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Granulins as Inflammatory Mediators in Alzheimer Disease

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Granulins as Inflammatory Mediators in Alzheimer Disease

by

Randi G. Reed

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Chemistry and Biochemistry

May 2015

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Abstract

Alzheimer disease (AD) is a neurodegenerative disorder characterized by severe memory deficit and cognitive decline among the elderly. This degeneration is caused by the aggregation and deposition of a protein called amyloid-β (Aβ) in the brain. Aggregation of Aβ causes neuroinflammation in addition to other toxic events. However, it is unclear whether inflammation from an external source, such as from a traumatic brain injury (TBI), could trigger Aβ aggregation. In this context, several pro-inflammatory mediators such as cytokines and chemokines have been suspects. It is now hypothesized that a group of proteins called granulins (Grns) are unique inflammatory mediators that can interact and modulate Aβ aggregation. Grns are a family of seven (A-G) small, cysteine-rich proteins that are proteolytically cleaved from a precursor protein called progranulin (PGrn) during neuroinflammation. Grns have been implicated in both AD and frontotemporal lobar degeneration (FTLD). Among the seven Grns, my work is primarily focused on GrnE. GrnE was recombinantly expressed in *E. coli* and purified using affinity chromatography. The structural characteristics were studied using several biochemical and biophysical techniques, such as sodium dodecyl sulfate (SDS) and native gel electrophoresis, circular dichroism (CD), and fluorescence spectroscopy. The collective data suggest that GrnE is an intrinsically disordered protein (IDP) and is able to dimerize at high concentrations. This is a novel finding because GrnE is not expected to be disordered due to its high degree of intramolecular disulfide bonds. Additionally, Grns C and F have been successfully expressed in *E. coli*.

Keywords: Alzheimer disease, granulin, progranulin, neuroinflammation

Acknowledgements

I would first like to express my gratitude to my adviser Dr. Vijay Rangachari for not only his help and guidance with this project, but also for his invaluable life lessons. Secondly, I give special acknowledgment to my graduate mentor, Gaurav Ghag, for his patience in guiding me through this research and for his endless encouragement. Lastly, I thank all of the people that made my time working on this project a fun and memorable one: Dexter Dean, Christopher Mullins, Tisha Sellers, and Ramya Murali.

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Chapter 1- Introduction

Alzheimer disease (AD) is a debilitating neurodegenerative disorder that leads to dementia and impaired cognitive function and memory. The acute neuronal loss that occurs in AD has been associated with the aggregation of a protein called amyloid-β (Aβ), which leads to the formation of toxic oligomers and fibrils. In addition to triggering neurotoxicity via different cellular mechanisms, the aggregates are also known to induce severe inflammation. Although little ambiguity remains about this mechanism, the reverse effect of inflammatory reactions in triggering Aβ aggregation and concomitant cognitive dysfunction is unclear. There is increasing clinical evidence to support inflammation-induced Aβ aggregation, which comes from the documented cases of traumatic brain injury (TBI). These responses are known to result in AD-type dementia in patients. It is known that about 50% of cases of AD related to frontotemporal dementia (FTD) are familial, or, in other words, genetic. Therefore, the remaining 50% of cases are the sporadic form of AD, which is affected by the environmental conditions of Aβ. Despite clinical and pathological evidence implicating inflammation-triggered neurodegeneration, the underlying molecular events involved in such mechanisms largely remain unclear.

In this regard, pro-inflammatory molecules involved in the cascade, such as chemokines and cytokines, have been the usual suspects, but a unique biomarker for inflammation-induced AD has not yet been identified. In this context, progranulin (PGrn), a protein which is known to play a role in the processes of wound healing and injury repair, is a key suspect mainly due to its involvement in neurodegenerative pathology, such as that seen in FTD. Among patients with AD, PGrn is upregulated in neuronal microglia surrounding Aβ plaques. During an inflammatory event, microglia are activated in the

brain, which in turn release proteases, such as neutrophil elastase, that cleave PGrn into seven smaller pro-inflammatory proteins called granulins (Grns A-G). Based on the known PGrn/Grn involvement in dementia and the pro-inflammatory nature of Grns, it is hypothesized that Grns may have unique interactions with Aβ. Although Grns are implicated in neurodegenerative diseases, their precise molecular interactions with Aβ have not yet been studied in the context of inflammation-triggered aggregation. In this context, GrnA and GrnB are being characterized in our laboratory. Our results indicate interactions with Aβ peptides (unpublished data). For this project, my research focus is on expressing Grns C, E, and F and specifically, characterizing GrnE. This research plans to accomplish the following:

1. Express Grns C, E, and F in *E. coli* and purify.

2. Characterize GrnE and compare those results to GrnB.

Chapter 2- Background

2.1 Alzheimer Disease and Amyloid-β

Alzheimer disease is characterized by memory impairment, loss of cognitive function, and altered behavior that can include paranoia and a loss of social appropriateness (*1*). Many studies via animal models have produced biochemical data that suggest AD is caused by the aggregation of Aβ, which forms toxic oligomers and fibrils (*2*). In familial forms of AD, it has been observed that mutations in amyloid precursor proteins (APP) or presenilins lead to the overexpression of Aβ (*3, 4*). However, the augmentation of Aβ aggregation by an environmental cause, such as a physical head injury, a viral infection, or severe stress, causing sporadic AD is not well-understood. Growing amounts of clinical evidence support that TBIs like these are the cause of inflammatory reactions that can trigger Aβ aggregation and later sporadic AD (*5*).

During inflammation, proteins such as α1-antichymotrypsin, interleukin-6 (IL-6), and C-reactive protein are released in the brain Studies show that plasma samples with increased levels of these inflammatory proteins occur before the onset of clinical AD (*6*). Furthermore, for the past 15 years, studies have shown that prolonged use of non-steroidal anti-inflammatory drugs (NSAIDS) have been fairly successful in preventing neurodegenerative diseases such as AD (*7*). This evidence supports the thought that neuroinflammation may be a cause of AD rather than only a consequence.

