Expression, Characterization, and Structural Studies of Anti-amyloid Antibody Fragments

by

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ABSTRACT

Alzheimer's disease is a major problem affecting over 5.7 million Americans. Although much is known about the effects of this neurogenerative disease, the exact pathogenesis is still unknown. One very important characteristic of Alzheimer's is the accumulation of beta amyloid protein which often results in plaques. To understand these beta amyloid proteins better, antibody fragments may be used to bind to these oligomers and potentially reduce the effects of Alzheimer's disease.

This thesis focused on the expression and crystallization the fragment antigen binding antibody fragment A4. A fragment antigen binding fragment was chosen to be worked with as it is more stable than many other antibody fragments. A4 is important in Alzheimer's disease as it is able to identify toxic beta amyloid.

To Darlene Woodyard

For believing I could do anything and picking me up every time I fell down

And to Lisa Colasurd

For being there every step of my academic career

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Dr. Brent Nannenga for giving me the opportunity to work in your lab and encouraging me toward my future success. Dr. Jeremy Mills for allow me to work in the lab as though I was one of your students. Dr. Nathan Henderson for taking the time to train me and encourage me when everything failed.

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INTRODUCTION

1.1 Alzheimer's Disease

Alzheimer's disease is a very serious neurodegenerative disease that often results in effects such as memory loss, confusion, hallucinations. Death is often a result of this disease by means of malnutrition, pneumonia, or inanition. (1) The disease is slow moving and can last many years after one is diagnosed.

1.1.1 Impacts

Alzheimer's disease effects an estimated 5.7 million Americans in 2018. By 2025 this number is projected to be 7.1 million, and by 2050 to 13.8 million. (1) Because of its high mortality rate, Alzheimer's disease is the sixth leading cause of death in the United States. (2) Many times, Alzheimer's disease effects not only the patients but their family as well as most patients survive four to eight years or even as long as twenty-five years following a diagnosis. (3) Because this disease effects the brain, these patients are often live in a state of dependency. With high healthcare costs, total lifetime cost of care being estimated at $341,840 in 2017, many patients are cared for by family members or other unpaid caregivers. This significantly effects the entire nation with an impact estimated to be $232.1 billion. (1)

1.1.2 Pathology

The two main features of Alzheimer's disease are the accumulation of amyloid beta-protein (Aβ) to form extracellular plaques and the accumulation of tau protein to form intracellular neurofibrillary tangles. Currently, it is hypothesized that the pathology of this disease is the change from nontoxic oligomers in the brain to toxic ones. However, this is simply a hypothesis, and the underlying cause of the neural death seen in Alzheimer's remains to be under investigation.

1.1.3 Amyloid Beta Protein

Aβ is produced through the cleavage of amyloid protein precursor (APP). (4) The cleavage of APP is done by three different proteases. These proteases are known as, α-,β-, and γ-secretases. Cleavage by β- and γ- secretases releases Aβ, whereas cleavage by α-secretase cleaves within the Aβ sequence. (4)

1.2 Antibody Fragments

There are several different classifications of antibody fragments depending on the size and part of the antibody that is being used. These antibody fragments are typically generated from the variable region of Immunoglobulin G (IgG) (see figure 1) These antibodies can be fragmented into the following main types: fragment antigen binding (Fab) and fragment crystallizable (Fc) (see figures 2 and 3) The Fab was used in this project as it includes the binding region of the antibody. The Fab can then be broken down into smaller subunits if desired, including single-chain variable fragments (scFv) and single domain antibody (sdAb). (see figure 4) (5)

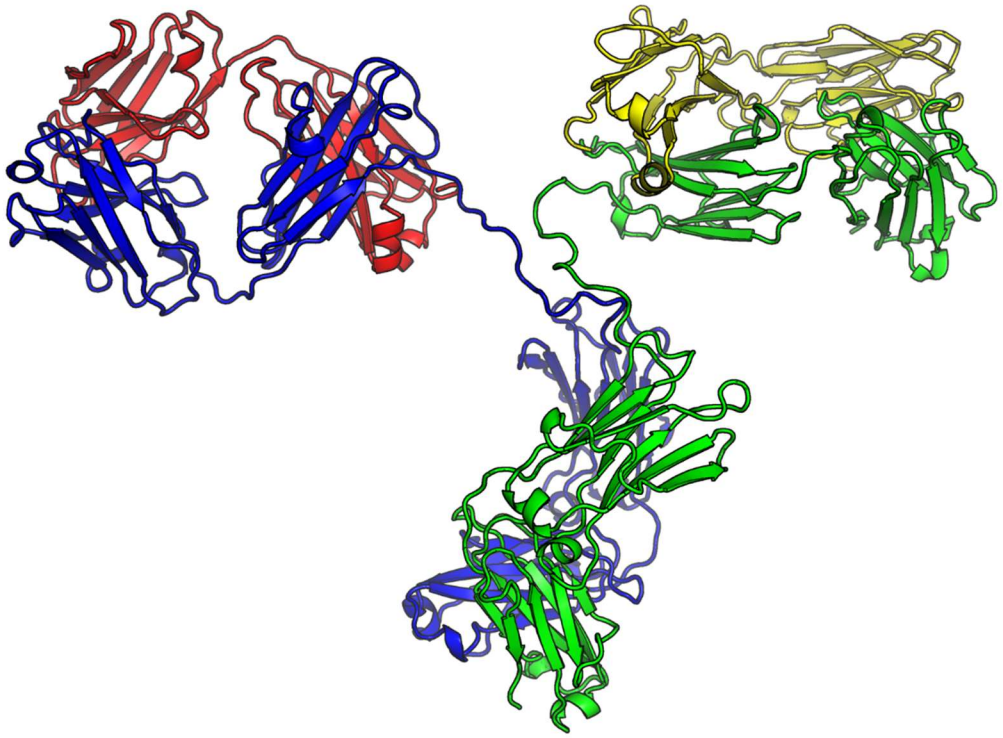

Figure 1: Immunoglobulin G. The two heavy chains are colored in green and blue. The two light chains are colored red and yellow. the structure shown is from PDB.

