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The production and purification of the 1,4-dihydroxy-2-naphthoate octaprenyltransferase enzyme from *Escherichia coli*

Master's thesis in the Biotechnology Master's Programme

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Gothenburg, Sweden 2012

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Abstract

The aim of the project was to produce, purify and crystallize the 1,4-dihydroxy-2-naphthoate octaprenyltransferase enzyme from *Escherichia coli*. This is a key enzyme in the biosynthesis of bacterial menaquinone and is encoded by the gene *menA*. Menaquinone is an electron carrier in the bacterial electron transport chain and constitute, together with phylloquinone, the available natural forms of Vitamin K. Since humans lack the genes for producing menaquinone, *menA* has strong potential as a new drug target for multi-drug resistant bacteria. Obtaining a crystal structure would serve as a valuable tool for further optimization of inhibitor molecules against the enzyme. The *menA* was cloned in a vector under control of the T7 promoter and transformed into the BL21*(DE3) strain. Protein production was done with shake-flask cultivation and induction with IPTG. Purifications included IMAC and size exclusion chromatography where different detergents were tested. The *menA* enzyme was successfully overproduced and it could be shown that it was also functional in the membrane. Initial purification revealed degradation of *menA*, both as precipitation when concentrating and as an obvious pattern on SDS-PAGEs. Specific protease inhibitors hindered the degradation to take place, yielding pure *menA* from the size exclusion chromatography. However, the protein concentration was too low to set up crystal plates. The growth conditions need to be optimized in order to obtain a larger amount of protein for structural studies.

Keywords: *menA*, drug target, BL21*(DE3), enzymatic activity, IMAC, size exclusion chromatography, precipitation, degradation

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I would like to thank my lab supervisor Annette Duelli for her fantastic support throughout the project. Also, I would like to thank Gergely Katona for having me as a master's thesis student in his research group. Furthermore, I would like to thank Martin Billeter for being my examiner.

Contents

1 Introduction

In the following paragraphs the background and the aim of the project will be described.

1.1 Membrane proteins

Membrane proteins are involved in a vast number of biological processes which include, but are not limited to, solute transport, signal transduction and the conversion of energy. A simple explanation of what the cell membrane is, is a lipid bilayer. According to the fluid mosaic model "the membrane is a fluid mixture of lipids and proteins" [1]. The proteins in the membrane are either peripheral or integral. Peripheral proteins are associated with the membrane on one side only by interacting with lipids or integral membrane proteins. An integral membrane protein spans through the whole membrane, often with several trans-membrane domains [1]. A third type of membrane protein has one part anchored in the membrane whilst the other part of the protein is directed into or out of the cell. A simplified view of the lipid bilayer, displaying the types of membrane proteins, is presented in Figure 1.
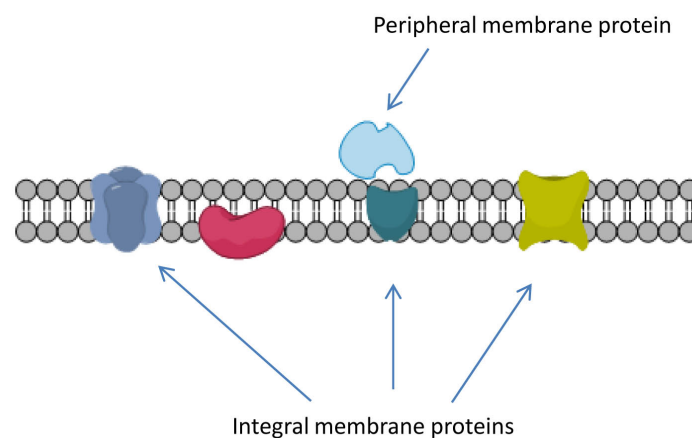
Figure 1: A simplified representation of a lipid bilayer, displaying the different types of membrane proteins (peripheral and integral). The red kidney-shaped protein is a protein with one part anchored to the membrane and the other part into the cell (third type of membrane protein).

The cell wall of gram-negative bacteria, such as *Escherichia coli*, is built up by two separate lipid bilayers. The cytoplasmic membrane (inner membrane) closest to the cytoplasm is separated from the outer membrane ("second lipid bilayer") by the periplasm [2].

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1.2 The electron transport chain

The electron transport chain is the intracellular process generating ATP through a series of electron transport events between different protein complexes. The process starts with reduced electron carriers, such as NADH, from the citric acid cycle. The reduced electron carrier is then reoxidized by the first enzymes in the electron transport chain. In eukaryotic cells the electron transport occurs in the inner membrane of the mitochondria whilst in prokaryotic cells the components of the electron transport chain are bound to the inner membrane of the cell surface[1].

In *E.coli*, which is a facultative anaerobic gram-negative organism, ubiquinone (coenzyme Q) and menaquinone each play a role in the electron transport chain. Ubiquinone acts as an electron carrier during aerobic growth whilst menaquinone is an electron carrier during anaerobic growth [3].

1.3 Menaquinone

Vitamin K exists in two natural forms, the plant form phylloquinone (PK or Vitamin K1) and the bacterial form menaquinone (MK or Vitamin K2)[4]. MK is an overall name for a family of 2-methylnaphtoquinones which differ from each other based on their side chain lengths. The side chains vary from 1 to 12 unsaturated isoprenoid residues [5]. PK and MK have the 2-methyl-1,4-naphthoquinone core in common whilst their side chains are different, PK having a phytyl and MK a prenyl side chain respectively [6].

It has been proven that PK is endogenously converted to MK-4 in humans by the UBIAD1 enzyme [4]. MK-4 is the most prevalent menaquinone available in humans [7]. The transformation of phylloquinone to MK-4 was first proven by the swiss scientist Carl Martius and later by several other scientists. Martius fed chickens with PK that was ^{3}H-labelled in the ring structure and with ^{14}C in the side chain. The result showed increased levels of MK-4 (only labelled with ^{3}H) in several organs such as the brain and the kidneys [8].

Vitamin K plays the role of a cofactor to the γ-glutamyl carboxylase (GGCX) which is an enzyme important for correct post-translational modifications of Vitamin K-dependent proteins. These proteins are coagulation factors, osteocalcin and matrix Gla protein. Furthermore, Vitamin K is a transcriptional regulator for the gene expression in bones [4]. Also, MK-4 has shown potential in the inhibition of cancer cell growth by making them differentiate or undergo apoptosis. The cancer types affected by MK-4 are liver, gut and leukaemia [8].

Humans cannot synthesize Vitamin K endogenously but are dependent on the Vitamin K that is provided through the diet and the MKs produced by bacteria in the large intestine. PK is the major source of Vitamin K through the diet and can for example be found in

green leafy vegetables and several fruits at high concentrations. MKs in our diet have not been as well studied but it has been proven to exist in animal livers and fermented foods from bacteria [8]

1.4 Menaquinone in *E.coli*

In *E.coli*, menaquinone plays an important role as major electron transporter during anaerobic growth. There are six genes which are responsible for the biosynthesis of menaquinone in *E.coli*; *menA*, *menB*, *menC*, *menD*, *menE* and *menF*. The *men*-genes are ordered in two clusters in the *E.coli* genome where *menB-F* constitute one cluster, *menA* and *menG* the other [9]. The isoprenoid side chain and the head group are synthesized separately [10]. The precursor of the head group is 1,4-dihydroxy-2-naphthoate (DHNA) and its synthesis starts with chorismate which is evolved in the branch point of the shikimate pathway [3][10].

The isoprenoid tail is added by the 1,4-dihydroxy-2-naphthoate octaprenyltransferase (*menA*) enzyme in a condensation reaction. The number of prenyl units added to the head group is species-specific since it depends on the length of the prenyl diphosphate produced in the organism. In *E.coli*, MK-8 is predominant[10]. *menA* converts DHNA to the membrane bound demethylmenaquinone which is a key step in the menaquinone biosynthesis[4] as it couples the head group to its side chain. Demethylmenaquinone is thereafter converted to menaquinone by *menG* by the addition of a methyl group to the naphthoate core [10]. The role of the *men*-genes is presented in Figure 2.
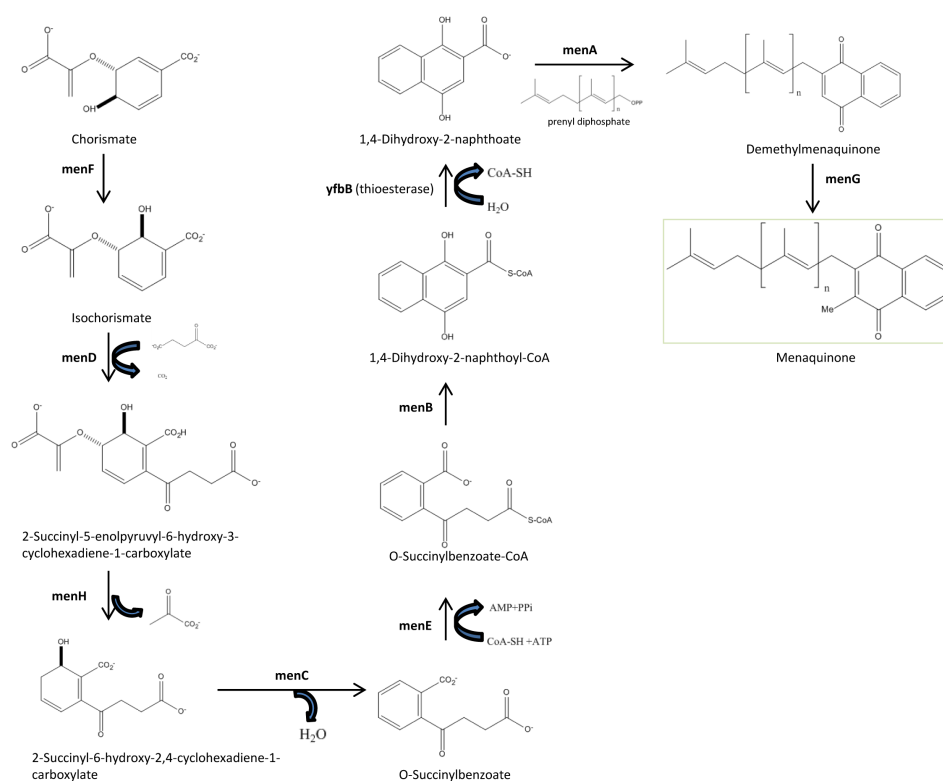
Figure 2: The biosynthesis of bacterial menaquinone in *E.coli* displaying the role of *menA*. *menA* converts 1,4-Dihydroxy-2-naphthoate to the membrane bound demethylmenaquinone by adding an isoprenoid side chain to it.

The first localization of the *menA* gene was said to be at around 78 map units on the *E.coli* chromosome. This was due to that *menA* was cotransducible with the *metB* and *argE* gene which are located in that region [11]. Later on, *menA* was localized to 88 map units on the *E.coli* chromosome and is 60% cotransducible with *metB* and 90% with the *glpK* loci [12]. The reporting of the *menA* genetic sequence revealed a "presumptive *menA*-encoded product in *Haemophilus influenzae*" and in *Synechocystis* species [12].

Other organisms, such as *Helicobacter pylori*, also produce menaquinone but lack orthologues to the *men* genes. This led to the discovery of an alternate menaquinone biosynthetic pathway called the futalosine pathway [10]. Chorismate is then converted to futalosine and by further enzymatic reactions to menaquionone [6]. Yet other organisms lack the *menG* gene and hence produces demethylmenaquinone as final quinone [6].

1.5 Menaquinone in the protein data bank

For *E.coli*, the available structures of enzymes leading to synthesis of menaquinone are the structures of *menB*, *menD*, *menF* and *menH*. Here, the structures of *menB* and *menD*

will be presented.

For *menB*, there are structures of both the apoenzyme and the *menB* enzyme co-crystallized with O-succinylbenzoyl-aminoCoA (OSB-NCoA). This is a more stable analogue of its true substrate, O-succinylbenzoyl-CoA (OSB-CoA). The use of this analogue revealed more information about the active site. In previous *menB* structures from other organisms the active site has been somewhat disrupted. The 2.0 $\dot{A}$ resolution structure of the *menB*-(OSB-NCoA) complex displays the position of all the amino acid residues involved in the active site [13]. The structure *menB*-(OSB-NCoA) is presented in Figure 3. For *menD*, the native protein could be crystallized [3] and the resulting 2.5 $\dot{A}$ crystal structure is presented in Figure 3.
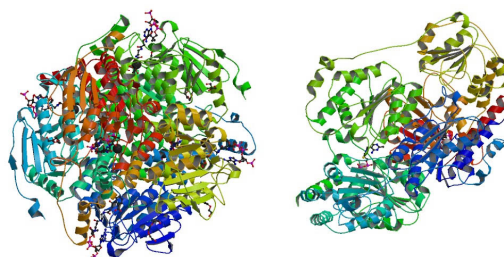

Figure 3: The structures of the *menB*-(OSB-NCoA) complex (left) [14] and the native *menD* enzyme (right) [15].

1.6 The importance of *menA*

The human electron transport chain does not use menaquinone and therefore it is an attractive drug target for many drug-resistant pathogens (such as methicillin-resistant *Staphylococcus aureus*). Studies have been done where a library of molecules was synthesized as possible inhibitors to the *menA* enzyme from *Mycobacterium tuberculosis*. Two molecules were identified as strong hits with minimum inhibitory concentrations (MIC) of 1.5 and 12.5 μg/ml. These molecules are also efficient inhibitors for other drug-resistant Gram-positive organisms such as *S.aureus* but inefficient for Gram-negative organisms. This is possibly explained by the fact that the majority of Gram-positive bacteria uses only menaquinone, not ubiquinone, as lipid-soluble electron carrier in the electron transport chain [9].

1.7 Aim of the project

The aim of this project is to produce and purify the 1,4-dihydroxy-2-naphthoate octaprenyltransferase enzyme from *E.coli* which is encoded by the gene *menA*. The organism chosen for this purpose is *E.coli*. Purified protein will be used to set up crystal plates with the ultimate goal to solve a crystal structure with X-ray crystallography. For simplicity, *menA* is the name that will be used from this point forward, both for the gene-and protein name.

2 Theory

The theoretical background to the methods employed in the master's thesis is presented in the following sections.

2.1 Cloning

The classical interpretation of the word clone is a "population of organisms that are genetically homogeneous" [1]. All bacterial cells in a colony belongs to a single clone since they all have been created from a single cell initially put on a Petri dish. However, the word cloning is also commonly used to describe the combination (ligation) of a gene with another DNA molecule, such as a plasmid. The product is referred to a recombinant DNA molecule and in the case with the plasmid, this is often inserted into a host cell. There it replicates along with the host cell's DNA. The cloning and transformation procedure are illustrated in Figure 4 .
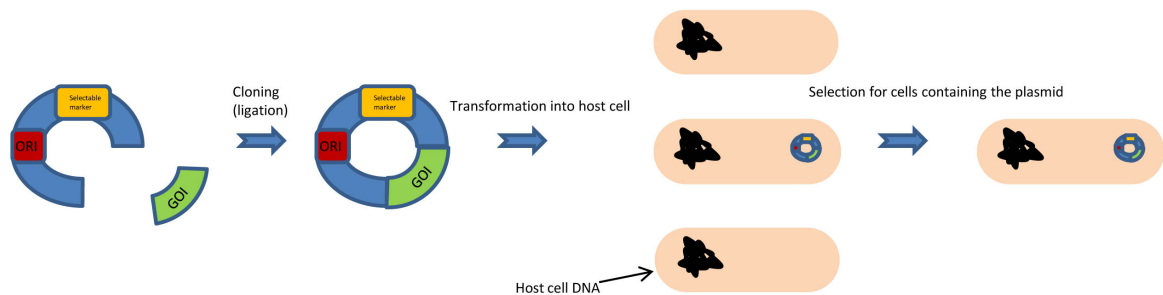

Figure 4: Schematic representation of the cloning of the gene of interest (GOI) into a vector and subsequent transformation into the host cell. Selection of plasmid-containing cells is usually done with growth in presence of the antibiotic for which the plasmid has resistance (selectable marker)

There are many steps involved in a successful cloning. First, the gene of interest (GOI) needs to be amplified. This is usually done with a technique called polymerase chain reaction (PCR) which was introduced in 1987. It is crucial that there is knowledge about the DNA sequence surrounding the GOI since primers that are target specific need to be synthesized . Primers are oligonucleotides that are complementary to a specific sequence of the GOI, one forward and one reverse. Both primers operate in the 5'to 3' direction.

