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The University of Southern Mississippi

Recombinant Expression and Purification of Amyloid-β in *E. coli*

by

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Abstract

Alzheimer's disease (AD) is a common neurodegenerative disorder that affects people older than 65 years [1,2,5]. It is characterized by the presence of extracellular plaque deposits that are seen specifically in the brains of AD patients [4,5]. These plaques are mainly comprised of amyloid-β (Aβ) peptide aggregates. Aβ plaque production and deposition is believed to drive AD pathogenesis. Studying these proteins is crucial to understanding aspects of AD in order to develop possible therapeutic treatments. Recombinant expression of Aβ can also provide a handle to introduce mutations in the protein to further study their structure-function relationships. However, synthetic Aβ monomer protein is expensive, which can become an obstacle when studying Aβ's biophysical and biochemical aspects. Therefore, in order to make it cost-effective, a standardization protocol for the expression and purification of Aβ from *Escherichia coli* (*E. coli*) cells is needed. Although expression of Aβ has been particularly difficult in the past, this project followed a recent report from Walsh et al. to express Aβ42 using inducible pET-Sac-Aβ(M1-42) plasmids. The *E. coli* cells were grown in LB media and collected. The cells were then lysed and Aβ isolated from inclusion bodies through sonication, centrifugation, urea solubilization. The urea-solubilized inclusion bodies were subjected to filtration through anion exchange chromatography using DEAE-cellulose beads. The monomer protein was separated from aggregates through size-exclusion chromatography. The identity monomeric Aβ was confirmed through MALDI-ToF MS. The pure monomer Aβ was then lyophilized and stored in HFIP (hexafluoro-2-propanol) until later use.

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Chapter I – Introduction

1.1. Alzheimer's Disease

The average life expectancy of humans has steadily increased with advancements in health care. Increase in life expectancy has unfortunately increased the risk of patients to Alzheimer disease (AD), which is a progressive neurodegenerative disorder that affects the elderly [1]. Genetics, lifestyle choices, and other environmental factors that progressively affect the brain are believed to be the main sources of AD [2].

AD is characterized as a form of dementia in which memory and cognitive losses occur in patients [3]. It is the most prevalent type of dementia and affects more than 50% of the American population [3]. AD among US citizens is the 6th leading cause of death and kills more people than breast and prostate cancer [1,3]. Although the definitive cause of AD is unknown, the symptoms and signs that are associated with the disease have been extensively studied to determine their origins as well as those who are most at risk. One common element of AD pathogenesis involves the brain, which contains pertinacious plaques made up of mainly amyloid-β (Aβ) protein aggregates [4,5,6].

1.2. Amyloid-β

A crucial factor in distinguishing AD from other types of dementia is the presence of amyloid-β (Aβ) plaque formation in the brain along with intracellular neurofibrillary tangles made of *tau* (**τ**) [6,7,8]. Dr. Alois Alzheimer, after whom the disease is named, was the first to discover these protein clusters. Specifically, the Aβ plaques that begin to form over time are not efficiently cleared from the brain, thus the neurons affected by the hard, insoluble Aβ plaques are not able to release adequate amounts of neurotransmitters and begin to degrade [1,6,8]. The **τ** tangles that are also considered as hallmarks of AD, essentially block the efficient transport of nutrients throughout the neuron [8].

