**Figure 36**). Therefore, **Figure 26B** indicates that *Pa*Aly7a-P2 is a properly folded protein.

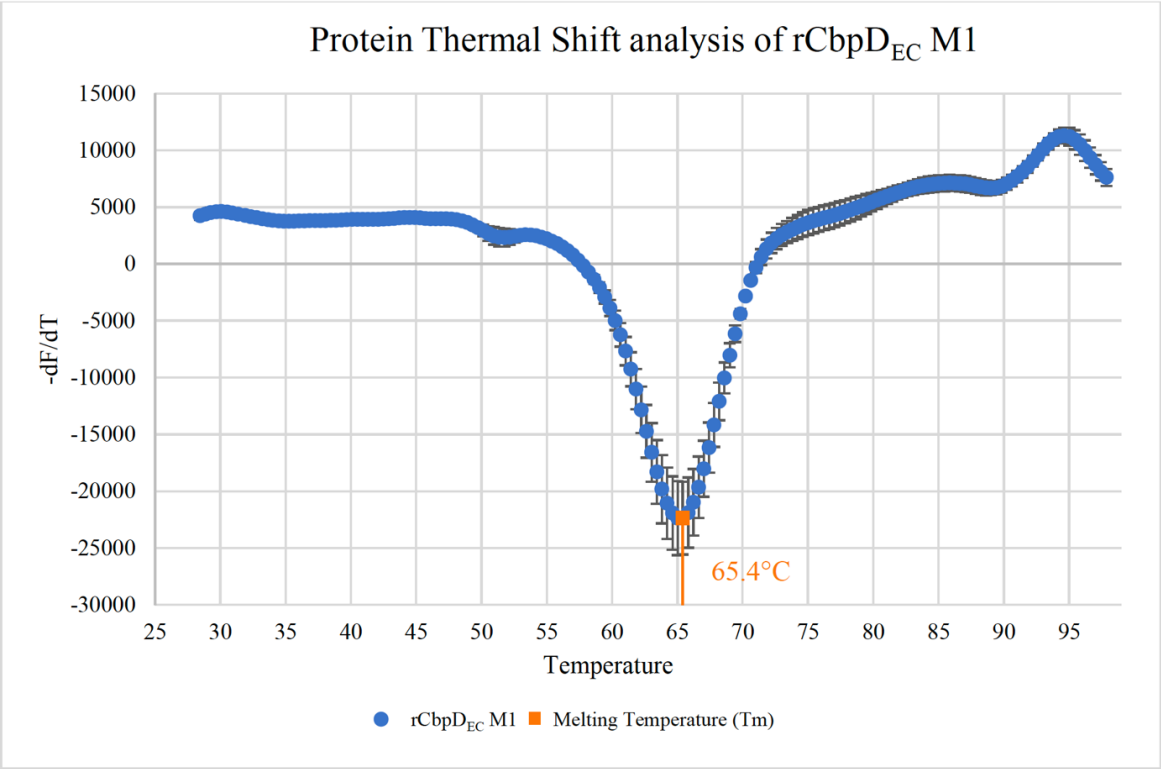

Figure 36. Protein melting point analysis of rCbpD$_{EC}$ M1*. The sample was mixed with orange dye and slowly heated from 25 to 100 ºC. The y-axis shows the first derivative of fluorescence (F) emission as a function of temperature (T), while the x-axis shows the temperature (T). The blue line represents the average score for the four replicas used, with standard deviation. The figure is obtained from (Golten, 2020).*

5.2 Alternative activity

5.2.1 Towards an alginate variant not tested

Given that the protein fraction collected in the SEC experiment showed the correct molecular weight and the promising melting curve indicated a correctly folded version of *Pa*Aly7a, an alternative explanation of the lack of activity observed for *Pa*Aly7a can be discussed. The enzyme might be active towards an alginate variant different from what was used in the experiments described in this thesis. There are several different types of alginates, and different alginate lyases often have specific activities (Zhu & Yin, 2015). Alginate can contain only mannuronic acids (polyM), guluronic acids (polyG), or a random mix of both (polyMG) (Haug & Larsen, 1966). In this thesis, the sodium alginate tested contained both mannuronic acid and guluronic acid, according to the producer (Sigma Aldrich). The alginate purified from *P.*

aeruginosa consisted mainly of acetylated polyM or polyMG (Davidson et al., 1977; Skjåk-Bræk et al., 1986).

All biochemical experiments are dependent on a positive control for experimental validation. In the current work, AMOR_PL7A was used as a positive control for the degradation of brown algae alginate. Indeed, activity for sodium alginate was observed for AMOR_PL7A using both the DNS reducing end assay and A_{235} monitoring the generation of double bond assays (**Figure 27** and **Figure 29**). These results were expected due to the previous characterization of AMOR_PL7A (Vuoristo et al., 2019). Vuoristo et al. (2019) showed that AMOR_PL7A had a higher affinity for polyM and sodium alginate than polyG and polyMG. AMOR_PL7A also showed degradation or prevention of mucoid biofilm formation biofilm by the strain PAU2. However, the purification of alginate from this strain showed no activity with AMOR_PL7A. The purification of alginate from *P. aeruginosa* was relatively crude, and a purity test of the alginate was never done. Therefore, the assays with purified alginate might not have given the correct results according to activity.

One of the conserved regions in the *Pa*Aly7a sequence might explain the lack of activity against sodium alginate and alginate purified from *P. aeruginosa*. Alginate lyases from *P. aeruginosa* consist of three conserved regions, and one of these is associated with substrate affinity; Q(I/V)H (Zhu & Yin, 2015). *Pa*Aly7a exhibits the sequence motif QIH, indicating that it is a guluronate-specific alginate lyase or a protein able to degrade polyMG (**Figure 9**). Proteins with the sequence motif QVH are more likely to be specific for ß-D-mannuronic acid (Zhu & Yin, 2015). Douthit (2004) also stated that PA1784 was a guluronic-specific protein, which indicates that polyG alginate should have been tested. In addition, Jagtap et al. (2014) proved that the alginate lyases OalA, OalB, and OalC from *Vibrio splendidus* were oligoalginate lyases. These proteins are classified as members of PL15 or PL17. However, not all proteins with the same activity have identical catalytic residues; for example, Alg17C was characterized as an enzyme with the identical reaction mode as PL15 alginate lyases. Nevertheless, Alg17C did not have the same catalytic residues for alginate as the other alginate lyase categorized as members of PL15 (Kim et al., 2012; Ochiai et al., 2010). The activity of *Pa*Aly7a could not be determined in this thesis, possibly explained by the lack of alginates tested.