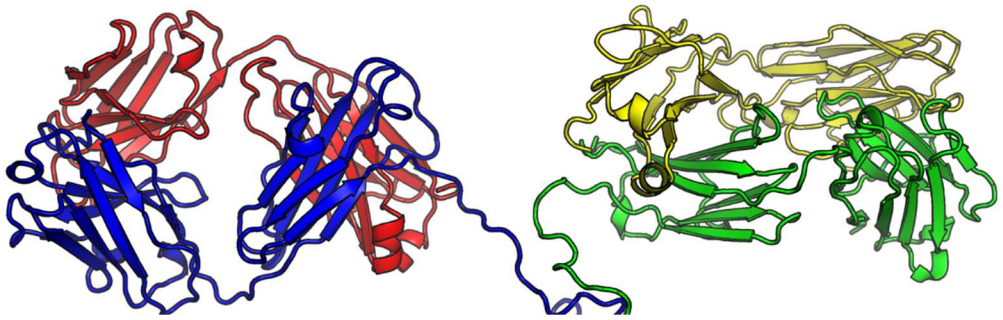

Figure 2: F(ab')$_2$ The two heavy chains are colored in green and blue. The two light chains are colored red and yellow This image is adapted from figure one.

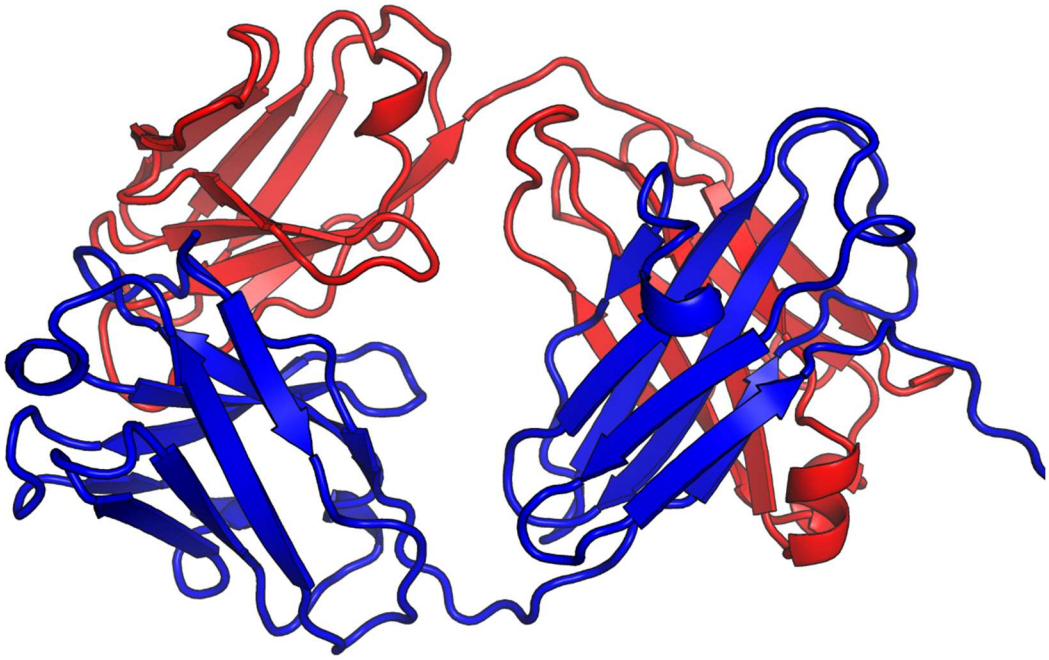

Figure 3: Fab, The heavy chain is colored blue. The light chain is colored red. This figure was adapted from figure 1

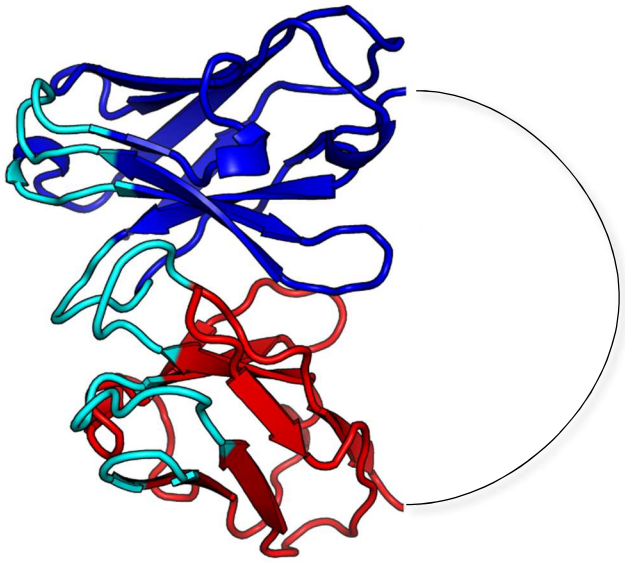

Figure 4: scFv, The heavy chain is colored blue and the light chain is colored red. The black line indicates where the serine and glycine linker connects to to chains. This figure was adapted from figure 1

1.3 Expression of Fragment Antigen Binding Antibody Fragments

There are several different ways that one can obtain a Fab. These include proteolytic digestion and expression in Escherichia coli cells. These antibody fragments are often difficult to obtain in high amounts, and several expressions are often necessary to obtain a sufficient amount.