Consequently, research has shifted its focus to the pathway formation of these lesions, specifically the Aβ plaques [1,7]. The Aβ peptide is generated by the sequential protelolytic processing of the amyloid precursor protein (APP) by γ and β secretase, which are endoproteases, resulting in Aβ protein fragments [6,8]. In healthy individuals, these fragments are normally cleared from the brain. Though the intrinsic function of Aβ in the body or brain is not well known, there is a higher concern for its recognizably different deposition in AD patients. The pathway mechanism of plaque formation in the brain is actively researched. There is evidence that these Aβ peptides products from cleaved APP can be toxic to other oligomer forms of the protein and cause them to form plaques, similar to prion activity [6,9]. From ongoing research concerning Aβ plaques in AD patients, one viable treatment has been documented to include drugs that inhibit the β and γ secretases from cleaving APP [6]. It is believed this potentially inhibits the formation of Aβ, despite concerning side effects [6,9]. Other approaches include simply attempting to remove the plaques from the brain [4,10]. Furthermore, it has become increasingly important to study all aspects of AD and these lesions. Therefore, identifying and characterizing the components of Aβ plaques and their mechanism of formation in the brain have become central to research encompassing AD epidemiology.

1.3. Expression of amyloid-β in the pET-Sac-Aβ(M1-42) plasmid

E. coli is an extremely versatile and well understood organism that has been used for decades to express proteins [11,12,13]. It has led to some groundbreaking discoveries in modern science and continues to be widely used in research labs around the world. It is economical and manageable because of its ease of cultivation, quick growth kinetics and transformation rates [12,13]. Additionally, the mechanism of homologous recombination in *E. coli* is very well understood and widely implemented in protein research [14]. This results in high yields of protein for efficient characterizations and further analyses [12].

E. coli strains have been engineered for certain types of protein expression. The specific BL21(DE3) strain of *E. coli* proves to be the best strain for the expression of the Aβ peptide[11,12]. This strain is a descendant of the taxonomic *Bacillus coli* strain, which has the standard name *E. coli B.* This strain is easy to control and contains elements that allow for recombinant protein expression[11].

The pET plasmid has the pMB1 replication origin [12]. The promoter in the plasmid incorporates characteristics that regulate which proteins are produced [7,11]. The plasmid is a replicon, which indicates a single replication origin that is used in the replication mechanism in the cell [11,12]. The replicon allows for the plasmid to replicate autonomously [11,12]. It is also where the copy number can be controlled, which refers to the average number of plasmid copies per cell [11].

The pET plasmid also includes the T7 promoter system, which is extensively used and very popular for expression of recombinant protein [7,11,12]. This system incorporates T7 RNA polymerase that binds to the T7 promoter and transcribes the the gene for the desired protein that is part of the pET plasmid. Transcription of the T7 RNA polymerase gene in the host genome is induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) [12]. T7 lysozyme co-expression, which is provided by the pLysS plasmid, is also important for the proper expression of the gene of interest. T7 lysozymes bind and inhibit T7 RNA polymerase activity so that the gene of interest is not expressed before induction with IPTG. This provides control of when the gene is expressed under optimal conditions[12]. Induction with IPTG causes the T7 RNA polymerase to accumulate and overcome the capacity of T7 lysozyme to degrade too much of it [12]. This catabolic repression or positive control of expression allows the T7 polymerase to transcribe the recombinant gene in a tightly controlled manner - the system does a good job at avoiding basal or default expression in the *E. coli* cell [7,12].

The selection marker is another characteristic that is important for the proper expression of a recombinant gene. Ampicillin resistance is the selection marker of choice for the recombinant expression of Aβ. The *bla* gene of the pET-Sac-Aβ(M1-42) plasmid confers ampicillin resistance [7,11,12]. It encodes β-lactamase, which hydrolyzes the lactam ring of ampicillin, allowing for cells that have successfully incorporated the pET-Sac plasmid with the gene of interest to survive. Two major setbacks have been encountered using this method, however. The first lies in mass production in that the cost may become a problem [12]. Another problem may involve β-lactamase degrading all of the ampicillin and allowing cells without the plasmid to grow [12]. The latter situation can be controlled through expression timing and technique explained in section 1.4.