5.2.2 Towards substrate not tested

A third explanation of the lack of activity towards alginate can be that the protein has evolved a different function and activity. This would also be in line with the data obtained by Douthit (2004), which did a molecular microbiological analysis of PA1167 and PA1784. In her research, PA1784 was suggested not to be an active alginate lyase since the mucoidy of the *P. aeruginosa* strains FRDl and FRDl153 with PA1784 overexpressed was unchanged. Other results support this conclusion. For example, the predicted structure of PA1784 differs in the active site compared to known active alginate lyase PA1167 (Yamasaki et al., 2004) (**Figure 13**). Based on the Alphafold2 model, the active site of PA1784 was tighter than the active site in PA1167, which might cause trouble for the protein when binding to alginate. In addition, PA1784 had a more negatively charged area around the active site than PA1167 (**Figure 13**). Alginate is negatively charged, and PA1784 might not bind to the alginate since two negatively charged molecules repel each other. Therefore, PA1784 might be adapted to a smaller and less negative substrate. This substantiates the hypothesis that PA1784 has activity against another substrate than alginate. However, the structure presented by Alphafold2 is a predicted structure based on the amino acid sequence (Jumper et al., 2021; Varadi et al., 2021). According to Alphafold2 own estimates, two areas are listed with a low or confident confidence (respectively 70 > pLDDT > 50 or 90 > pLDDT > 70, of 100). These areas are around the active site and might therefore be incorrect. A correct structure of the protein could have been obtained by crystallization (McPherson & Gavira, 2014). However, a protein with high quality and in a high concentration with no aggregation is needed to crystallize a protein. To get that, the problem with protein expression in this thesis needs to be fixed.

Identifying the activity of a protein with another function than expected might be challenging. However, the STRING analysis showed that there was a high possibility, based on data, that PA1784 had a physical or functional association with the DNase eddB (**Figure 14**), which might imply that *Pa*Aly7a can degrade DNA. Prasad et al. (2009) showed that other lyases also could cleave DNA through β-elimination: for example, the human DNA polymerase θ possesses 5′-dRP lyase activity (Prasad et al. (2009). DNA is an essential part of the *Pseudomonas* biofilm, so an assay with *Pa*Aly7a and DNA was tested. The results from the assay showed that DNA was less visible over time with *Pa*Aly7a-P2 added (**Figure 35**). As a control, *Pa*Aly7a-P2 was analyzed with a proteomics analysis. The result identified a DNase as one of ten proteins with the highest intensity in the sample (**Table 25**). Therefore, the DNA degradation was probably due to the DNase caused by contamination.

Alginate lyases have a debated role in the degradation of *P. aeruginosa* biofilm. Lamppa and Griswold (2013) showed that the activity of the proteins did not affect the biofilm degradation but rather the proteins themselves. On the other hand, Mahajan et al. (2021) showed that *Ca*Aly, *Vsp*AlyVI, *Fsp*AlyFRB, and SA1-IV were able to inhibit biofilm formation in the *Pseudomonas* strain 2843. Alkawash et al. (2006) also found that alginate lyase from *B. circulans* ATCC 15518 cooperated with gentamicin to increase the elimination of mucoid biofilm of *P. aeruginosa*. As stated in these studies, further testing on alginate lyase is essential, and *Pa*Aly7a might be a part of that. However, the activity of *Pa*Aly7a first needs to be determined, and then the protein can be tested as a possible treatment for mucoid biofilm. In this thesis, only *in vitro* testing has been proceeded. However, when the protein is characterized, testing with sputum from patients could determine if *Pa*Aly7a has the potential to disrupt *P. aeruginosa* biofilm in the lungs of patients with CF.

5.3 Concluding remarks and further work

The focus of this study was to test the activity of *Pa*Aly7a from *P. aeruginosa* PA14. The aim was to analyze this enzyme biochemically, investigate the possibilities for new methods for degrading alginate, and possibly identify a new method for treating mucoid biofilm of *P. aeruginosa* in patients with CF. Therefore, *Pa*Aly7a was produced and purified to test the protein biochemically.

*Pa*Aly7a showed no signs of activity when tested with different activity assays. Based on the protein melting point results, *Pa*Aly7a-P2 was probably correctly folded. Therefore, the lack of activity might be due to the lack of the correct substrate. The STRING analysis and the DNA-degradation assay indicated that *Pa*Aly7a had activity against DNA, a vital part of the *P. aeruginosa* biofilm. However, a DNase was identified in the protein sample, probably due to contamination. Therefore, to test this experiment further, *Pa*Aly7a should be purified with ion-exchange chromatography or hydrophobic interaction chromatography to hopefully remove the DNase. The DNA-degrading assay tested in this thesis was relatively crude. Therefore, an activity assay such as the Kunitz assay, which determines the number of free nucleotides released by measuring the absorbance at 280 over a time, could have been tested (Kunitz, 1950). If the result from this second experiment showed promising results, *Pa*Aly7a should be further tested with eDNA from *P. aeruginosa* biofilm for an opportunity to identify a new treatment for mucoid biofilm.

Out of curiosity, the AMOR_PL7A was tested on the agar plate biofilm degradation assay. The AMOR_PL7A showed promising results with the mucoid strain PAU2. AMOR_PL7A has been thoroughly characterized by (Vuoristo et al., 2019), but no results are shown with AMOR_PL7A tested with the mucoid strain PAU2. Further work on AMOR_PL7A and PAU2 can give new methods for degrading alginate.

