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Expression, Purification and Characterization of Complement Factor H and Apolipoprotein E

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In humoral innate immune response, the complement system is a central player. It attacks immediately microbes and foreign particles invading the human body. It is constantly active in plasma and can easily be activated on self surfaces and thereby trigger local inflammation if efficient regulators are missing. Complement factor H (CFH) is one of the main complement regulators in plasma and it has been recently shown to interact with apolipoprotein E, a multifunctional protein mostly associated with lipid metabolism thereby reducing complement activation in plasma.

This Bachelor thesis project was initiated to learn more about the mechanism of binding between Complement factor H (CFH) and apolipoprotein E (apoE). The experiments were performed at Helsinki University, Research Programs Unit, Department of Bacteriology and Immunology. The laboratory work was done as a part of Jokiranta group's research.

The aim of this study was to express a recombinant fragment of CFH domains 5-7, obtain high-expressing CFH 5-7 *Pichia Pastoris* clone and isolate apoE from human very low density lipoprotein (VLDL) and intermediate density lipoprotein (IDL) particles. These isolated molecules will be used in a study investigating the role of CFH and apoE in reducing complement mediated inflammation at early stages of atherosclerosis development.

In this thesis the cloning, production and purification of CFH fragment containing three domains (CFH 5-7) and apoE isolation from human VLDL and IDL particles are described. The CFH 5-7 expression process was performed successfully with a maximum protein purity expression. The total amount of the expressed CFH 5-7 (~400 µg) was sufficient to perform further experiments. Further optimization of the plasmid transformation procedure will be needed to obtain high-expressing CFH 5-7 *Pichia Pastoris* clone in the future.

Keywords	Factor H, apolipoprotein E, expression, complement system

Tekijä(t) Otsikko Sivumäärä Aika	Mikael Gromyko Komplementtifaktori H:n ja apolipoproteiini E:n ilmentäminen, puhdistaminen ja karakterisointi 34 sivua + 2 liitettä 25.5.2018
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Komplementtisysteemi on humoraalisen synnynnäisen immuunivasteen keskeinen toimija. Se hyökkää välittömästi niitä mikrobeja ja vieraita aineita vastaan, jotka tunkeutuvat elimistöön. Se toimii plasmassa jatkuvasti, ja sitä voidaan aktivoida helposti itsepinnoilla ja siten se laukaisee paikallisen tulehduksen, jos tehokkaat säätävyysmekanismit puuttuvat. Komplementtifaktori H (CFH) on yksi tärkeimmistä komplementtisäätelytekijöistä plasmassa, ja sen on viime aikoina osoitettu olevan vuorovaikutuksessa apolipoproteiini E:n kanssa, joka on useimmiten lipidien metaboliaan liittyvä multifunktionaalinen proteiini, prosessissa, joka vähentää komplementin aktivoitumista plasmassa.

Tämä tutkimus aloitettiin, koska haluttiin tutkia sitoutumismekanismia komplementtifaktori H:n (CFH) ja apolipoproteiini E:n (apoE) välillä. Tutkimuksia suoritettiin Helsingin yliopiston Bakteriologian ja Immunologian tutkimusohjelmayksikössä. Laboratoriotyö tehtiin Jokiranta-ryhmän tutkimuksen osana.

Opinnäytetyön tavoitteena oli ilmentää komplementtifaktori H:n 5–7 domeenia, saada CFH 5–7:n korkeailmentävä *Pichia pastoris* -klooni ja eristää apoE:ta ihmisen hyvin alhaisen tiheyden lipoproteiinin (VLDL) ja välituotetta lipoproteiinin (IDL) partikkeleista. Näitä eristettyjä molekyylejä käytetään tutkimuksessa, jossa selvitetään CFH:n ja apoE:n roolia ateroskleroosin alkuvaiheessa komplementtivälitteisen tulehduksen vähentämisessä.

Tässä työssä on kuvattu CFH:n 3-domeenin fragmentin (CFH 5–7) kloonaus, tuotanto ja puhdistus ja apoE:n eristäminen ihmisen VLDL- ja IDL-partikkeleista. CFH 5–7:n ilmentymisen menetelmä suoritettiin onnistuneesti. Tätä varten maksimimäärä puhtainta proteiinia ilmennettiin. CFH 5–7:n kokonaismäärä (~400 µg) oli riittävä lisäkokeille. Lisää plasmidimuunnosmenettelyn optimointia tarvitaan, jotta saataisiin korkeaa määrää ilmentävä CFH 5-7 Pichia Pastoris -klooni tulevaisuudessa.

Avainsanat	Komplementtifaktori H, apolipoproteiini E, ilmentäminen, komplementtijärjestelmä

Contents

Abbreviations

AD	-	Alzheimer`s disease
AGE	-	agarose gel electrophoresis
aHUS	-	atypical hemolytic uremic syndrome
AMD	-	age related macular degeneration
AP	-	alternative complement pathway
apoE	-	apolipoprotein E
CFD	-	complement factor D
CFH	-	complement factor H
CFHL-1	-	complement factor H-like protein 1
CFI	-	complement factor I
CP	-	classical pathway
CPN	-	carboxypeptidase N
CR1	-	complement receptor 1
CRIg	-	complement receptor of the immunoglobulin family
CRP	-	C-reactive protein
CVD	-	cardiovascular disease
DAF	-	decay accelerating factor
DDD	-	dense deposit disease
IDL	-	intermediate density lipoprotein
HCV	-	hepatitis C virus
HDL	-	high-density lipoprotein
HIV	-	human immunodeficiency virus
HLP	-	hyperlipoproteinemia
HSPG	-	heparan sulfate proteoglycan
LB	-	Luria-Bertani (medium)
LP	-	lectin pathway
MAC	-	membrane attack complex
MBL	-	mannan-binding lectin
MCP	-	membrane cofactor protein
PAMP	-	pathogen associated molecular pattern
SCR	-	short consensus repeat
SDS-PAGE	-	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TP	-	terminal pathway
UCF	-	sequential ultracentrifugation

VLDL	-	low-density lipoprotein
YPDS	-	yeast extract peptone dextrose sorbitol medium or plate
Zeo^R	-	Zeocin-resistant

1 Introduction

The immune system is complex, sensitive, and organized system that helps all living beings to manage foreign material penetrating our body. Immunity can be classified into two subsystems called acquired and innate immunity. The main differences between them is that acquired immune response requires time to recognize the antigen and to produce specific antibodies, whereas innate immune response can be activated rapidly without antigen recognition and antibody production. The term 'complement system' was introduced by Paul Ehrlich in 1899 but was first described 10 years earlier by Hans Bucher while demonstrating the ability of heat labile substance to kill bacteria in blood serum without use of antibodies, hence complementing use of antibodies. The evolutionary origin of antibody-independent complement activation (alternative pathway, AP), is much more ancient than that of classical antibody-dependent system (classical pathway, CP). In addition to the AP the third complement activation system, lectin pathway (LP), also seems to have an ancient origin [1; 13; 14.]

The overall goal of this work was to express and purify enough apolipoprotein E (apoE) and complement factor H (CFH) proteins for determination of binding mechanism of these proteins, using X-ray crystallography. The successful determination of connection between CFH and apoE proteins might lead to a breakthrough in treatment or hindering the development of Alzheimer`s disease (AD), atherosclerosis and age related macular degeneration (AMD).

This final work was done under the supervision of Dr Karita Haapasalo-Tuomainen, Ph.D, researcher at Jokiranta group, Haartman institute, Department of Bacteriology and Immunology. The Jokiranta group is primarily interested in alternative pathway of complement (AP), specifically, in the mechanism, which allows destruction of invading microbes while leaving host organism intact. The overall goal of the project is to establish how the regulator of alternative complement pathway, CFH, interacts with apoE via domains 5-7, 'marking' lipids, connected with apoE protein, to not be destroyed during AP.

2 Theoretical Background

2.1 Function of Complement System

The main goal of complement system is to recognize targets and get activated through organized interactions between complement proteins that advance or stop the activation process. The importance of the complement system can be illustrated through deficiencies of complement components in humans that are associated with diseases such as age related macular degeneration (AMD) and atypical hemolytic uremic syndrome (aHUS) [1; 15.]

The most obvious purpose of the complement system is to defend against infections. The complement system attacks invading microbes directly causing immediate partial destruction of the targets and generate target-bound opsonization of C3b molecules, thus enhancing phagocytosis. Also, complement system generates substances that enhance cellular migration, or chemotaxis, and inflammatory response by releasing C3a and C5a anaphylatoxins, thus enabling innate immune response [1.]

Another important role of complement system lies in removal of immune complexes and cellular debris from the body. Complement system attacks wreckage and immune complexes following their capture by erythrocytes and transportation to be destroyed in liver or spleen. Necrotic and apoptotic cells are also cleared by complement system [1; 16.]

Some complement receptors recognize certain cleavage products of the complement activation pathways, thus becoming expressed on lymphocytes. This plays an important role in linking adaptive and innate immunity [1.]

2.2 Recognition

The complement system has three specific recognition strategies to separate self from non-self structures:

Complement system recognizes non-self targets directly. Usually, innate immunity can identify certain patterns appearing on micro-organisms, so called pathogen associated

molecular patterns (PAMPs). Such process is mediated by complement components as C1q, mannan-binding lectin (MBL) and ficolin [1; 17.]

Also, a plasma protein complement factor H (CFH) that acts as a complement regulator of AP, plays an important role in distinguishing self from non-self cells. Partially, CFH recognizes self cells by binding to surface deposited C3b and self surface sialic acids and glycosaminoglycans [1; 18.]

To prevent complement attack, host cells express membrane-bound complement regulatory proteins. On apoptotic and necrotic cells these surface regulators are missing and are therefore attacked by complement. It has been proposed that phagocytosis of apoptotic and necrotic cells requires two signals. Complement component C1q receives signals from nucleic acids exposed by apoptotic cells, whereas loss of surface complement regulators acts as missing self signals. This process promotes complement activation and opsonization of the target leading to destruction of the cell [1; 19.]

2.3 Activation

After differentiating self from non-self targets using described three recognition mechanisms the complement becomes activated. The activation may progress through three pathways: classical pathway (CP), alternative pathway (AP) and lectin pathway (LP). Upon activation, all three pathways end the same way: with the generation of the active enzyme, C5-convertase, initiating activation of the terminal pathway (TP) and formation of the membrane attack complex (MAC) [1.]

2.4 Alternative Pathway (AP)

AP along with other two possible complement pathways is highly dependent on component C3 since all major processes are possible only through activation of this molecule. C3 is a 185 kDa plasma protein composed of two chains, called α and β chains. Activation of AP is spontaneous and is initiated via formation of metastable C3(H_2O) by the process of low rate hydrolysis of C3 in human plasma. Following its formation, C3(H_2O) can bind factor B within milliseconds thus leaving it exposed to cleavage by factor D following formation of C3(H_2O)Bb complex, also known as C3-convertase. Fluid phase C3 is then cleaved by this complex into two fragments: C3a and C3b. Freshly formed C3b can then attach onto any available biological surfaces within a

short time, whereas smaller fragment C3a (9 kDa) is released and serves as an anaphylatoxin and chemotactic factor [1; 20; 21.]

Complement factor D (CFD) is a 26 kDa serine protease. During AP factor D cleaves single chain factor B only when it is connected to C3(H_2O) or C3b. This cleavage is possible due to conformational change in factor B during the C3(H_2O)(C3b) complex formation, which leaves factor B exposed to activation by factor D. When cleaved, factor B is divided into fragments Ba (30 kDa), which is released into liquid phase, and Bb (60 kDa), which serves as serine protease in newly formed C3bBb complex [1; 22.]