1.3.1 Proteolytic Digestion of Immunoglobulin G

As a Fab can be very difficult to express, the most common type of expression is generally using proteolytic digestion. In this type of expression, the complete IgG protein is expressed which is then subsequently degraded by a protease. The type of protease used for this degradation depends on whether one wants two separate Fab fragments or one $F(ab')_2$ fragment, two Fab fragments joined by disulfide bonds. The most common

protease used to produce two separate Fab fragments is papain. This is a thiol-type protease that when incubated with IgG molecules and a reducing agent. The peptide bonds of the hinge region are digested and three distinct fragments are formed. This is the ideal way to digest the IgG molecules if one desires to have the individual Fab fragments and also an intact Fc fragment. Once the digestion is complete, the Fc and Fab fragments can be separated and purified. Another popular type of protease that used for IgG digestion is pepsin. Pepsin is an acid type protease that digests the Fc portion of an IgG molecule leaving an intact $F(ab')_2$ molecule.

1.3.3 Escherichia coli

Escherichia coli (*E. coli*) is one of the more common ways that Fabs are currently expressed. One is able to insert the desired deoxyribonucleic acid (DNA) into the *E. coli* cell to obtain a Fab with the desired light and heavy chains. The Fab is produced in the nucleus of the cell then moved to the periplasm where it is easily retrieved through periplasmic lysis. This entails the cell being broken open so that the protein is able to be purified.

1.4 Research Objectives

The objective of this research was to express and crystalize the A4 Fab. This Fab was focused on as it identifies one of the toxic oligomeric forms of Aβ. These antibody fragments are needed as identification of these proteins as proved to be an impossible task on their own. These difficulties are due to the fact that the toxic oligomer Aβ are usually found only at trace levels and are constantly changing in structure making

crystallography very difficult. When using a Fab, these obstacles are mitigated by helping to purify the oligomers and generating a large and stable antigen complex that is more easily crystalized enabling structure determination

Chapter 2

EXPRESSION OF A4

2.1 Expression

2.1.1 Liquid Culture

For this experiment the Fab was expressed using *E. coli*. To begin the expression of A4, liquid culture was performed. An overnight culture was started using 50 mL of 2xYT containing 0.05 mg/mL kanamycin (Kan). This culture was placed in the shaker at 37°C at 250 rpm for approximately sixteen hours, or once the overnight culture reached a high density. This culture was then transferred into six separate 1 L cultures, contained 0.05 mg/mL Kan. This culture was grown until an optical density at 600 nm (OD_{600}) absorbance was approximately 1. Once this OD_{600} was achieved, expression was induced with arabinose, and the culture was left in the shaker for sixteen to twenty hours.

2.1.2 Auto-Induction on Solid Media

After mediocre, unrepeatable results were obtained using liquid media for the expression of A4, solid media was tried. In hopes of yielding a higher expression, autoinduction was tried. This was done as A4 is known to be toxic to the cells. Therefore, if auto-induction is used, more cells are able to grow before the expression is induced with arabinose. This allows for more cells to be present therefore there are more cells to produce the wanted antibody. To begin this expression, two liters of 2xYT were made. In one liter, 15 g/L of agar was added, and to the other, 7.5 g/L of agar was added. After autoclaving, the media was allowed to cool, and 0.2% g/L arabinose and 0.05 mg/mL

Kan were then added into the media with 7.5 g/L agar. The same amount of Kan was then added to the media with 15 g/L agar. The media containing 7.5 g/L agar was poured into the plate first and allowed to solidify. After that layer was completely cool, the second layer was poured with the media containing 15 g/L agar. This was then allowed to cool. After both layers were completely solidified, the plate was streaked using 1 mL of an overnight culture. The plate was then left for seven days at room temperature under a hood. After seven days, the plate was removed and the colonies that grew on the plate where scraped off.

2.1.3 Auto Induction in Liquid Culture

As some protein was obtained through auto induction on solid media with a higher repeatability, one would conclude that auto induction would be the expression of choice. Because of the time necessary and the limited amount of A4 that was expressed when using solid media however, it was not a feasible way to obtain a significant amount of A4. Therefore, autoinduction using liquid culture would be desired. Although this is more time intensive than expression in a regular liquid culture, the hope was to finally be able to express a reasonable amount of A4 in a repeatable manner. To begin auto induction in liquid culture, one 100 mL overnight culture using 2xYT was made with 0.05 mg/mL Kan. After approximately sixteen hours of growth, the culture was evenly divided into two parts and spun down at 6,000 rpm for fifteen minutes. The pellets were then resuspended into separate 1 L 2xYT cultures with 0.05 mg/mL Kan. This was then left to grow overnight or about twenty hours. These cultures were then separated into six parts and spun down at 6,000 rpm for fifteen minutes. Each pellet was then resuspended

in 1 L of 2xYT with 0.05 mg/mL Kan, 0.05% g/L glucose, and 0.2% g/L arabinose. These cultures were then left to grow for sixteen to twenty hours.