The expression and purification of *Pa*Aly7a turned out to be problematic. For further research on the protein, the formation of inclusion bodies should have been prevented. A possible solution to avoid developing inclusion bodies could have been to transform the plasmid into *E. coli* Arctic Express (DE3). *E. coli* Arctic Express (DE3) can be incubated at temperatures from 4-12 °C and still produce active proteins since it contains two co-expressed cold-adapted chaperonins (Hartinger et al., 2010). Incubation at 4-12 °C can prevent the formation of inclusion bodies since standard incubation temperatures can impair the formation of active proteins. Hartinger et al. (2010) tried many different approaches when they wanted to express the corresponding gene *fumI* in *E. coli*, but due to inclusion bodies, few of them produced enough protein. The best solution for their protein was to use Arctic Express (DE3). An important issue with this strain is that the chaperones can co-purify with the protein of interest and, therefore, interact with the protein's activity and stability (Belval et al., 2015). Another solution could have been to refold the inclusion bodies produced with three different steps. The first step is to isolate and wash the inclusion body, the second step is to solubilize the aggregated protein, and the last step is to refold the solubilized protein (Clark, 2001). Singh et al. (2015) listed four different steps for protein recovery. Their list included the first three steps, plus purifying the refolded protein. However, both articles mentioned that refolding inclusion bodies was a complicated procedure and may have a meager recovery yield.

The role of alginate lyase activity in degrading *P. aeruginosa* biofilm is still discussed, but scientists agree that further studies on the subject can participate in the discovery of new treatments for *P. aeruginosa* infections. *P. aeruginosa* is a well-studied bacterium, but there are still several unanswered questions about the bacteria and the proteins produced by the bacteria. This thesis discovered no new method for degrading alginate nor treating mucoid biofilm of *P. aeruginosa* with *Pa*Aly7a. However, new insights on the protein *Pa*Aly7a and AMOR_PL7A were presented. Further knowledge about alginate lyase and *P. aeruginosa* can be obtained by continuing this research.

6 References

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7 Appendix A

7.1 Laboratory Equipment and Materials

Table A 1. Laboratory Equipment and Materials. *An overview of which equipment has been used in this study with the supplier of the equipment.*

Category	**Equipment**	**Supplier**
Appliances	Certoclav Sterilizer, A-4050	CertoClav
	Freezer, -20 °C	Bosch
	Freezer, -80 °C	Sanyo
	Incubator 37 °C, New Brunswick™ Scientific Innova 44	Eppendorf
	Incubator, 37 °C	Termaks
	Laminar Flow Workbench, Telstar AV-100	Azbil Telstar, S.L.
	Microwave Oven, MD142	Whirlpool
	Milli-Q® Direct Water Purification System, Direct 16	Merck Millipore
	Refrigerator, 4 °C	Bosch
	Safe 2020 Laminar flow workbench	Thermo Fisher Scientific
Centrifuges	Allegra X-30R	Beckmann Coulter
	Avanti™ J-26S XP	Beckman Coulter
	Benchtop Centrifuge 5418R	Eppendorf
	Sorvall LYNX 6000	Thermo Fisher Scientific
	Heraeus™ Multifuge™ X1R	Thermo Fisher Scientific
	F21-8 X 50Y	Thermo Fisher Scientific
	JLA 8.1 rotor	Beckman Coulter
	Microcentrifuge, Heraeus™ Pico™ 21	Thermo Fisher Scientific
	Microcentrifuge, MiniStar	VWR
	Plate spin II	Kubota
	Vacufuge plus	Eppendorf
Filtration Equipment	Membrane dry vacuum pump/compressor, VCP 80	VWR

	Multiscreen®HTS 96-Well Plates, 0.45 µm Durapore Membrane	Merck Millipore
	Multiscreen®HTS Vacuum Manifold	Merck Millipore
	Syringe Filtration Unit, Filtropur S 0.2 µm PES Membrane	Sarstedt
	Ultrafiltration Unit Vivaspin® 20, 10,000 MWCO PES	Sartorius
	Vacuum Filtration Systems, 0.2 µm PES Membrane	VWR
	Vivaspin 20 centrifugal concentrators 3 000 MWCO	Sartorius
Gel Equipment	8-Well Comb, and 15-Well Comb	Bio-Rad
	Bench top UV Transilluminator	UVP
	Criterion™ IEF Precast gels	Bio-Rad
	Electrophoresis Cell, Mini-PROTEAN® Tetra Vertical	Bio-Rad
	Electrophoresis System, Mini-Sub® Cell GT Horizontal	Bio-Rad
	Gel Casting Tray	Bio-Rad
	Gel Imagine System, Gel Doc™ EZ	Bio-Rad
	Power Supply, PowerPac™ 300	Bio-Rad
	Power Supply, PowerPac™ Basic	Bio-Rad
	Precast SDS Gels, Mini-PROTEAN® TGX Stain-Free	Bio-Rad
	Stain-Free Sample Tray	Bio-Rad
	UV Sample Tray	Bio-Rad
Instruments and Columns	ÄKTA Pure	GE Healthcare Life Sciences
	ÄKTA Start	GE Healthcare Life Sciences
	Bransonic® Ultrasonic Cleaner, Branson 3510	Sigma-Aldrich
	Cell density meter, Ultrospec 10	Biochrom

	HiLoad® 16/600 Superdex® 75 pg	GE Healthcare Life Sciences
	IMAC Column, HisTrap™ HP, 5 mL	GE Healthcare Life Sciences
	MP Biomedicals™ FastPrep -24™ Classic Instrument	Thermo Fisher Scientific
	NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer	Thermo Fisher Scientific
	NanoPhotometer® C4 spectrophotometer	Implen
	Orbitrap Exploris™ 480 Mass Spectrometer	Thermo Fisher Scientific
	pH Meter, 913	Metrohm
	QBD2	Grant
	SBB Aqua 5 plus	Grant
	SimpliAmp™ Thermal Cycler	Thermo Fisher Scientific
	Sonics Vibra-Cell™ Ultrasonic Processor, VC750	Sonics & Materials, Inc.
	Spectrophotometer, BioPhotometer D30	Eppendorf
	Thermal cycler, SimpliAmp	Thermo Fisher Scientific
	ThermoMixer® C	Eppendorf
	UltraFlextreme MALDI-TOF-MS	Bruker
	Varioskan LUX	Thermo Fisher Scientific
	Water bath 22-65 °C, Julabo 5A	Julabo
Miscellaneous Equipment	Automated Pipettes	Thermo Fisher Scientific
	Boiling water bath, SBB Aqua 5 plus	Grant
	Cuvettes, Disposable	Eppendorf
	Glassware	Duran Group
	Inoculation Loops, 10 µL, blue	Sarstedt
	Magnet, Teflon Stirring Bar	SP Scienceware
	Magnetic Stirrer, RCT Basic	IKA
	Petri dishes, 90 mm	Heger
	Pipette Refill Tips	Thermo Fisher Scientific and VWR
	qPCR StepOnePlus™	Applied Biosystems