2.2 Granulins and Neuroinflammation

As mentioned earlier, Grns are generated from their precursor protein, PGrn. PGrn is found in high levels, along with its messenger RNA, in peripheral blood in patients with AD (*8*). Human PGrn is a 68.5 kDa protein that is rich in cysteine and is made up of seven and a half smaller Grn domains (Grns A-G and a half Grn P) (*Figure 1*) (*9*). These Grns are generated when proteases, such as neutrophil elastase, are released in large quantities by microglia during inflammation and act on PGrn by cleaving it at specific linker regions (*10*). The cleavage sites have been mapped, although the specific proteolytic enzyme(s) that acts on the sites in Grns F, B, and A is unknown. Each Grn is ~6 kDa and contains a motif of 12 cysteine residues consisting of four central pairs flanked by a single pair on each terminus.
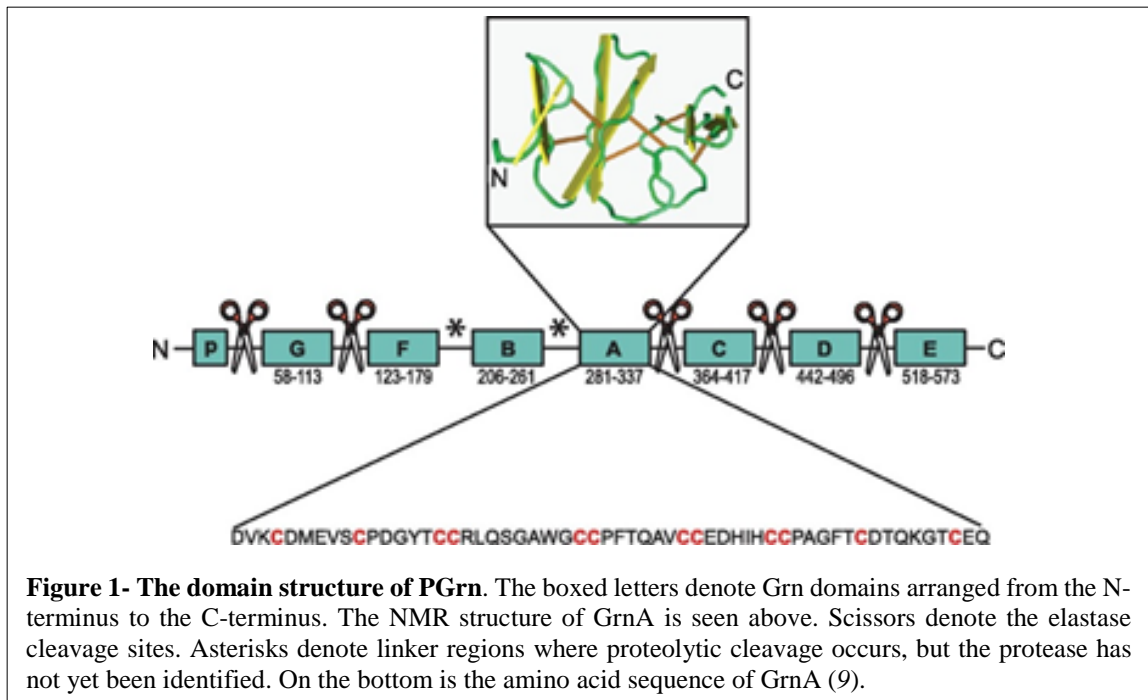

Figure 1- The domain structure of PGrn. The boxed letters denote Grn domains arranged from the N-terminus to the C-terminus. The NMR structure of GrnA is seen above. Scissors denote the elastase cleavage sites. Asterisks denote linker regions where proteolytic cleavage occurs, but the protease has not yet been identified. On the bottom is the amino acid sequence of GrnA (*9*).

The homeostasis between PGrns and Grns is maintained by serine leukocyte protease inhibitor (SLPI), which prevents elastase from cleaving PGrn (*11*). Grns stimulate epithelial cells to secrete interleukin-8, which initiates an immune response. However, PGrns have no such effect, but rather inhibit the spreading, degranulation, and respiratory burst of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) (*10*). These contrasting effects, the anti-inflammatory and pro-inflammatory of PGrns and Grns, respectively, could be responsible for the involvement of microglia in either a deficient or an overactive response to injury and the later development of AD or other types of dementia (*12, 13*).

PGrns are known to play a role in many categories of biological function, including growth-factor-like activities, modulation of immune responses, and neuronal effects (*9*). Notably, PGrn is heavily involved in wound repair, predominantly in epithelial and hematopoietic cells (*14*). As discussed earlier, PGrns/Grns also play an important role in neurodegeneration. The PGrn gene is located on chromosome 17 in humans, and it has been shown that mutations in this gene can result in a familial form of dementia known as FTD and specifically frontotemporal lobar degeneration (FTLD) (*13*).

Since PGrn expression is upregulated in microglial cells that are localized with Aβ (*12*), this overexpression could play a critical role in the response to brain injury, neuroinflammation, and neurodegeneration. Depositions of Aβ plaques trigger inflammatory cascades, which activate the microglia to secrete various pro-inflammatory mediators that may affect Aβ aggregation. Acute inflammation triggered by other factors like a TBI activates the microglia that secrete elastases and other proteases, which in turn cleave PGrn to produce pro-inflammatory Grns (*11*). These Grns could then interact with

extracellular Aβ by either exacerbating or mitigating its aggregation and modulating the onset of AD, depending on the extent of the initial inflammatory response.