1.4. Protocol for bacterial expression of recombinant Aβ peptide

Researchers typically rely on synthetic proteins to investigate hypotheses. However, synthesizing a protein takes time, is expensive, and requires specialized equipment. Therefore, researchers who deal with proteins usually opt to form protocols specific to the protein they are analyzing in order to have recombinant protein readily at hand. Recombinantly expressed proteins are convenient for labeling and can be easily manipulated, which is very attractive for researchers who study Aβ. Walsh and his team have standardized a protocol for expression and purification of recombinant Aβ peptides from *E. coli* cells using pET-Sac-Aβ(M1-40) and the pET-Sac-Aβ(M1-42) plasmids [7]. The Aβ(1-40) and Aβ(1-42) coding sequences were inserted into the pET vector to produce the Met-Aβ(1-40) and Met-Aβ(1-42) expression sequences [7]. The expression and purification protocol described provided solutions to some problems posed by synthetic Aβ. The Aβ(1-40) and Aβ(1-42) peptides were produced quickly, inexpensively, and without special equipment. The resulting recombinant Aβ was then purified and confirmed through size exclusion chromatography (SEC) and mass spectrometry, respectively [7].

For expression of Aβ(M1-42) in bacteria, heat shocked *E. coli* strain was used, BL21(DE3) pLysS Star [7]. They were transferred onto ampicillin and chloramphenicol-containing LB (Luria Bertani) plates and left overnight [7]. The cells grew until the optical density at 600 nm (OD_{600}) reached a value of approximately 0.6 at which the culture was induced with IPTG and allowed to grow. Walsh's results showed that incubating the culture after induction with 0.1-0.2 mM IPTG for approximately 3-4 hours. The allotted incubation time produced the optimal concentration of protein in which not too much was expressed that would cause rapid aggregation and not too little was expressed that would not produce a useable quantity. The cells were then centrifuged and frozen for storage.

The team then resuspended the frozen cell pellet in 100 mL of 10 mM Tris/HCl pH 8.0 and 1 mM EDTA (buffer A) on ice, sonicated for 2 minutes, and centrifuged for 10 minutes at 18,000 g. This was done three times. The SDS-PAGE results ultimately indicated that Aβ was still in the inclusion bodies. So, after the third pellet was produced, it was solubilized in 8 M urea for 12 hours, sonicated and centrifuged as previously done [7].

The supernatant that was collected after urea solubilization was subjected to a slough of purification techniques that started with incubation with DEAE-cellulose beads. The beads and protein slurry were agitated slowly for 20 minutes to encourage Aβ binding. The mixture was then vacuum filtered via a Büchner Funnel. Furthermore, the mixture was washed with buffer A followed by a series of NaCl concentration buffers. The SDS-PAGE showed that the elution fractions that were washed with 50-125 mM NaCl contained the highest Aβ purity [7]. Those fractions were centrifuged in a 30 kDa filter that filtered out any aggregated Aβ and larger proteins. The clean samples were then lyophilized and stored.

The purified Aβ samples were analyzed through mass spectrometry and sequencing. Size-exclusion chromatography (SEC) was performed in order to separate the monomers from any aggregates that may have formed [7]. Walsh also performed a Thioflavin T (ThT) binding

assay, using a transmission election microscope, to analyze recombinant Aβ aggregation compared to synthetic Aβ [1,7].

Although useful data was obtained, the protocol is not perfect. The fault lies in its tedious nature. Also, although Walsh was able to produce decent amounts of recombinant Aβ40, he was less successful in producing good amounts of recombinant Aβ42 [7]. Therefore, this project aimed to standardize a protocol for the expression and purification of the recombinant pET-Sec-Aβ(M1-42) plasmid in the *E. coli* strain BL21 pLysS(DE3) [1,7,11]. Furthermore, this protocol can be used to produce monomer Aβ for various uses in the lab.