2.2 Purification

2.2.1 Periplasmic Lysis

To obtain the Fab from the *E. coli* cells, a periplasmic lysis must be performed. Once the cultures grew for their specified time, the samples were centrifuged at 6,000 rpm to remove the full cells from the media. The supernatant was then poured off and the pellets were resuspended in periplasmic lysis buffer (PPLB). The PPLB used contained, 20% g/L sucrose, 30 mM Tris(hydroxymethyl)aminomethane hydrochloride pH 8 (Tris), and 1 mM ethylenediaminetetraacetic acid (EDTA). The volume of buffer used was an excess of four times the weight of the pellets. To this PPLB, 1 mM phenylmethane sulfonyl fluoride (PMSF), and lysozyme were also added. This was stirred for one hour before being centrifuged at 6,000 rpm for 15 minutes to remove the full cells. The supernatant was poured off and spun down again at 18,500 rpm for 15 minutes. The two pellets were combined and resuspended in the same amount of PPLB and PMSF as the previous resuspension. The supernatant that resulted was placed in a flask with 2mM MgCl, 0.5 mM CaCl, and deoxyribonuclease (DNAse), and stirred for 15 minutes. This was then filtered using a syringe filter with a pore size of 0.2 μm. This prepared the lysate to be loaded onto three different columns to ensure a high final purity of the A4.

2.2.2 Protein G

After being filtered, the lysate was run over three 1 mL protein G columns in succession, using 20 mM $NaPO_4$ at a pH of 7 as the buffer. Once all the lysate was loaded onto the columns, the columns were washed with 0.1 M glycine pH 2.7 into fourteen 0.5 mL tubes that each contained 100 µL of Tris pH 9. These tubes were then tested for presence of protein using a Bradford protein assay. The tubes that tested positive for protein were then pooled together and dialyzed in 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.5 and 10 mM NaCl. This buffer will be referred to as buffer A. This dialysis was done in preparation for ion-exchange chromatography.

2.2.3 Ion-exchange Chromatography

Once the sample dialyzed for an hour or more the sample was loaded onto a Mono S® cation exchange column using buffer A. Once the entire sample was loaded onto the column, a buffer (buffer B) containing 20 mM MES pH 5.5 and 1 M NaCl was used to elute the A4 from the column. This elution was done by linearly increasing the concentration of buffer B to buffer A ratio so that 100% buffer B would be achieved in eight minutes (see figure 5). The large Gaussian peak shown in the chromatogram was collected.

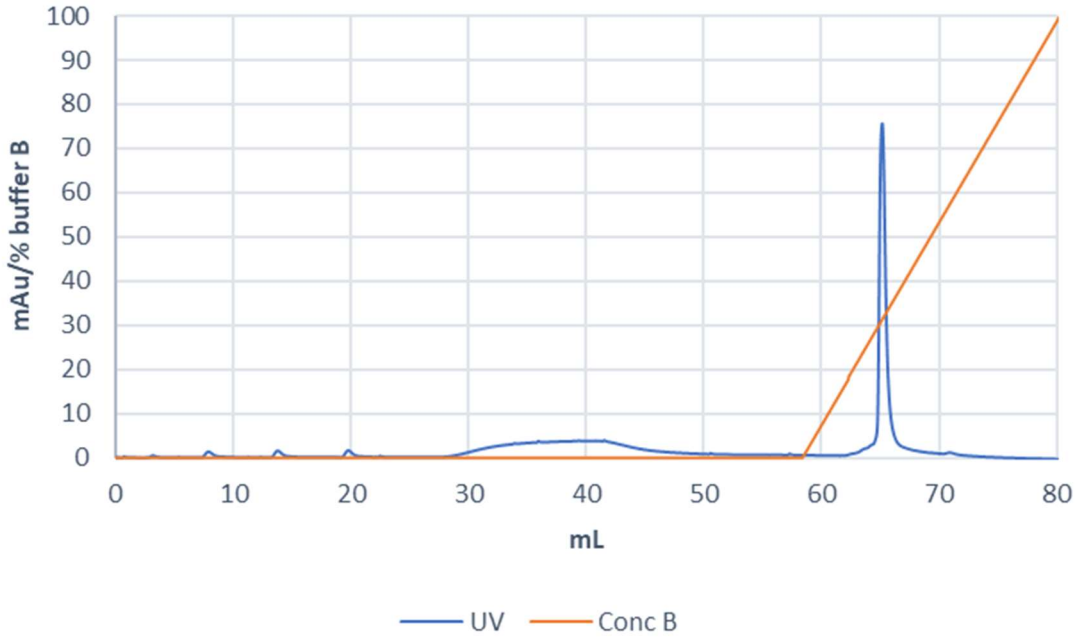

Figure 5:Ion-exchange Chromatogram with a linear gradient (measured using the conductivity of the solution – shown in orange). The UV absorabance at 280 shows that the Fab elutes from the column in a single sharp peak at approximately 30% buffer B.