The cleaved C3b can covalently bind to any target surface due to exposed thioester group. The continuation of AP may occur on any target's surface where C3b is bound to. At this point C3b can interact with factor B or CFH, starting molecular process of self/non-self differentiation of AP. After the formation of C3-convertase properdin, or factor P, which is the only positive regulator of complement activation can further stabilize the C3bBb complex, leading to the formation of C3bBbP. Development of AP is achieved at this stage by cleavage of fluid phase C3 to produce enough C3b, which enables forceful opsonization of the target. C3bBbP is a stable compound, which may bound to an additional C3b causing formation of surface bound C5-convertase C3bBbC3b that cleaves fluid phase C5 to C5b, thus activating the TP [1; 23.] (Figure 1)

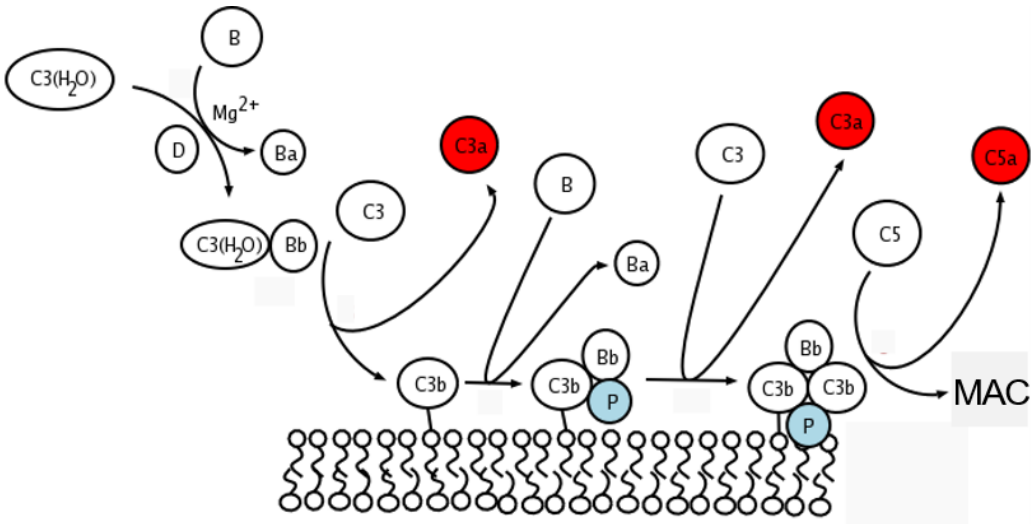

Figure 1: Alternative complement pathway. C3(H_2O) binds factor B and gets exposed to cleavage by factor D following formation of C3(H_2O)Bb complex, C3-convertase, which is then cleaved into C3a and C3b particles. C3b then gets attached onto the cell membrane. C3b is then further stabilized by properdin and factor B, leading to the formation of C3bBbP, which can further react with C3b resulting in the formation of surface-bound C5-convertase.

2.5 Terminal Pathway (TP)

C5 is structurally related to C3 molecule, consisting of α (~140 kDa) and β (~80 kDa) chains, linked together with disulfide bonds. C5 does not have exposed thioester group during conversion of C5 to C5a and C5b, so it cannot covalently bind to target surfaces. Because of that, C5 covalently binds to C3bBbC3b complex and becomes exposed for cleavage by Bb. After cleavage, C5 releases a powerful anaphylatoxin, C5a, in the fluid phase while the C5b fragment remains attached to C3b in the complex. Following C5b binding, two single chain proteins C6 (~104 kDa) and C7 (~92 kDa) bind to C5b in sequence. The attachment of C7 to C6 leads to a conformational change in the C5b67 complex, resulting in its release from the C5-convertase to fluid phase. Following the release, the majority of C5b67 complexes become inactivated by S-protein or by C8. However, small number of complexes can reach the membrane and connect tightly to the target membrane [1; 24.]

Component C8 is a molecule that has similarities with C6 and C7 and is comprised of α (64 kDa), β (64 kDa) and γ (22 kDa) chains. The C8 α subunit is the first complement protein that penetrates the cell membrane. C8 has a two-fold role: it inhibits attachment

of C5b67 in the fluid phase to the membrane and promotes MAC formation when complex is attached to the surface. When C8 is attaching to the surface-attached C5b67 complex, it deepens the attachment to the membrane and enables binding of the last complement component C9 to C8. C9 is a single chain protein (69 kDa) like C6, C7 and C8. While attached to the complex through C8, C9 becomes unfolded and serves as a binding site for up to 12-16 additional C9 components that together form a transmembrane pore [1; 25.]

2.6 Regulation

In the AP activation cascade C3b must attach to the target's surface within a short period of time to avoid degradation in fluid phase. Also, formed C3-convertases are vulnerable to degradation in the absence of properdin. However, complement activation requires more efficient means of control because of the explosive propagation due to efficient amplification steps of the system. Without specific regulation activation of AP can become harmful even for the host and consume components needed for activation of AP, leading to acquired complement deficiency and complement inactivity [1.]

The specific regulators for AP include complement receptor 1 (CR1), decay accelerating factor (DAF) and CFH, factor H-like protein 1 (CFHL-1), carboxypeptidase N (CPN), factor I (CFI), properdin (factor P), clusterin, S-protein (vitronectin), membrane cofactor protein (MCP), complement receptor of the Immunoglobulin family (CRIg) and CD59 (protectin). CFH, CFHL-1, CPN, properdin, clusterin and S-protein are fluid phase regulators, while CR1, DAF, MCP, CRIg and CD59 are membrane-associated regulators. Most of those specific regulators consist of four or more short-consensus repeat (SCR) domains arranged in row. Each SCR is a round domain generally consisting of ~ 60 amino acids [1.]

2.7 Complement Factor H (CFH)

The activation of AP seems to be linked to comparatively great amount of polyanions on the cell surface, including glycosaminoglycans or sialic acids. These structures prefer binding to CFH rather than factor B. CFH is a cofactor that is required for the inactivation of C3b by factor I. CFH also contributes to reduced production C3-convertases as it inhibits binding of factor B to C3b. Furthermore, binding of CFH on C3 convertase accelerates the decay of AP convertases [1; 26.] (Figure 2)

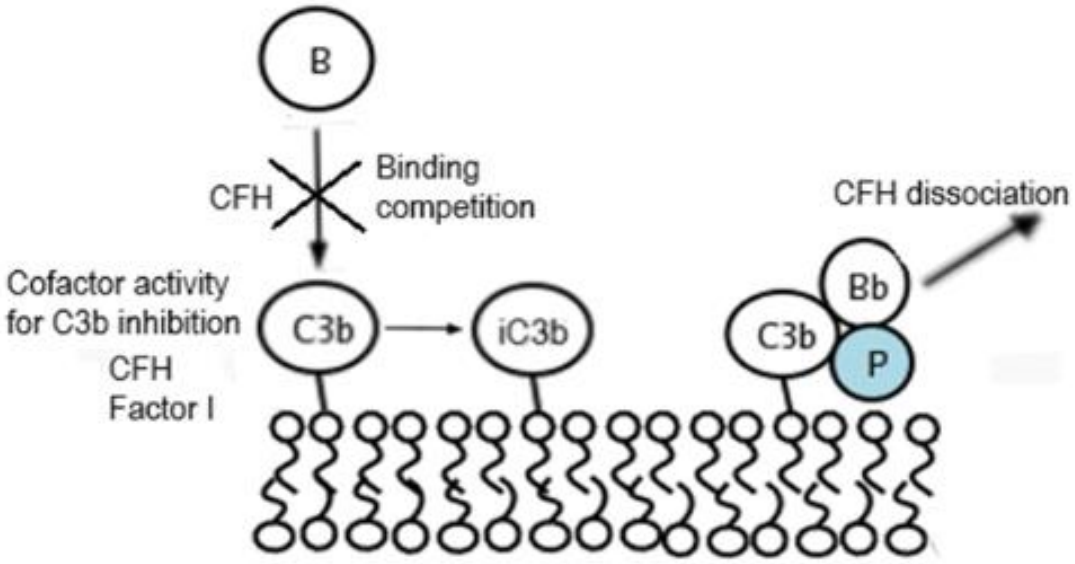

Figure 2: Alternative pathway regulation by complement factor H. CFH acts as a cofactor for factor I mediated inactivation of C3b to iC3b. In addition, CFH competes with factor B for binding on surface bound C3b and accelerates the dissociation of C3-convertase.

CFH is a single-chained (~155 kDa) glycoprotein consisting of 20 short consensus repeat (SCR) domains. (Figure 3) The first four domains of CFH serve as a cofactor and decay acceleration centers. The main surface recognition activities are associated with domains 19-20. CFH is known to interact with C3b by both domains 1-4 and 19-20. The principle of self vs non-self differentiation is based on interaction of CFH with C3b via domains 19-20 and its interaction with surface-bound glycosaminoglycans or sialic acids, which are not usually present on pathogen surfaces. CFH also reacts with heparin via domains 7 and 20 [1; 27.]

Mutations or polymorphisms in CFH are known to be related to kidney diseases characterized by kidney malfunction due to uncontrolled complement activation. Mutations in domains 19-20 lead to aHUS, while mutations in amino-terminal domains may lead to dense deposit disease (DDD). Also, many CFH polymorphisms have been described: most CFH polymorphisms are connected to aHUS, but one polymorphism in domain 7, Y402H, is associated with age-related macular degeneration (AMD), the main cause of visual loss of the elderly in industrialized countries. This polymorphism has been shown to affect ability of factor H to bind to heparin and C-reactive protein (CRP), from which CRP is a biomarker of inflammation. The Y402 polymorphism also affects binding

of CFH to necrotic cells, leading to the increase or reduction of complement activation [1; 2.]

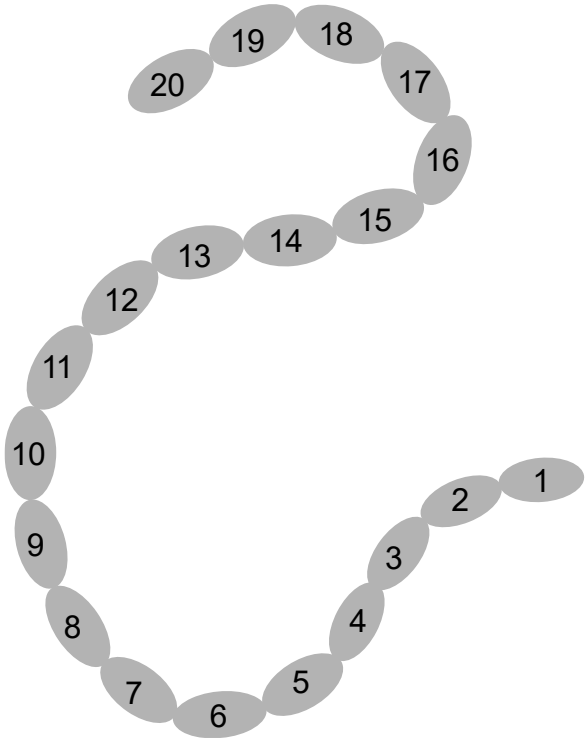

Figure 3: CFH structure. Domains 1-4 serve as a cofactor and decay acceleration centers. Domains 19-20 are associated with surface recognition. Both domains 1-4 and 19-20 are associated with CFH-C3b interaction. Domains 7 and 20 are associated with heparin binding.

In addition to self-structures several microbes are known to bind CFH to allow complement regulation on their surfaces. This is one of the mechanisms of selective microbial alternative pathway evasion mechanisms. Most microbes bind CFH via two regions of this protein, domains 5-7 and 18-20 [1; 28.]