The PCR reaction starts with heat-denaturation of the DNA containing the GOI (above 90° C). Thereafter the temperature is lowered to in between 40-60° C so that the primers can anneal to the specific DNA sequence. This temperature is chosen based on the individual melting temperature of the primers, hence the wide temperature range. The temperature is then raised again to the specific working temperature of the DNA-polymerase

that will carry out the extension. *Taq* polymerase has its optimal working temperature at 72 ° C. Nucleotides, dNTPs, are added in the PCR mix and used by the DNA polymerase for extension. The denaturation-annealing-extension cycle is usually repeated more than 30 times, each cycle increasing the amount of GOI amplified. PCR is conducted in an automated temperature-regulating device [1] [16]. One cycle of the PCR reaction is presented in Figure 5.
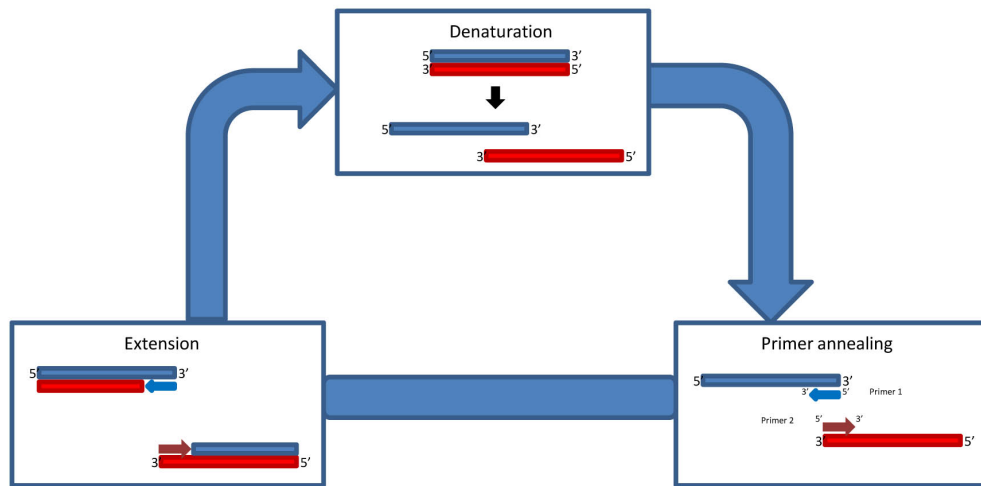

Figure 5: Presentation of one PCR-cycle, displaying the denaturation, primer annealing and extension by a DNA polymerase. Inspiration for the picture was taken from [16]

The second cloning step includes a vector or another DNA molecule which the GOI can be inserted into. Usually, plasmids and bacteriophage chromosomes are used as vectors since they contain an origin of replication (ORI). This means that they independently replicate in the host cell after transformation. Third, it is important to have some selection that facilitates identification of those bacteria that contain the cloned gene. Introducing an antibiotic resistance gene in the vector is a popular selectable marker. Growth in the presence of this antibiotic ensures survival only of the cells that have taken up the plasmid. There are special expression vectors available that control transcription and translation to ensure high-level expression of the GOI.

To facilitate purification, the GOI is often supplemented with a tag inserted directly adjacent to its DNA sequence. Histidine tags are often used, usually 6 or 8, and these codons are inserted adjacent to either the C- or N-terminus of the GOI. The histidine tag is tightly bound to the nickel ion in an affinity column, see section IMAC [1].

2.2 Induction with IPTG

The T7 RNA polymerase is a very effective polymerase for transcription. Compared to the *E.coli* RNA polymerase it is five times faster in the elongation step. Therefore it is competent for high-level expression of a gene of interest. In the BL21*(DE3) cells, the gene for T7 RNA polymerase is under control of the *lacUV5* promoter. Without inducer, the polymerase is not expressed. However, adding an inducer such as isopropyl-β-D-thiogalactopyranoside (IPTG) ensures expression of the polymerase [17].

The GOI is cloned in a vector as to make it under control of the T7 promoter. When the vector is successfully transformed into the BL21*(DE3) cells, induction with IPTG gives access to the T7 RNA polymerase. The polymerase then transcribes the gene of interest when interacting with the T7 promoter. Hence, this is a way to control gene expression and at the same time receive high-level expression. The benefit is that the induction can be set to a certain cell density, which increases the protein yield.

2.3 Microbial growth

The definition of growth in microbiology is "an increase in the number of cells" [2]. The cell division of *E.coli* has been described elsewhere and will not be further addressed in this section. The generation time, i.e. the time needed to double the amount of cells, is dependent on the growth media and the culturing conditions such as incubation temperature. When cell cultivation is taken place in a flask it is referred to as a batch cultivation since everything that is needed for growth is provided from the beginning. This microbial cultivation takes place in three different phases, starting with the *lag phase*, followed by the *exponential phase* and the *stationary phase*. These three phases constitute the growth cycle of the microbial population.

The *lag phase* is the phase when the micro-organisms are adapting to the cell media and growth conditions. A lag can be seen when a culture is shifted from a rich media to a poorer media. It can also be seen if there is a lack of essential metabolites in the cell culture since then enzymes need to be biosynthesized in order to produce these metabolites.

The *exponential phase* is the phase in which each cell divides to become two. Exponential growth is usually the phase in which the cells are in their healthiest state. That makes this state attractive for studying cell components, such as enzymes.

The *stationary phase* is reached after a certain time of exponential growth, often as a result of a deficiency in an essential nutrient or of accumulation of a toxic waste product. There is no net cell production in this phase; hence the growth rate is zero. The stationary phase is followed by the death phase in which the cells die. The typical behaviour of a growing bacterial population is shown in the graph presented in Figure 6.
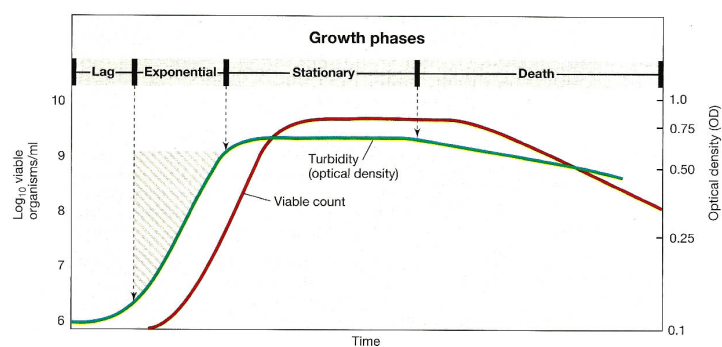
Figure 6: The typical growth of a bacterial population displaying each individual growth phase (lag, exponential and stationary). Copied from [2].

Determination of the number of cells in the population is often done by measuring the optical density (OD) of the cell culture. Cell suspensions scatter light and the more cloudy (turbid) the suspension, the more light is scattered. Turbidity measurements indicate the total cell mass which in turn can be coupled to the number of cells in the population. When subjecting the cells to light in a spectrophotometer "the unit of the turbidity measurement is OD"[2]. The spectrophotometer measures the unscattered light at a certain wavelength, usually 540 nm (green) or 600 nm (orange) for bacterial populations.

E.coli is a mesophile organism and for most strains the optimum growth temperature is $39°$ C . However, the survival temperature is as high as $48°$ C and the minimum $8°$ C [2].

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2.4 Centrifugation

Centrifugation is a technique that is used for sedimentation of biological structures or molecules through acceleration in a centrifugal field. This allows separation of different parts of the cell structure. The focus here will lie on preparative centrifugation and ultracentrifugation.

Preparative centrifugation

Preparative centrifuges include large-capacity low-speed centrifuges which are often the first steps of centrifugation to achieve cellular and subcellular fractionation. Differential centrifugation is a technique which uses differences in sedimentation rate of biological particles to separate them. Practically it means a stepwise increase of the centrifugal field in order to separate different biological structures into the pellet and the supernatant. The largest molecules or structures end up at the bottom of the tube whilst the smaller particles stay in the supernatant. The pellet is often resuspended and centrifuged again to ensure a pure sample.

Ultracentrifugation

Ultracentrifugation is centrifugation at very high speed, up to 250 000 g. For membrane proteins, ultracentrifugation is used just after cell disruption and subsequent centrifugation, to obtain the cell membrane. Ultracentrifugation is also used to pellet the unsolubilized membrane proteins after incubation in solubilization buffer [16].

2.5 Cell disruption

There are a number of available cell disruption methods in which the cell wall is destroyed. Bacteria have rigid cell walls and are usually disrupted with either a press, such as the French Press, or with sonication. The French Press is presented in Figure 7.

The cell suspension is passed through a small orifice by a piston-type pump with applied high pressure (around 1500 psi). When the cells emerge from the high-pressure chamber they experience a drastic pressure drop which make them burst. It is not unusual that several passes through the French Press are needed in order to effectively lyse all the cells.

Sonication is a method that uses sound waves for cell disruption. The sonicator probe is inserted into the cell suspension and subjects the cells to sound waves (<20 kHz) for a short time period [16].
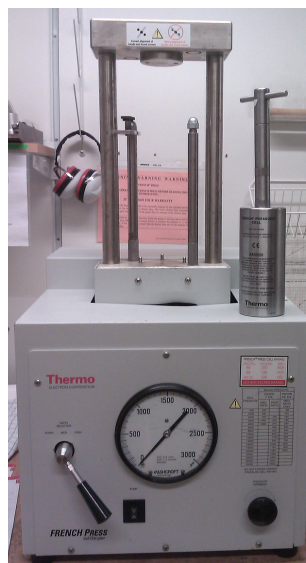
Figure 7: The features of a French Press.

2.6 Proteases in *E.coli*

There are more than 20 proteases identified in *E.coli* to this date. The proteases can be found in the cytoplasm, inner membrane, periplasm and in the outer membrane. The types of proteases present are serine proteases, metalloproteases and ATP-activated proteases [18].

Complete EDTA-free protease inhibitor cocktail tablets (Roche) contain inhibitors for serine-and cysteine proteases but not for metalloproteases. Since *E.coli* contains metalloproteases as well, this discovery led to the usage of the two other protease inhibitors. Pefabloc (Sigma-Aldrich) is an irreversible serine protease inhibitor and 2,2'-Bipyridyl (Sigma-Aldrich) is a metalloprotease inhibitor with strong chelating power of Fe^{2+}.

2.7 Solubilization of membrane proteins

The study of membrane proteins requires extraction from its natural environment in the lipid bilayer. Since the transmembrane domains of membrane proteins are hydrophobic they need to be shielded when extracted into an aqueous solution. For this purpose, detergents are used which assemble around the hydrophobic structures of membrane proteins. Detergents are built up by a polar head group and a non-polar (hydrophobic) tail. In aqueous solutions they form micelles which are spherical structures where the tails are pointing inwards and the heads are forming the edge of the sphere. Solubilization of membrane proteins is hence possible due to the similarity of the true lipid bilayer in the cell membrane, formed by the detergents. There are several different types of detergents, mainly sorted into four major groups; *ionic detergents*, *non-ionic detergents*,

bile acid salts and *zwitterionic detergents*. Examples of each type of detergent are presented in Figure 8, Figure 9, Figure 10 and Figure 11. Inspiration to the pictures came from [19].
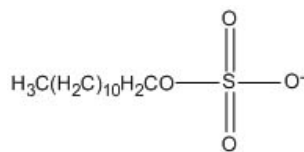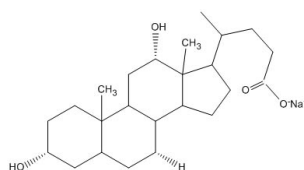

Figure 8: SDS is an ionic detergent.

Figure 9: Sodium deoxycholate is a bile acid salt.

Ionic detergents have a head group with a net charge and a tail region consisting of a hydrophobic hydrocarbon chain. The critical micelle concentration (cmc), i.e. the minimum concentration of detergent for micelle formation, is dependent on the net effect of the repulsive head group and the hydrophobic interactions in the tail region. Solubilization of membrane proteins is very effective with ionic detergents but is often accompanied by some protein denaturation. *Bile acid salts* are ionic detergents but differ in the sense that their backbone is built up by steroidal groups. However, instead of forming micelles they form kidney-shaped aggregates [19].

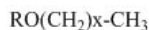$$RO(CH_2)x\text{-}CH_3$$

$$R = \text{glucose, } x = 7$$

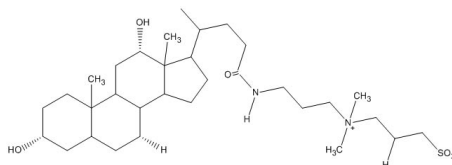**Figure 10:** OG is a non-ionic detergent.

Figure 11: CHAPS is a zwitterionic detergent.

Non-ionic detergents contain uncharged hydrophilic head groups. Many membrane proteins have successfully been solubilized in non-ionic detergents due to their non-denaturing and mild nature. Also, isolation of the membrane protein is usually in its

biologically active form with this type of detergent. *Zwitterionic detergents* are a combination of ionic and non-ionic detergents, more deactivating than non-ionic detergents [19]. Generally, detergents are classified as either harsh or mild where harsh detergents are those that with high probability denature proteins and mild not. In a recent study it has been shown that both harsh detergents (such as SDS) and mild detergents (such as DDM) interact with individual transmembrane segments "in a similar manner" [20]. It is suggested that the denaturing power of the harsh detergents may be due their interaction and disruption of non-membranous sections of the protein. Furthermore, it is proposed that these non-membranous sections likely are important for the formation of the transmembrane segments of the protein and a disruption is expected to denature the whole protein [20].

2.8 Chromatography

The basic principle of chromatography is separation of analytes from a mixture. The chromatographic system consists of a stationary phase and a mobile phase. The stationary phase can be a solid, a gel, a liquid or a mixture of solid/liquid. The mobile phase is either liquid or gaseous.

FPLC

Fast protein liquid chromatography, or FPLC, is one of the most popular variants of chromatography for purification of proteins. The components of a typical FPLC system are shown in Figure 12.
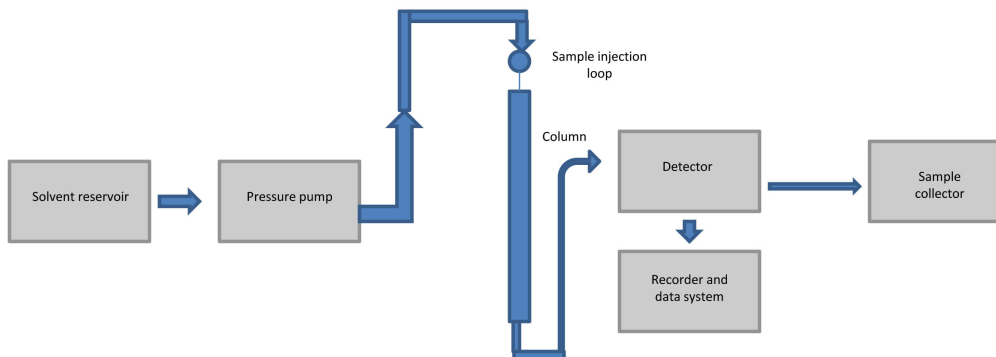

Figure 12: The components of a typical FPLC system. Inspiration for the picture was taken from [16].

Immobilized metal affinity chromatography (IMAC)

For the purification of His-taggged proteins, nickel (Ni^{2+}) is immobilized on a column. Nickel binds strongly to the imidazole rings of the histidines. Elution of the protein is

done with imidazole at a concentration that has to be decided for each individual protein. Elution occurs when imidazole conquers out the binding of histidine to nickel.

This method can be performed either manually with Ni-NTA resin and column or by attaching a HisTrap to a FPLC system that runs automatically [16].

Size exclusion chromatography (SEC)

The SEC technique separates molecules based on their size and shape. The stationary phase is built up by cross-linked polymers such as dextran which creates a pore network. The largest molecules are eluted first since they cannot transfer through the pore network. The smallest molecules interact with the polymer matrix and travel at a slower rate, hence elutes last from the column[16].