1.5. Focus of research

Since Aβ is such a significant aspect of AD, it is necessary to have pure forms of the protein. This research aims to standardize a protocol for expression and purification of Aβ in its monomer form. Recombinant Aβ will then be readily available for experiments. The recombinant form of Aβ is convenient for labeling and can be easily manipulated for different experiments. This can provide flexibility in Aβ and ease of sequencing. There are also economical implications to manually expressing and purifying recombinant monomer Aβ rather than buying synthetic forms of it. Once a protocol is standardized, it can lower the cost and time it takes to produce. It can also ensure the purity of the protein, improving the quality of the experiments it is used for. Therefore, this research project focused on expressing Aβ in the pET-Sac plasmid of *E. coli* and purifying Aβ monomer through SEC and verification through MALDI.

Chapter II - Methods and Materials

1.1. Growth of *E. coli* cells containing Aβ42 plasmid

First, an overnight culture was made by adding 80 µL of a 100 µg/µL stock of ampicillin to 80 mL of sterile LB media in a baffled flask. Cells from a glycerol stock of transformed *E. coli* cells were scraped with a pipet tip and added to the sterile LB media in the baffled flask, swirled, and incubated in a shaker-incubator overnight for approximately 20 hours at 37º C. While the overnight culture was growing, two 1L batches of fresh LB media were made in 2L-baffled flasks, autoclaved at the liquid 20 setting, meaning that the fresh LB media was placed under a pressure of 20 atmospheres above sea level pressure, raising the temperature and effectively sterilizing the media. Once cooled, 1 mL of 100 µg/µL of ampicillin was added to each fresh 1L media and swirled to properly distribute the antibiotic. Ampicillin was used to select for the ampicillin resistant cells that contained the pET-Sac plasmid, as previously explained. Half of the overnight culture was transferred to each of the media flasks, which now had ampicillin mixed in, and swirled. The media and overnight mixtures were then incubated at 37º C until the optical density at 600 nm of each reached a value around 0.3 to 0.6 and then induced with 500 µL of 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and grown for approximately 4 hours. These cell cultures were then transferred into culture bottles, balanced, and centrifuge at 10,000 rpm for 20 minutes at 4º C. The supernatant was dispensed and the cell pellets were stored in the same bottles at -20º C for later use in the purification steps.

1.2. Protein extraction

A cell pellet in one of the culture bottles was resuspended in 20 mL of 20 mM Tris buffer with a pH 8.0 and 1mM EDTA. The cell suspension was transferred to a 50-mL conical tube and kept on ice. The cells were sonicated at 80% power (16/20) four times with 90 seconds on and approximately 1.5 minutes off in between each round. The break was extended

whenever frothing occurred. The sonicated cells were transferred into centrifugation tubes, and the tubes balanced and centrifuged at 18,000 x g for 10 minutes at 4º C. The supernatant was immediately poured into a separate conical tube. The pellet, which was composed of mainly inclusion bodies, was resuspended and incubated in 20 mL of ice-cold 8 M urea for approximately 15 minutes at room temperature (~23º C). The inclusion bodies in the urea suspension were periodically triturated with a micro spatula every 5 minutes during the incubation period. The urea-solubilized inclusion bodies were then centrifuged again at 18,000 x g for 10 minutes, as previously done. Then the second supernatant was collected in a 50-mL conical tube and placed back on ice.

1.3. Protein purification

Before filtration could be started, the second supernatant was dialyzed in order to make the urea concentration negligible. This was done by making three 2L buffers that contained 20 mM Tris pH 8.0 buffer and 1 mM EDTA. The second supernatant was poured into a dialysis membrane and placed in the first dialysis buffer for approximately 2 hours and the buffer was changed every 2 hours. The dialyzed protein solution was then diluted with approximately 20 mL of a solution containing 20 mM Tris buffer with a pH of 8.0 and 1mM EDTA.