2.8 Apolipoprotein E (apoE)

Apolipoprotein E (apoE) is a 34 kDa glycoprotein, associated with normal and altered lipid metabolism, where it transports lipids, vitamins as well as cholesterol into the lymph system and then into blood from hepatic cells (hepatocytes), which are the basic metabolic cells in liver. ApoE is an important protein for anti-microbial defense, oxidative stress and inflammation. ApoE can also be produced in the brain astrocyte cells and is the best expressed apolipoprotein in the cerebrospinal fluid [3.]

Structurally, apoE molecule consists of N-terminal domain (amino acids 1-191) and C-terminal domain (~206-299), joined by a protease-sensitive loop. (Figure 4) The N-terminal domain is a bundle of four helixes containing receptor-binding region and the heparan sulfate proteoglycan (HSPG) binding region. The C-terminal domain consists of amphipathic α-helix regions showing high affinity for lipid binding and apoE self-associating region [3.]

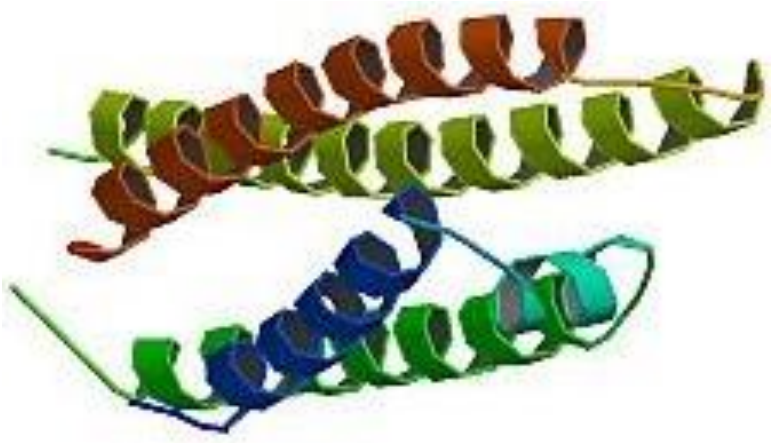

Figure 4: Apolipoprotein E structure. The N-terminal include the receptor binding region (green helix), which includes HSPG binding region and three other helixes (deep blue and light blue), one of which is not illustrated. The C-terminal domain include lipid-binding domain (yellow) and apoE self-association region (red) [29.]

ApoE occurs in humans in three structurally and pathologically different isoforms: apoE2, apoE3 and apoE4. Because of their structural differences, three apoE isoforms have shown different binding to lipoproteins. ApoE2 is a genetic variant of apoE, associated with a condition called type III hyperlipoproteinemia (HLP), associated with increased cholesterol and decreased high-density lipoprotein (HDL) levels. ApoE3 is contributing to protection against cardiovascular disease (CVD). ApoE4 isoform is the risk factor in the development of Alzheimer's disease (AD), the neurodegenerative disorder that ultimately leads to dementia. ApoE4 isoform is also connected to hepatitis C virus (HCV) and human immunodeficiency virus (HIV) [3.]

Majority of apoE in plasma is produced by the liver and the molecule is mainly associated in very low-density lipoprotein (VLDL) or intermediate density lipoprotein (IDL) particles. Approximately 5-10% of apoE is, however, produced by macrophages. The endogenous production of apoE by macrophages in blood vessel walls has been shown to be critical in prevention and healing of atherosclerotic plaques. Atherosclerosis is a disease

characterized by chronic inflammatory response in the walls of arterial blood vessels where the complement system plays an important role. It has been previously shown that CFH domains 5-7 bind apoE and thereby reduces complement activation in plasma. [2] Interestingly, the common polymorphisms Y402H in CFH domain 7 that is associated with age-related macular degeneration (AMD) a disease that is significantly associated with occurrence of atherosclerosis [3.]

3 Aims of the Study

The goal of this work was to express up to 10 mg of proteins CFH 5-7 and isolate apoE for X-ray crystallography and for biochemical assays aiming to study the role of apoE - CFH interaction in atherosclerosis. The main goal of CFH 5-7 expression was divided into two tasks: obtain new CFH expressing *Pichia pastoris* clone, and check CFH 5-7 expression parameters using already made *Pichia* clone and try to express necessary amount of CFH 5-7. Theory and workflow of the methods used to achieve work objectives are explained in paragraphs 4 and 5.

4 Cloning, Expression and Purification of Complement Factor H

There are many different protein expression systems, which are designed to produce multiple copies of required protein in a host cell and *Pichia pastoris* (Invitrogen) is one of such systems. *Pichia pastoris* has many of the advantages of eukaryotic expression systems. It is faster, easier to use, less expensive and more efficient comparing to other eukaryotic expression systems, such as mammalian cell culture. *Pichia pastoris* can be used to express proteins inside the cell (intracellular expression) or in the supernatant (extracellular expression). The main advantage of expressing proteins as secreted proteins during extracellular expression is that *Pichia pastoris* secretes very little amounts of non-target proteins, leading to the increase of target proteins in the expression medium [4.]

4.1 General Characteristics of *Pichia pastoris*

Pichia pastoris is a methylotrophic yeast, capable of using methanol as its only carbon source. The process of methanol metabolism includes oxidation of methanol to formaldehyde, using molecular oxygen by the enzyme called alcohol oxidase, generating

hydrogen peroxide in the process. This process taxes place within peroxisome, a specialized cell organelle which separates toxic hydrogen peroxide by-products away from the rest of the cell. It is necessary for *Pichia pastoris* to generate large amounts of the alcohol oxidase, because this enzyme does not tolerate molecular oxygen well [5.]

For this work a wild-type *Pichia* X-33 strain was used. This strain is useful for selection on Zeocin and large-scale growth. It uses yeast extract peptone dextrose (YPD) liquid media or agar plate for growth at 30 °C. Growth in temperatures above 30 °C significantly hinders protein expression or might even lead to cell death. It is advisable to add 0.5 – 1 % of methanol to the media every day to induce protein expression in *Pichia*, when using plates or medium containing methanol, to compensate for loss because of evaporation or consumption [5.]

4.2 Vector selection

Selection of the vector appropriate for protein expression is the first step in protein expression in *Pichia*. There are two expression vectors available to promote the expression of protein of interest in *Pichia pastoris*: pPICZ vector, which is used for intracellular protein expression and pPICZα, used for secreted expression (Invitrogen). These vectors provide high-level expression of the gene of interest in *Pichia* regulated by methanol induction. Each vector contains the Zeocin resistance gene, which allows vector-containing *E. coli* and *Pichia* organisms to grow in the presence of antibiotic Zeocin providing positive selection of these organisms [5.]

pPICZα is a terminal fusion vector (Figure 5), available in three reading frames: pPICZα A, pPICZαB and pPICZα C. The vector used in this work, pPICZα B (3597 bp), was previously tested for CFH expression in *Pichia pastoris*.

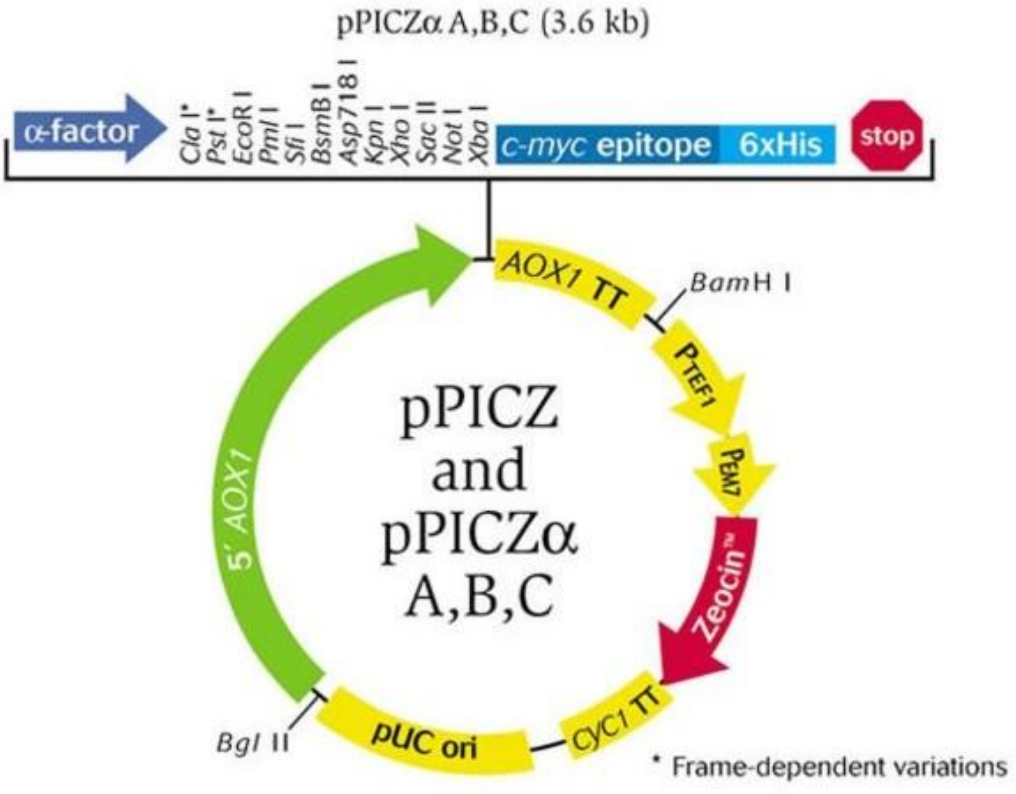

Figure 5: Contents of pPICZα vector. The vector contains AOX 1 promoter region, α-factor secretion signal, multiple cloning site with 12 unique restriction sites including *Pst I* restriction site, which was used for this work; C-terminal myc epitope tag, C- terminal polyhistidine (6*His) tag, AOX1 transcription termination (TT) region, TEF1 promoter region, EM7 promotion region, Zeocin resistant (*Sh ble*) gene, CYC1 transcription termination (TT) region and pUC origin region [4.]

4.3 Obtaining *E. coli* strain containing vector with gene of interest

After choosing an appropriate vector, next step is to obtain *E. coli* clone that contain vector with the gene expressing protein of interest. The gene coding for the protein of interest needs to be ligated into vector. The vector then is transformed into *E. coli* strain and the successful transformation is confirmed by growing developed *E. coli* onto Low Salt LB plate in the presence of Zeocin. That allows positive selection of *E. coli* clones containing the insert, due to the presence of Zeocin resistant gene in the vector [5.]

E. coli strain that was used in this work is called TOP10 (TOP10 Competent Cells, Thermo Fisher Scientific). TOP10 *E. coli* strain have a high transformation efficiency and they are recombination deficient (recA) and endonuclease A deficient (endA). *E. coli*

containing vector is grown in Luria-Bertani (LB) medium with lower than usual amount of salt, 0.5% instead of 1%. Use of higher than required salt concentration will inactivate the expression vector. The *E. coli* clone containing CFH 5-7 expression vector was obtained by Dr. Karita Haapasalo-Tuomainen prior to the beginning of this work [5.]

4.4 Plasmid transformation into *Pichia Pastoris*

Obtained Zeocin-resistant (Zeo^R) *E. coli* colonies all contained the expression vector with the inserted gene necessary for the plasmid transformation into *Pichia*.

4.4.1 Plasmid DNA extraction and purification

It is required to extract *E. coli* plasmid DNA containing the insert and linearize it prior to transformation into *Pichia* using electroporation. If isolated plasmid DNA contains contaminants it is required to purify them using commercial kit. The GeneJET PCR purification system is commonly used to purify DNA from PCR reaction mixture but can also be used for different enzymatic reaction mixtures. After the purification, it is required to check plasmid DNA for complete purification using AGE. The desired amount of plasmid DNA for continuation of the work is 5 – 10 µg [4.]