2.2.4 Gel Filtration Chromatography

To ensure a high amount of purity of the sample gel and to verify the identity of the sample gel filtration chromatography was utilized. Because of size of the pores for each individual column once the elution data is verified, one can verify the identity of the sample each subsequent run. Using the absorbance that is read at 280 nm, one is able to distinguish an approximate amount of protein in each sample. The higher the absorbance the higher the amount of protein that is present. In the gel chromatograms shown in figures 6, 7 and 8, one is able to clearly see that the liquid expression with auto induction has the most amount of protein present. This outcome is hypothesized to be because the antibody is toxic to the cells, and therefore if a larger number of cells are grown before the cells are induced, the larger number of cells there will be to produce A4 before

ultimately dying. Although the liquid culture did give a larger amount of A4 than that of the solid media it should be mentioned that the amount of liquid culture often fluctuated between a sufficient amount and none. The solid media however generated a smaller amount of A4 each time however it was consistent in the amount that it would produce.

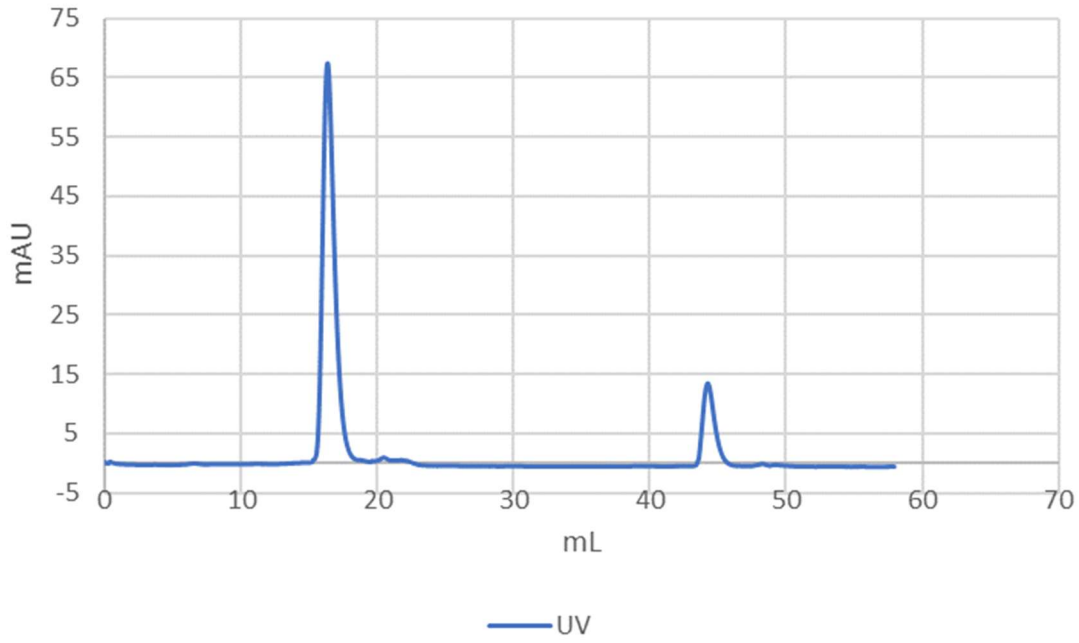

Figure 6: Gel Chromatogram from Liquid Expression using an S200 sizing column. The sizing trace shows a sharp peak at approximately 16 mL which is the appropriate position for a molecule that is the size of A4.