For anion-exchange chromatography, 2 mL of DEAE (diethyl-amino-ethyl groups) cellulose beads that were gently cleaned and pre-equilibrated with a solution containing 20 mM Tris buffer with a pH 8.0 and 1mM EDTA were added to a 0.2 μm pore size Nalgene filter connected to a vacuum pump along with the dialyzed protein. The mixture was incubated for approximately 45 minutes at room temperature with intermittent agitation to ensure protein-bead interaction. Then, the protein and beads slurry on the filter was filtered through and collected as flow through 1. Approximately 50 mL of buffer A, was added to the filter and let sit for 2 minutes before collecting it in another glass bottle labeled as flow

through 2. This removed any remnants of the denaturing agent. Then, 50 mL of a 25 mM NaCl solution made with 20mM Tris pH 8 buffer and 1mM EDTA was then added to the filter and let sit for another 2 minutes. The pump was turned on to collect any nonspecific proteins in the wash fraction. Then, another 50 mL salt solution containing 750 mM of NaCl was added to elute out Aβ. This was repeated two more times. Finally, a 50 mL strip solution containing 1M of NaCl was added to the Nalgene filter to collect the strip fraction. To gather the used beads, 20% ethanol was added to the filter. The beads were stored at 4º C for future use.

The fraction containing the most protein was then filtered through a 30 kDa molecular weight cutoff Amicon filter by centrifugation at 9,500 x g for 30 minutes. This step allowed for larger proteins to concentrate on the filter and Aβ to be filtered out into the resulting solution.

The filtered solution that contained Aβ was lyophilized, which involved distributing the filtrate into 1.5 mL microfuge tubes and placing them in a lyophilizer. Then, 50 μL of Hexafluoroisoproponol (HFIP) was added to resuspend the lyophilized protein. The resuspended protein was subjected to size exclusion chromatography (SEC) in order to remove any aggregates and collect only monomer Aβ. Finally, matrix-assisted laser desorption/ ionization (MALDI) analysis was performed on the sample in order to confirm the presence of monomer Aβ protein.

Chapter III – Results

1.1. Aβ(M1-42) expression and anion exchange chromatography

Aβ(M1-42) was expressed using the pET-Sac plasmid in the *E.coli* strain BL21(DE3). Protein purification from the crude extract was performed using anion exchange chromatography and fractions were collected at each step of the purification and

characterized using SDS-PAGE and silver stained to check for presence of protein. The Aβ42 protein has a mass of 4645 Da. The band migration proceeded as expected considering the very basic pH of the Aβ peptides. The band that represented Aβ, therefore, was clearly seen between the 3.5 and 10 kDa standard bands on the gels. The elution fractions contained a band in the expected region (Figure 1, Lanes 8, 9, 10). The protein was also seen in the strip fraction (Figure 1, Lane 11), which indicated that a higher salt concentration in the elution fraction was necessary. To confirm that the observed bands were indeed composed of Aβ42, immunoblotting was performed using a novel monoclonal antibody specific to the N-terminus of the protein. The immunoblot image showed that the strip fraction contained the highest yield of Aβ. The second and third elution fractions contained a small amount of protein.