2.3 Research Objectives

The purpose of this research is to express Grns C, E, and F and to specifically characterize GrnE. The objectives to accomplish this goal are:
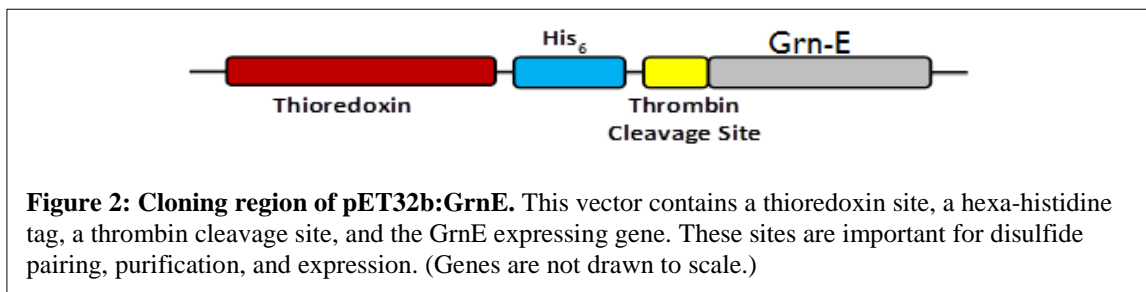
1) Expression of Grns C, E, and F in *E. coli* cells.

2) Purification using affinity chromatography and high performance liquid chromatography (HPLC).

3) Characterization of GrnE using gel electrophoresis and biophysical tools such as fluorescence and circular dichroism spectroscopy.

4) Comparison of the results obtained for GrnE to data of GrnB.

Chapter 3- Methodology

3.1 Expression

The following describes specifically the expression of GrnE. The other Grns are expressed using these same methods.

The gene for GrnE and the other Grns were synthesized commercially (GENEWIZ Inc, NJ). The GrnE gene was inserted into a pET32b plasmid that was readily available in the lab. The cloning region (*Figure 2*) contains a thioredoxin tag which is known to promote disulfide bonds, a hexa-histidine (His_6) tag for Ni^{2+} affinity purification, and a thrombin cleavage site upstream from the GrnE gene to separate GrnE from the rest of the fusion protein after expression. The plasmid also contains an ampicillin resistance gene to selectively grow the cells containing the plasmid.

Figure 2: Cloning region of pET32b:GrnE. This vector contains a thioredoxin site, a hexa-histidine tag, a thrombin cleavage site, and the GrnE expressing gene. These sites are important for disulfide pairing, purification, and expression. (Genes are not drawn to scale.)

To confirm the presence of the correct gene, the plasmid was extracted from the cells using a Zyppy™ Plasmid Miniprep Kit, and then was used in a restriction digestion. The enzymes XhoI and XbaI were used with 10x BSA, 10x NEB4 Buffer, sterile water, and ~300 ng/μL of plasmid DNA, with a total volume of 10 μL. Each sample was run on a 0.8% agarose gel and stained with ethidium bromide. Two distinct bands at 650 base pairs and 4500 base pairs would confirm plasmid insertion.

Upon confirmation, small scale expression was carried out. Cultures of 6 mL were grown in LB broth and cell growth was measured by recording the optical density (OD) at 600 nm on a Cary 50 UV-Vis spectrophotometer (Agilent Technologies). When the OD reached 0.5-0.7 AU (absorbance units), half of each culture was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.9-1.0 mM and allowed to grow for four additional hours, prompting overexpression of the fusion protein, GrnE-trxA. The cells were then spun down and the pellets washed by resuspending in 750 μL sterile water. Both induced and un-induced samples were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). An intense band at ~20 kDa observed in the induced samples would confirm expression of GrnE-trxA. For large scale expression, 1-2 L of sterile LB broth were inoculated with overnight cultures of cells and grown until the cell density was between 0.5-0.7 AU. The cells were then induced using 0.9-1.0 mM IPTG for four hours, after which they were harvested by centrifuging at 14,000xg for ten minutes and were either used immediately or stored at -20 °C until use.

3.2 Purification

The purification of GrnE was first performed similarly to the protocols standardized for Grns A and B in our laboratory. The cells were resuspended in column equilibration buffer (*Table 1*) and had phenylmethylsulfonyl fluoride (PMSF) added to inhibit proteases. The cells were lysed by sonication (6 cycles of 20 seconds with 1 minute intervals on ice) before subjecting to centrifugation at 9,500xg for 15 minutes. The supernatant was then loaded on to the Ni^{2+}-NTA affinity column that was kept pre-equilibrated with loading buffer. The hexa-histidine tag on the fusion protein bound to the nickel beads (HisPur™ Ni-NTA resin, Thermo Scientific), allowing the other components of the supernatant to

flow through. Urea was added to the washes in decreasing amounts to enable unfolding of the protein and facilitate the binding of the His-tag to Ni^{2+}. The washes were also made with buffer containing steadily increasing concentrations of imidazole. Imidazole is a compound that has a higher affinity for nickel than histidine has and hence, the imidazole washes removed non-specific binding proteins before the wash with the highest imidazole concentration (500 mM, Table 1) was finally run through, which allowed the purified protein to elute.

Wash	Imidazole (mM)	Urea (M)	Total volume (mL)
Column Equilibration	10	6	100
Wash 1	80	4	100
Wash 2	100	2	100
Elution buffer	500	0	20

Table 1: Purification buffers with their imidazole and urea concentrations. Each also contained 50 mM Tris-HCl and 300 mM NaCl and had their pH adjusted to 6.5.

The column was finally stripped with 15 mL of a 300 mM NaCl, 20 mM Tris-HCl, 100 mM EDTA solution. This strips anything that might have remained bound to the beads and readies the column for the next purification.