4.4.2 Plasmid DNA digestion

Prior to transformation into *Pichia*, *E. coli* plasmid DNA needs to be linearized for successful electroporation. Plasmid DNA can be linearized using DNA restriction enzyme *Pme I* that cut the expression vector at 5' AOX1 promoter region, resulting in linearized DNA molecule [4.]

4.4.3 Electroporation

Electroporation is a method of DNA transfection into cells that utilizes high-voltage electric shock to the cell, resulting in the permeability increase of the cell membrane allowing DNA molecules to be introduced into the cell. Electroporation is the preferred method for transformation of *Pichia pastoris* with pPICZα because it does not destroy

the cell wall of *Pichia*. Usually, it provides 1000 – 10000 transformants per µg of digested plasmid DNA [6.]

4.4.4 Positive selection and cultivation of *Pichia* with CFH 5-7 colonies

After incubation, electroporated cells are spread on YPDS agar plates, YPD agar plates containing sorbitol, with different added Zeocin concentrations and are then incubated at + 30 °C for 3 – 10 days. Grown colonies are transferred onto a new YPDS plate, containing Zeocin, allowing for certain positive selection of Zeocin resistant clones. Obtained *Pichia* growth contain the inserted vector due to acquired resistance to Zeocin. The preference is given to the CFH 5-7 expressing *Pichia* clone grown in the highest Zeocin concentration plate [4.]

4.5 CFH 5-7 expression

After obtaining the CFH 5-7 expressing *Pichia* clone, it is possible to start expression of the CFH 5-7. For protein expression in recombinant *Pichia* strains buffered complex glycerol (BMGY) medium and buffered minimal methanol (BMM) medium are needed. BMM and BMGY are buffers used for secreted protein expression, but BMGY is the medium used for *Pichia* growth prior to the expression, while BMM is used for the expression itself. BMGY medium differs from BMM because it contains yeast extract and peptone for stabilization of secreted proteins and prevention of proteolysis of secreted proteins.

It is important, that during the expression the culture volume does not exceed 30 % of total flask volume to allow for adequate aeration during methanol induced protein expression [4.]

4.5.1 Purification of CFH 5-7 protein using affinity chromatography

Affinity chromatography is a powerful tool for the purification of the specific proteins. The basic principle is that specific ligand is immobilized to a solid support of a column matrix. A solution containing the protein of interest is passed through the column and the protein in question reacts with the ligand, while other proteins are washed through the column.

The ability of CFH to bind to heparin by its 5-7 domains allows for selective binding of expressed CFH 5-7 in the Heparin column. Heparin is a member of a glycosaminoglycan family (5 – 30 kDa) of carbohydrates, consisting of repeated, differently sulfated disaccharide units. Heparin column is filled with cross-linked agarose beads, which are covalently bound to heparin, giving high capacity and performance for protein coupling. The binding process is stable in the pH range 5 – 10 and at the maximum speed of 1 ml/min. Filtered supernatant is run through HiTrap Heparin column, which can be operated using either peristaltic pump or chromatography system. It is possible to isolate up to 3 mg of CFH 5-7 in one Heparin column [7.] The schematic representation of CFH 5-7 isolation system used in this work is presented below. (Figure 5)

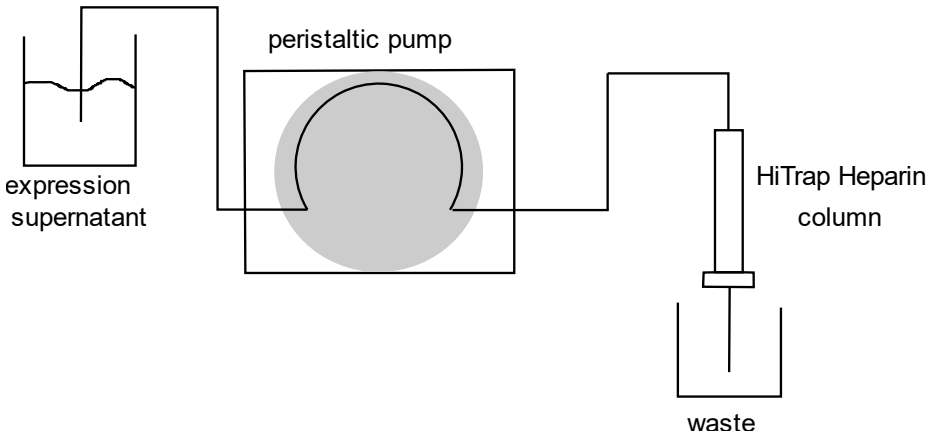

Figure 5: Heparin column isolation system. The expression supernatant is run through the Heparin column using the peristaltic pump.

4.5.2 Salt gradient elution

Recovery of the column-bound protein is achieved by changing salt concentration of the column solution. This is achieved by first purifying the column with filtered Aqua Milli-Q water, filtered 20 % ethanol solution and 75 mM PBS at the flow of 1 ml/min. Then the CFH 5-7 can be eluded by running 2 M NaCl/75mM PBS with the elution detection at 280 nm. Salt molecules compete for binding to heparin with the CFH 5-7, resulting in elution of CFH 5-7 from the column in fractions.

CFH 5-7 containing fractions must be checked for sample purity. The CFH 5-7 purity check is achieved by separating eluded charged molecules in the elution solution by their

molecular masses using 10 % SDS-PAGE and staining them using Coomassie Instant Blue.

4.5.3 Dialysis and protein concentration measurement

CFH 5-7 fractions need to be dialyzed to remove any unwanted molecules from the elution solution. Dialysis is used in the removal of salt molecules and small contamination proteins. The process of dialysis is based on random movement of the molecules that leads to the movement of molecules from high-concentration to low-concentration areas. Mixture, containing protein molecules is then transferred into semi-permeable membrane, allowing for diffusion of molecules with small molecular weight through membrane pores but restricting diffusion of big protein molecules into high amount of dialysis buffer, PBS. Slide-A-Lyser Dialysis Cassette is a dialysis system that was used for this work. It is composed of low-binding cellulose and is a hermetically sealed sample dialysis chamber allowing for efficient CFH 5-7 dialysis.

After dialysis, concentration of CFH 5-7 in the sample is measured using Qubit Fluorometer (Life Sciences). Qubit Protein Assay is an easy, fast and accurate way to measure protein concentration. Qubit provides an accurate protein concentration measurement from 12,5 µg/ml to 5 mg/ml. It is required to prepare three (0, 200 and 400 µg/ml) standards before preparing an aliquot of the isolated CFH 5-7 sample for concentration measurement [8.] Expressed CFH 5-7 can then be stored at –20 °C until further use.

5 Isolation of apoE

ApoE is a lipid-binding protein resulting in formation of lipoproteins, so the quantitative isolation of apoE from human blood requires large amount of lipids. Hypertriglyceridemia is a condition that results in elevating numbers of fatty molecules, triglycerides, in human blood, so isolation of apoE from hypertriglyceridemia type IV or type V patients plasma is the most productive. It is required to dialyze samples before continuing with isolation. Isolation of apoE from human plasma occurs in several stages: lyophilization, delipidation, solubilization and size-exclusion chromatography [9; 10.]

5.1 Lyophilization

Dialyzed IDL-VLDL samples are very diluted, so the excess water needs to be removed from the samples. Lyophilization, or freeze-drying, is the process that allows water to sublime from the solid phase directly to the gas phase. It is achieved by freezing samples in dry ice to the lowest point possible, usually -80 ^{o}C quickly to avoid formation of ice crystals. Frozen samples are then put in freeze-dryer, Heto FD 2.5 to lower the sample pressure, resulting in sublimation of water from the sample and concentration of apolipoproteins in the sample, including apoE.

5.2 Delipidation

Water-free IDL-VLDL samples need to be removed of lipids, resulting in the isolation of lipid-binding apolipoproteins in the supernatant. Delipidated samples are washed several times with methanol using low-speed centrifugation (1000 x g) before proceeding with solubilization, resulting in the formation of apolipoprotein pellet at the bottom of the flask. It is important to not allow the apolipoprotein pellet to dry and to proceed with solubilization of the apolipoproteins quickly.

5.3 Solubilization

Pelleted apolipoproteins need to be prepared for gel filtration chromatography by reducing protein disulphide bridges and solubilized. Since apolipoproteins do not dissolve in normal conditions, homogeneous molecular dispersion of apolipoproteins in the solution is achieved by dissolving them in several substances that increase solubility of apolipoproteins [12.]

5.4 Gel filtration chromatography

ApoE can be separated from other apolipoproteins in the solubilized apolipoprotein solution by gel filtration through a gel consisting of spherical ~30 mm beads containing pores and allowing for specific size distribution thus separating apolipoproteins according to their size using gel filtration chromatography [13]. Characteristics and major functions of apolipoproteins are presented below. (Figure 6)

Name	Chromosome	Amino acids	Mol wt (kDa)	Structural role	Function
A-I	11	243	29	HDL	LCAT activator
A-II	1	77	17 (dimer)	HDL	LPL regulator, LCAT and CETP cofactor
A-IV	11	396	44	Chylomicrons, HDL	LCAT activator
A-V	11	366	41	Chylomicrons, VLDL, HDL	LPL activator, chylomicron assembly
C-I	19	57	7	Chylomicrons, VLDL, HDL	LCAT activator
C-II	19	79	9	Chylomicrons, VLDL, HDL	LPL activator
C-III	11	79	9	VLDL, chylomicrons, HDL	LPL inhibitor, VLDL assembly
B-100	2	4536	500	VLDL/IDL/LDL	LDL-R ligand
B-48	2	2152	240	Chylomicrons and remnant particles	Structural component of chylomicrons
D	3	169	33		Transport of small lipophilic molecules
E	19	299	34	Chylomicrons, VLDL, remnants	LDL-R ligand for chylomicron remnants; LRP ligand
M	6	188	26		Transport of small lipophilic molecules
(a)	6	Variable	200–800	Lp(a)	

Figure 6: Apolipoproteins. In the sample may be present: apoA-V, apoC-I, apoC-II, apoC-III, apoB-100, apoE. [30]

5.5 Dot blot

Isolated fractions can be checked for successful apoE isolation using dot blot procedure with anti-apoE antibody. Dot blot is a simplified version of the Western blot technique. To analyze the successful apoE isolation from the mix using dot blot technique, a small aliquot of the separated fractions is applied to the nitrocellulose membrane as a dot and then spotted using staining of the membrane with anti-apoE antibody.

After isolation, it is possible to check apoE isolation purity using 10 % SDS-PAGE and stain with the Coomassie Instant Blue. If apoE sample contains contaminants it is possible to use specific binding of apoE to heparin to isolate apoE using HiTrap Heparin column and salt gradient elution as in CFH isolation.

6 Materials and Methods

6.1 Plasmid transformation

The sample used for plasmid transformation was ready-made CFH 5-7 T54.6 402_{Tis} PicZαB TOP10 *E. coli* clone.

The sample was grown in 50 ml Low Salt LB medium at +37 °C over-night in the presence of Zeocin (Invitrogen) and plasmid DNA was isolated using GenElute HP Plasmid

Miniprep Kit's protocol (Sigma Aldrich) or QIAprep Spin Miniprep Kit protocol (Qiagen). Isolated plasmid DNA was then checked for purity using 1% agarose gel electrophoresis (AGE). The presence of the plasmid DNA in the agarose gel was confirmed by screening the gel using GelDoc Molecular Imager (BIO-RAD).

The GeneJET PCR purification kit (Thermo Fisher scientific) was used for plasmid DNA purification after isolation. The concentration of the isolated plasmid DNA was established using Nanodrop ND-1000, Thermo Fisher Scientific.