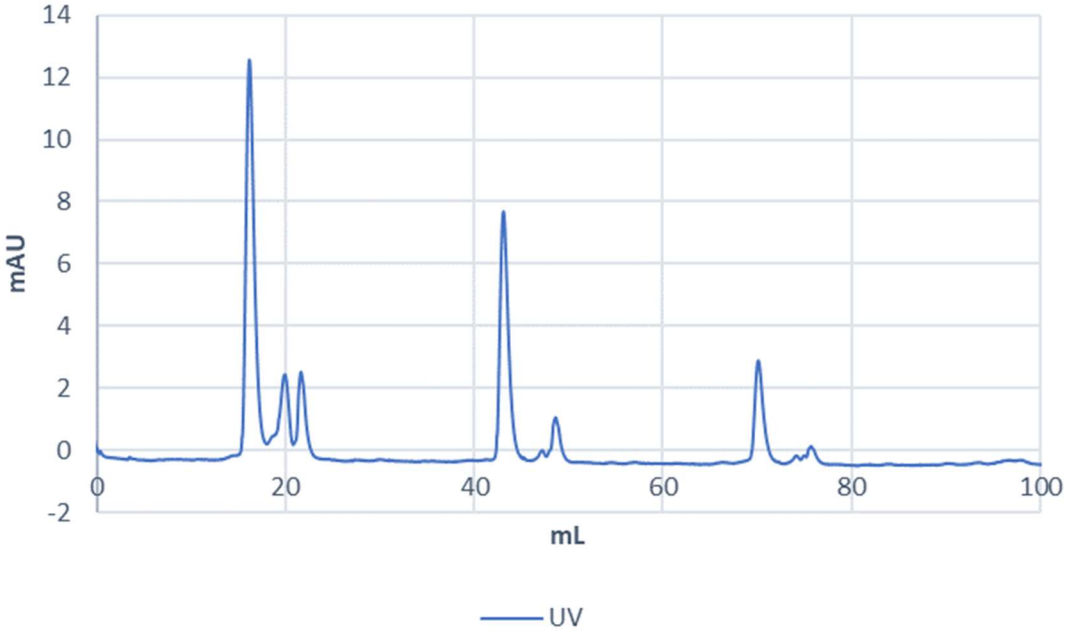

Figure 7:Gel Chromatogram from Auto-induction on Solid Media

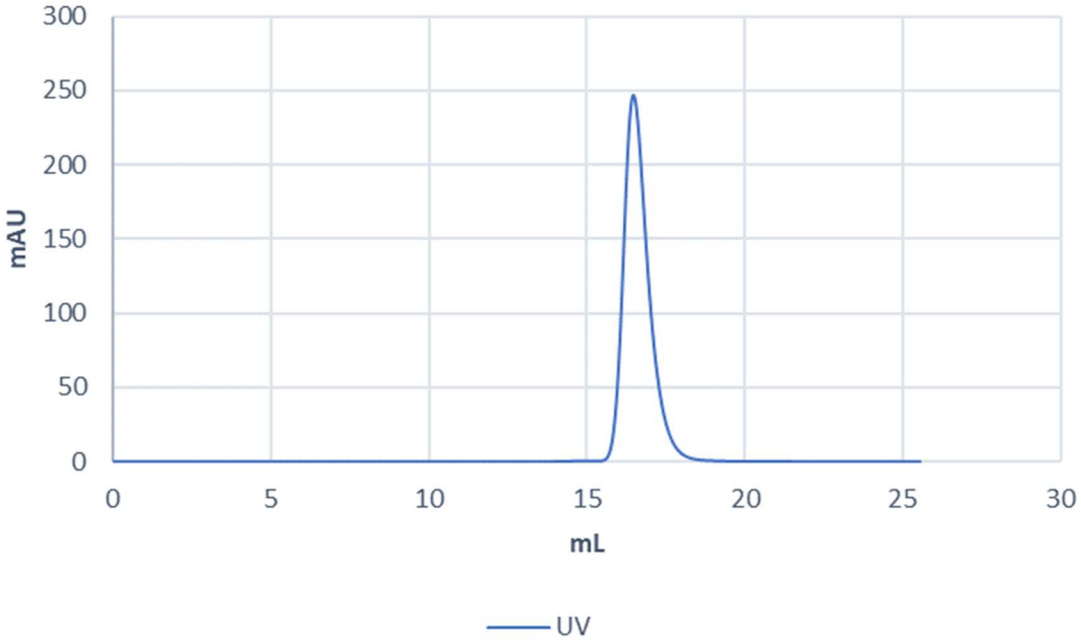

Figure 8::Gel Chromatogram from Auto-induction in Liquid Media. Again a single peak at approximately 16 mL is seen, however these expression conditions show a much larger amount of purified A4.

Chapter 3

CRYSTALLOGRAPHY

3.1 Protein Crystallization

Once A4 was purified and concentrated one was able to begin screening for ideal crystallization conditions. The purification and identity of the protein was demonstrated using an SDS-PAGE gel as is shown in figure 9.

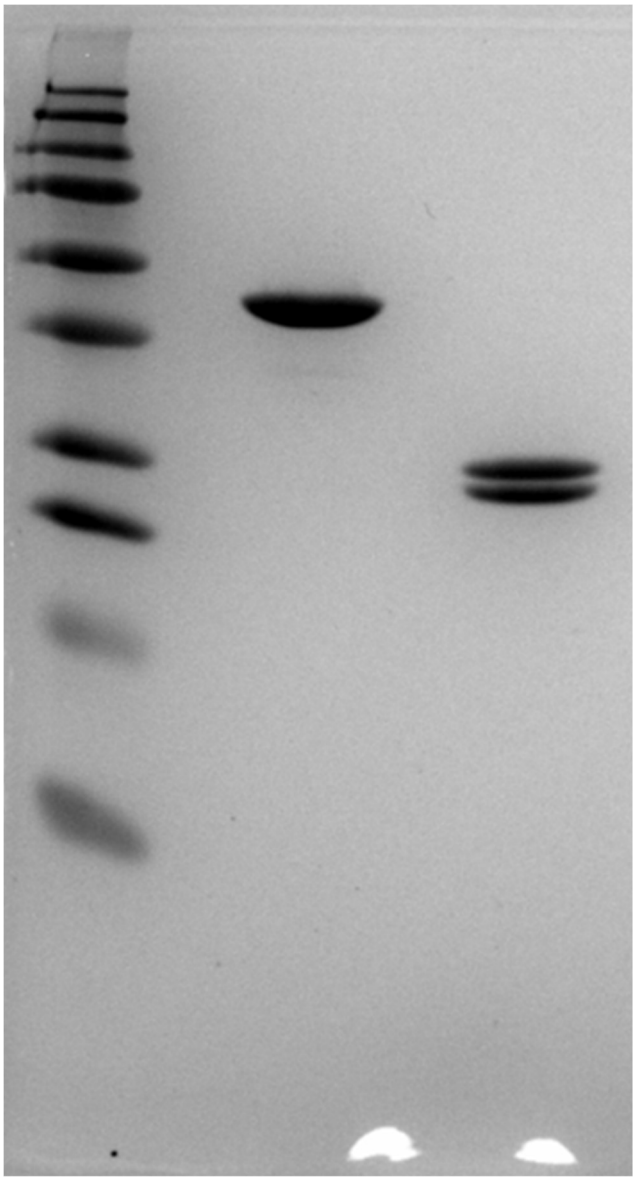

Figure 9: SDS-PAGE Gel of purified A4. Far left lane, molecular weight ladder, middle lane is non- reduced A4 showing the expected molecular weight of ~48kDa, and the far right lane is reduced A4 showing the expected positions of the individual dissociated heavy and light chains. This gel demonstrates that the purification protocol produces very pure A4.