The eluted fraction containing GrnE-trxA was confirmed by polyacrylamide gel electrophoresis (PAGE). The fraction was then reduced with 5 mM tris(2-carboxyethyl)phosphine-HCl (TCEP-HCl), followed by a 10x dilution in a 50 mM Tris-HCl, 300 mM NaCl pH 6.5 buffer in an effort to facilitate only intramolecular disulfide bond formation. Dialysis was then performed using 10kDa MWCO (molecular weight cut off) Spectra/Por® dialysis membranes (Spectrum Labs) in 5 L of 0.2 mM Tris-HCl, 0.5

mM NaCl buffer. Buffer was replaced four times after every two hours to remove any remaining imidazole, TCEP-HCl, or salts. After drying in a vacufuge and resuspending the fusion protein in 2 mL of sterile water, thrombin cleavage was initiated by incubating the protein with thrombin enzyme (1 U thrombin/200 µg protein) overnight in order to cleave GrnE from the thioredoxin fusion. Finally, cleaved GrnE was purified using a LiChroCART C-18 reverse phase high performance liquid chromatography (HPLC) column (Merck, Germany) on an AKTA FPLC system (GE Healthcare, Buckinghamshire). GrnE was fractionated with a gradient of 30-90% acetonitrile containing 0.1% trifluoroacetic acid. The fractionation resulted in the protein separating from thioredoxin in an undigested form along with isoforms of GrnE, eluting in fractions 8 and 9. The fractions were dried in a vacufuge and later resuspended in 100 µL of 20 mM Tris, 0.010% NaN$_3$.

3.3 Biophysical Characterization of GrnE

3.3.1 SDS-PAGE

Fractions from HPLC were run on an 8-16% SDS-PAGE gel. Each fraction had reduced, non-reduced, and non-reduced/non-boiled samples. This was performed to discern any information about GrnE's oligomeric state.

3.3.2 Native PAGE

GrnE samples of concentrations 25 and 50 µM were run on a native gel. Native gels contain no SDS, allowing proteins to run at their native conformation. Running buffer (10X) consisted of 25 mM Tris base and 192 mM glycine. The pH was adjusted to 11.3. Since the predicted pI of GrnE is 8.05, the buffer must have a higher pH so that the protein

has a net negative charge and migrates to the positive electrode during electrophoresis. A 20 μM Aβ42 was run alongside GrnE as a control.

3.3.3 Ellman's Assay

Ellman's assay was performed on GrnE fractions to determine the concentration of free cysteine residues. This was accomplished by adding Ellman's reagent, 5-5'-dithiobis-2-nitrobenzoic acid (DTNB), to a protein where it reacts with free thiols, which cleave the disulfide bond in DTNB. The result is TNB$^-$, which ionizes to TNB^{2-} in water. This product has a yellow color which was quantified using UV spectroscopy. Different concentrations of GrnE fractions were used in these reactions. The volumes used are described in Table 2.

GrnE	6.25 μL protein	1.25 μL DTNB	62.5 μL Tris, pH 6.5
Blank	6.25 μL water	1.25 μL DTNB	62.5 μL Tris, pH 6.5

Table 2: Reactions made for Ellman's assay.

The reactions were incubated at room temperature for 15 minutes before the absorbance was measured at 412 nm. The extinction coefficient of the reagent, 14150 M^{-1}cm^{-1}, was then used to measure the amount of free cysteine residues present.

3.3.4 Circular Dichroism

Circular dichroism (CD) spectroscopy is a technique in which the CD (difference of absorption between left-handed and right-handed circularly polarized light) is measured over a range of wavelengths. The resulting spectrum provides information about a protein's secondary structure based on its minimum absorbance wavelength. CD spectra of fractions at concentrations of 2 μM and 20 μM were obtained using a Jasco J-815 spectrometer

(Jasco Inc, Easton, MD). The samples were monitored in a continuous scan mode from 260 to 198 nm with a scanning speed of 50 nm/min with a data integration time of 8 s, 1 nm bandwidth, and the data pitch of 0.1 nm. Each data set was an average of three scans.

3.3.5 ANS Binding Assay

Anilinonaphthalene-8-sulfonate (ANS) is a compound that non-specifically binds to hydrophobic amino acid residues. When bound, it excites at 380 nm which can be measured using a fluorometer, here a Cary Eclipse spectrometer (Agilent Inc). Scans were obtained by exciting the samples at 380 nm and scanning from 410-600 nm with both excitation and emission slits set at 10 nm. Every data set was an average of three scans. An ANS assay was performed on decreasing amounts of GrnE by titrating the highest concentration with 500 mM ANS and 20 mM Tris, pH 6.5 (keeping these concentrations constant). A negative control was also performed under the same conditions using bovine serum albumin (BSA), a protein that only slightly dimerizes (*15*).

3.3.6 Intrinsic Tryptophan Assay

Each granulin has a tryptophan in its amino acid sequence which can be used as a probe to characterize the protein by fluorescence spectroscopy. Tryptophan fluorescence is sensitive to its environment. As tryptophan gets buried within the hydrophobic pocket of a protein, its fluorescence increases. This change can be monitored as a function of protein concentration, indicating a conformational change. The change in fluorescence intensity was monitored over decreasing amounts of Grn by titrating the protein with 20 mM Tris-HCl buffer, pH 6.5. This was performed on the Cary Eclipse spectrometer with emission spectral scans between 320-400 nm arising from the intrinsic tryptophan
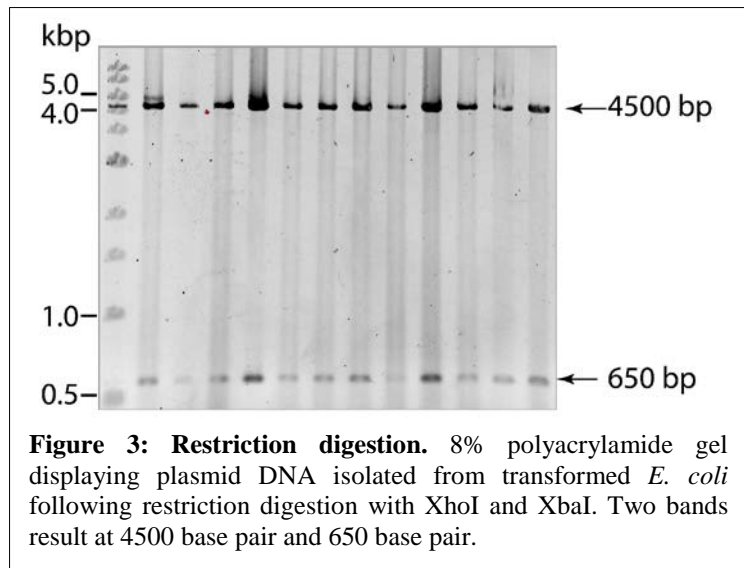
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fluorescence being measured by exciting the samples at 280 nm. The excitation and emission slits were set at 10 or 20 nm. As in the CD spectroscopy and ANS assay, each data set was the average of three scans.