Obtained amount of plasmid DNA was digested. To perform DNA digestion, it was first required to prepare restriction mix: (Table 1)

Table 1: Plasmid DNA digestion. During the first plasmid DNA linearization attempt the excessive amount of the *Pme I* restriction enzyme was used (30 U)

Reagent	Amount
E. coli plasmid DNA/water	60 µl (5 – 10 µg)
10x NEB buffer (Biolabs)	7 µl
Pme I restriction enzyme (Thermo Fisher Scientific)	3 µl (20 U)
Total volume	70 µl

The plasmid DNA linearization was then performed by incubating the digestion mix at +37 ^{o}C for 1 h and then heat inactivating it by incubation at +65 ^{o}C for 20 minutes. Linearized plasmid DNA was then purified from the mix components using JeneJET purification kit protocol (Fermentas). The success of the linearization was confirmed by running 1 % AGE with 1 kb DNA ladder (GeneRuler).

Before starting the electroporation procedure, 500 ml of *Pichia pastoris* was grown in YPD medium for 2 days to a final overall density of the culture OD_{600}= 1.3 – 1.5. Grown *Pichia* cells were then concentrated by centrifuging and resuspending cells in decreasing amount of ice-cold water and ice-cold 1 M sorbitol to a final volume of ~1,5 ml.

80 µl of the concentrated *Pichia* cells were then mixed with linearized plasmid DNA and transferred to 0.2 cm electroporation cuvette (BIO-RAD). After 5 minutes incubation period the electroporation mix was pulsed at the resistance of 400 Ω, the capacitance of 25 µF and the voltage of 1,5 kV, using Bio-rad Gene Pulser. Immediately after the

electroporation, 1 ml of ice-cold 1 M sorbitol was added, and the mixture was incubated for +30 °C for 1 - 2 hours.

After incubation, the 10, 100 and 200 µl of electroporated cells are spread on YPDS agar plates with added 100, 500 and 2000 µg/ml Zeocin and incubated at +30 °C for 5 days.

6.2 CFH 5-7 expression

The expression was performed from ready-made X-33 *Pichia* Pastoris clone containing CFH 5-7 expressing vector. Obtained *Pichia* sample was grown on YPDS plate at +30 ^{o}C overnight. Grown *Pichia* colonies containing the insert were used for the CFH 5-7 expression.

The amount of protein-expressing *Pichia Pastoris* was increased by inoculating certain *Pichia* colony in 5 ml BMGY medium, containing 100 µg/ml Zeocin (Invitrogen), growing over-night at +30 °C and then repeating growth in 100 ml and 400 ml (for expression parameters check) or 1000 ml (for scale-up expression) of BMGY medium. Grown cells were harvested by centrifugation, using Avanti J-26 centrifuge (Beckman Coulter) at 1,500 – 3,000 x g for 5 minutes at room temperature and the cell pellet was resuspended in BMM medium to OD_{600} = 1.0 – 1.2 to induce expression.

Expression mix was then grown in Innova 4330 refrigerated shaking incubator (New Brunswick Scientific) at 29,5 °C, 250 rpm for 5 days with daily methanol inductions up to 1,0 % of the total expression mix.

After expressing *Pichia* with insert for appropriate amount of time, the expression supernatant was collected by centrifuging expression cell mix 3 times at 1.500 - 3,000 x g for 15 minutes at room temperature using Avanti J-26 centrifuge and disposing of the cell pellet. The pH of the expression supernatant was adjusted to 6.5 using 0.2 M Na_2HPO_4. During scale-up CFH 5-7 expression it was decided to check pH of the expression mix daily before methanol induction and keep pH at 6.0 with 1 M K_2HPO_4, because of the susceptibility of the expression inducing enzymes, proteases, to the low pH that might lead to a complete inactivation of the process. The expression supernatant was filtered using 0.8 and 0.2 µm vacuum filters (Thermo Scientific).

Purification of CFH 5-7 protein was performed by running the expression supernatant through the HiTrap Heparin column (Healthcare) at the speed of 0.5 ml/min over-night for expression parameter check and 1.0 ml/min during the scale-up expression.

After CFH 5-7 isolation in the Heparin column, CFH 5-7 was separated from the Heparin column using Äkta salt-gradient elution purifier. CFH 5-7 was eluded by washing the Heparin column with the 2M NaCl/75mM PBS. The fractions containing the CFH 5-7 were checked for purity by running them in 10 % SDS-PAGE gel. The gel was stained with the Coomasie Instant Blue (Invitrogen).

The collected CFH 5-7 expression fractions were then pooled together into two samples (High and Low concentration) and dialyzed using Slide-A-Lyser Dialysis Cassettes. Dialysis was performed by loading CFH 5-7 containing samples into Slide-A-Lyser Dialysis Cassettes (Thermo Scientific) and placing the cassettes in 4l of PBS overnight at +4 °C.

The concentration of dialysed CFH 5-7 samples was measured using Qubit Fluorometer and the total amount of the protein present in the sample was calculated based on the concentration provided.

6.3 ApoE isolation

The process of apoE isolation from isolated intermediate-density lipoproteins (IDL) and very low-density lipoproteins (VLDL) plasma samples was performed from H296 and H333 plasma samples, obtained from Dr. docent Matti Jauhiainen. The IDL and VLDL were isolated in H296 and H333 samples by sequential ultracentrifugation (UCF) at density 1.006 – 1.019 g/ml prior to the beginning of this work.

Obtained IDL-VLDL samples were filtered to remove clumps and dialyzed using regenerative cellulose (RC) Dialysis Membrane at density <1.019 g/ml in 0.01% EDTA at pH 7.4.

Dialyzed IDL-VLDL samples were then lyophilized by freezing dialyzed samples in dry-ice with ethanol for ~30 minutes, while inverting the tube to let ice crystals form on the side. Frozen IDL-VLDL samples were transferred to the Heto FD 2.5 lyophilizer and the

pressure was lowered to less than 10 mbar, resulting in the sublimation of water from the samples.

Water-free IDL-VLDL samples were then delipidated by adding ~30 ml of chloroform – methanol, 2:1, v/v to the ~50 mg of the sample and incubated for 1h at +4 °C. Resuspended 3 times in 20 ml methanol by centrifuging at 1000 x g and resuspending to pellet apolipoproteins.

Pelleted apolipoproteins were then solubilized by resuspending the pellet in ultrapure 6 M guanidine (minimal absorbance at 280 nm) (Sigma-Aldrich), 0.1 M Tris, 0.01% EDTA (pH 7.4) and 1% 2-mercaptoethanol over-night at room temperature. Any insoluble material was removed by low-speed centrifugation (300 x g for 10 min) and preserved for repeated solubilization.

The solubilized apoE samples (~3 ml) were separated from other apolipoproteins and detected at A_{280} nm by running apoE samples through the PBS cleaned Hiload Superdex 16/600 200 pg column (Healthcare), with the flow of 1 ml/min.

To analyze the successful apoE isolation from the mix using dot blot technique, a small aliquot of the separated fractions is applied to the nitrocellulose membrane (Sigma-Aldrich) as a dot, blocked for 2 h at room temperature using 5% milk/PBS and then spotted by staining the membrane with polyclonal anti-apoE Rabbit lgG 107 Bl XIII lgG antibody diluted 1:10,000 in 1 % milk/PBS overnight at +4 °C. Isolated fractions were detected with the secondary antibody IRDye 800CW Donkey anti-Rabbit lgG diluted 1:10,000 on the anti-apoE stained membrane using Li-COR Odyssey Infrared Imaging System.

7 Results

7.1 Obtaining high CFH 5-7 expressing *Pichia* clone

In total, two attempts to transform *E. coli* plasmid DNA into *Pichia Pastoris* were made. The goal of the first attempt was to check described *E. coli* plasmid DNA transformation parameters. Because of the insufficient growth time of the E. coli grown in 50 ml Low Salt LB medium, the total calculated mass of the isolated plasmid DNA was only 1.56 µg

(31.2 µg/ml). Even though according to the described procedure at least 5 µg of plasmid DNA was needed for successful transformation, it was decided to continue with electroporation using obtained amount of plasmid DNA. After 5 days incubation period, four electroporated *Pichia* colonies that grew on YPDS plates containing Zeocin were obtained. They were grown overnight on a new YPDS plates with 100 µg /ml Zeocin and checked for protein expression by growing them overnight in 50 ml of BMGY medium and resuspending in 100 ml of BMM medium. Expression supernatant was collected and analyzed using SDS-PAGE after 9 days of expression. All 4 *Pichia* clones did not show CFH expression and were discarded.

Second attempt to obtain high FH 5-7 expressing *Pichia* clone was performed using the same plasmid DNA transformation procedure as the first attempt. After the first failure to produce sufficient amount of *E. coli* plasmid DNA the E. coli was grown in 50 ml of Low Salt LB medium for 2 days to obtain sufficient amount of *E. coli* growth. The total calculated mass of the isolated *E. coli* plasmid DNA was 83.8 µg (167.6 µg/ml). The aliquot of plasmid DNA containing calculated amount of 10.02 µg *E. coli* plasmid DNA was used for the electroporation. After 5 days incubation period, only one electroporated *Pichia* colony grew on YPDS plates containing Zeocin. It was stored at -20 ^{o}C to be checked for CFH 5-7 expression in the future by my supervisor.

7.2 CFH 5-7 expression

In total, two successful attempts were performed in order to express CFH 5-7. The protein expression was performed, as described in 6.2. The primary goal of the first expression attempt was to check described expression parameters. For that purpose, the insert-containing *Pichia* clone was grown in the final volume of 400 ml BMGY medium and then expressed for 5 days in BMM medium with daily methanol additions. The successful isolation of the CFH 5-7 in the Heparin column was confirmed by eluding a small volume of CFH 5-7 from the Heparin column (~200 ml) by running 50 ml of 2 M NaCl/ PBS through and analyzing the eluded CFH 5-7 sample by running it in 10 % SDS-PAGE gel and staining it using Coomassie Instant Blue. (Figure 7)