This was done using a CrystalMation Phoenix RE robot from the company Rigaku along with 96-well sitting-drop vapor diffusion trays to identify crystallization condition for A4. Three course screen crystallization kits from Hampton Research (PEG-Ion, Index, and Crystal) were tested with A4 at 3.6 mg/mL in 10 mM (4-(2-hydroxyethyl)-1-

poperazineethanesulfonic acid sodium hydroxide (HEPES-NaOH) pH 7.5 and 75 mM NaCl. Each condition was tested at two protein to well ratios (2:1 and 1:1) giving 576 total conditions screened. Two hits were identified for follow-up screening: Index condition # 68 [0.2 M NH_4SO_4, 0.1 M HEPES pH 7.5, 25% polyethylene glycol (PEG) 3350] at a 2:1 protein to well ratio and Crystal #93 [10 mM Nickel(II) chloride hexahydrate, 0.1 M Tris pH 8.5, 20% PEG monomethyl ether (MME)] also at a 2:1 protein to well ratio. While Index condition #68 was not reproducible when scaling up to the larger 24-well plate format, the latter was amenable to optimization on a larger scale (see figure 10). A total of four follow up trays were set up and a total of forty crystals were tested for diffraction at the synchrotron, with a current best condition containing 18% PEG MME, 12.5 mM $NiCl_2$, 0.1 M Tris pH 8.5. Prior to flash freezing under liquid nitrogen and diffraction data collection, crystals were transferred to mother liquor with the PEG MME increased to 40% w/v.

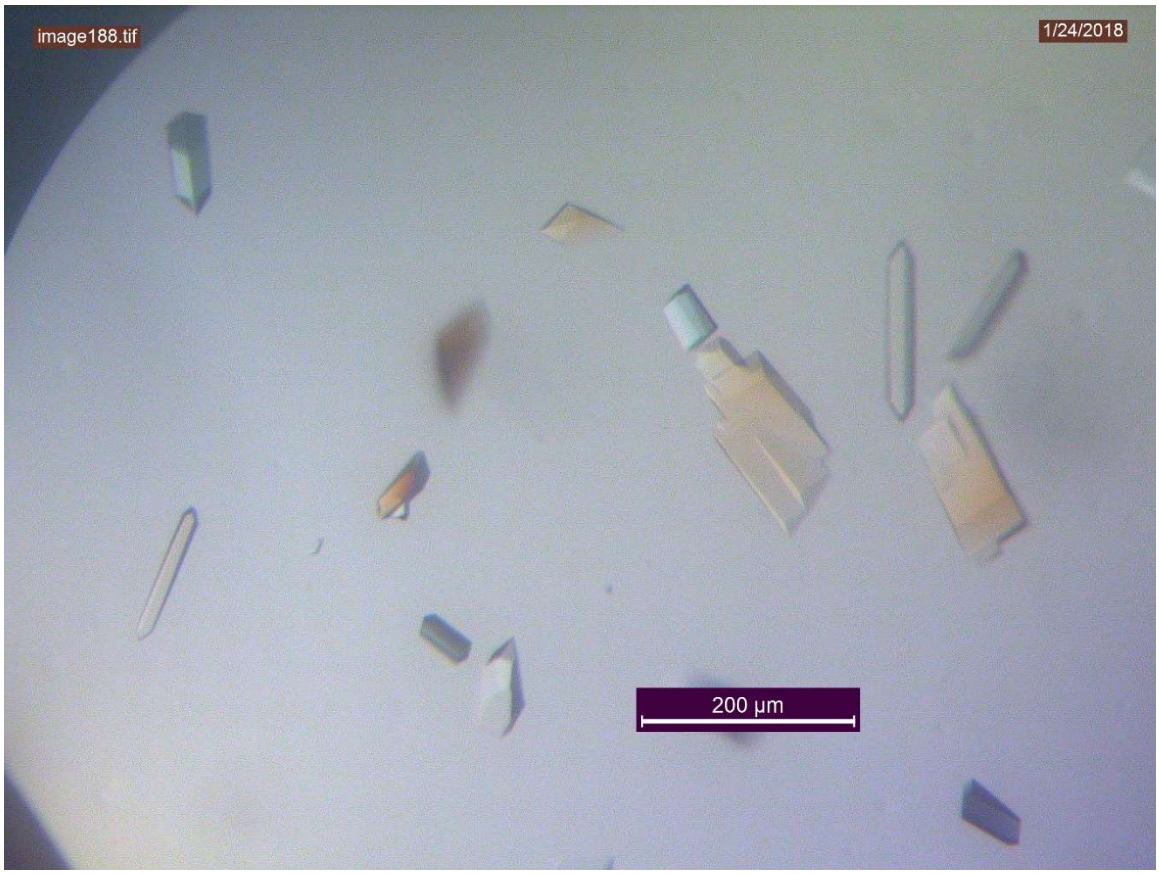

Figure 10: A4 Crystals from 24 Well Screening. Crystals are approximately 100um in size and grow as individual crystals.