Chapter 4- Results and Discussion
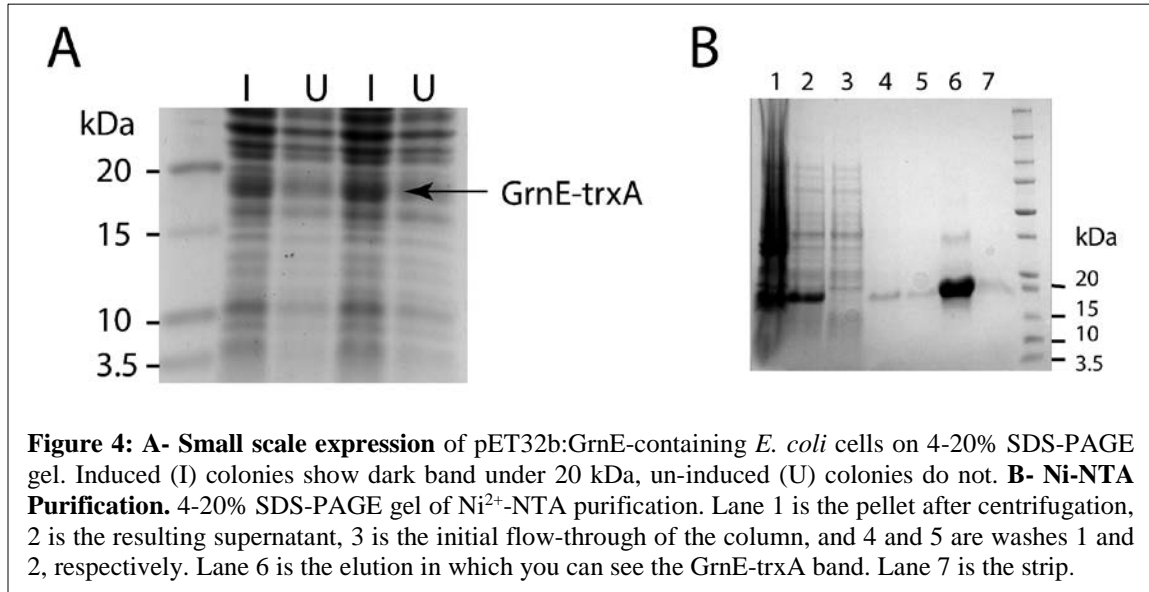
4.1 Recombinant Expression of GrnE

After transforming competent *E. coli* cells with the pET32b:GrnE plasmid, confirmation of the presence of the gene in the extracted plasmids was initiated by a digestion using restriction enzymes. Digestion by the restriction enzymes XhoI and XbaI should result in two bands: one at ~650 base pair and one at ~4500 base pair. These bands were indeed observed when the isolated plasmids were digested with the endonucleases (*Figure 3*).

Once the presence of the insert was confirmed in the plasmid, the encoded protein was expressed on a small

Figure 3: Restriction digestion. 8% polyacrylamide gel displaying plasmid DNA isolated from transformed *E. coli* following restriction digestion with XhoI and XbaI. Two bands result at 4500 base pair and 650 base pair.

scale. Both induced and un-induced culture samples were run on a 4-20% SDS-PAGE gel (*Figure 4A*). As seen in the figure, a band just under 20 kDa is more prominent in the induced than in the un-induced samples. This corresponds to expression of GrnE.

The positively expressing colonies were then used for expression on a large scale as described in the methods. Aliquots of each step of recombinant protein affinity purification (pellet, supernatant, flow-through, wash 1, wash 2, elution, and strip) were electrophoresed on a 4-20% SDS-PAGE gel (*Figure 4B*). An intense band at ~20 kDa in the elution confirmed the purification of the GrnE-trxA fusion protein.

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Figure 4: A- Small scale expression of pET32b:GrnE-containing *E. coli* cells on 4-20% SDS-PAGE gel. Induced (I) colonies show dark band under 20 kDa, un-induced (U) colonies do not. **B- Ni-NTA Purification.** 4-20% SDS-PAGE gel of Ni^{2+}-NTA purification. Lane 1 is the pellet after centrifugation, 2 is the resulting supernatant, 3 is the initial flow-through of the column, and 4 and 5 are washes 1 and 2, respectively. Lane 6 is the elution in which you can see the GrnE-trxA band. Lane 7 is the strip.
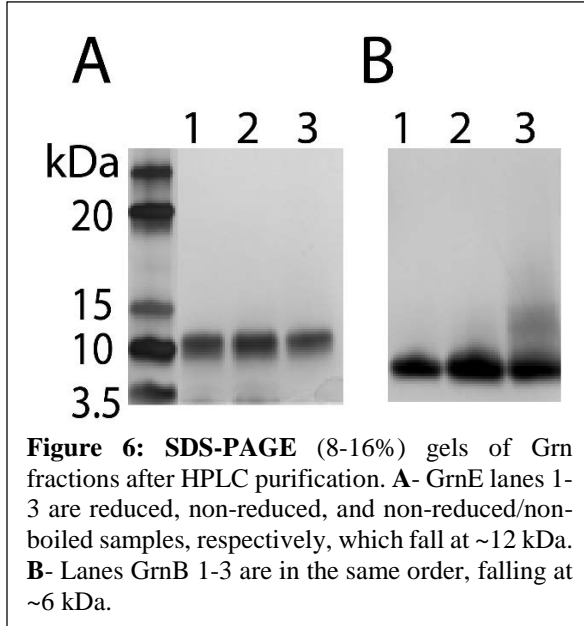
The eluted protein was then reduced, diluted, and dialyzed before being digested with thrombin overnight to later be purified by HPLC. The profiles of both GrnE and GrnB can be seen in Figure 5.