	Scale, Entris 3202I-1S	Sartorius
	Scalpel, Stainless Steel Surgical Blade No. 10	Swann-Morton
	Vortex, MS2 Minishaker	IKA
	µCuvette®	Eppendorf
Tubes, Vials, and Plates	CellStar™ Tubes, 15 and 50 mL	Greiner Bio-One
	Centrifugal bottle 1 L	Thermo Fisher Scientific
	Centrifuge Tubes, Nalgene®, Oak Ridge, Style 3119, 25 mL	Sigma-Aldrich
	CryoPure tubes, 1.8 mL	Sarstedt
	Culture tubes, 13 mL	Sarstedt
	Eppendorf tubes, 1.5 and 2.0 mL	Axygen
	Injection needle LC 0.7 mm	GE Healthcare
	MALDI-plate mtp 384 ground steel	Bruker Corporation
	MicroAmp™ Adhesive Optical covers	Applied Biosystems
	MicroAmp™ Optical 96-well reaction plate	Applied Biosystems
	Micro test plate, 96-well, slip-on lid, flat base, PS, transparent	Sarstedt
	Nunc® MicroWell™ 96-well Polystyrene Plate	Thermo Fisher Scientific
	PCR Tubes, 0.2 mL	Axygen®
	Screw Cap Micro Tubes, 2 mL	Sarstedt
	Snap Ring Micro-Vials, 0.3 mL, and Snap Ring Caps	VWR

7.2 Chemicals

Table A 2. Chemicals. *The table shows every chemical used in this study and the supplier of the chemical.*

Chemical	**Supplier**
10 x NEBuffer r3.1	New England Biolabs
2,5-Dihydroxybenzoic acid (DHB)	Sigma-Aldrich
3,5-Dinitrosalicylic acid ($C_7H_4N_2O_7$)	Sigma-Aldrich
Acetic acid Glacial	VWR
Acetonitrile (ACN)	Honeywell
Agar Powder	VWR
AmBic (Ammonium bicarbonate)	Sigma-Aldrich
Ampicillin disodium salt	Sigma-Aldrich
Antifoam 204	Sigma-Aldrich
Arabinose	Merck
Ascorbic Acid	Sigma-Aldrich
Bacto™ Tryptone	Becton, Dickinson, and Co.
Bacto™ Yeast Extract	Becton, Dickinson, and Co.
BgLII	New England BioLabs
Brain Heart Infusion	Oxoid
Calcium Chloride	Sigma-Aldrich
Calf intestine alkaline phosphatase	Invitrogen
Chlorin	Orkla
cOmplete™, EDTA-free Protease Inhibitors	Rocher
Coomassie G-250	Bio-Rad
Dithiothreitol (DTT) ($C_4H_{10}O_2S_2$)	Invitrogen or Sigma
DNA Gel Loading Dye, 6X	New England Biolabs
DNase 1	Thermo Fisher Scientific
EcoRI	New England BioLabs
Ethanol (C_2H_6O) absolute	VWR
Ethylenediaminetetraacetic acid (EDTA) ($C_{10}H_{16}N_2O_8$)	Merck
Gel Loading Dye purple	New England BioLabs
Glc1 – Glc6 Stock Solution, 1.0 mg/mL	Made in lab
Glucose ($C_6H_{12}O_6$) (Glc1)	Fluka Analytical

Glycerol ($C_3H_8O_3$), 85 %	Merck
Hydrochloric acid (HCl)	Merck
IEF Anode buffer 10X	Bio-Rad
IEF standards 4.45-9.6	Bio-Rad
Imidazole ($C_3H_4N_2$)	Sigma-Aldrich
Iodoacetamide (IAA)	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Isopropyl b-D-1-thiogalactopyranoside (IPTG) ($C_9H_{18}O_5S$)	Sigma-Aldrich
Kanamycin ($C_{18}H_{36}N_4O_{11}$)	Sigma-Aldrich
Lysozyme	Sigma-Aldrich
M9 minimal salts 2x	Gibco
Methanol	Honeywell
Monopotassium phosphate (KH_2PO_4)	Merck
NcoI	New England BioLabs
NEBuffer 2.1	New England BioLabs
NuPAGE® LDS Sample Buffer (4X)	Invitrogen - Thermo Fisher Scientific
NuPAGE® Sample Reducing Agent (10X)	Invitrogen - Thermo Fisher Scientific
peqGREEN DNA/RNA Dye	Peqlab
Phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich
Phosphoric Acid (PA)	Sigma-Aldrich
Potassium sodium tartrate tetrahydrate ($C_4H_4KNaO_6 \cdot 4H_2O$)	Sigma-Aldrich
Quick load 1Kb	New England BioLabs
RedTaq 2x Master	VWR
S.O.C Medium	Invitrogen
SeaKem® LE Agarose	Lonza
Sodium acetate anhydrous (CH_3COONa), BioUltra	Sigma-Aldrich
Sodium alginate	Sigma-Aldrich
Sodium chloride (ClNa)	VWR
SYPRO Orange dye (8x)	Thermo Fisher Scientific

Trifluoroacetic acid (TFA)	Sigma-Aldrich
Tris-Acetate-EDTA (TAE) Buffer (50X)	Thermo Fisher Scientific
Tris-Glycine-SDS (TGS) Buffer (10X)	Bio-Rad
Trypsin	Thermo Fisher Scientific
Tryptone soya broth	Oxoid – ThermoFisher Scientific
UltraPure™ DNase/RNase-Free Distilled Water	Thermo Fisher Scientific
XhoI	New England BioLabs