2.9 Electrophoresis

Electrophoresis is a method used for separating charged molecules based on size. Many biological molecules, such as nucleic acids and peptides, are naturally charged due to presence of ionisable groups. By applying an electric field these molecules will either move towards the anod or the cathode, determined by the net charge of the molecule (positive net charge towards anod, negative net charge towards cathode). The velocity of the molecules travelling through the pore network is determined by the net force exerted on the molecule. The potential gradient E (voltage divided by the distance of the electrodes) exerts a force of Eq Newton where q is the charge of the molecule in Coulombs. Retardation of the molecule's movement occurs through frictional resistance which is dependent on many parameters, including size and shape of the molecule. The velocity of the molecule can be described with the following equation

$$v = \frac{Eq}{f} \tag{1}$$

where f is the frictional coefficient. The term electrophoretic mobility μ, which is the velocity divided by the field strength, is frequently used

$$u = \frac{v}{E} \tag{2}$$

and explains how charged molecules are separated based on their individual electrophoretic mobility. Equally charged molecules will separate if they have a difference in e.g. molecular size due to their different frictional forces. Smaller molecules pass more easily through the pore network of the gel whilst larger molecules are more affected by the frictional resistance and hence move slower.

Electrophoresis is always accompanied by an appropriate buffer to ensure a constant state of ionization of the separating molecules.Typically, proteins are separated on poly-

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acrylamide gels on vertical gel units connected to a power pack. Nucleic acids are often separated on agarose gels on a horizontal gel unit connected to a power pack [16].

SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is the most common method for separating proteins based on size. It is an important qualitative tool as it is often used to address protein content after individual purification steps such as IMAC and SEC. Sample buffer added to the proteins prior to loading onto the gel contains SDS which denatures the protein. SDS is strongly negatively charged which means that all proteins also end up with a negative charge; all original protein charges are hence cancelled.

The gel consists of cross-linked polyacrylamide and is formed by polymerization of acrylamide. This reaction is a free-radical catalysis and is initiated by the addition of N,N,N',N'-tetramethylenediamine (TEMED). The bottom part of the gel consists of the separating gel and at the top there is a stacking gel. The stacking gel contains the wells and makes sure that the protein sample is assembled to a sharp band before entering the separating gel [16]. The setup for SDS-PAGE is illustrated in Figure 13.
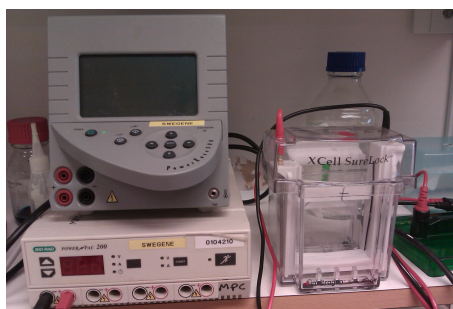
Figure 13: The common setup of an SDS-PAGE system. A powerpack is coupled to the electrophoresis unit.

Agarose gel electrophoresis

Agarose gels are often cast at an agarose concentration between 0.8-1%. A 0.8% gel is suitable for separating 0.5-10 kilobases (kb) of DNA. Preparation of the gel involves dissolving agarose in gel buffer by boiling and casting the gel in an appropriate container. The gel is completely covered in excess buffer before the samples are loaded in the wells.

The samples are prepared by adding loading dye, usually bromophenol blue, to visualize the sample and to be able to know where the electrophoresis front is while running the gel [16].

Western blotting

Western blotting is the technique of transferring protein to a nitrocellulose membrane for further detection of your specific protein with antibodies. The proteins are transferred to the membrane through electroblotting in which the gel (SDS-PAGE) and the membrane are compressed in a cassette which is submerged in buffer and subjected to an electrical field. The current transfers the proteins from the gel onto the nitrocellulose membrane.

The membrane is thereafter incubated with the primary antibody that binds specifically to the protein of interest if available on the blot. A common method is to then use an enzyme-linked secondary antibody which is specific to the primary antibody. The blot is then incubated in substrate solution and the substrate is converted by the enzyme to a coloured product that precipitates on the blot[16].

3 Methods

Buffer and growth media recipes can be found in the Appendix section.

3.1 Primer design and PCR

Primer design

For the 927 base pair (bp) *menA* gene, primers (Invitrogen) were designed;

Forward: 5' ATGACTGAACAACAAATTAGCCGAACTCA 3'
Reverse: 5' TTATGCTGCCCACTGGCTTAGGAATAT 3'

The T_m of the primers are 57.62 and 58.02 $^{\circ}$ C for forward and reverse primer respectively.

PCR reaction

E.coli genomic DNA was extracted from BL21*(DE3) cells with the GenElutePlasmidMini-prepKit(Sigma) according to the manufacturer's instructions with some modifications (see Appendix B1) and used as template DNA in the PCR reaction. The Phusion High-Fidelity DNA Polymerase (FINNZYMES) was used which creates blunt end DNA with minimal error. DNA(228 ng), primers(0.5 μM, Invitrogen), dNTP (0.2mM, Fermentas), Phusion DNA Polymerase (0.4 U, FINNZYMES), HF Buffer (FINNZYMES), DMSO and H_2O were included in the PCR mix according to the manufacturer's instructions. The initial PCR was done with a temperature gradient [57.1 , 57,9, 58,7, 59,5 $^{\circ}$ C] based on the T_m of the primers. Analysis of the PCR product was done with gel electrophoresis (stained with SYBR Safe DNA gel stain (Invitrogen)) and the PCR reaction was repeated with the appropriate primer annealing temperature. Extraction of the *menA* gene was done with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

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3.2 Cloning and transformation

For successful cloning into the pEXP5-NT/TOPO Expression Kit (Invitrogen) addition of adenine (A) to the 3' ends of the insert is crucial. The vector and its properties is represented in Figure 14.
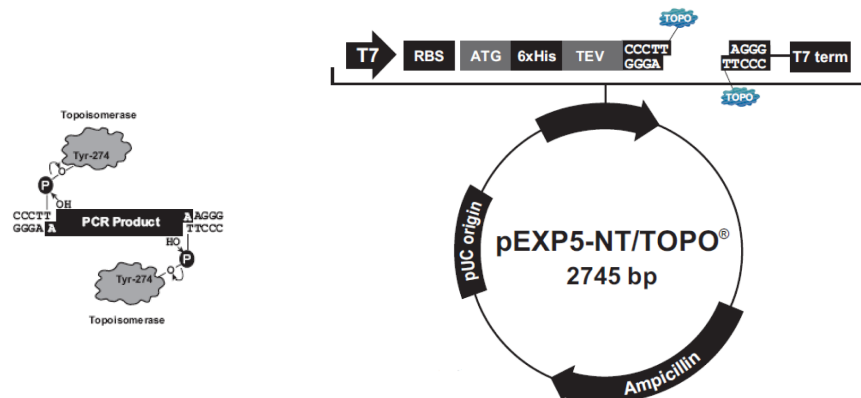

Figure 14: The features of the pEXP5-NT/TOPO vector (Invitrogen). Copied from the manual from Invitrogen.

For the 3'A addition, *Taq*-polymerase was used (1.25 u) with *menA*(748 ng), dNTP (10mM), 10x*Taq*Buffer. Incubated at 72°C for 20 min and subsequent cloning into the pEXP5-NT/TOPO expression vector according to the manufacturer's instructions with some modifications (see Appendix B2). Transformation into TOP10 OneShot *E.coli* cells with plasmid and control vector (pUC19) was followed by plating on LB-Amp plates (50μg/ml) according to the manufacturer's instructions with some adjustments (see Appendix B3). Colonies were regrown in LB media (1 %glucose, 50μg/ml Amp) at 37°C, 200 rpm for approximately 5h, followed by plasmid isolation (GenElute Plasmid Mini-prep Kit, Sigma) according to the manufacturer's instructions. Plasmid size was analyzed with gel electrophoresis and promising plasmids were sent for sequencing (Eurofins MWG/Operon).

Transformation into BL21* (DE3) cells was performed according to the manufacturer's instructions with some modifications (see Appendix B4). Cells were grown in preheated LB media (1 % glucose, 50μg/ml Amp) at 37°C, 200 rpm over night (O.N).

3.3 Test expression

The initial test expression was made to investigate if protein production occurred at all. Cells were grown in LB media (1 % glucose, 50μg/ml Amp) at 37°C, 200 rpm until OD$_{600}$ reached 0.83. Cells were aliquoted in E-flasks and induced at 0, 0.5 and 1mM IPTG, each concentration grown at 25, 30 and 37 °C. Cell samples were taken each hour,

spun down and analyzed with Western blotting (ECLPlus Western Blotting Detection System (GE Healthcare))(100V, 1h). Primary antibody (6xHis Monoclonal Antibody Albumin free, 0.5mg/ml, Clontech) and secondary antibody (w402B Antimouse IgG HRP Conjugate, 1mg/ml (Promega)) were diluted in blocking buffer prior to use.

Two test expression trials in 24-well plates were performed according to Table 1 and 2 with autoinduction and IPTG induction respectively, grown at 37 °C and 200 rpm. Two types of media were tested, LB and 32Y.

For the autoinduction plate, samples were taken at a number of timepoints and the 22 h after induction sample was analyzed with Western blotting. Cell culture was added in each well to reach an initial OD_{600} of 0.1.

Table 1: Expression conditions for autoinduction. Total well volume is 1ml. A1 is 0.01% lactose,A2 0.02 % lactose and A3 is 0.04% lactose. B1 is 0.05% glucose and B2 is 0.1% glucose. C is sterile MQ water, D is $MgSO_4$.

well: A1-D1	well: A2-D2	well: A3-D3	well: A4-D4	well: A5-D5	well: A6-D6
LB, A1,B1,C	LB,A2,B1,C	LB,A3,B1,C	LB,A1,B2,C	LB,A2,B2,C	LB,A3,B2,C
32Y,A1,B1,C	32Y,A2,B1,C	32Y,A3,B1,C	32Y,A1,B2,C	32Y,A2,B2,C	32Y,A3,B2,C
32Y,A1,B1,C,D	32Y,A2,B1,C,D	32Y,A3,B1,C,D	32Y,A1,B2,C,D	32Y,A2,B2,C,D	32Y,A3,B2,C,D
LB,C,IPTG	32Y,C,IPTG	32Y,C,D,IPTG	LB,C	32Y,C	32Y,C,D

For the IPTG-plate, induction was done at approximately OD_{600}=0.6 for the LB wells. Sampling was done 12 h after induction according to the growth protocol from Truglio *et al*[21]. Further analysis with Western blotting as previously described.

Table 2: Expression conditions for IPTG induction. Total well volume is 1ml. E1 is 0.05% glucose, E2 is 0.5% glucose and E3 is 1% glucose. F1 is 0.5mM IPTG, F2 is 1mM IPTG. C is sterile MQ water and D is $MgSO_4$.

well: A1-D1	well: A2-D2	well: A3-D3	well: A4-D4	well: A5-D5	well: A6-D6
LB,C,E1,F1	LB,C,E2,F1	LB,C,E3,F1	LB,C,E1,F2	LB,C,E2,F2	LB,C,E3,F2
32Y,C,E1,F1	32Y,C,E2,F1	32Y,C,E3,F1	32Y,C,E1,F2	32Y,C,E2,F2	32Y,C,E3,F2
32Y,C,D,E1,F1	32Y,C,D,E2,F1	32Y,C,D,E3,F1	32Y,C,D,E1,F2	32Y,C,D,E2,F2	32Y,C,D,E3,F2
LB,C,E1	32Y,C,E1	32Y,C,D,E1	LB,C	32Y,C	32Y,C,D

3.4   Cell growth and membrane preparation

All cell cultivations, independent of small-or large scale, included inoculation of BL21*(DE3) freeze cultures (containing plasmid) in prewarmed LB media (1 %glucose and 50 μg/ml Amp). The cells were grown at 37 °C, 200 rpm until OD_{600} reached approximately in between 0.6->1. Induced with IPTG (1mM) and grown (30° C, 200 rpm) for a certain time period, between 1 and 19 h.

For the large-scale production, an O.N culture was cultivated (BL21*(DE3) freeze cultures in prewarmed LB media as described above) to be used for inoculation in the 5 L

E-flasks (6 E-flasks in total).

Membrane preparation included several centrifugation steps as described below.

Cell harvest (5000xg, 15min, 4 °C, Beckman Coulter J-20) was followed by resuspension in cell lysis buffer (0.2 g cells/ml buffer) and cell lysis with the French press three times at 1500 psi. Unlysed cells and cell debris were pelleted (15000xg, 30min, 4 °C,Beckman Coulter J-20) and the supernatant ultracentrifuged (45000 rpm, 3h, 4°C, Beckman Coulter Optima L90K) to obtain the cell membrane. In the first membrane preparation, samples were taken at each step from before harvest to ultracentrifugation.

The first prepared membrane was diluted in membrane resuspension buffer at different dilutions (1, 1:1, 1:3, 1:7, 1:15, 1:31, 1:33, 1:63, 1:127, 1:255, where non-diluted (1) means 0.25 g/ml) to determine which was appropriate. Cell samples from every step, from before harvest to lysed cells, were also prepared. Western blotting was performed as previously described, with the samples from above, to identify a suitable membrane dilution and to investigate if the protein is inserted into the membrane. For every other membrane preparation the membrane was resuspended in membrane resuspension buffer at 0.25g/ml.

3.5   Solubilization screen

An initial solubilization screen with different detergents, added to 20 μl membrane, was performed according to Table 3. This was achieved by solubilization of the samples for 1.5h at +10° C on a rolling table and further analysis with Western blotting. The aim was to determine appropriate detergents for the *menA* enzyme.

Table 3: Pipetting schedule for the solubilization screen. 20xCMC is the detergent concentration in each case. 20 μl membrane was added to each detergent.

	10 x membrane resuspension	Detergent amount	Water	CMC
	buffer without glycerol			
DDM	2 μl	2 μl (10%)	16μl	0.0087%
OG	2 μl	13.6μl(20%)	4.4μl	0.68%
CHAPS	2 μl	9.8μl (20%)	8.2μl	0.49%
LDAO	2 μl	6.4μl (10%)	11.6μl	0.0032%
FC-12	2 μl	2μl (10%)	16μl	0.047%
OM	2 μl	16μl (20%)	2μl	0.8%
DM	2 μl	3.5μl (10%)	14.5μl	0.087%
NG	2 μl	4μl (20%)	14μl	0.20%
OTG	2 μl	5.6μl (20%)	12.4μl	0.28%
SDS	2 μl	8μl (10%)	10μl	
NO DETERGENT	2 μl		18μl	

3.6   Immobilized Metal Affinity Chromatography (IMAC)

The membrane was solubilized in solubilization buffer in a 1:1 volume relationship for 1.5 h at +10°C on a rolling table and was followed by ultracentrifugation (50000rpm, 30 min, 4°C). The ultracentrifuged membrane was incubated with NiNTA-agarose resin

(QIAGEN) (for preparation see Appendix) O.N for the manual IMAC purification. The detergents that have been used for the IMAC purifications are DDM, DM, LDAO and FC-12. For the solubilization, 20xCMC detergent concentration was used and for purification 2xCMC, except from LDAO where 0.5% was used for solubilization and 0.1% for purification.

Solubilized membrane was subjected to IMAC purification, either manually or with the HisTrapTM HP 1ml (GE Healthcare) column on the $\ddot{A}KTA$ system. The manual IMAC started with an initial equlibration in Buffer A followed by three wash steps (20, 40 and 80 mM imidazole). Elution was done with 100% Buffer B (500 mM imidazole). Approximately 5 bead volumes were used for each step and fractions of 100 μl collected.

The HisTrap purification included equilibration with Buffer A and three wash steps (20, 40 and 60 mM imidazole) and elution was done in a stepwise manner, gradually reaching the final concentration of 500 mM imidazole. Approximately 5 column volumes were used for each step and 500 μl fractions were collected. For the HisTrap purification, which was performed five times in total, the two last times new protease inhibitors (PIs) (0.025 mM 2,2'-Bipyridyl and 0.05mM Pefabloc) were added both to the solubilization buffer and to Buffer A. Previously, only complete EDTA-free protease inhibitor tablets (Roche) had been used.

The features of each individual IMAC purification is given in Table 4.

Table 4: The individual IMAC purifications.