Figure 5: HPLC profiles. GrnE elutes at fractions 8 and 9 while GrnB elutes in fractions 9 and 10, an indication that the biophysical characteristics of these two proteins may be different.
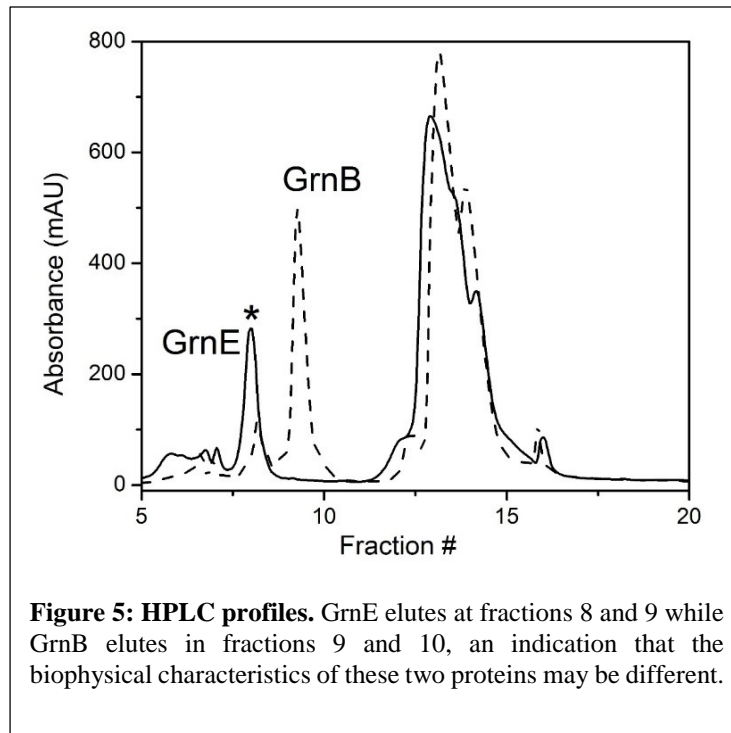
4.2 Biophysical Characterization of GrnE and GrnB

4.2.1 SDS-PAGE

Figure 6: SDS-PAGE (8-16%) gels of Grn fractions after HPLC purification. **A**- GrnE lanes 1-3 are reduced, non-reduced, and non-reduced/non-boiled samples, respectively, which fall at ~12 kDa. **B**- Lanes GrnB 1-3 are in the same order, falling at ~6 kDa.

Reduced, non-reduced, and non-reduced/non-boiled samples of GrnE were run on an 8-16% SDS-PAGE gel as seen in Figure 6A. The bands correspond to ~12 kDa, the expected weight of a dimer. However, in lane 1, the reduced band also falls in this dimer range. Considering that all the disulfide bonds should have been reduced in the denaturing gel, the protein is expected to run as a monomer corresponding to ~6 kDa. This is the first indication that GrnE may have a potential to dimerize.

4.2.2 Native PAGE

In order to unambiguously characterize the dimer formation, GrnE was then run on a native PAGE gel at pH 11.3 (*Figure 7A*). Concentrations of 25 and 50 μM GrnE were run alongside a control of 25 μM Aβ42. The Aβ resulted in three bands, a 4.5 kDa monomer, a 9 kDa
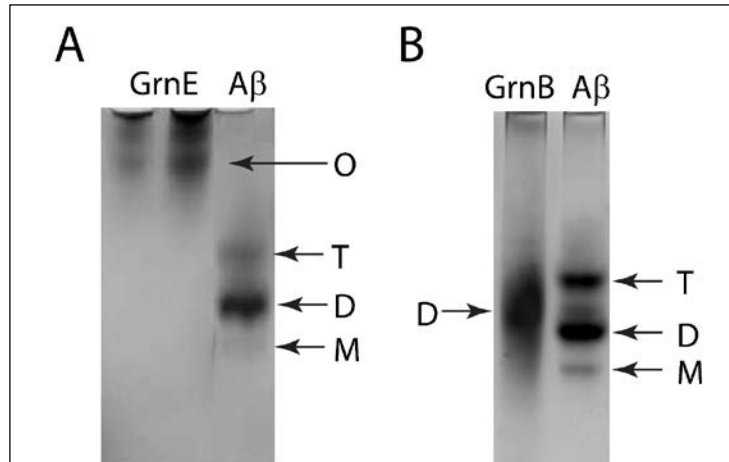