8 Appendix B

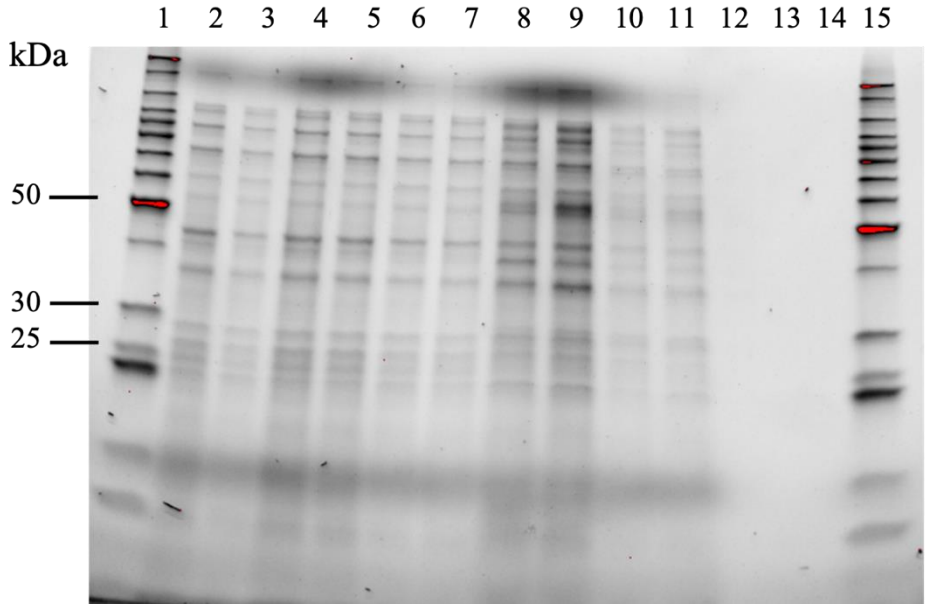


Figure B 1. Small-scale expression trials for pBAD+PA14_41500 incubated in LB and TB medium*. The lanes show the following samples: 1, 15: the protein ladder Benchmark, 2: culture without arabinose in TB, 3: culture without arabinose in LB, 4: 0 hours incubation in TB medium with 0.01 % arabinose, 5: 0 hours incubation in TB medium with 0.1 % arabinose, 6: 0 hours incubation in LB medium with 0.01 % arabinose, 7: 0 hours incubation in LB medium with 0.1 % arabinose, 8: overnight incubated in TB medium with 0.01 %, 9: overnight incubated in TB medium with 0.1 %, 10: overnight incubated in LB medium with 0.01 %, 11: overnight incubated in LB medium with 0.1 %.*

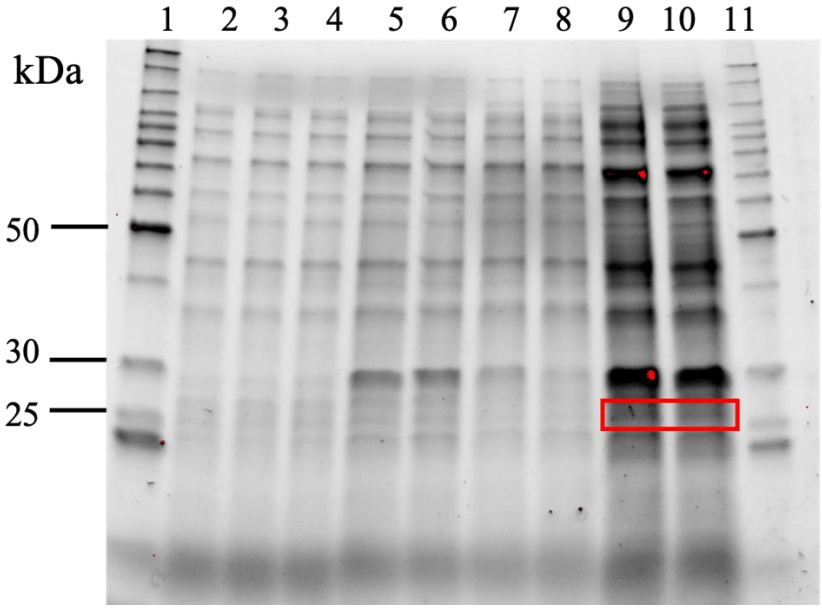


Figure B 2. Small-scale expression trials for pET+PA14_41500 in LB medium*. BL21 with pET+*PA14_41500 *lysate. The lanes show the following samples: 1, 11: the protein ladder Benchmark, 2: culture without IPTG, 3: 0 hours incubation with 1 mM IPTG, 4: 0 hours incubation with 0.2 mM IPTG, 5: 3 hours incubation at 37 °C with 1 mM IPTG, 6: 3 hours incubation at 37 °C with 0.2 mM IPTG, 7: incubated at 37 °C overnight with 1 mM IPTG, 8: incubated at 37 °C overnight with 0.2 mM IPTG, 9: incubated overnight at 20 °C with 1 mM IPTG, 10: incubated overnight at 20 °C with 0.2 mM IPTG*