Run	Type of IMAC	Solubilization detergent	Purification detergent	Protease inhibitor
1 Manual		DDM	DDM	PI tablet
2 Manual		DM	DM	PI tablet
3 Manual		DM	LDAO	PI tablet
1 HisTrap		DM	LDAO	PI tablet
2 HisTrap		DM	DM	PI tablet
3 HisTrap		LDAO	LDAO	PI tablet
4 HisTrap		LDAO	DM	Specific
5 HisTrap		FC-12	FC-12	Specific

Concentration determination of all the fractions from the manual runs was done with the NanoDrop Spectrophotometer (SWAB) at 280nm. The fractions were then analyzed with SDS-PAGE (NuPAGE 4-12% Bis-Tris Gel (Life Technologies),1xMES running buffer, 200V for 30 min) and initially also with Western blotting. Fractions containing the *menA* protein were pooled and concentrated in Vivaspin 500, 10 kDa MWCO tubes (Sartorius Stedim biotech) at 4000xg, 4°C. Concentration measurements were thereafter done with a theoretical extinction coefficient (54430 $M^{-1}cm^{-1}$) at 280 nm using ProtParam. For the HisTrap purifications the fractions taken from the corresponding protein peak were analyzed on SDS-PAGE. The protein-containing fractions were then pooled

and concentrated as previously described.

3.7 Desalting the fractions from IMAC

The elution fractions containing the *menA* enzyme from one of the manual IMAC purifications were passed through a PD-10 desalting column (GE Healthcare) according to the manufacturer's instructions. 500 μl aliquots were collected and the absorbance of the fractions was measured. Buffer A was used as buffer both for washing the column and for sample elution. The samples were put on an SDS-PAGE for further analysis.

3.8 Precipitate testing

The precipitate was collected by centrifugation at 13000xg (Biofuge, Kendro Laboratory Products) for 15 min, 4 °C with the pooled fractions from the second HisTrap purification. The precipitate was resuspended in a solution containing 0.2% LDAO, 17% glycerol and aliquoted in different tubes. Salts and buffers were added to a final concentration of 100 mM and with 0.1% LDAO. Detailed information of a precipitate solubility screen has been described elsewhere [22].The content in each tube is presented in Table 5.

Table 5: The different salts and buffers tested together with the resuspended precipitate.

Salts	Buffers
LiCl	MES
$MgCl_2$	HEPES
KCl	TAPS
$CaCl_2$	
NH_4Cl	
NaCl	

The aliquots were analyzed on an SDS-PAGE as previously described.

3.9 Coupling degradation to cell growth

A small-scale cell culture was grown aerobically at 37° C, 200 rpm until induction at an OD_{600} value of approximately 1. The temperature was then lowered to 25° C, 200 rpm and samples were taken at certain time points after induction. Samples were run on SDS-PAGE and analyzed with Western blotting. The aim was to investigate if the degradation occurs at a certain time point of growth.

3.10 Size exclusion chromatography (SEC)

The protein sample, after IMAC, pooling and concentrating, was loaded onto a Superdex TM 200 column (GE Healthcare) with 1.5 MPa as highest pressure. Buffer A was used as mobile phase together with the protein sample. In the first experiment, Buffer A with 0.1% LDAO and without the specific protease inhibitors (but with PI tablets) was used. In the second experiment Buffer A with 0.1% FC-12 with the specific PIs was used.

3.11 Enzymatic activity (membrane)

The end-point enzymatic activity test was used to test *menA* activity in the membrane. The kit used was the EnzChek Pyrophosphate Assay Kit (Molecular Probes).

DHNA (1,4-dihydroxy-2-naphthoic acid) is converted to demethylmenaquinone by *menA* in the presence of an isoprenoid diphosphate (in this case farnesyldiphosphate (F-DPTA)). The reaction releases PPi which in turn is converted to two equivalents of Pi by the pyrophosphatase enzyme available in the kit. In the presence of Pi the substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) is converted to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by the PNP enzyme available in the kit. The amount of Pi consumed is detected as an increase in the absorbance at 360 nm and can hence be coupled to enzyme (*menA*) activity. The idea of using farnesyldiphosphate as precursor for the isoprenoid side chain came from an article by Melzer and Heide [23].

Three types of membranes were tested for enzymatic activity; membrane prepared without the specific PIs (but with PI-tablets), membrane prepared with the specific PIs and non-induced membrane. The non-induced membrane was produced in order to establish the baseline enzymatic activity.

One unit of membrane means 10 μl membrane (suspended in membrane resuspension buffer). Each membrane was tested with both 1 and 2 units in the reaction.

3.12 Anaerobic growth

Anaerobic growth was compared to aerobic growth in a simultaneous experiment. After induction (37° C, 200 rpm) the cell culture was divided into two Falcon tubes, one completely filled (anaerobic; 15 ml cells in 15 ml Falcon tube) and the other one just slightly filled (aerobic;15 ml cells in 50 ml Falcon tube). The temperature was lowered to 30° C. To the anaerobic tube NO_3 was added (0.2%) as new terminal electron acceptor instead of O_2. Cell samples were taken, prepared and put on an SDS-PAGE and analyzed through Western blotting. A non-induced sample was used as a reference. Inspiration to test for anaerobic growth was provided in the article by Unden and Bongaerts [24].

In the same Western blot, cell samples from another anaerobic (only) batch was analyzed as well. This was a large-scale batch performed in a 5L E-flask completely filled with media (supplemented with 0.2 % NO_3) and with a slow stirring rate (75 rpm) to ensure anaerobic growth. The temperature up to induction was 37° C and thereafter lowered to 30° C.

4 Results

In the following sections the results from the experiments will be presented.

4.1 PCR

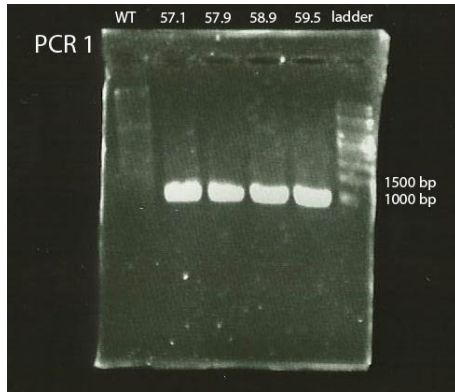The PCR products from the temperature gradient test are presented in Figure 15.

Figure 15: Gel picture of the PCR temperature gradient of the primers to determine appropriate annealing temperature.
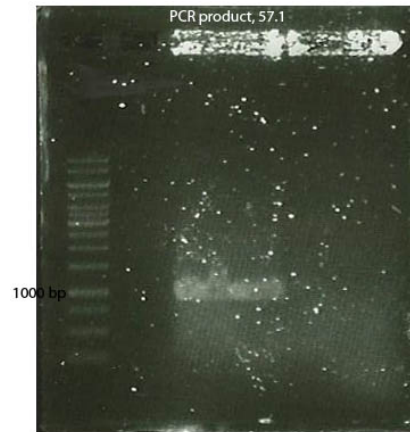

Figure 16: Confirmation of *menA* size and its presence after choosing 57.1 ° C as primer annealing temperature.

Any temperature seems appropriate for *menA* amplification since the four bands are equally visible. 57.1 ° C was chosen as primer annealing temperature and the resulting gel picture, after the second PCR amplification, is presented in Figure 16.

[*menA*]: 44ng/μl after gel extraction from the gel in Figure 16.

4.2 Cloning and transformation

Ligation of the *menA* gene with the pEXP5-NT/TOPO expression vector and subsequent transformation into TOP10 cells and LB-Amp plate spreading did not succeed immediately. The number of colonies formed were few and of small size. Increasing the cell plating volume on the LB-Amp plates and decreasing the *menA*:vector concentration relationship (from 1:1 to 0.5:1) increased the number of colonies. However, it should be noted that there were never many colonies on any of the plates. The first plasmid size analysis is presented in Figure 17.
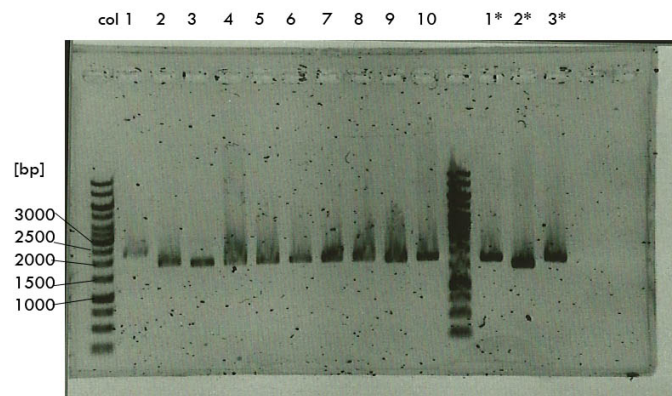
Figure 17: Plasmid size analysis with agarose gel electrophoresis. The construct should be 3672 bp. The numbers present the different colonies which were analyzed.

The basepair (bp) length of the insert combined with the vector should be 3672 bp. None of the bands corresponds to the right size but the ones closest are colonies 1, 1* and 3*. However, sequencing of these plasmids together with 6 other different plasmids (not shown in figure) gave no correct insertion. Changing the conditions for the 3'A end addition (20 min instead of 10 min in 72 °C) gave more colonies on the LB-Amp plates and the plasmid sizes are shown in Figure 18.
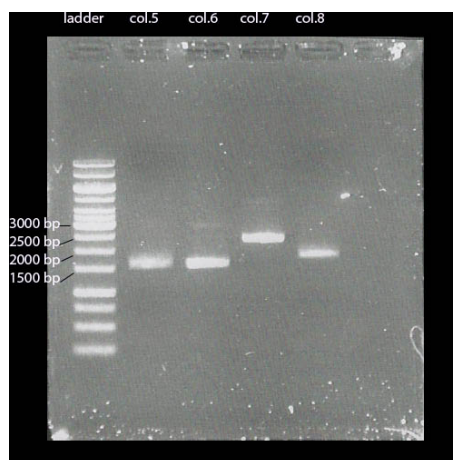

Figure 18: Plasmid size analysis after changing to 20 min instead of 10 min with the 72°C *Taq*-polymerase reaction. Col stands for colony.

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Plasmid number seven looked promising since it has several bands. This indicates presence of both linearised and round plasmid which typically is seen on agarose gels loaded with circular DNA. Sequencing of the plasmid from this clone confirmed presence of the *menA* gene inserted into the vector in the right direction. Therefore it can be concluded that for the *menA* gene to be inserted in the pEXP5-NT/TOPO vector, 20 min 3'A addition at 72 ° C is needed for success.

Transformation into BL21*(DE3) One Shot cells was successful when preheated (37° C) LB media was used for both initial and subsequent growth.

4.3 Test expression

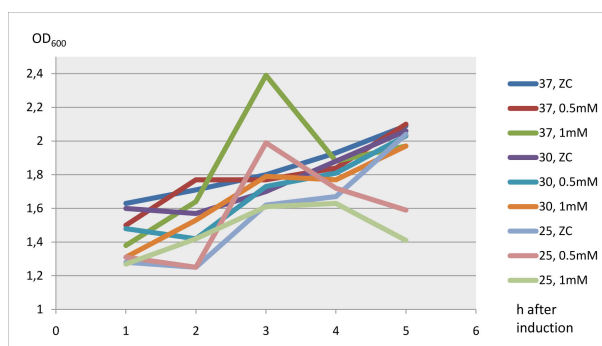The growth curve of the initial test expression (after induction) is presented in Figure 19.

Figure 19: Initial test expression. Monitoring of the optical density after induction. ZC stands for zero control, 0.5 and 1 mM is the IPTG concentration

The cells are growing in a similar manner, independent of temperature and IPTG concentration.The Western blot for the initial test expression is presented in Figure 20.

Protein production can be confirmed and it occurs shortly after induction with IPTG. The strongest protein production is at 2h after induction at 25 °C and 30°C. The test expression (24-well plate) with autoinduction at the investigated timepoint did not give any sign of protein production except from the LB well induced with IPTG. The IPTG test expression (24-well plate) yielded the growth curve presented in Figure 21.
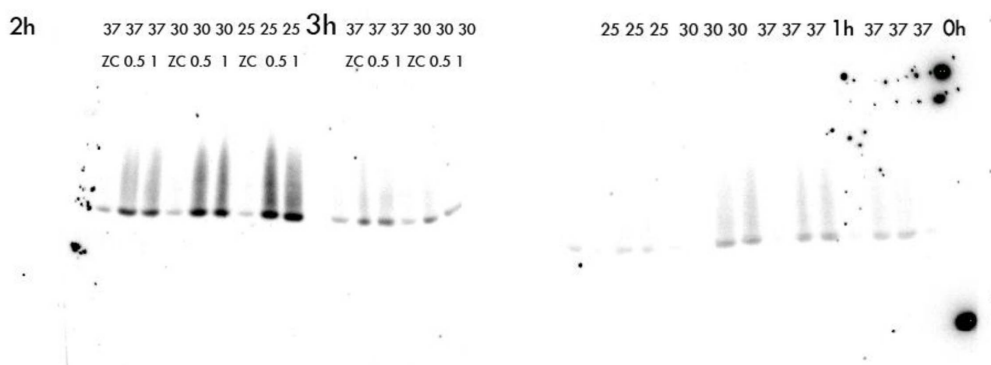
Figure 20: Western blot of the initial test expression. Samples from the 0 h–>3h after induction. ZC stands for zero control, 0.5 and 1 stands for mM concentration of IPTG.
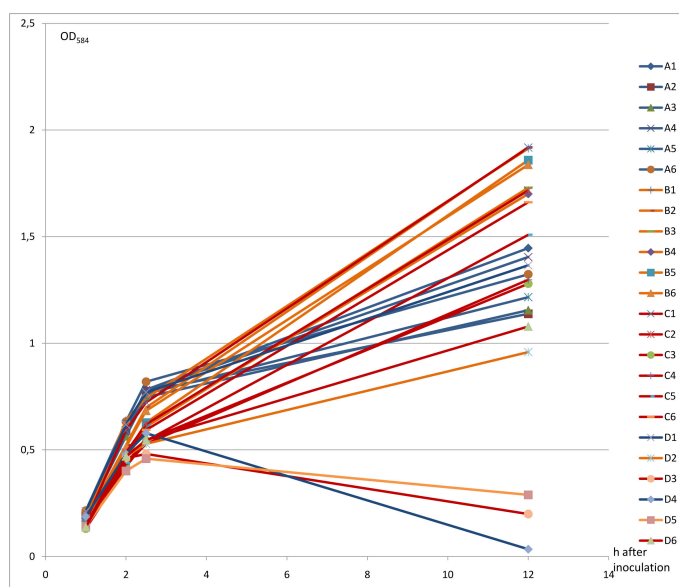

Figure 21: 24-well IPTG test expression. Optical density followed after induction. The blue lines represent LB media, the orange contain 32Y and the red 32Y supplemented with $MgSO_4$.

The cells grow in a similar manner. The Western blot for the IPTG test expression plate (LB wells only, A1->A6 in Table 2) is presented in Figure 22.

Protein production is only seen within the LB wells of this test expression, no Western blot detection was apparent with 32Y media. Furthermore, it could be concluded that 0.5–>1 % glucose and 0.5–>1mM IPTG are good conditions for protein production. From this point forward LB media was used with 1% glucose and induced with 1mM IPTG. 50 μg/ml Ampicillin was used as before.
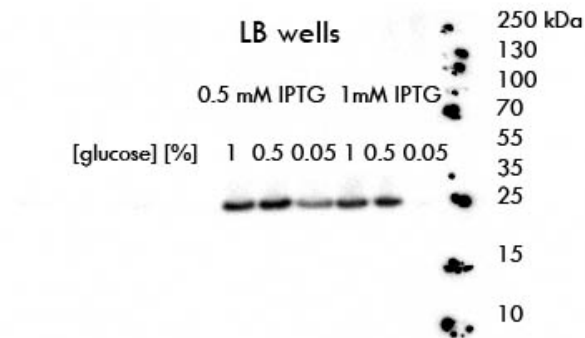
Figure 22: Western blot of the 24-well IPTG test expression. LB wells (A1->A6) with different glucose-and IPTG concentrations.

The *menA* protein centers close to the 25kDa band. In reality, the molecular weight (MW) for *menA* is 33.59 kDa but since the protein ladder consists of soluble proteins and not membrane proteins this MW-misbehaviour can be expected.

4.4   Cell growth and membrane preparation

The OD_{600} of the O.N. culture varied between 1.8–>3.5 for the different cultures used for membrane preparation.