Figure 7: Native PAGE. A- Lanes 1 and 2 are 25 μM and 50 μM GrnE, respectively. Lane 3 is 25 μM Aβ42, resulting in a monomer (M, 4.5 kDa), dimer (D, 9 kDa), and a trimer (T, 13.5 kDa). **B**- Lane 1 is 100 μM GrnB. Lane 2 is 25 μM Aβ42, also resulting in a monomer (M), dimer (D), and a trimer (T).
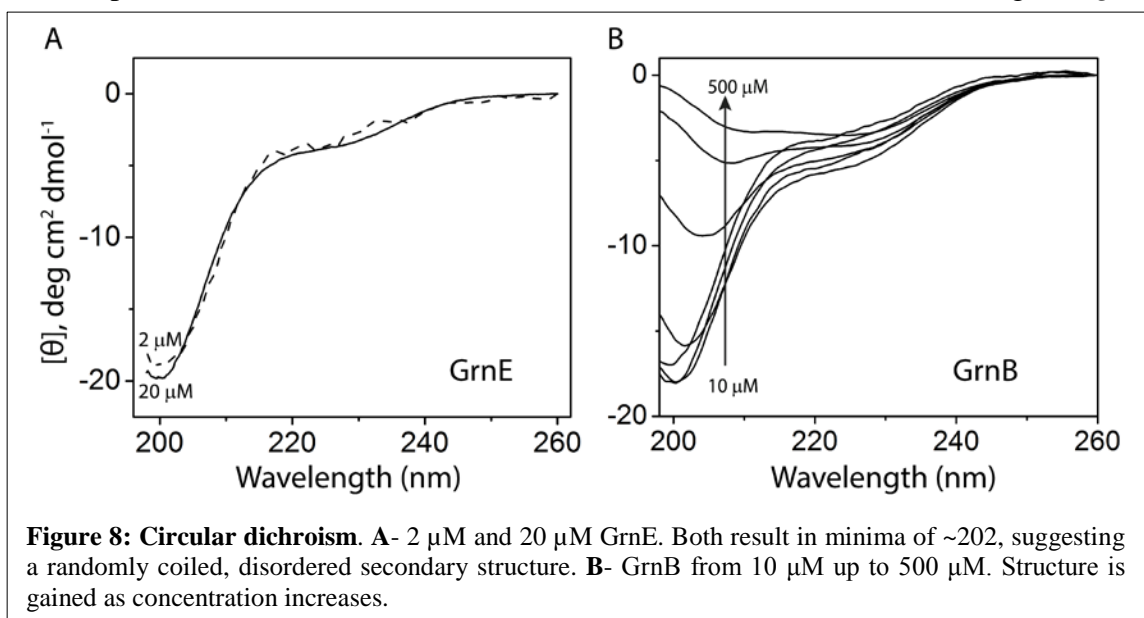
dimer, and a 13.5 kDa trimer. The GrnE bands migrated significantly less than all three of

the Aβ bands, suggesting that GrnE forms oligomers. On the other hand, GrnB (100 µM)

shows a dimeric band approximately corresponding to 11 kDa (*Figure 7B*) when compared

with Aβ, confirming that GrnB indeed forms a dimer.

4.2.3 Ellman's Assay

By calculating the concentration of cysteine residues in the sample of GrnE and

comparing it to the absorbance obtained at 412 nm, the percentage of free cysteine residues

was found to be ~7%. This low percentage suggests that the majority of the protein is in its

physiological form.

4.2.4 Circular Dichroism

Two concentrations of GrnE, 2 and 20 µM, underwent CD spectroscopy. The result

was a random coil with spectra minimum at ~200 nm (*Figure 8A*). This lack in structure

could explain the conundrum seen earlier in the reduced lane of the SDS-PAGE gel (*Figure*

Figure 8: Circular dichroism. **A**- 2 µM and 20 µM GrnE. Both result in minima of ~202, suggesting a randomly coiled, disordered secondary structure. **B**- GrnB from 10 µM up to 500 µM. Structure is gained as concentration increases.
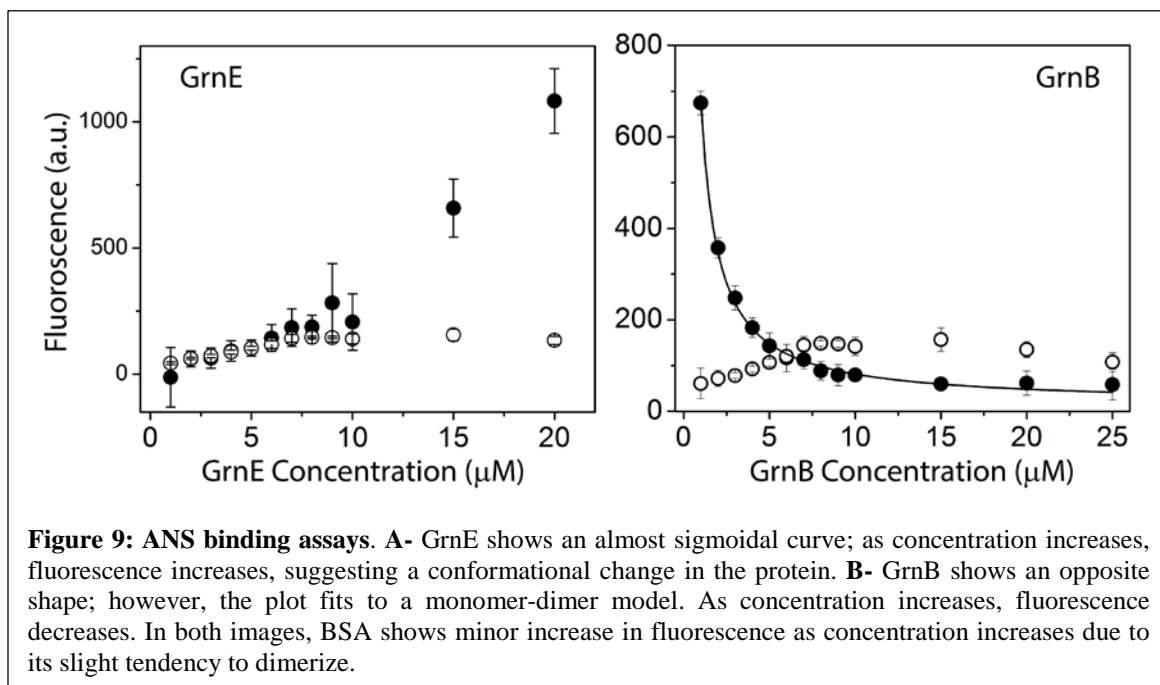
6A). Intrinsically disordered proteins (IDPs) are known to run slightly higher than their true

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molecular weights on gels (*16*) and have little to no structure at low concentrations (*16*, *17*). The CD spectra of GrnE at low concentrations show similar results to those of GrnB (*Figure 8B*), which has been characterized as an IDP (*unpublished data*). GrnB, at low concentrations, is unstructured, but gains structure as concentration increases.