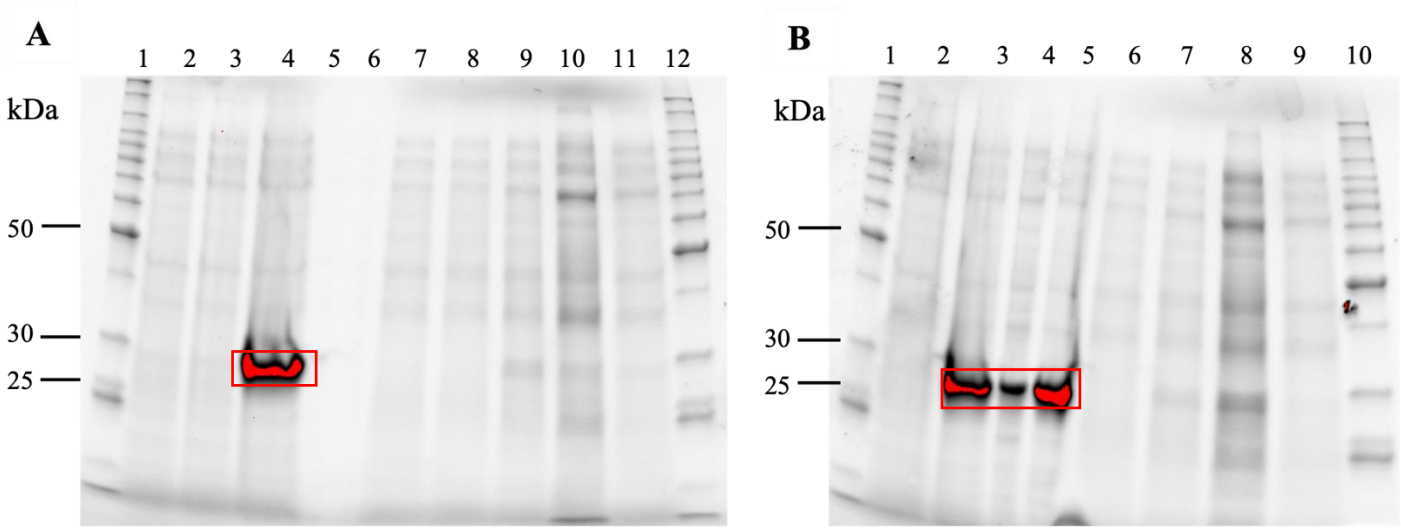


Figure B 3. Small-scale expression trials with pET*+PA14_41500 *in TSB medium. *BL21 with pET*+PA14_41500 *incubated in: (A) 0.2 mM IPTG. In lanes 2-6, the total protein samples were added, while in lanes 7-11 lysate samples were added. The lanes show the following samples: 1, 12: the protein ladder Benchmark, 2, 7: non-induced culture, 3, 8: 0 hours incubation with IPTG, 4, 9: 3 hours incubation at 37 °C with IPTG, 5, 10: incubated at 20 °C overnight with IPTG, 6, 11: incubated at 37 °C overnight with IPTG. Panel (B) shows the following samples incubated with 1 mM IPTG. In lanes 2-5, the total protein samples were added, while in lanes 6-9 lysate samples were added. The lanes show the following samples: 1, 10: the protein ladder Benchmark, 2, 6: non-induced culture, 3, 7: 3 hours incubation at 37 °C with IPTG, 4, 8: incubated at 20 °C overnight with IPTG, 5, 9: incubated at 37 °C overnight with IPTG.*

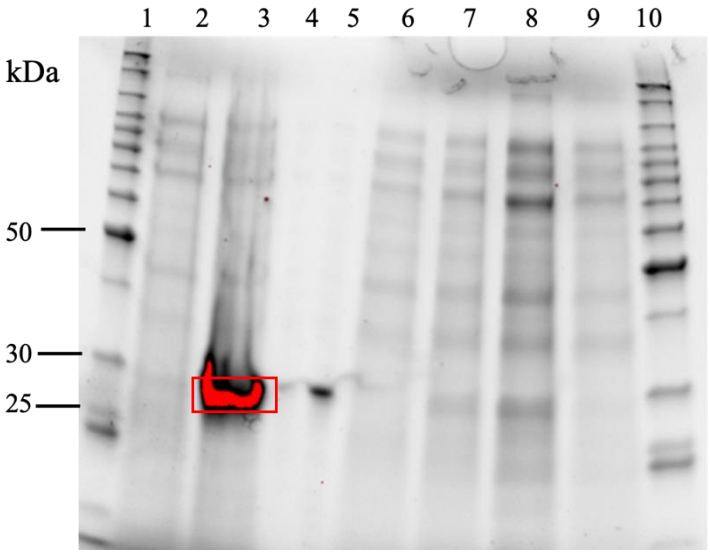


Figure B 4. Small-scale expression trials with pET*+PA14_41500 *in BHI medium. *BL21 with pET*+PA14_41500 *incubated in BHI medium with 1 mM IPTG was incubated at 20 or 37 ºC. In lanes 2-5, the total protein samples were added, while in lanes 6-9 lysate samples were added. The lanes show the following samples: 1, 10: the protein ladder Benchmark, 2, 6: non-induced culture, 3, 7: 3 hours incubation at 37 °C with IPTG, 4, 8: incubated at 20 °C overnight with IPTG, 5, 9: incubated at 37 °C overnight with IPTG*

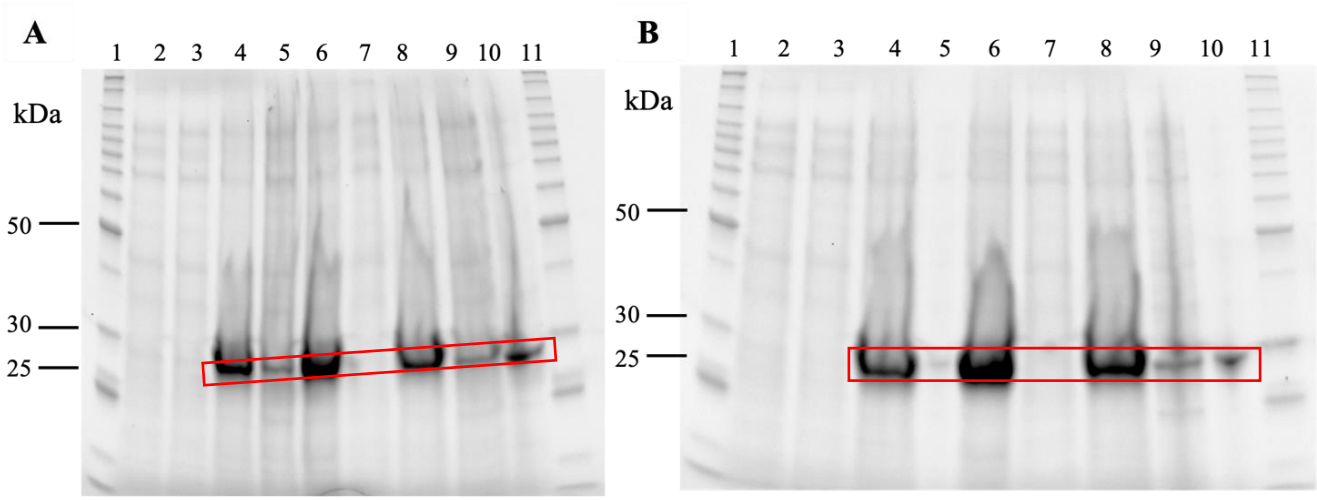


***Figure B 5. Total protein samples of pET*+PA14_41500.** *BL21 with pET+*PA14_41500 *incubated in (A) BHI medium and (B) TSB with 1 and 0.2 mM IPTG. In lanes 2-5, cultures incubated with 0.2 mM IPTG were added, while in lanes 6-9 cultures with 1 mM were added. The lanes show the following samples: 1, 11: the protein ladder Benchmark, 2: non-induced culture, 3, 7: 0 hours incubation with IPTG, 4, 8: 3 hours incubated at 37 °C with IPTG, 5, 9: incubated at 20 °C overnight with IPTG, 6, 10: incubated at 37 °C overnight with IPTG.*

9 Appendix C

Expression of *Pa*Aly7a with the pNIC-CH vector.