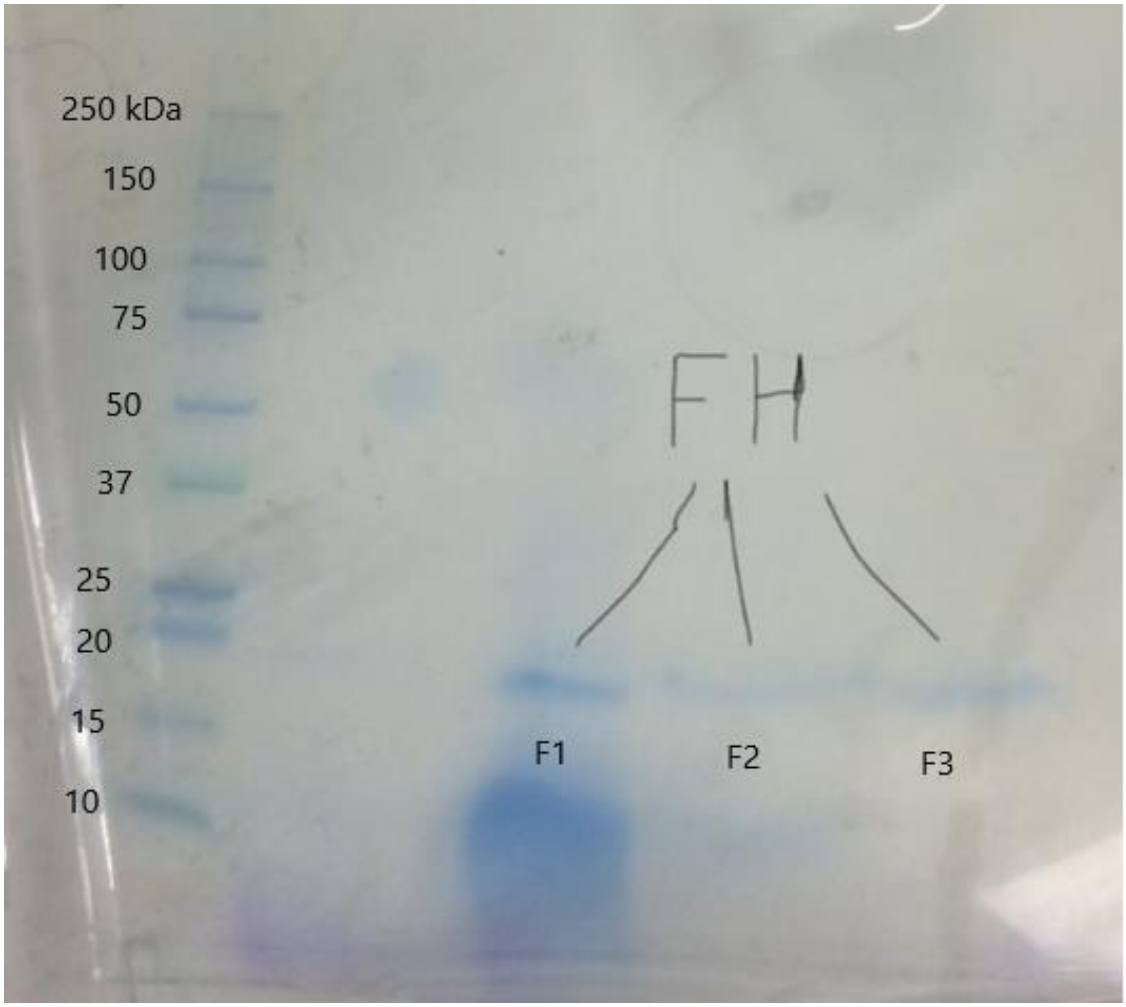

Figure 7: SDS-PAGE of the Heparin column sample, containing the Kaleidoscope Precision Plus Protein Standards (left) and eluded CFH 5-7 (right). The eluded samples clearly show the isolation of the CFH 5-7 size band (~19 kDa).

The remaining amount of the CFH 5-7 isolated into the new Heparin column was separated from using Äkta salt-gradient elution purifier. (Figure 8) The fractions containing the CFH 5-7 were analyzed for purity using 10 % SDS-PAGE gel and stained using Coomassie Instant Blue. The fractions appeared to be contaminant-free. (Figure 9) After the 10 % SDS-PAGE purity check the fractions containing the largest amount of CFH 5-7 (A7, A8, A9, A10) were pooled together, forming High Concentration sample and the rest (A6, A11, A12, A13) were pooled together to form Low Concentration sample.

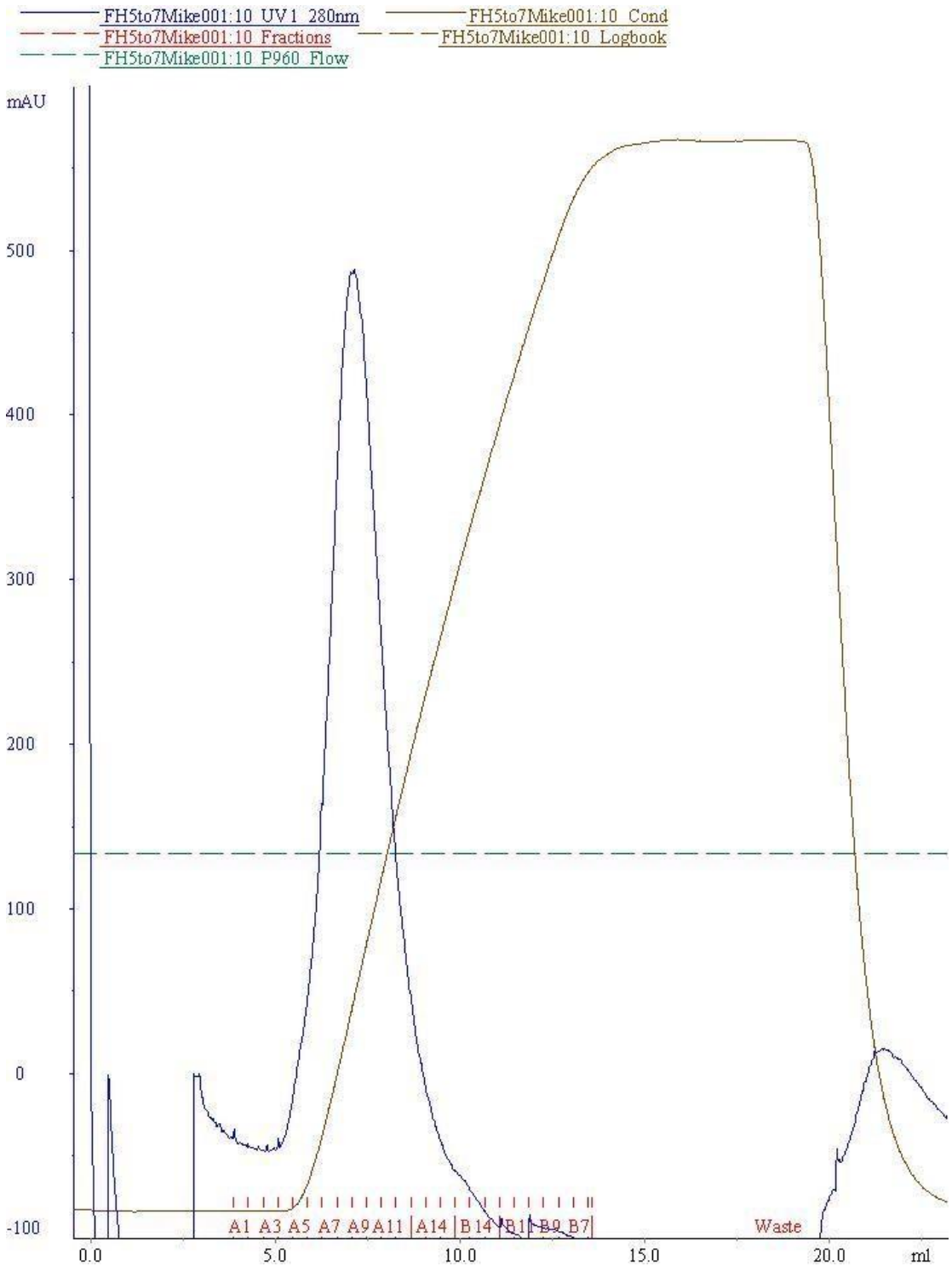

Figure 8: CFH 5-7 salt gradient elution. Fractions A4 – A13 contained the expressed CFH 5-7.

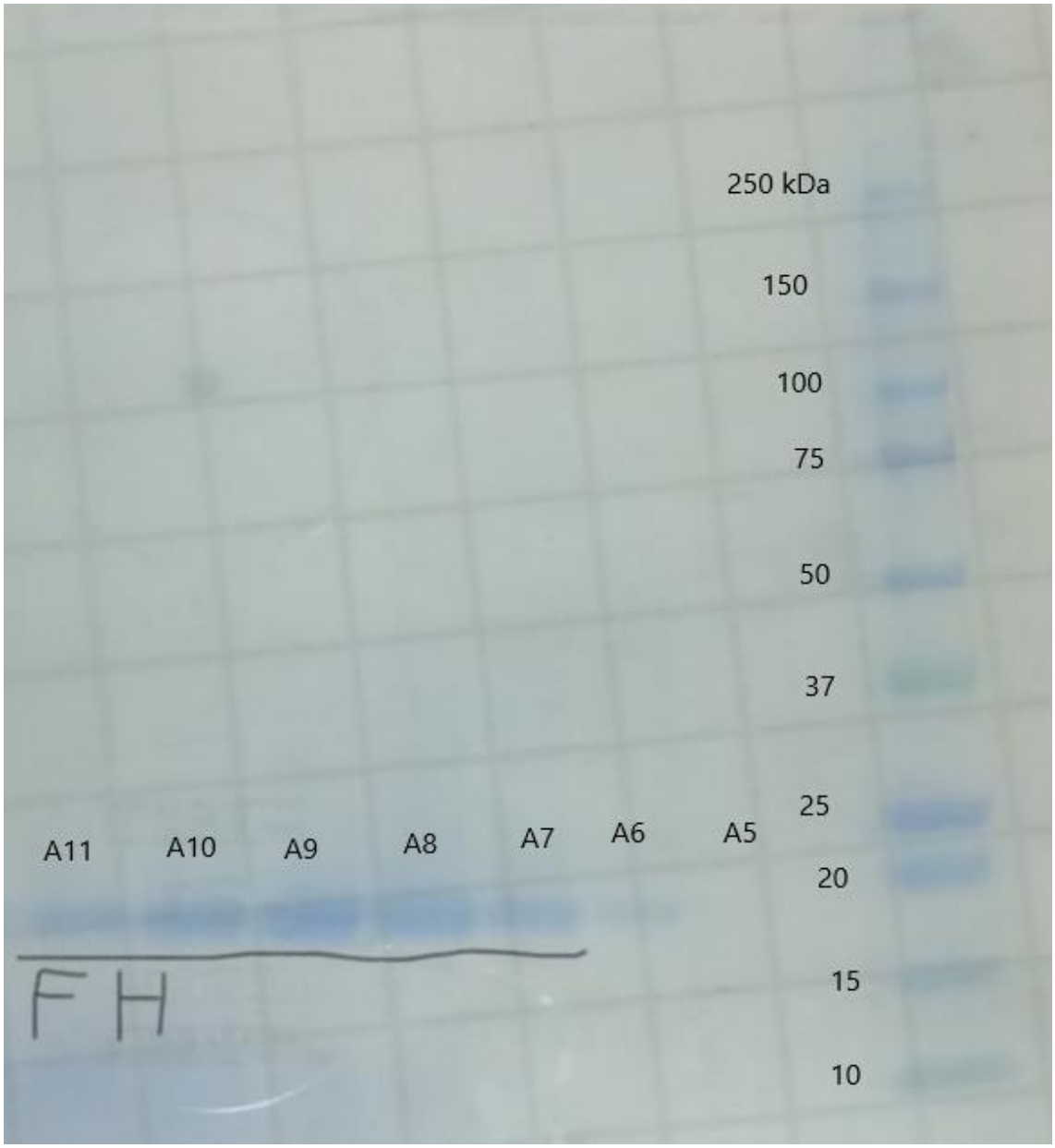

Figure 9: Purity check of the CFH 5-7 protein expression solution isolated from Heparin column on 10% SDS-PAGE gel, containing the Kaleidoscope Precision Plus Protein Standards (right) and separated CFH 5-7 fractions (left).

After the first successful CFH 5-7 isolation, it was decided to scale-up CFH 5-7 expression. For that purpose, the *Pichia* clone was grown in 1l of BMGY medium and expressed for 5 days in BMM medium with daily methanol additions. It was also decided to keep the pH of the expression medium at 6.0 - 6.5 by adding 0.2 M Na_2HPO_4 to not allow inactivation of the expression vector.

After performing salt gradient elution, the fractions containing the CFH 5-7 (Figure 10) were analysed for purity using SDS-PAGE. The fractions containing the largest amount

of CFH 5-7 (A7, A8, A9, A10) were pooled together, forming High Concentration sample and the rest (A6, A11) were pooled together to form Low Concentration sample.