4.2.5 ANS Binding Assay

ANS binding assays were performed on GrnE along with a BSA negative control. As the concentration of GrnE increased, ANS fluorescence also increased (*Figure 9A*). This change was the opposite of the ANS data for GrnB (*Figure 9B*), which can be fitted to a monomer-dimer model. In contrast, although GrnE showed significant concentration-dependent changes, the data could not be fitted to a dimer model perhaps due to the

Figure 9: ANS binding assays. **A-** GrnE shows an almost sigmoidal curve; as concentration increases, fluorescence increases, suggesting a conformational change in the protein. **B-** GrnB shows an opposite shape; however, the plot fits to a monomer-dimer model. As concentration increases, fluorescence decreases. In both images, BSA shows minor increase in fluorescence as concentration increases due to its slight tendency to dimerize.

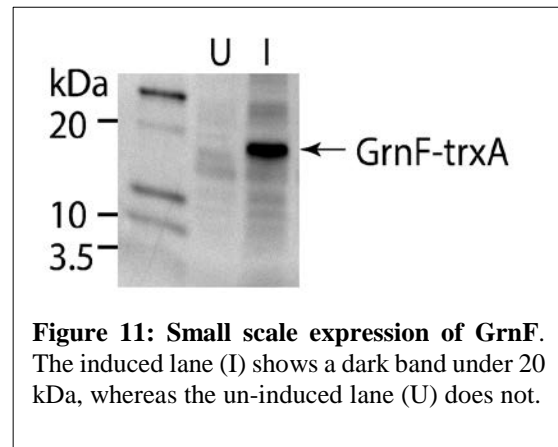formation of oligomers as indicated by the native gel data (*Figure 7A*).

4.2.6 Intrinsic Tryptophan Assay

An intrinsic tryptophan assay was performed on GrnB, but not on GrnE because not enough of the protein could be purified to perform enough trials. In Figure 10, it can be seen that as concentration of GrnB increases, fluorescence increases. This suggests a conformational change as the tryptophan

Figure 10: Intrinsic tryptophan assay. As GrnB increases in concentration, fluorescence increases.

residue becomes more buried in the hydrophobic pocket of the protein. This data is in agreement with the ANS binding assay data.
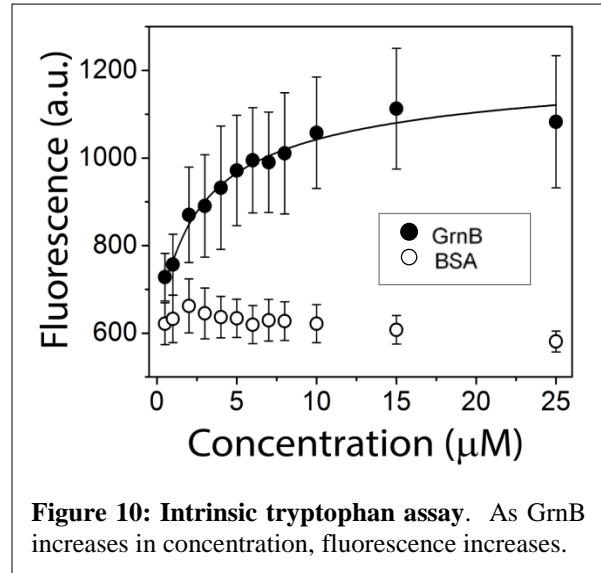
4.3 Recombinant Expression of Other Grns

Cloned genes for Grns C and were transformed via the same methods described, and GrnF has been expressed on a small scale. Figure 11 confirms that GrnF is overexpressing; future plans include scaling up its expression to obtain pure protein for biophysical and biochemical characterization.

Figure 11: Small scale expression of GrnF. The induced lane (I) shows a dark band under 20 kDa, whereas the un-induced lane (U) does not.

Chapter 5- Conclusions

5.1 Grn Conclusions

In the first part of this project, expression and purification of GrnE, was optimized. This took many trials and significant modification of the protocol to accomplish. After expression, GrnE was purified using the protocol already generated for GrnB. However, it was quickly discovered that the two proteins could not be purified using the same method, perhaps due to the differences in their structure. Therefore, the protocol underwent several modifications (over 7 rounds of purification) before GrnE was finally purified in a large enough amount to move forward. GrnE was not found in the elution until the imidazole concentration was increased over twice the amount described in the original GrnB protocol.

Based on the CD data obtained (*Figure 8*), it was clear that GrnE is an IDP because this class of proteins lacks three-dimensional conformation and secondary structure. The SDS-PAGE gel findings also support this notion because in the reduced lane from Figure 6A, the protein was shown at a molecular weight higher than what was expected, which is another characteristic of IDPs. GrnE and GrnB seem to share this characteristic of disorder while having only 40% sequence similarity. What is surprising, however, is the difference in ANS binding data (*Figure 9*). The granulins both showed a transition, but an opposite transition. This indicates that while they both undergo concentration-dependent changes, those changes may be conformationally different.

In line with the second part of this project, the genes for Grns C and F have been transformed successfully and expression of GrnF has been achieved. It is important to note

that all of these Grns (C-G) have different yields and levels at which they express, most likely due to their somewhat low sequence similarity.

The inference that GrnB and GrnE are both IDPs is novel as they have a high degree of disulfide bonds. In fact, to the best of our knowledge, Grns are the only family of IDPs containing six disulfide bonds.

5.2 Future Directions

GrnE needs to undergo more probing for its dimerization characteristics by more ANS binding assays and intrinsic tryptophan fluorescence assays. When characterized, GrnE must then have its effects with Aβ characterized by an array of fluorescence and turbidity assays.

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