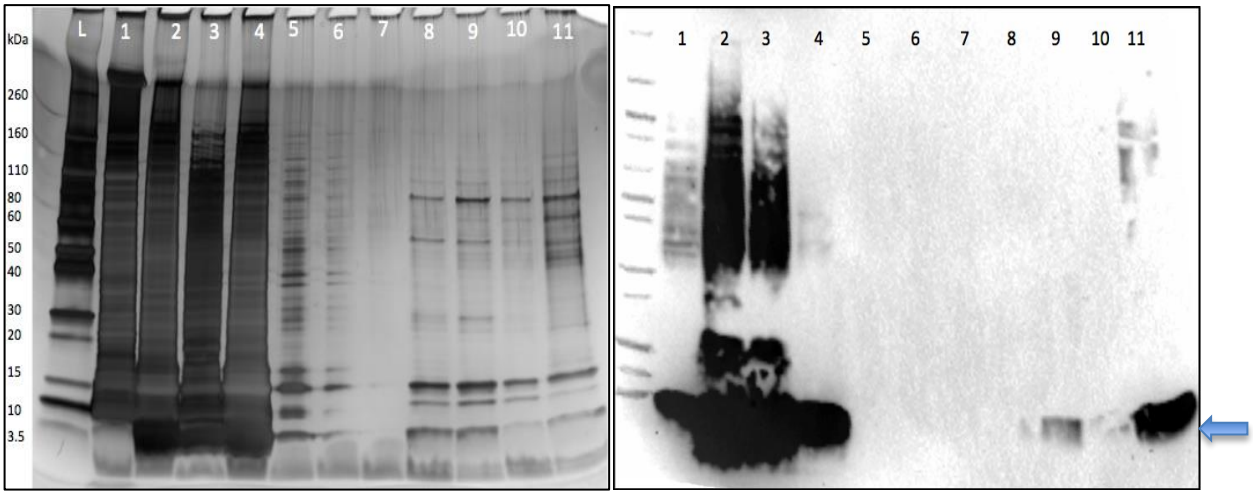


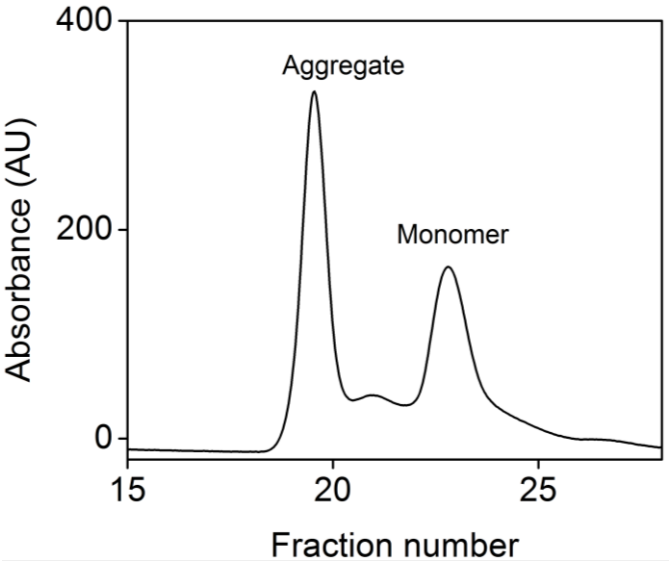
Figure 1: Indication of Aβ was seen at the 4.5 kDa band. The SDS-PAGE images shown include samples of the fractions taken throughout the purification protocol. All fractions were electrophoresed on 4-20% polyacrylamide gel. The fractions presented in the lanes include 1) and 2) supernatant 1 and pellet 1, respectively. Lanes 3) and 4) represent fractions taken after the centrifugation of the urea-solubilized inclusion bodies and labeled supernatant 2 and pellet 2, respectively. Lanes 5) and 6) represent the first and second flow through fractions. Lane 7) represents the wash fraction washing with 25 mM NaCl wash buffer. Lanes 8), 9), and 10) represent the three elution fractions eluted using 750 mM NaCl buffer. Lane 11) represents the strip fraction. The lanes in both images have the same label. The image on the left was visualized by silver staining while the image on the right was visualized through western blotting.

When the cell pellet was sonicated at 80% as mentioned in the methods section, the cell membranes were expected to be lysed and release the cell's contents along with the inclusion bodies that were expected to harbor Aβ. Lanes 1 and 2 of Figure 1 show the contents of the supernatant and pellet of the first round of sonication, confirming that the pellet contained the

inclusion bodies that harbored Aβ. Lanes 2 and 3 of Figure 1 show the contents in supernatant and pellet after the pelleted inclusion bodies were solubilized in urea and centrifuged, confirming that urea indeed solubilized the inclusion bodies and release Aβ. The presence of Aβ in the first pellet (Figure 1, Lane 2) and the second supernatant (Figure 1, Lane 3) were confirmed through a Western Blot analysis. The bands between 3.5 kDa and 10 kDa were indicative of the presence of Aβ, illustrated by the arrows in Figure 1. Furthermore, the silver stain image showed Aβ in all three elution fractions (Figure 1, Lanes 8, 9, and 10) along with the strip fraction (Figure 1, Lane 11).