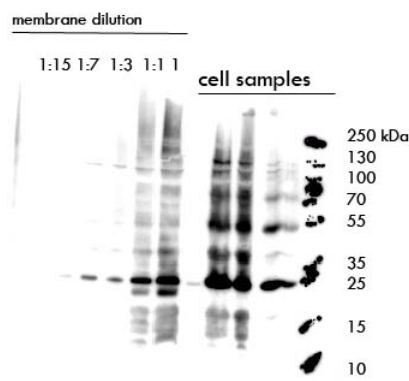The result from the membrane dilution series is presented in Figure 23.

Figure 23: Membrane dilution series, resuspended in membrane resuspension buffer. Cell samples means before harvest (closest to the ladder), unlysed cells, lysed cells, lysed cells centrifuged. The ultracentrifuged sample (supernatant above membrane) was loaded right after the membrane dilution samples.

From this, 1:1 was determined as the membrane dilution for the following experiments. Other dilutions (1:3) would also be appropriate due to the strong Western signal. Presence of Western blot bands in the membrane dilutions indicate that the *menA* enzyme is inserted into the membrane.

The OD_{600} for induction, the OD_{600} at harvest point and membrane yield for the large-scale productions (not anaerobic) are presented in Table 6.

Table 6: Cell cultivation conditions and membrane yield of the individual large-scale runs.

Culture	OD(600) induction	Growth time after induction (h)	OD(600) harvest	membrane yield (g)
6L	0.9	19	3.65	3.42
6L	0.89	1	1.2	4.08

4.5 Solubilization screen

The result from the solubilization screen is presented in Figure24.
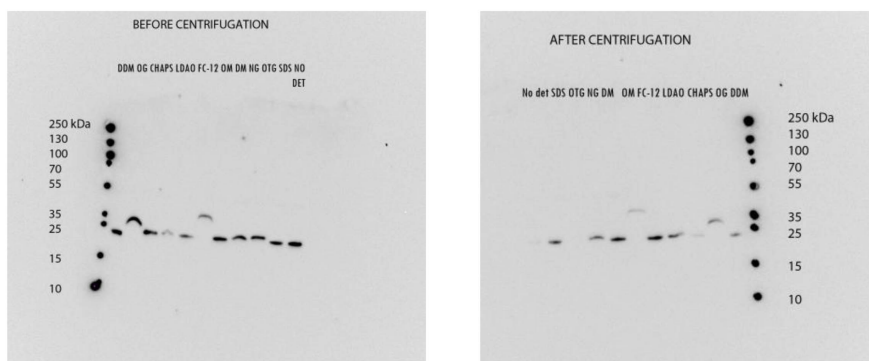

Figure 24: The solubilization screen: before and after ultracentrifugation of solubilized membrane. No det stands for No detergent.

This solubilization screen makes it clear that DM, FC-12,LDAO and possibly DDM are suitable solubilization detergents for *menA* since strong Western bands close to the 25kDa band are seen after ultracentrifugation. OG and OM are not appropriate detergents due to some kind of aggregate formation as can be seen with the higher molecular weight bands for those detergents.

4.6 IMAC purification

The features of the individual IMAC purifications can be found in Table 4. Some of the features will also be repeated in this section for clarity. Furthermore, in the manual

IMAC purifications, no specific protease inhibitors (only PI tablets) were added to the buffers.

The manual IMAC purifications

The absorbance measurements from the manual IMAC purification fractions are presented in Table 7, 8 and 9. The corresponding SDS-PAGEs are presented in Figure 25, 26 and 28. A stands for Buffer A, 20, 40, 80 stands for mM concentration of imidazole in the wash buffer and E stands for elution (500mM imidazole).

Table 7: The first manual IMAC purification. Absorbance measurements with DDM as detergent. A stands for Buffer A, 20, 40 and 80 means mM imidazole concentration in the different wash steps and E stands for elution (500mM imidazole). The number in brackets is each individual fraction collected.

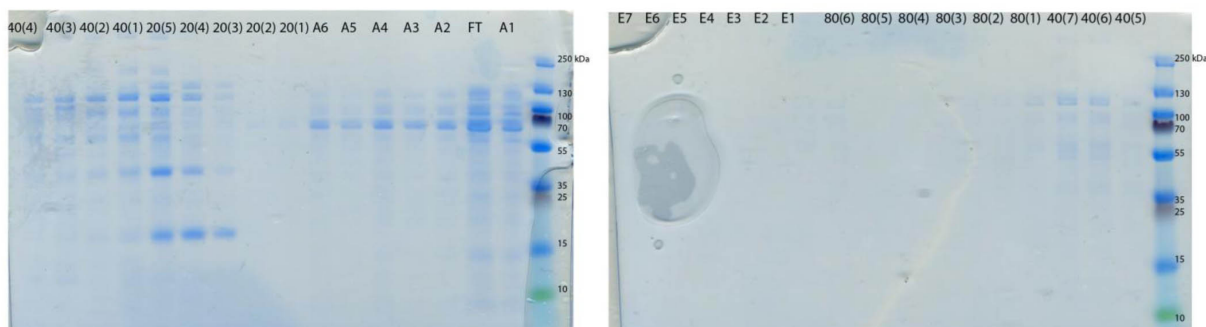| A (1-->10)	20(1-->10)	40(1-->10)	80(1-->6)	E(1-->8)
3.78	0.2	0.17	0.49	0.25
1.84	0.14	0.29	0.21	0.57
1.32	0.16	0.35	0.19	0.66
1.48	0.26	0.41	0.17	0.69
1.17	0.10	0.38	0.17	0.75
0.95	0.28	0.39	0.18	0.75
0.35	0.19	0.41		0.76
0.21	0.12	0.38		0.75
0.19	0.12	0.38		
0.19	0.11	0.38		

Figure 25: The first manual IMAC purification. The number in brackets means the fraction number of each step.

The result from the first manual purification was not promising due to the fact that the elution bands are very weak (merely visible in the pictures). It is impossible to identify the *menA* protein in any of the wash steps or elution steps. Probably, the protein was lost during the ultracentrifugation after solubilization since much pellet was formed. Furthermore, after re-calculation of the solubilization protocol it seems that too little detergent (DDM) was added which most likely can explain the lack of protein in the elution bands.

Table 8: The second manual IMAC purification. Absorbance measurements with DM as detergent. A stands for Buffer A, 20, 40 and 80 means mM imidazole concentration and E stands for elution (500mM imidazole). The number in brackets is each individual fraction collected.

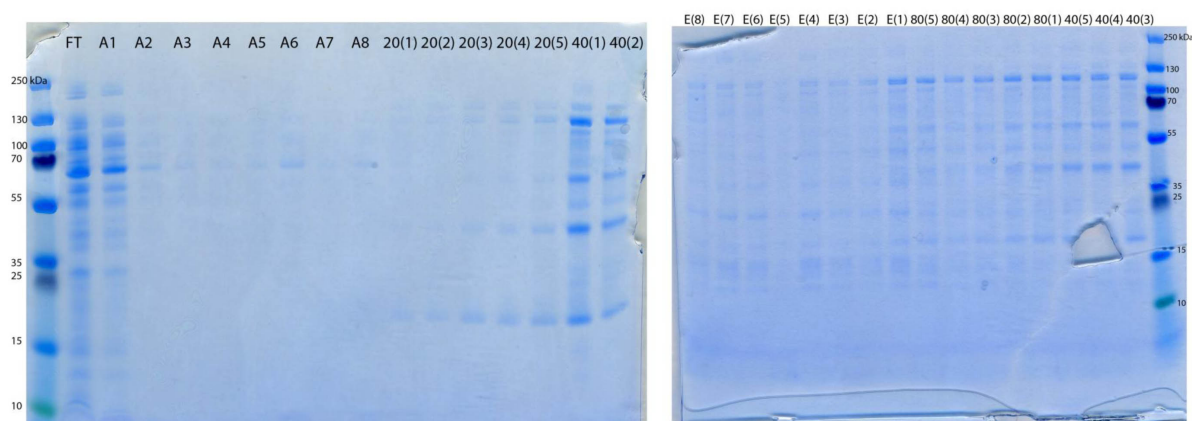| A1-->A10	20(1)-->20(5)	40(1)-->40(5)	80(1)-->80(5)	E(1)-->E(8)
5.7	0.45	0.68	0.57	0.94
1.25	0.32	0.56	0.49	0.97
0.89	0.35	0.46	0.48	0.99
0.73	0.39	0.42	0.50	1.04
0.70	0.39	0.45	0.48	0.94
1.13				0.97
0.54				0.99
0.63				1.04
0.33				
0.28				

Figure 26: The second manual IMAC purification. The number in brackets means the fraction number of each step.

In the second manual purification, there are many separated bands in each well in the elution part (E-wells) of the gel which means that the *menA* protein is not eluted alone. The wells with the 80mM wash and the elution wells seem to contain the *menA* protein. This is based on an available band close to the 25kDa band. However, to confirm this theory a Western blot was performed. The result is presented in Figure 27.
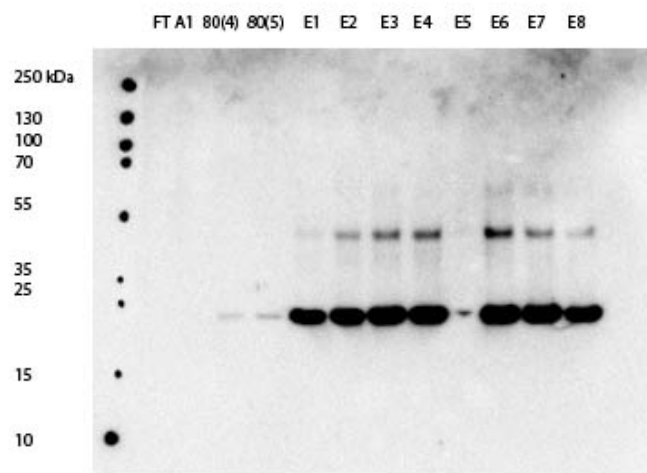
Figure 27: Western blot of the second IMAC purification (DM). FT stands for flow through. The number in brackets means the fraction number.

The Western blot supports the theory and further reduces the protein-containing fractions to the elution bands only. Furthermore there seems to be a higher molecular weight band present, suggesting aggregation or dimer formation. The elution fractions were pooled, separated by SDS-PAGE and sent for mass spectrometry to confirm protein identity. Mass spectrometry identified *menA* in the 25 kDa band. The pooled sample was concentrated which revealed protein precipitation. *menA* could not be concentrated to more than 0.93 mg/ml with 10 μl left.

Table 9: The third manual IMAC purification. Absorbance measurement with DM as solubilization detergent and LDAO as purification detergent.A stands for Buffer A, 20, 40 and 80 means mM imidazole concentration and E stands for elution (500mM imidazole). The number in brackets is each individual fraction collected.

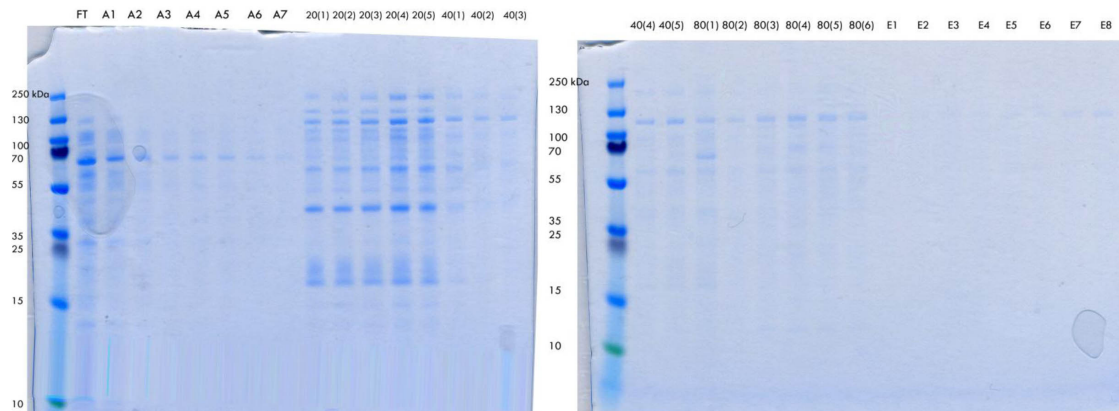| A1-->A8	20(1)-->20(6)	40(1)-->40(5)	80(1)-->80(8)	E(1)-->E(8)
3.6	0.45	0.39	1.41	0.89
1.74	0.39	0.43	0.35	0.95
1.44	0.48	0.29	0.33	0.96
1.28	0.53	0.32	0.38	0.95
1.05	0.52	0.30	0.35	0.96
0.64	0.49		0.35	0.97
0.51			0.33	0.99
0.45			0.34	0.97

Figure 28: The third manual IMAC purification. The number in brackets means the fraction number of each step.

There are no bands visible in the elution fractions of the third manual IMAC purification. Furthermore, there is no evidence of presence of *menA*. However, a Western blot was done with samples FT, A1, 20(3), 20(4), 40(5), 80(4)–>80(6), E1–>E8. The idea was that a variation of samples could give a clear sign at what stage the protein is eluted. The result is presented in Figure 29.
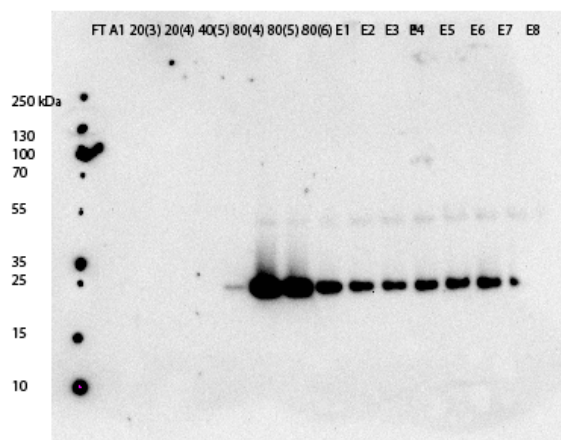
Figure 29: Western blot of the third IMAC purification (DM+LDAO). FT stands for flow through. The number in brackets means the fraction number.

The protein is indeed present in the elution bands and in two of the 80mM imidazole fractions. Concentration of the protein-containing samples (80(5)->E(8)) revealed pos-

sible precipitation of the protein. The protein concentration reached a maximum of 0.5 mg/ml, with 50 μl left, when concentrated. Efforts to resuspend the precipitate failed.

IMAC purification with HisTrap on the *ÄKTA*

This section will present the results from the IMAC purifications with the HisTrap. PI tablets were added to the buffer for the first three experiments. The last two experiments contained specific protease inhibitors. The detergents used in each case can be found in Table 4.

The HisTrap purifications without serine-and metalloprotease specific inhibitors
The chromatogram from the first HisTrap purification is presented in Figure 30 and the resulting SDS-PAGE in Figure 31.
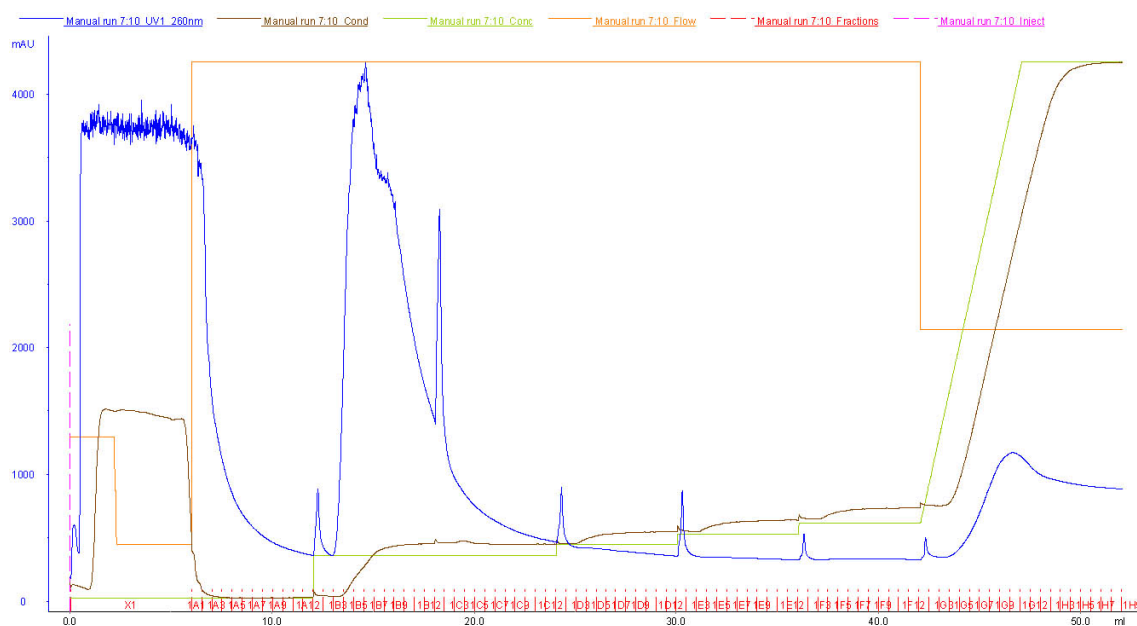

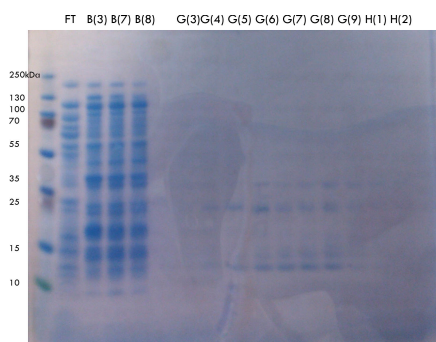**Figure 30:** The chromatogram from the first His-Trap purification.