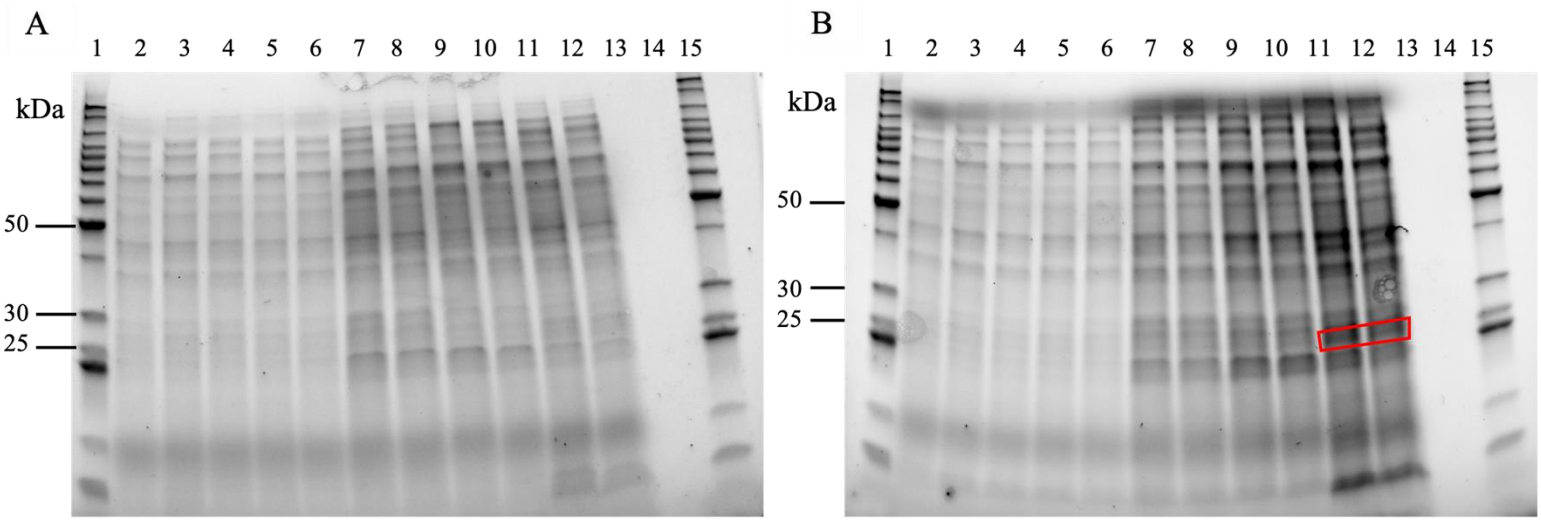


Figure C 1. Small-scale expression trials for pNIC-CH in LB and TB medium*. BL21 with pNIC-CH+*PA14_41500 *incubated in: (A) LB medium and (B) TB medium. The lanes show the following samples: 1, 15: the protein ladder Benchmark, 2: culture without IPTG, 3, 4: 0 hours incubation with 0.2 mM IPTG, 5, 6: 0 hours incubation with 1 mM IPTG, 7: 3 hours incubation at 37 °C with 0.2 mM IPTG, 8: 3 hours incubation at 37 °C with 1 mM IPTG, 9: incubated at 37 °C overnight with 0.2 mM IPTG, 10: incubated at 37 °C overnight with 1 mM IPTG, 11: incubated overnight at 20 °C with 0.2 mM IPTG, 12: incubated overnight at 20 °C with 1 mM IPTG.*