1.2. Size-Exclusion Chromatography

Subsequent purification of the lane that contained the highest concentration of protein was performed using SEC to obtain monomeric Aβ42. The species eluting near the void volume of the column, fraction 16, was suspected to contain aggregate Aβ, also termed Aβ42 aggregates. This is represented by the peak corresponding to elution fractions 16, 17, and 18 in Figure 2. The protein bands in the fractions collected from the SEC were imaged by silver staining to show that fraction 23 indeed contained the monomeric form of Aβ while fraction 16 through 21 contained Aβ aggregates.



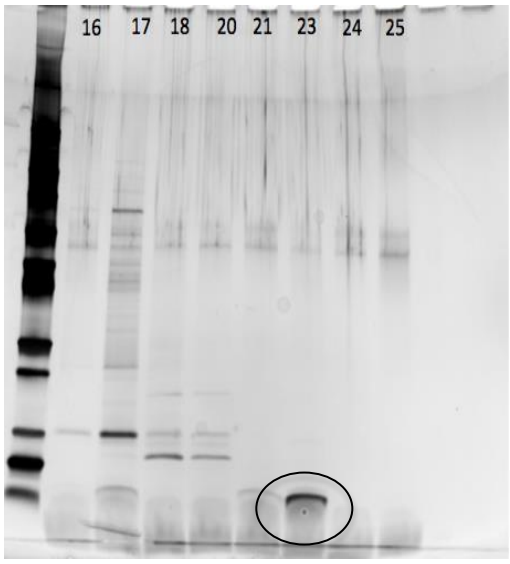


Figure 2: The image on the left shows the SEC profile of the concentrated fraction from lane 11. The x-axis measures the molecular weight (g/mol) of the proteins while the y-axis measures the number of molecules. The image on the right shows the fractions 16, 17, 18, 20, 23, 24, and 25 that were taken from SEC and run on a gel. This image was visualized through silver staining.

The silver stain image indicated that the majority of pure monomer Aβ eluted at fraction 23, confirming the second peak as monomer in the SEC profile.

1.3. MALDI-ToF MS Profile

Confirmation of the observed band being Aβ42 was obtained using MALDI-ToF MS(matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The theoretical mass of Aβ42 is 4645 Da. A peak corresponding to the m/z ratio of 4644.9 was seen on the spectrum indicating the presence of Aβ protein. This peak was indicative of only the monomeric form of Aβ.

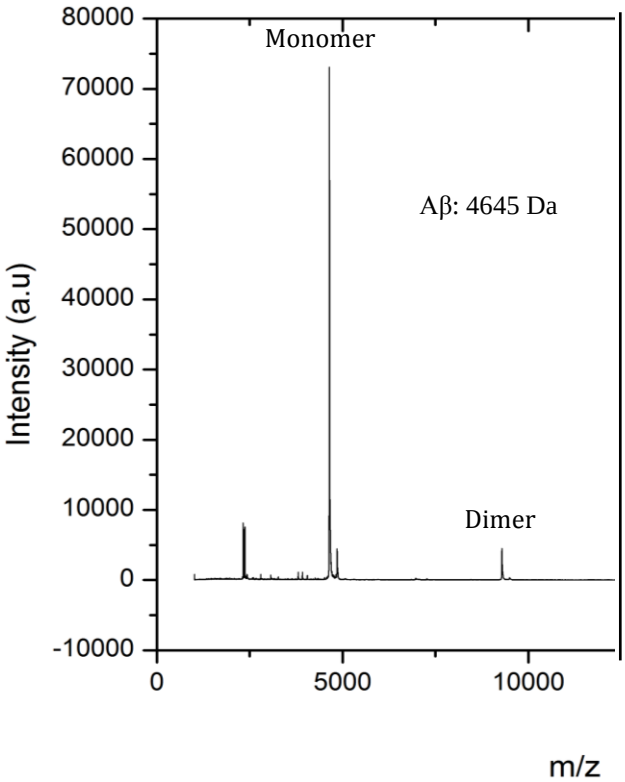


Figure 3: Fraction 23 was subjected to matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-ToF MS) in order to identify the presence of monomer Aβ.