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Figure 31: The SDS-PAGE from the first HisTrap.The corresponding fractions are indicated above each well.

The result from the separation by the SDS-PAGE reveals bands with equal intensity as the *menA* band but with lower molecular weight which is the typical sign of degradation. The G(4)–> G(9) fractions were pooled and concentrated. However, precipitation occurred almost immediately. The protein concentration reached 0.5 mg/ml with 1 ml left.

The chromatogram and SDS-PAGE of the second HisTrap purification are presented in Figure 32 and Figure 33.
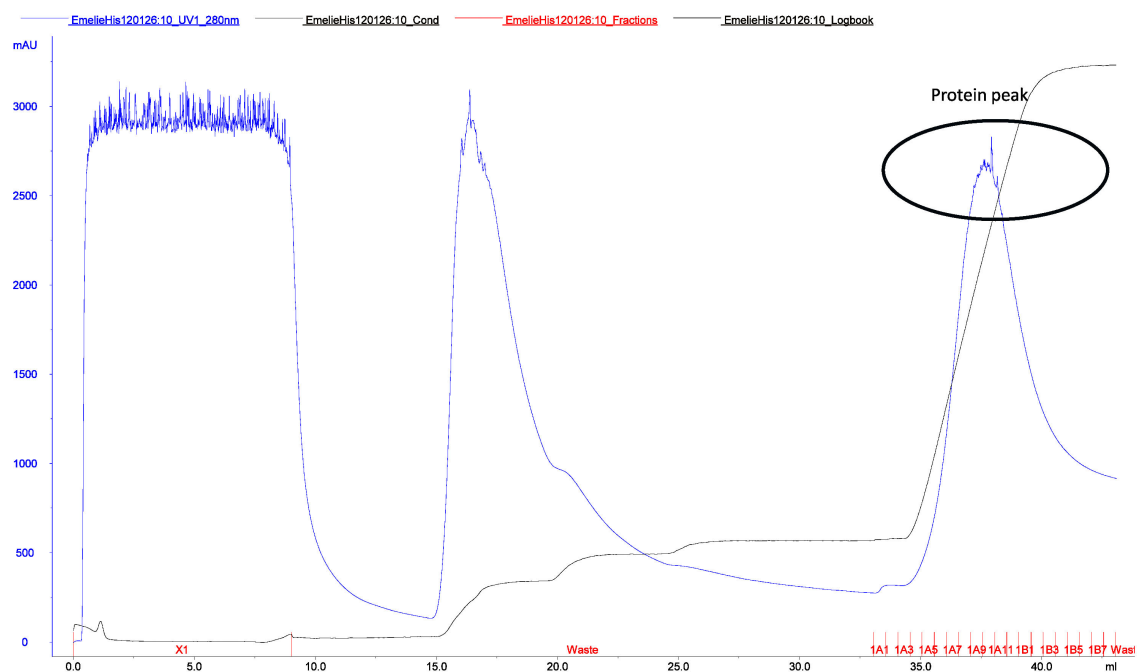

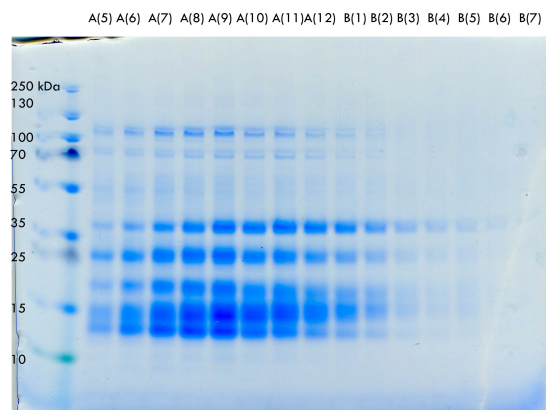**Figure 32:** The chromatogram from the second His-Trap purification.

Figure 33: The SDS-PAGE from the second His-Trap purification.The corresponding fractions are indicated above each well. Note the clear sign of degradation as the equal-intensity bands above and below the *menA* band (25 kDa)

Bands with equal intensity as the *menA* band but with lower molecular weight are available, again displaying a typical degradation pattern (more visible here than in the previous SDS-PAGE). All samples were pooled and concentration of the sample proceeded until precipitation. The precipitate was collected and tested, see section "Precipitate testing". Further concentration of the supernatant, after removal of precipitate, yielded a protein concentration of 2.55 mg/ml with 250 μl left. The chromatogram and SDS-PAGE from the third HisTrap purification are presented in Figure 34 and Figure 35.
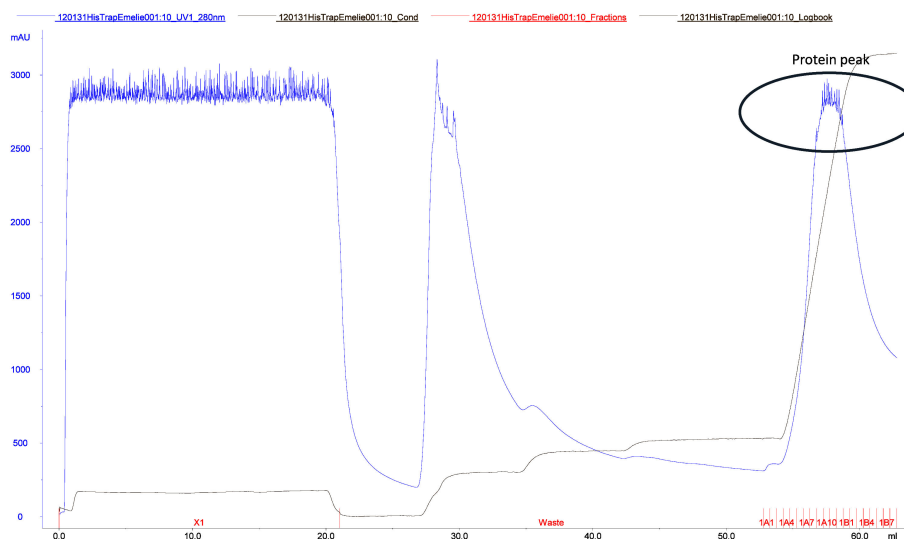

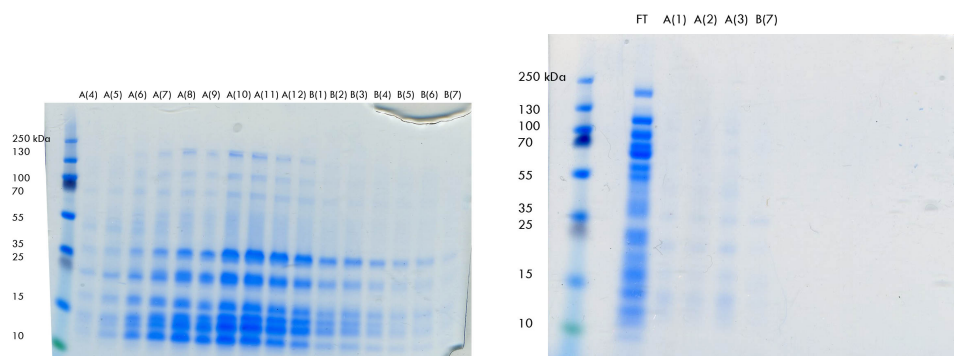**Figure 34:** The chromatogram from the third His-Trap purification.

Figure 35: The SDS-PAGE from the third His-Trap purification. The corresponding fractions are indicated above each well.

Again, the degradation pattern is obvious. The A(4)–>B(7) samples were pooled, pressed through a 0.2 μ m filter (VWR) and concentrated. A small amount of protein was lost when filtering (2.5 mg/ml before–>1.9 mg/ml after filtering). The protein concentration could thereafter reach 3.7 mg/ml with 250 μl left. The protein sample was loaded onto the Superdex 200.

The HisTrap purifications with serine-and metalloprotease specific inhibitors

The result from the first HisTrap purification with the specific protease inhibitors is presented in Figure 36. LDAO was used as detergent for solubilization, DM for purification. The SDS-PAGE shows that the amount of protein loaded on the gel is very low. However, there are no or few degradation bands since there are no bands with equal intensity as *menA* with a lower molecular weight. The reduced amount of these low molecular weight bands could indicate a less degree of degradation. A(5)–>A(12) were pooled, filtered and concentrated. The protein was concentrated to 1.1 mg/ml with 250 μl left. Protein precipitation did not occur.
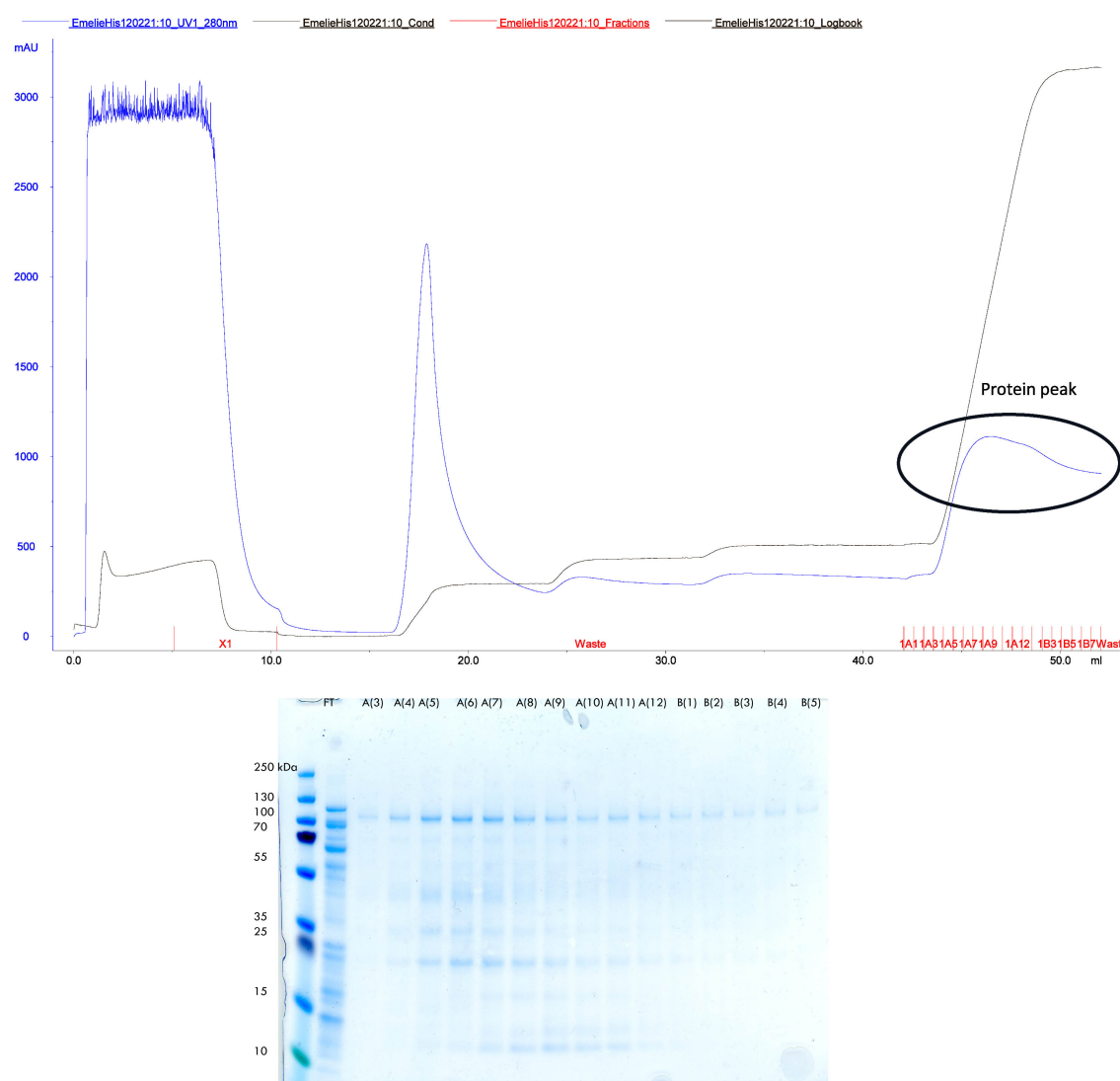
Figure 36: The chromatogram and SDS-PAGE from the first HisTrap with the new protease inhibitors.The corresponding fractions are indicated above each well.

The result from the second HisTrap purification in this category is presented in Figure 37. FC-12 was used as detergent, both for solubilization and purification. The SDS-PAGE has no degradation pattern and indicates a strong presence of *menA*. The A(4)–>A(11) fractions were pooled, filtered and concentrated. While concentrating the sample it turned yellow. The protein concentration started at 0.7 mg/ml with 3 ml sample and reached 5.6 mg/ml with 200 μl sample left after concentrating. This means that a large fraction of the protein amount is lost when concentrating. The sample was loaded onto the Superdex 200 column.
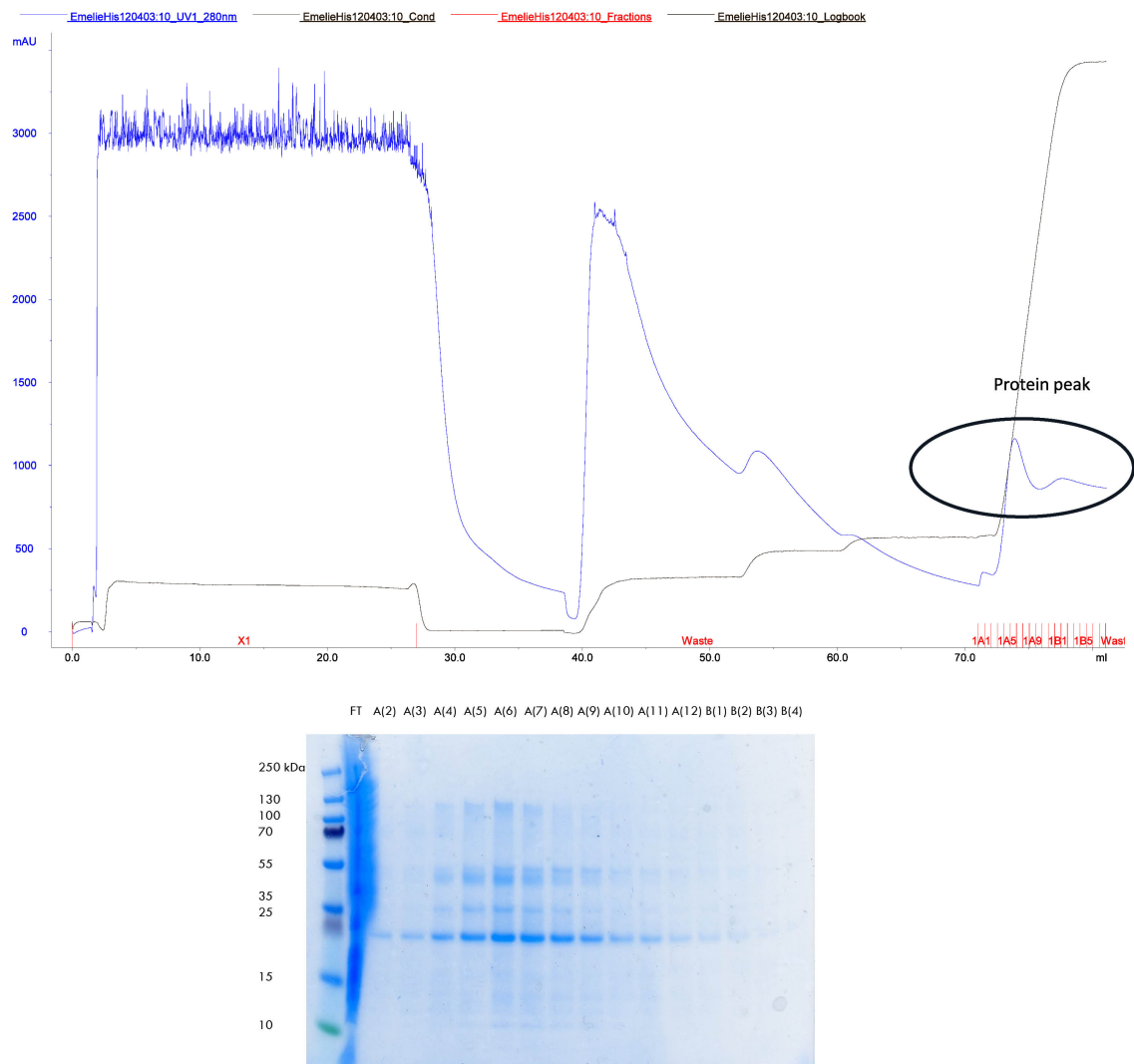
Figure 37: The chromatogram and SDS-PAGE from the second HisTrap with the new protease inhibitors.The corresponding fractions are indicated above each well.