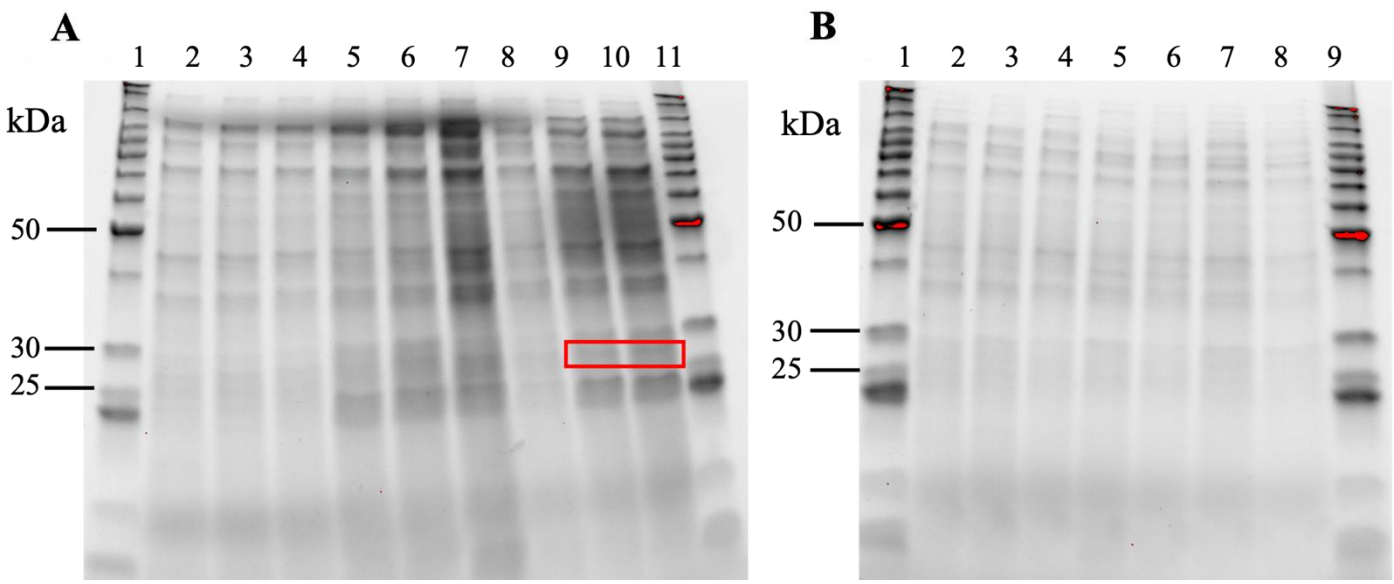


Figure C 2. Small-scale expression trials for pNIC-CH in BHI and M9 medium. *BL21 with pNIC-CH+*PA14_41500 *incubated in: (A) BHI medium. The lanes show the following samples: 1, 11: the protein ladder Benchmark, 2: cell extract before adding of IPTG, 3: 0 hours incubation with 0.2 mM IPTG, 4: 0 hours incubation with 1 mM IPTG, 5: 3 hours incubation at 37 °C with 0.2 mM IPTG, 6: 3 hours incubation at 37 °C with 1 mM IPTG, 7: incubated at 20 °C overnight with 0.2 mM IPTG, 8: incubated at 20 °C overnight with 1 mM IPTG, 9: incubated overnight at 37 °C with 0.2 mM IPTG, 10: incubated overnight at 37 °C with 1 mM IPTG. (B) shows BL21 with pNIC-CH+*PA14_41500 *incubated in M9 medium. The lanes show the following samples: 1, 9: the protein ladder Benchmark, 2: culture without IPTG, 3: 0 hours incubation with 0.2 mM IPTG, 4: 0 hours incubation with 1 mM IPTG, 5: incubated at 20 °C overnight with 0.2 mM IPTG, 6: incubated at 20 °C overnight with 1 mM IPTG, 7: incubated overnight at 37 °C with 0.2 mM IPTG, 8: incubated overnight at 37 °C with 1 mM IPTG.*

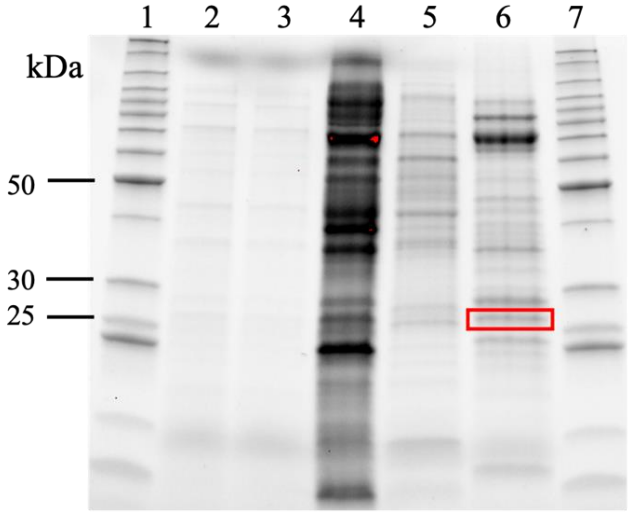


Figure C 3. SDS-PAGE analysis of purified* PaAly7a *on IMAC. *The SDS-PAGE gel shows analysis of expression and purification of* PaAly7a *from* E. coli *BL21 containing pNIC-CH+*PA14_41500. *The lanes show the following samples: 1, 7: BenchMark™ protein ladder, 2: cell extract before adding IPTG, 3: the sample 0 hours with 1 mM IPTG, 4: the flow-through, 5: the wash fraction, 6: the eluted fraction.*

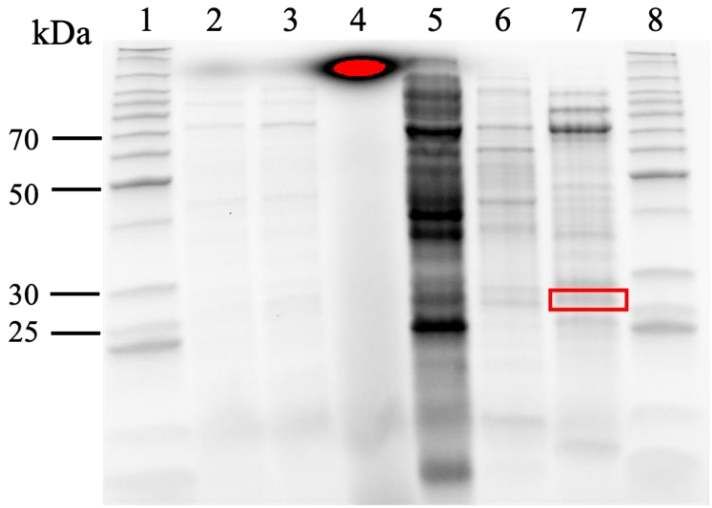


Figure C 4. SDS-PAGE analysis of purified* PaAly7a *from pNIC-CH+*PA14_41500 *on IMAC with DTT. *The SDS-PAGE gel shows analysis of expression and purification of* PaAly7a *from* E. coli *BL21 containing pNIC-CH+*PA14_41500 *incubated with LDS and DTT. The lanes show the following samples: 1, 8: BenchMark™ protein ladder, 2: the cell extract before adding IPTG, 3: the sample 0 hours after the adding of 1 mM IPTG, 4: the sample from the medium after incubation without sonication and centrifugation, 5: the flow-through, 6: the wash sample, 7: the eluted sample.*

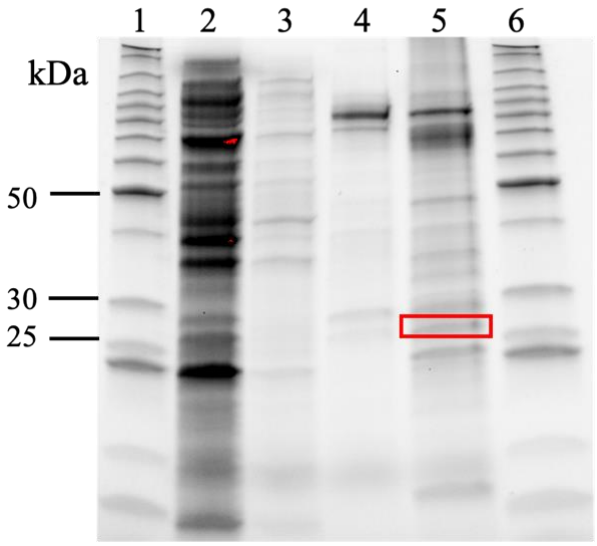
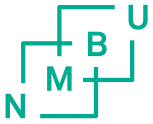


Figure C 5. SDS-PAGE analysis of purified flow-through with 0.1 % sodium alginate from IMAC. *The flow-through from* ***Figure C 4.*** *was incubated with sodium alginate until a final concentration of 0.1 % and then purified once more on IMAC. The following lanes show the samples: 1, 6: BenchMark™ protein ladder, 2: the flow-through, 3: the wash sample, 4: the elution sample, 5: the eluted sample from* ***Figure C 4.*** *(not added sodium alginate).*



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