The correct mass of the Aβ monomer species was confirmed through analysis of the MALDI-ToF profile.

Chapter IV – Discussion

The increasing interest in the presence and function of Aβ in the pathogenesis of AD has prompted more properties of this species to be uncovered. The studies that lead to Aβ characterizations and discoveries were conducted using synthetic Aβ, which is expensive to make and purchase. The use of synthetic Aβ may have limited the progress and quality of some studies. Thus, developing a standardized recombinant Aβ expression and purification protocol is economically beneficial in its use in the lab. Moreover, the implementation of the well-studied *E. coli* expression system can provide high yields of monomer Aβ. It is also a useful system in expressing purposefully-generated mutant or altered recombinant Aβ peptides for certain projects. Furthermore, this expression and purification protocol is economical, quick, and does not require specialized equipment.

Purifying recombinant human proteins is known to cause issues concerning the conservation of its integrity in bacterial expression systems. Isolating monomer Aβ can be quite difficult because it is susceptible to aggregation. Aβ is intrinsically disordered and does not have a definitive secondary structure, making it frustratingly difficult to purify. Coincidentally, a few issues were encountered while standardizing this purification protocol. One such issue involved the solubilization of the protein. The protein would precipitate in the dialysis membrane during the dialysis step of the protocol. This was solved by increasing the volume of urea added to solubilize the inclusion bodies in the step before the dialysis. This proved beneficial in that it decreased the amount of protein that would precipitate. Another issue encountered involved the spontaneous aggregation of the Aβ peptides. Aβ is susceptible to rapid aggregation at high concentrations over time [7]. Thus Aβ aggregation ensued when the protein was left longer than a day, despite being temporarily stored at 4º C. Completing the purification protocol swiftly solved this problem. Furthermore, aggregation became a setback after the same Nalgene filter was used multiple times. Aβ aggregates along with any

remnants of the DEAE beads had essentially clogged the filter, rendering it useless. A new filter was required to overcome this obstacle. However, a situation that may become an issue is the cost of the Nalgene filters and affinity beads used for purification. The scaling up of the protocol may become slightly expensive, thus leading to economical delays for low-budget labs.

The data in Figure 1 indicated that the use of urea is crucial in accessing the Aβ peptides enclosed in inclusion bodies. The deposition of the peptide in inclusion bodies is beneficial to the purification process in that it allows for efficient clearing of bacterial proteins and debris after sonication without harming or losing much of the protein. Aβ can be purified after solubilization of the inclusion bodies in urea, simplifying the protocol. Although protein was lost throughout the process, the end result of purification yields approximately 10 mg of Aβ monomer (Figures 2 and 3) and can be increased once the protocol is optimized. The protocol could be improved by implementing sonication after triturating the urea-solubilized inclusion bodies. This would ensure the complete release of all Aβ protein into the solution, further increasing its yield. It would also be beneficial to decrease the incubation time of the protein solution and DEAE beads since most of the Aβ peptides eluted in the strip. The use of the pH 8.0 Tris buffer guarantees that Aβ has a negative charge, ensuring that it has a high affinity to the positively charged DEAE resin beads.

The procedure described for expressing and purifying recombinant Aβ peptides is inexpensive and efficient. It can be completed in an appropriate amount of time without the use of specialized equipment. This protocol can be manipulated to suit the project at hand. Further modifications of the protocol will continue in order to develop an optimized and standardized purification procedure for Aβ monomers.

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