4.7 Desalting the fractions from IMAC

The result from the desalted IMAC fractions is presented in Figure 38.
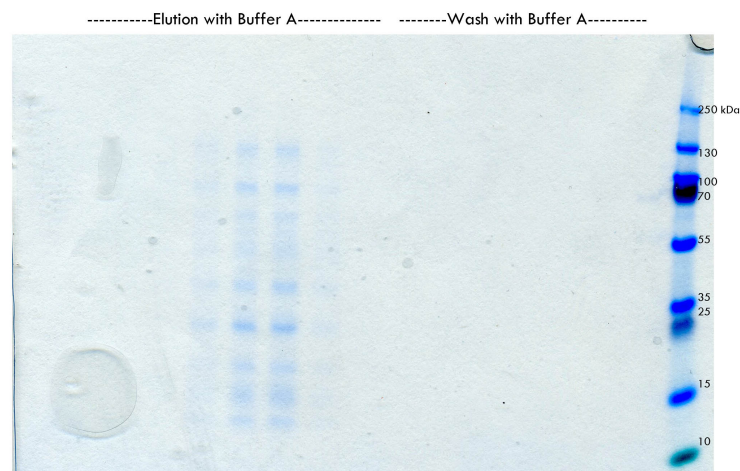

Figure 38: The SDS-PAGE of the wash-and elution fractions from the desalting column

Further concentration of the protein revealed precipitation which indicates that desalting does not hinder precipitate formation.

4.8 Precipitate testing

The SDS-PAGE for the precipitate testing is presented in Figure 39.

S=supernatant
S1=supernatant+ $MgCl_2$
S2=supernatant+NaAc
S3=supernatant+TAPS
P=precipitate, before aliquot
P1=LiCl
P2= $MgCl_2$
P3=KCl
P4=$CaCl_2$
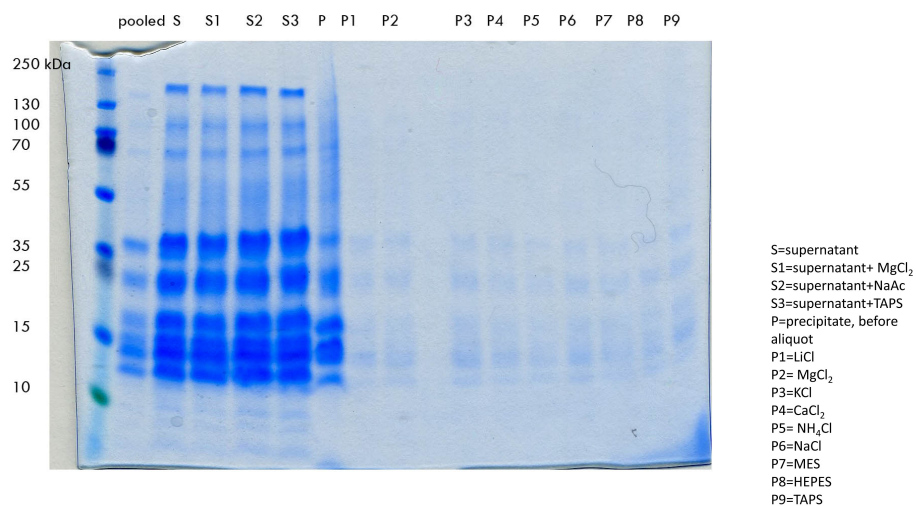P5= NH_4Cl
P6=NaCl
P7=MES
P8=HEPES
P9=TAPS

Figure 39: The result from the precipitate testing. P stands for precipitate, S stands for supernatant.

There seems to be very little protein present in the different precipitate aliquots and it looks like *menA* and degraded protein only.

4.9 Coupling degradation to cell growth

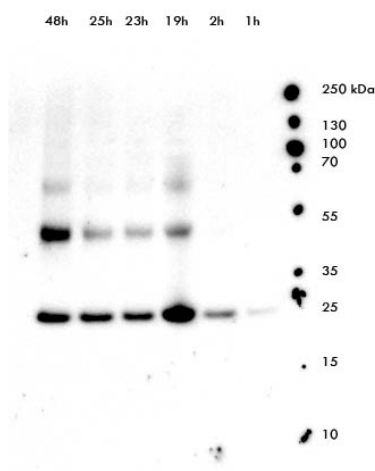The Western blot from the cell samples prepared to analyze the degradation pattern is presented in Figure 40.

Figure 40: The Western blot with cell samples taken at different time points after induction (indicated in the figure) to map the degradation pattern.

The highest *menA* production is at 19h after induction with IPTG. However, at 19 h and after that time-point, aggregated (or dimer formation of) *menA* can be seen as the higher molecular weight band above the native *menA* band. No degradation pattern is visible in this Western blot.

4.10 SEC purification

The result from the first gel filtration purification is presented in Figure 41.

Since there are a number of peaks there are many proteins available. All of the fractions containing protein were separated by SDS-PAGE. The result is presented in Figure 42. Based on the appearance of the SDS-PAGE, *menA* is present but not pure enough for crystallization. There are signs of degradation on the SDS-PAGE.
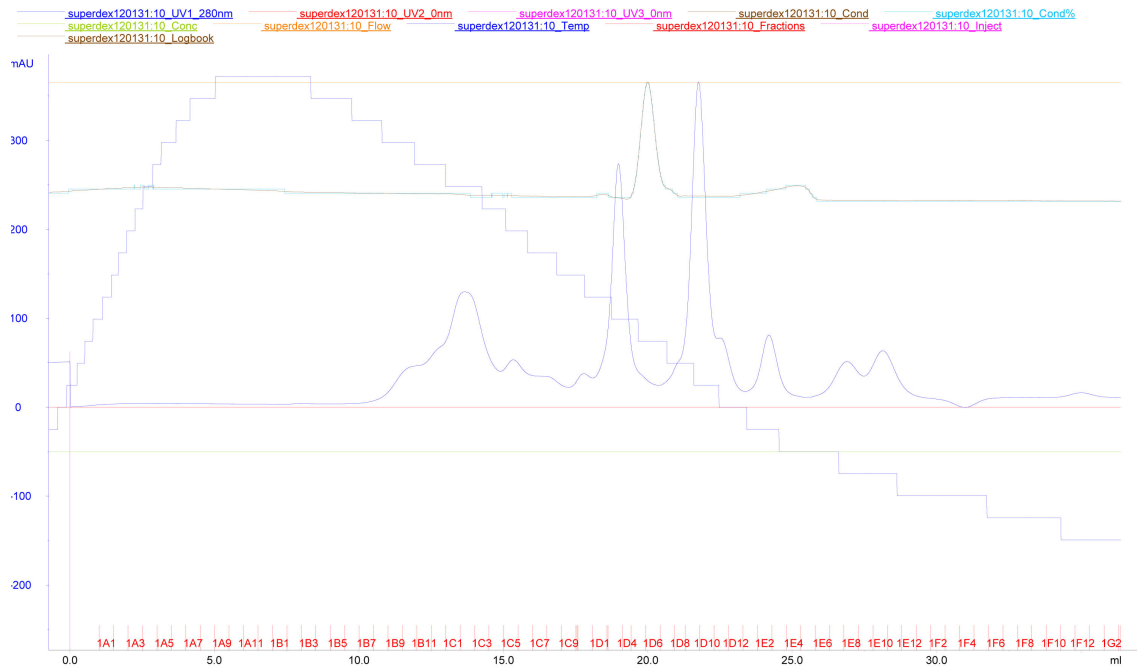
Figure 41: The chromatogram of the first Superdex200 purification.
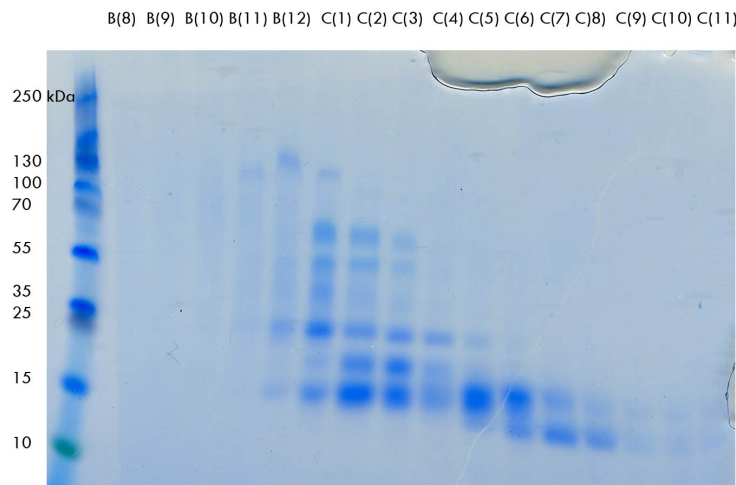
Figure 42: The SDS-PAGE of the first Superdex200 purification. The corresponding SEC fractions are indicated above each well.

The result from the second gel filtration purification is presented in Figure 43. The chromatogram reveals two peaks and the corresponding fractions were separated by SDS-PAGE. The aggregation (or dimer) pattern is present in the fractions corresponding to the first peak (eluted first from the column, the B-samples). Some of the fractions, in particular C1 and C2, of the second peak contain pure *menA*. Furthermore, no

Figure 43: The chromatogram and SDS-PAGE of the second Superdex200 purification.
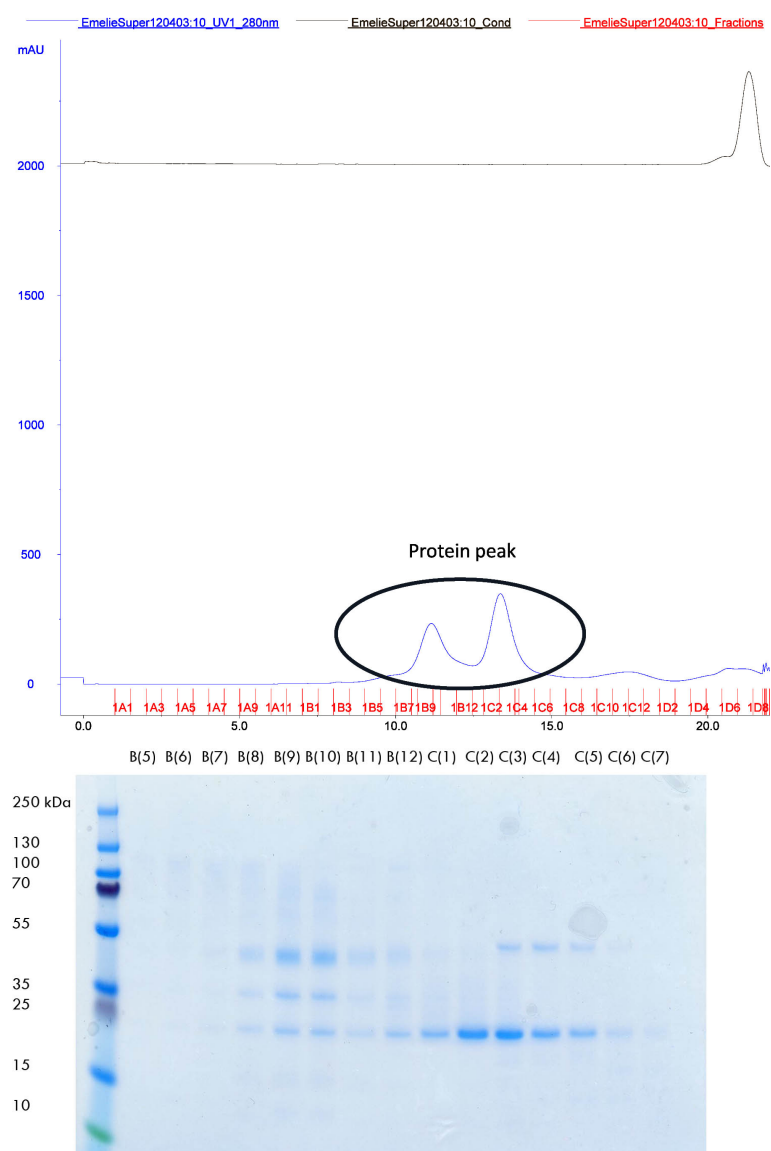
degradation pattern is visible in any of the fractions. B9+B10 and C1+C2 were pooled separately and concentrated. However, the concentration could not reach more than 3 mg/ml (with 30 μl left) and hence could not be used to set up crystal plates with. No precipitation occurred.

4.11 Enzymatic activity (membrane)

The absorbance measurements for the three different types of membranes is presented in Table 10.

Table 10: The absorbance measurements at 360 nm for the different membranes to determine *menA* activity. M1 is the membrane without specific PI, M2 is the membrane with specific PI and M3 is the non-induced membrane. 1 unit corresponds to 10 μl membrane.

unit/M	M1	M2	M3
1 unit	0.53	0.4	0.205
2 units	0.97	0.82	0.615

The result shows that *menA* is overproduced and that the overproduced part is active in the membrane. Comparing the results with one unit of membrane it can easily be seen that the overproduced membrane contain approximately double amount of *menA* compared to the non-induced. It should however be noted that even though the enzyme is active in the membrane, there is no guarantee for its activity in the purified form.

4.12 Anaerobic growth

The Western blot performed to compare aerobic and anaerobic cell growth in respect to the production of *menA* is presented in Figure 44.

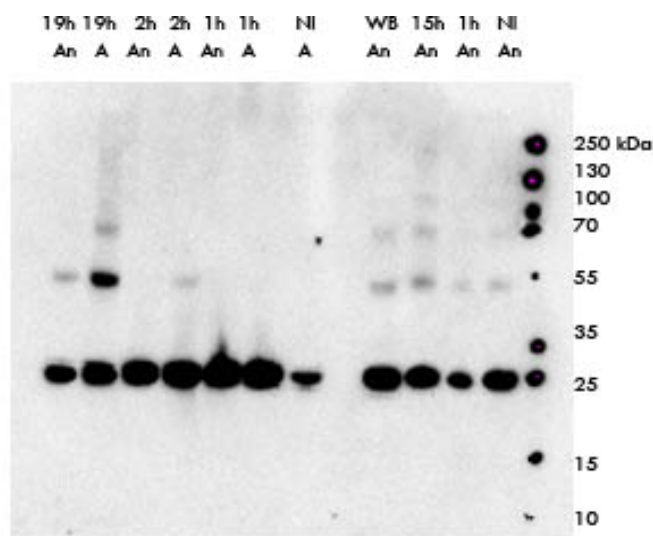**Figure 44:** The Western blot with cell samples taken from anaerobic and aerobic growth. The fractions closest to the protein ladder is from another batch (large-scale anaerobic growth) with anaerobic growth only. A stands for aerobic, An for anaerobic, NI for non-induced and WB for cells resuspended in wash buffer.

Again, the aggregation pattern is visible at 19h of growth after induction. However,

for the large-scale anaerobic growth aggregation is visible already in the non-induced sample which suggests that it occurs earlier than 19h. To establish the exakt time-point of aggregation, more cell samples need to be investigated in the time-point in between 1-19 h after induction.