3.2 Crystal Diffraction Data Collection and Structure Determination

Diffraction data from A4 were collected at the Berkeley Center for Structural Biology (BCSB) from the Advanced Light Source (beamline 5.0.2) on a PILATUS3 6M detector (see figure 10). A 240° wedge of data (1° oscillations) was collected at 1.00003 Å from a crystal cooled to 100 K in cryo-stream. Data were indexed, refined, integrated, and scaled using iMosflm from the CCP4i package (6). A reasonable structure solution was obtained by molecular replacement using the program Molrep (7). The search model consisted of the 5c8 Fab split into two ensembles, the first consisting of the constant domain of both heavy and light chains and the second the variable domains of the two

chains. All models were refined using Refmac5 (8) and model building was carried out using the program Coot (9).

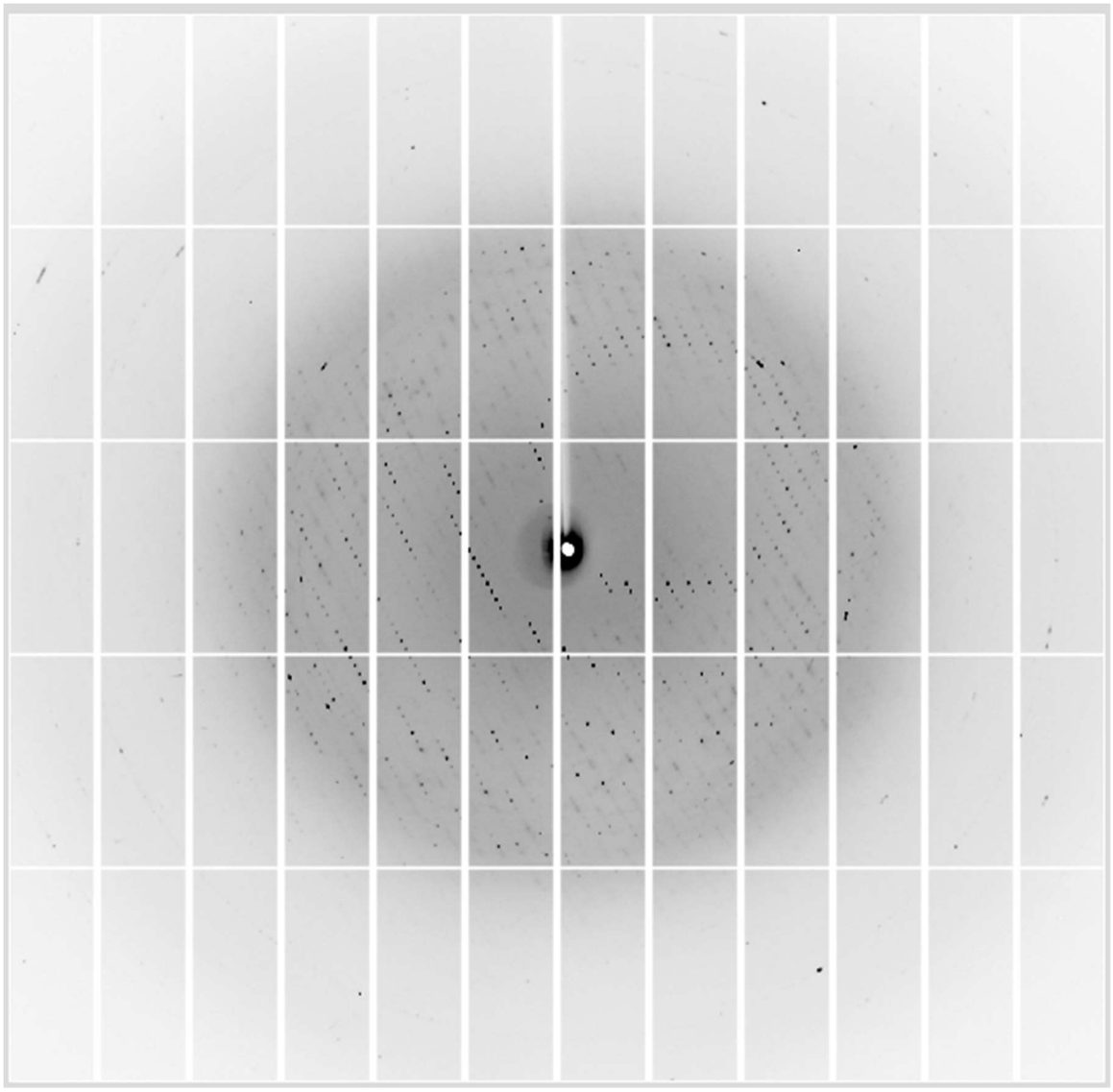

Figure 11:Diffraction Pattern for A4. The diffraction pattern shows well-separated and sharp reflections that extend to approximately 2.2Å.

Chapter 4

CONCLUSION AND FUTURE WORK

Alzheimer's disease is a very significant problem in this country. Aβ is a very large part of this disease and needs to be researched further. Future work for this project includes solving the structure of A4 in hopes of understanding more about the structure of Aβ. This would include the optimization of the crystal growing parameters. Some other future work includes the optimization of the expression of two other anti-amyloid Fab fragments C6T and E1. Once these are expressed in a repeatable manner, one would then be able to set up crystal tray and continue solving the structures of these as well.

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