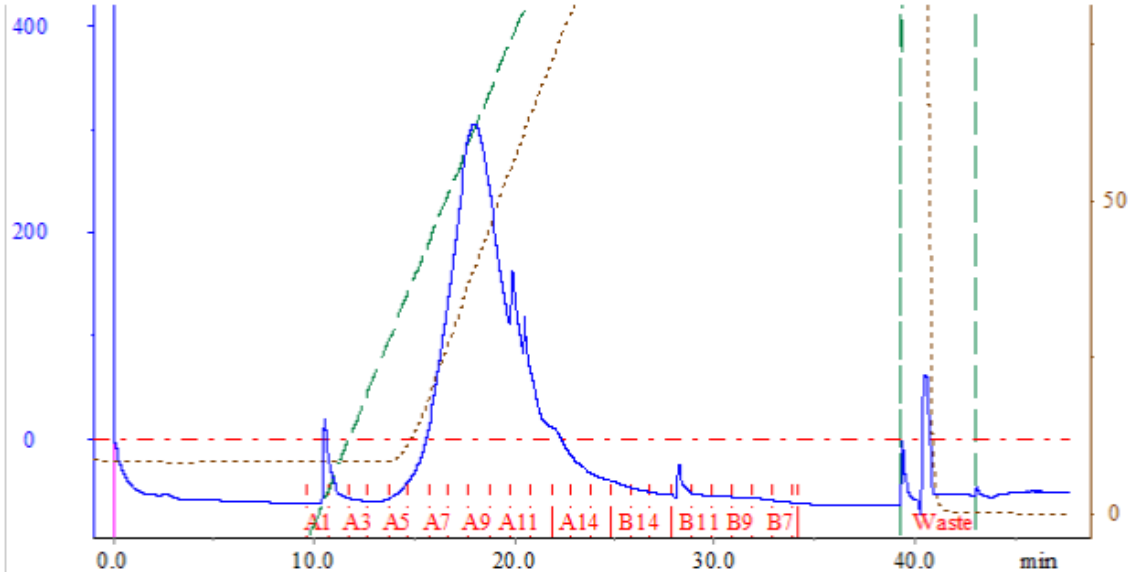

Figure 10: Second CFH 5-7 salt gradient elution. Fractions A6 – A11 contained the expressed CFH 5-7.

The concentrations of the expressed High and Low Concentration CFH 5-7 samples measured using Qubit Fluorometer during the first expression attempt were 76.4 µg/ml and 130.0 µg/ml and the during the second expression attempt were 61.2 µg/ml and 138 µg/ml. The total calculated amount of the expressed CFH 5-7 from total amount of 1.4 l *Pichia* growth was 398.86 µg.

7.3 ApoE isolation

It was attempted to separate apoE from ~3 ml of solubilized IDL-VLDL H296 and H333 samples using gel filtration chromatography by running IDL-VLDL sample supernatant through the Hiload Superdex 16/600 200 pg column and detect separated apoE at A_{280} nm using Äkta purifier. (Figures 12, 13)

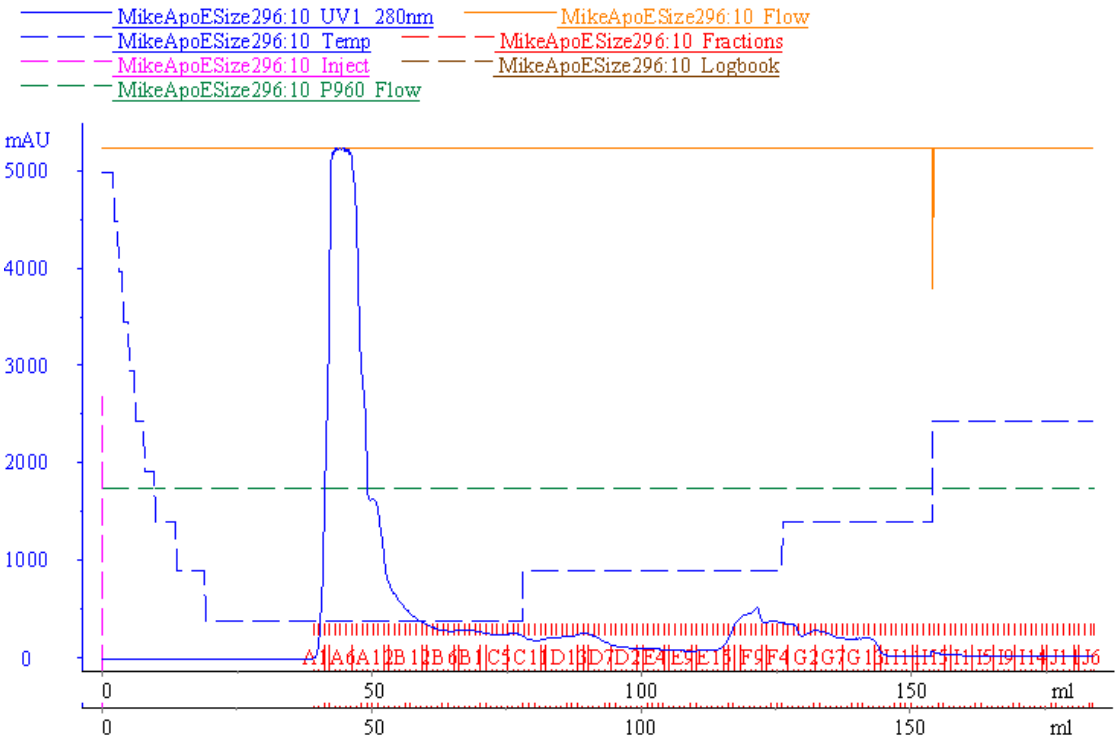

Figure 12: Gel filtration chromatography of H296. Based on the dot blot analysis it was established that apoE containing fractions are A1-D9.

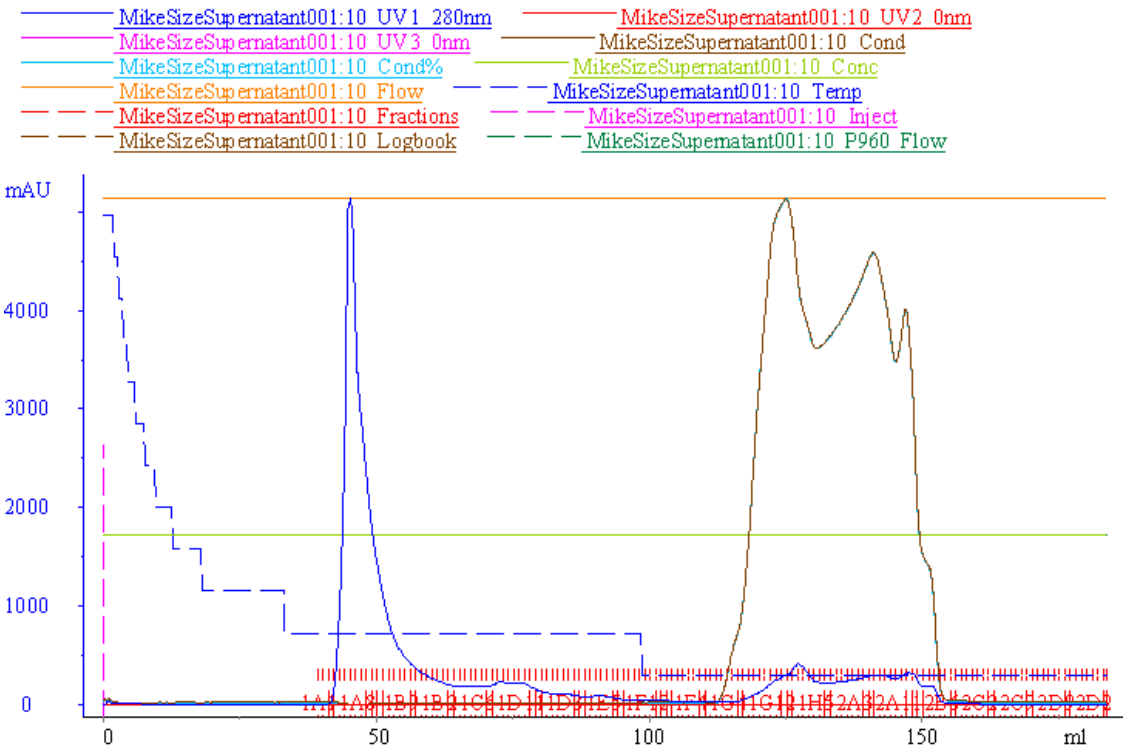

Figure 13: Gel filtration chromatography of H333. Based on the dot blot analysis it was established that apoE containing fractions are A4 – D2.

The separated apolipoprotein fractions were then analyzed using dot blot technique. (Figure 14)

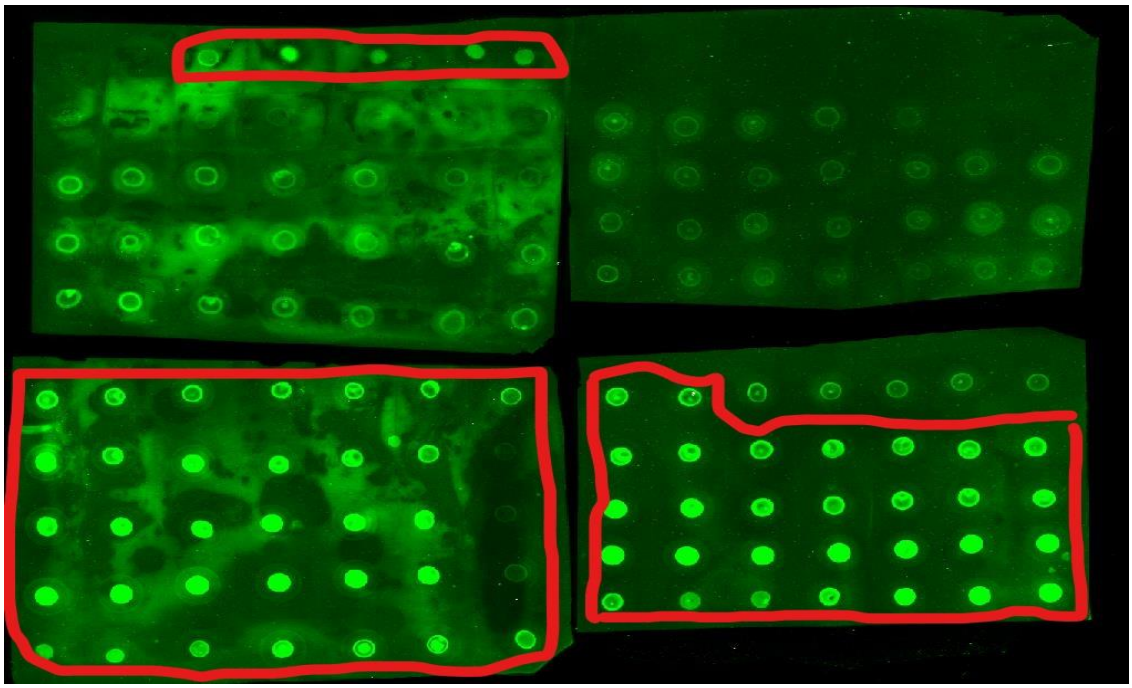

Figure 14: Dot blot of the isolated H296 (left) and H333 (right) fractions. Fractions containing apoE are highlighted.