5 Discussion and conclusions

5.1 Discussion

The topics included in the Results section will be discussed in the following sections.

Cloning and transformation

Out of the large number of different plasmids that were sent for sequencing, only one had *menA* inserted into the vector in the right direction. The reasons to why the ligation was not successful in most plasmids can be many. Since the 3'A addition is an obligation for gene insertion the most probable answer is that something went wrong at this stage. A proof for this theory arose when the incubation time in 72 ° C was changed to 20 min from 10 min. The plasmid with the *menA* inserted in the right direction came from the 20 min incubation.

Transformation into the BL21* (DE3) cells succeeded only when the media was pre-heated. The reason behind this fact is hard to decide. Maybe it is the strain as such that needs heated medium for survival or that the uptake of the plasmid gives other demands on growth.

Cell growth

The growth of the BL21* (DE3) cells with the plasmid is affected by the presence of the plasmid. This is illustrated by the fact that the OD_{600} of an O.N. culture never reached more than 2.8 (small scale O.N. culture, used for inoculating the big flasks, could reach an OD_{600} of 3.5). The slower growth indicates that much of the cell's machinery is focussed on producing protein which of course is beneficial. On the other hand, this extra stress for the bacterial system can mean death of cells that cannot handle it.

Also, prewarming of the media before inoculating it with freeze cultures is important. It has been observed that the cell's OD_{600} increased faster when pre-heating the media. This indicates that the BL21* (DE3) cells (with plasmid) are not very stable as freeze cultures but it is beneficial that a simple method as pre-heating the media solves the problem.

Membrane preparation and solubilization

The first membrane dilution series, see Figure 23, could confirm presence of *menA* both in the cell samples and in the resuspended membrane with little loss of total protein

amount. This suggests that most of the overproduced part of *menA* is also inserted into the membrane.

Solubilization of the *menA* enzyme out of the membrane has been successful with DM, LDAO and FC-12. Successful in this sense means that the protein has been visible after solubilisation and IMAC purification. However, there is no direct measure of effectiveness of each individual detergent. The only effectiveness you can measure is how strong the protein has bound to the column (or resin) for each type of detergent. This can be done by comparing the flow through (FT) fractions with the wash-and elution step within and between SDS-PAGEs. What can be concluded is the fact that some *menA* is lost in the solubilization step. This is due to the weaker *menA* bands after centrifugation compared to before centrifugation of the solubilized membrane.

Purification

For the manual IMAC purifications, the *menA* enzyme is hard to distinguish on the different SDS-PAGEs. However, the Western blots for the same SDS-PAGE show high levels of *menA*, indicating the importance of Western blotting as identification tool. Furthermore, the SDS-PAGEs indicate that the *menA* enzyme is not eluted alone but with several other proteins as well. It might depend on the interaction between the histidine residues and the NiNTA resin, that it is not strong enough. Precipitation occurred in every manual run when the protein was pooled and concentrated.

For the purifications with the HisTrap (without the specific inhibitors) the bands on the SDS-PAGEs are much stronger. Also, it is easier to follow where (at what imidazole concentration) the *menA* protein is eluted. However, since precipitation occurred again the method as such (manual or HisTrap) does not seem to affect the fact that precipitation occurs. What could be distinguished from the HisTrap purifications is the obvious degradation pattern on the SDS-PAGEs. The equal-intensity bands at and around the *menA* band could indicate that a large part of the total protein amount is degraded. The first Superdex200 run revealed many peaks, indicating that the protein sample was not homogeneous. That was further confirmed with the SDS-PAGE after the first Superdex200 (see Figure 42).

Before the new (specific) protease inhibitors were tested, the experiments "Desalting the fractions from IMAC" and "Precipitate testing" were performed. Desalting was tested in order to investigate if imidazole was the reason for degradation or aggregation. Desalting did not hinder precipitation and analysis of the precipitate revealed the content to be only *menA* and its degradation residues.

With the specific protease inhibitors the outcome from the purification was drastically changed. The first HisTrap (with the specific PIs) purification gave a low yield of *menA* but there was no sign of degradation on the gel. Furthermore, there was no sign of

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precipitation when pooled and concentrated. The second HisTrap purification (with the specific PIs) gave strong bands on the SDS-PAGE and could reach a high concentration when pooled and concentrated. The Superdex200 profile for this sample revealed two peaks. The SDS-PAGE showed that two fractions (C1 and C2) contained pure *menA* enzyme. Unfortunately there was too little protein available to set up crystal plates with. More large scale productions need to be done in order to establish whether the protein production is enough for setting up crystal plates. If it is not, some modifications need to be done with the growth conditions or even exchange expression vector.

FC-12 is the detergent that should be chosen for purification due to the presence of pure *menA* on the SDS-PAGE after SEC with that detergent. On the other hand, it would be interesting to see if DM or LDAO gives the same pure pattern. It might also be an idea to solubilize in DM and purify with FC-12, to investigate if the binding to the HisTrap column (Ni $^{2+}$) is stronger with DM than with FC-12.

Degradation and aggregation

It is obvious that the degradation pattern, and hence precipitation, vanishes when the specific protease inhibitors are used. This is an indication of the importance of using protease inhibitors that target every possible protease in the *E.coli* cell. Other proteins might not be affected by the proteases but as has been shown, *menA* is truly affected by the presence of proteases. Luckily, this could be overcome by exchanging protease inhibitors.

The degradation pattern could not be coupled to a certain time point of growth, judged by the Western blot in Figure 40, where there is no sign of degradation. The lack of degradation pattern can be explained simply by its non-existence or by the fact that the His-tag is not included in the degraded protein.

The aggregation (or dimer) pattern could be coupled to 19 h of growth after induction. It could however be earlier than 19 h but most definitely after 1h. This time span is too large to precisely locate the time-point of aggregation/dimmer formation. An additional experiment with cell samples taken every hour in between 1 and 19 h after induction would solve the mystery.

Enzymatic activity

There is strong evidence of the activity of *menA*, both in the non-induced membrane and in the two variants of induced membrane. This means that the over-produced fraction of *menA* is inserted into the membrane and that it is active. It would be beneficial to create a BL21 strain with *menA* deleted to compare enzymatic activity of its membrane to the other membranes produced.

<u>Anaerobic growth</u>
Interestingly, there seems to be no difference in the production level of *menA* between anaerobic and aerobic growth. This is proved by the same intensity of the *menA* band for anaerobic and aerobic growth at each time-point respectively. However, since the cell growth for the anaerobic batches are slower, the total protein yield/L culture will be less with anaerobic growth. Furthermore, it seems like 1h after induction has the highest level of *menA* which suggests that this is the best time to harvest the cells. Also, it has less sign of aggregation compared to the later time points.

Comparing in between the batches it seems like the non-induced samples also contain *menA* which suggests induction by something in the media. The amount of the protein is much higher for the anaerobic non-induced sample compared to the aerobic non-induced cell sample.

5.2 Conclusions

The conclusions that can be drawn are,

- for successful 3'A addition on the *menA* gene, *Taq*-polymerase need to work for 20 min at 72 ° C.

- the *menA* gene is cloned into the pEXP5-NT/TOPO Expression Vector in the right direction, confirmed with plasmid sequencing. This was achieved when the above point was performed.

- pre-warmed growth media (LB) has positive effects on growth, especially when freeze cultures are used. Also, it is a necessity for transforming into BL21*(DE3) One Shot cells.

- the *menA* protein is successfully inserted into the membrane. There seems to be minimal loss of protein from the harvesting point to the membrane resuspension step.

- the maximum membrane yield is obtained when harvesting 1 h after induction with IPTG.

- the overproduced part of the *menA* protein is functionally active in the membrane.

- there is no difference in the amount of produced *menA* when comparing aerobic to anaerobic growth.

- the *menA* protein is eluted with IMAC purification starting at an imidazole concentration around 80 mM, both for the manual and HisTrap runs.

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- the *menA* protein is not eluted alone from the IMAC purifications since presence of other proteins can be seen on the SDS-PAGEs after IMAC.

- the precipitation contains *menA* and degraded *menA* only.

- desalting the protein after IMAC does not hinder precipitate formation.

- precipitation/degradation can be avoided with the use of the specific protease inhibitors.

- the *menA* protein is pure when purified on a gel filtration column with FC-12 as buffer detergent. However, the concentration is too low to proceed with crystal plates set-ups.

5.2.1 Future work

The immediate future work would be to perform more large-scale productions in order to obtain enough pure *menA* to set up crystals. It would be beneficial to optimize the growth conditions so that there is enough protein from one large-scale (6L) production.

To design an *E.coli* strain with the *menA* gene deleted would also be of great scientific value. The activity of *menA* from this membrane would be good to have in comparison with the three other membranes prepared in this project.

Reconnecting to the aim

The aim of the project was fulfilled to a great extent. The *menA* enzyme was successfully produced in *E.coli* and after many trials also successfully purified. No crystal plates could be set up due to the too low concentration of pure *menA* and hence, no crystals could be obtained.

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Appendices

A   Growth media and buffers

A.1   Growth media

LB media
1 % tryptone (peptone)
0.5 % yeast extract
1 % NaCl
pH 7.0

LB-agar
1 % tryptone (peptone)
0.5 % yeast extract
1 % NaCl
1.5 % agar
pH 7.0

32Y media
0.8 %tryptone (peptone)
3.2 %yeast extract
1 %NaCl
pH 7.0

A.2   Buffers

Wash buffer
20 mM Hepes
150 mM NaCl

pH 7.4

Membrane resuspension buffer
20mM Hepes
150 mM NaCl
17 %glycerol
4mM β-mercaptoethanol
2.5 mM DTT

pH 7.4

Cell lysis buffer
20 mM Hepes
300mM NaCl

4mM β-mercaptoethanol
2.5 mM DTT
0.05mM Pefabloc
0.025 mM 2,2'-Bipyridyl

pH 7.4

Solubilization buffer
20mM Hepes
150 mM NaCl
Detergent (20xCMC)
4mM β-mercaptoethanol
0.05mM Pefabloc
0.025 mM 2,2'-Bipyridyl
2 mM DTT

pH 7.4

Buffer A
20mM Hepes
150 mM NaCl
Detergent (2xCMC)
8.5 %glycerol
4mM β-mercaptoethanol
0.05mM Pefabloc
0.025 mM 2,2'-Bipyridyl
0.5 mM DTT

pH 7.4

Buffer B
20mM Hepes
150 mM NaCl
Detergent (2xCMC)
8.5 %glycerol
4mM β-mercaptoethanol
0.5 mM DTT

500 mM imidazole

pH 7.4

MES Buffer
1M Tris
1M MES
2% SDS
20mM EDTA

pH 7.3

10xPBS, 1L recipe
80g NaCl (Mw 58.44 g/mol)
2g KCl (Mw 74.55 g/mol)
6.1g Na_2HPO_4 (Mw 141.96 g/mol)
1.9 g KH_2PO_4 (Mw 136.09 g/mol)

pH 7.4

Blocking buffer
5 % dry milk in Wash Buffer(0.2% Tween-20 in PBS)

B   DNA isolation- and Transformation protocols

B.1   Genomic DNA isolation

- cells from 1-5 ml O.N. culture were pelleted and the supernatant discarded.

- resuspended in 200 μl Resuspension Solution by pipetting up and down or vortex.

- 200 μl of Lysis Solution was added, inverted and then vortexed (vortex only for genomic DNA, for plasmid NOT). Was let to clear for approximately 5 min.

- 350 μl Neutralization Solution (S3) was added and inverted 4-6 times to mix.

- cell debris was pelleted for 10 min at max speed.

- 500 μl Column Preparation Solution was added to a binding column in a collection tube. Spun at 12000xg for 1 min, flow through (FT) discarded.

- the cleared lysate was transferred to the binding column, spun for 1 min and FT discarded.

- 750 μl of Wash Solution was added to the column and spun for 1 min, FT discarded.

- 1 min additional spin to dry column.

- column was transferred to a new collection tube.

- 100 μl Elution Solution or MQ water was added and spun for 1 min to elute the DNA.

B.2 Cloning reaction

- 1.5μl 3'A-amplified PCR product (*menA*) was mixed with 1 μl salt solution,2.5 μl Water, 1 μl NT Topo vector.

- mixed gently and incubated for 25 minutes in RT.

- immediately transferred to ice.

B.3 Transformation into One Shot TOP10 cells

- 2 μl (1 μl of pUC19 control plasmid) of the cloning reaction was added to the vial of One Shot TOP 10 cells and incubated on ice for 15-30 min.

- Heat shock for 30 s, 42 ° C and then immediately transferred to ice.

- 250 μl of RT S.O.C medium was added to each vial and incubated for 1h, 37 ° C and 220 rpm.

- 100 μl of the above bacterial cultures was added to LB-Amp(50 μg/ml) plates and incubated O.N at 37 ° C without shaking.

B.4 Transformation into One Shot BL21*(DE3) cells

- 2 μl (8.4 ng) of plasmid (with *menA* inserted) was added directly into the vial and mixed gently.

- incubated on ice for 30 min.

- heat-shocked the cells for 30 s at 42 ° C and immediately transferred to ice.

- 250 μl of prewarmed LB media (instead of S.O.C media as in protocol) was added directly to the vial. Incubated at 37 ° C, 200 rpm for 50 minutes (not 30 min as specified in the protocol).

- The vial of cells were added to 10 ml prewarmed LB media (suppl. with 1% glucose and 50 μg/ml Amp. Incubated O.N. at 37° C, 200 rpm.

C   Solubilization

C.1   Ni-NTA preparation

The following protocol was used for preparation of Ni-NTA agarose for IMAC purification.

- the bottle of Ni-NTA agarose (QIAGEN) was shaken.

- approximately 100-150 μl of the Ni-NTA agarose was added to a 2ml collection tube. Centrifuged for 2 min at 5000xg.

- the liquid was discarded and 1 ml of MQ water added. Mixed and centrifuged for 2min, 5000xg.

- the MQ was discarded and 1ml of Buffer A was added. Mixed and centrifuged for 2min, 5000xg. Buffer discarded and repeated twice.

- the Ni-NTA resin was mixed with the solubilized membrane and incubated on the rolling table (+10 ° C) over night.

C.2   Detergents

The detergents used for solubilization and purification are presented in Figure 45-Figure 48
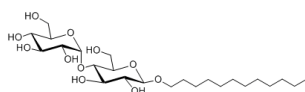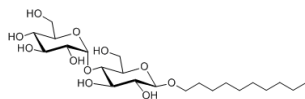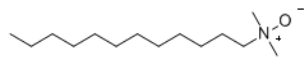

Figure 45: DDM
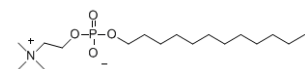
Figure 46: DM

Figure 47: LDAO

Figure 48: FC-12

D   DNA-and protein ladders

The DNA-and protein ladders utilized are presented in Figure 49 and Figure 50
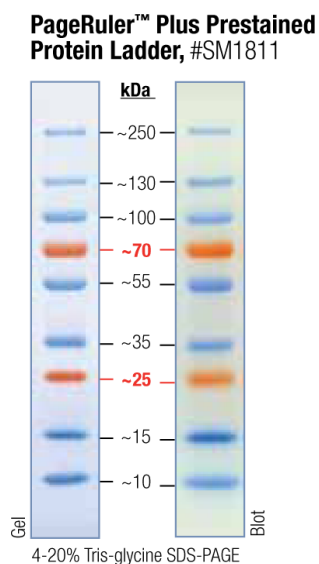

Figure 49: The PageRulerPlus Prestained Protein Ladder (Fermentas) used for SDS-PAGE.
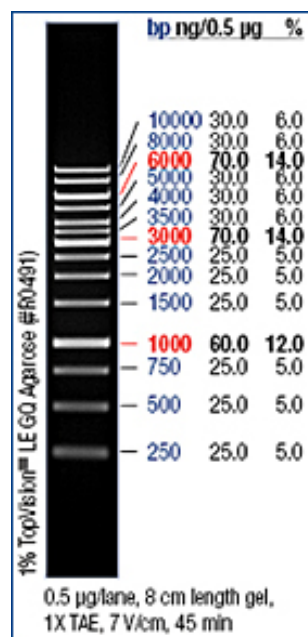
Figure 50: The GeneRuler ikb DNA Ladder (Fermentas) used for DNA gel electrophoresis.

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