The dot blot was the last thing that was managed to do for the apoE isolation on the time of this project, but a small amount of apoE was isolated and checked for purity by running the samples in the 10 % SDS-PAGE gel by my supervisor. (Figure 15)

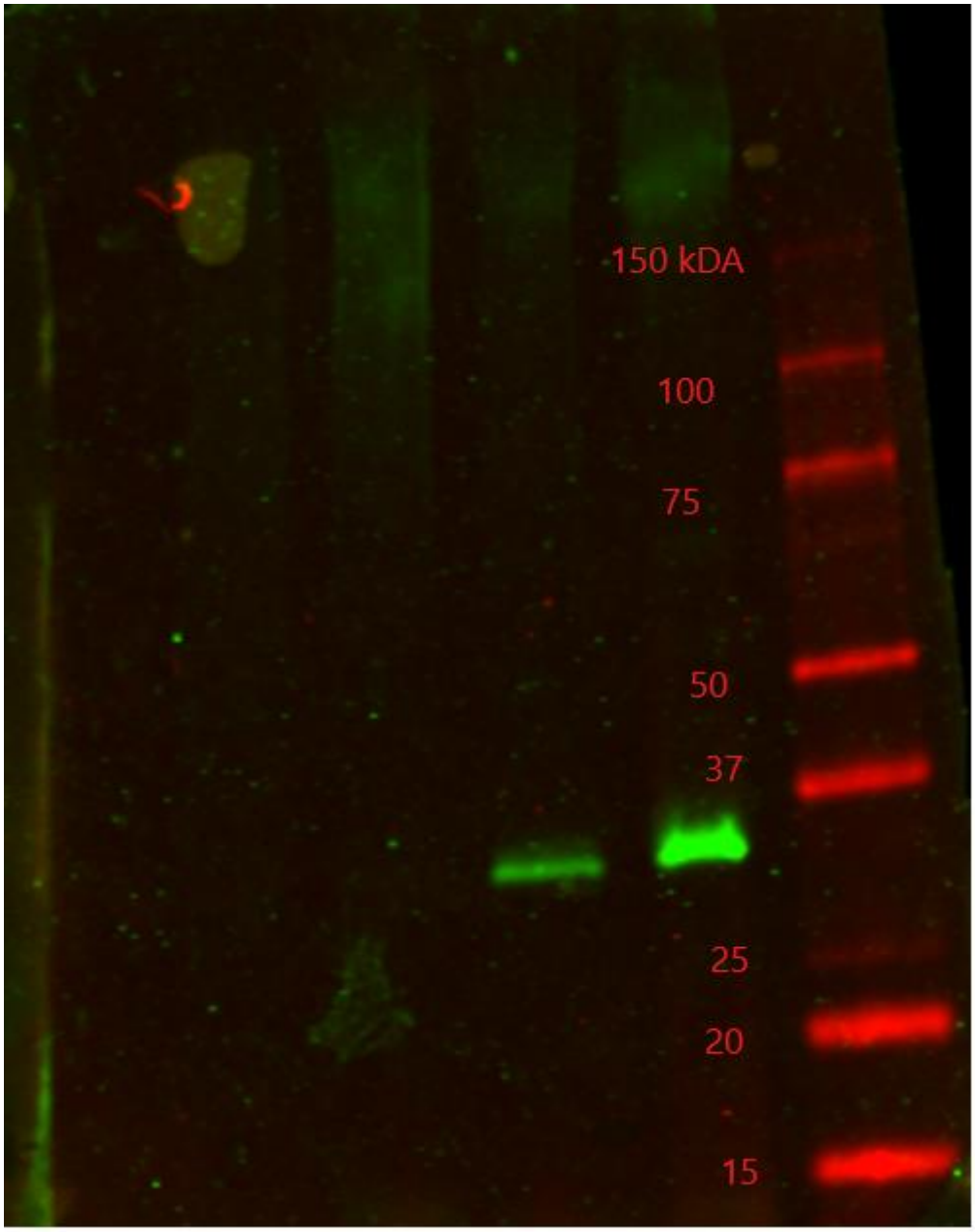

Figure 15: SDS-PAGE of the purified apoE (~34 kDa). H333 (left), H296 (middle) and Kaleidoscope Precision Plus Protein Standards (right).

8 Discussion

The processes of CFH 5-7 expression and apoE isolation in this experimental work proved to be more challenging and time-consuming than initially anticipated. Several experimental attempts were made to obtain the desired result, which indicates the need for precision and vigilance when performing the procedures.

Two attempts to transform CFH expressing vector into *Pichia pastoris* were made. The first attempt was not successful, even though four *Pichia* strains were grown in the presence of Zeocin, they did not show production of CFH 5-7 when performing the expression. During the second attempt one *Pichia* strain grew in the presence of Zeocin, but the remaining amount of time for this work was not sufficient to check it for CFH 5-7 expression. My laboratory work time was already at an end, so it was not possible to check obtained *Pichia* clone for CFH 5-7 expression.

In total, three attempts to express CFH 5-7 from ready-made *Pichia pastoris* clone were performed. First two expression attempts were successful. The quality of the expressed CFH 5-7 was enough for the planned biochemical experiments, but larger amounts of protein were required for X-ray crystallography. The third expression attempt failed due to the contamination of the expression mix: at the end of the five days expression it was not possible to purify the supernatant of CFH 5-7 expression because of the apparent BMGY or BMM medium contamination. The third protein expression sample was discarded.

During this experimental work the expression and purification of high quality CFH 5-7 was successfully performed, which indicates that processes of CFH 5-7 expression and isolation from *Pichia* work fine. The expressed high-quality CFH 5-7 fragments are the absolute requirements for the future assays. Although, the amount of expressed CFH 5-7 was not enough for x-ray crystallography, the purity of the expressed protein met required standards. In addition to producing these proteins for the laboratory use I re-wrote the experiments and recipes for the required buffers and media. These will be in great need for the new students and researchers performing the same experiments.

The success of CFH 5-7 expression was at least encouraging to my supervisor. It was decided to use the expressed CFH 5-7 for another laboratory work that requires high-quality CFH 5-7 without any contaminants. The plan for continuation of this work was to obtain a high CFH 5-7 expressing *Pichia* clone with the same purity levels. The process of plasmid DNA transformation into *Pichia* will also require some optimization in the future.

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BMGY medium recipe

Definition:

- Prepare 1 M Potassium Phosphate buffer (pH 6.0):

  Combine 132 ml of 1 M K_2HPO_4 and 868 ml of 1 M KH_2PO_4, confirm that pH = 6.0±0.1 (if pH needs to be adjusted, use phosphoric acid or KOH). Sterilize by autoclaving and store at RT.

- Prepare solutions 1 and 2

Recipe: solution 1	Amount / 1l
Bact. Yeast extract	10 g
Bact. Peptone	20 g
Biotin	2 ml/l = 0.4 mg
Glyserol	10 ml
1 M Potassium Phosphate buffer (pH 6.0) (keep in RT)	100 ml
Aq. dest.	adjust to 900 ml

Sterilization: Autoclave 15 min at +121 °C.

Recipe: solution 2	Amount / 1l
YNB = yeast nitrogen base*	13.4 g
Aq. steril.	100 ml

Make sure YNB contains ammonium sulfate

Filtrate solution 2 with Millipore (filter size – 0.22 µm)

- Combine solutions 1 and 2

Storage: fridge at +4 °C

Biotin dilution: dissolve 20 mg of Biotin / 100 ml of sterilized water and sterile filtrate (filter size – 0.22 µm). Store in fridge.

BMM medium recipe

Definition:

- Prepare 1 M Potassium Phosphate buffer (pH 6.0):

  Combine 132 ml of 1 M K_2HPO_4 and 868 ml of 1 M KH_2PO_4, confirm that pH = 6.0±0.1 (if pH needs to be adjusted, use phosphoric acid or KOH). Sterilize by autoclaving and store at RT

- Prepare solutions 1 and 2

Recipe: solution 1	Amount / 1l
Biotin	2 ml/l = 0.4 mg
Methanol	5 ml
1 M Potassium Phosphate buffer (pH 6.0) (keep in RT)	100 ml
Aq. dest.	adjust to 900 ml

Sterilization: Autoclave 15 min at +121 °C

Recipe: solution 2	Amount / 1l
YNB = yeast nitrogen base*	13.4 g
Aq. steril.	100 ml

Make sure YNB contains ammonium sulfate

Filtration with Millipore (filter size – 0.22 µm)

- Combine solutions 1 and 2

Storage: fridge +4 °C

Biotin dilution: dissolve 20 mg of Biotin / 100 ml of sterilized water and sterile filtrate (filter size – 0.22 µm). Store in fridge.

1 M Sorbitol recipe

- For 1l: dissolve 18,21 g of D-sorbitol in 100 ml of water
- Adjust to 1l with water
- Filter, using vacuum filters

YPDS agar plate recipe (+ Zeocin)

- For 1l: dissolve the following in 900 ml of water:
  10 g yeast extract
  182.2 g sorbitol
  20 g peptone
- Add 20 g agar
- Autoclave for 20 min on liquid cycle
- Add 100 ml of 20 % Dextrose
- Cool solution to 60 ºC (and add 1.0 ml of 100 mg/ml Zeocin, if needed)
- Store YPDS plates at 4 ºC, **in the dark** (1-2 weeks)

YPD medium recipe (+ Zeocin)

- For 1l: dissolve in 900 ml water:
  10 g yeast extract
  20 g peptone
- Autoclave for 20 min on liquid cycle
- Add 100 ml of 20 % Dextrose
- Cool solution to 60 ºC (and add 1.0 ml of 100 mg/ml Zeocin, if needed)
- Store YPDS plates at 4 ºC, **in the dark** (1-2 weeks)

0.2 M Na_2HPO_4 recipe

- For 1l: dissolve 28,39 g of Na_2HPO_4 in 100 ml of water
- Adjust to 1l with water
- Filter, using vacuum filters

1 M K_2HPO_4 recipe

- For 1l: dissolve 228,23 g of $K_2HPO_4*3H_2O$ in water
- Adjust to 1l with water
- Filter, using vacuum filters

***E. coli* Low Salt LB medium recipe**

- For 1 liter, dissolve the following in 950 ml deionized water:
  10 g tryptone
  5 g Yeast Extract
  0.5 % NaCl
- Adjust pH of the solution to 7.5 with 1N NaOH and bring the volume up to 1 liter
- Autoclave for 20 minutes at 15 lb/sq. in and 121 ^{o}C
- Let cool to 55 ^{o}C (and add Zeocin to 25 µg/ml final concentration, if needed)
- Store at 4 ^{o}C in the dark (1-2 weeks)

AGE:

Prepare 1 l electrophoresis buffer (1x TBE) to fill the electrophoresis tank and to cast the gel:
For this we take 100 ml of 10x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) stock solution in an Erlenmeyer flask and make the volume to 1000 ml by adding 900 ml of distilled water. The 1x working solution is 8.9 mM Tris-borate-EDTA (TBE)
It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and the gel preparation.

Prepare a solution of agarose in electrophoresis buffer at an appropriate concentration:
For this weight 1.5 grams of agarose to 150 ml of electrophoresis buffer.

Loosely plug the neck of the Erlenmeyer flask. Heat the slurry in a microwave oven until the agarose dissolves. The agarose solution can boil over very easily so keep checking it. It is good to stop it after 45 seconds and give it a swirl. It can become superheated and NOT boil until you take it out whereupon it boils out all over your hands. Wear gloves and hold it at arm's length. Use insulated gloves or tongs to transfer the flask/bottle into a water bath at 55°C. When the melted gel has slightly cooled, add 6 µl of 0.04 µg/ml Midori Green (Nippon Genetics), fluorescent dye used for staining nucleic acids. Mix the gel solution thoroughly by gentle swirling.

While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel.

Pour the warm agarose solution into the mold.
(The gel should be between 3 - 5 mm thick. Check that no air bubbles are under or between the teeth of the comb.)

Allow the gel to set completely (30-45 minutes at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off the electrophoresis buffer. Mount the gel in the electrophoresis tank.