OPTIMIZATION OF SDD-AGE AS A METHOD TO STUDY AMYLOID CONVERSION OF HUMAN RECOMBINANT PRION PROTEIN

by

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DEDICATION

TO MY MOTHER AND FATHER

ACKNOWLEDGEMENTS

I would like to thank Dr. Steve Whitten for his guidance in the completion of this work and the training he offered during my time in his lab. I am deeply indebted to him for fostering a passion for quantitative biology in me that I will carry throughout my scientific career. Additionally, I would like to thank my committee members Dr. Walter Rudzinski and Dr. Rachell Booth, for their advice on this thesis project and their continued support during my doctoral studies. I also want to thank my fellow student Reagan Meredith, I would not have been able to complete this work without his help. Finally, I would like to thank my friends and family for their continuous support.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

Many common diseases are caused by amyloid proteins[1]. Amyloid structures are β sheet rich, protease resistant, and can form polydisperse insoluble fibers[2]. Due to these properties, biochemical/biophysical studies of amyloid have been hampered. One technique that is able to study amyloid is Semi Detergent Denaturing-Agarose Gel Electrophoresis (SDD-AGE)[3]. The work presented here shows the optimization of several parameters such as gel thickness, electrophoretic conditions, and capillary transfer method. Through this optimization of SDD-AGE we show that it can be used to 1) study a recombinant human amyloid system and 2) achieve a higher resolution than has been previously reported.

CHAPTER I

Introduction

I.1 Amyloid

The intracellular environment presents proteins with many possible binding partners (ligands), which can include peptides, lipids, nucleic acids, charged ions, and other proteins. The ability to faithfully recognize the correct ligand is critical to a protein's proper cellular function. To recognize ligands a protein needs to adopt the correct 3-dimensional structure, or the "native" state. Many common diseases are the consequence of improper ligand recognition, such as in cancer where due to aberrant signaling caused by a mutated protein, normal cell proliferation is affected. Other diseases that result from a protein binding to an abnormal ligand are the amyloid diseases (Table 1).

Table 1. Human amyloid disease. Table of common human amyloid diseases and the proteins that form the amyloid[1].

Disease	Variant protein	Amyloid
Alzheimer's disease	AβPP, PS1/2	Aβ
Hereditary cerebral hemorrhage with amyloidosis, Dutch type	AβPP	Aβ
Hereditary cerebral hemorrhage with amyloidosis, Icelandic type	Cystatin C	ACys
Familial British Dementia	BriPP	ABri
Familial Danish Dementia	BriPP	ADan
Parkinson's disease	α-synuclein	ASyn
Frontotemporal lobar degeneration (FTLD)-tau	Tau	ATau
Gerstmann-Sträussler-Scheinker	PrP	APrP
Creutzfeldt-Jacob Disease	PrP	APrP
Amyotrophic lateral sclerosis	SOD1	ASOD1
Transthyretin familial amyloidosis	TTR	ATTR
Hereditary lysozyme amyloidosis	Lysozyme	ALys
Hereditary fibrinogen A α-chain amyloidosis	Fibrinogen α-chain	AFib
Hereditary ApoAI/II amyloidoses	Apolipoprotein AI/II	AApoAI/II
Finnish hereditary amyloidosis	Gelsolin	AGel

Amyloid diseases are caused by the conversion of a protein from the native state to an amyloid state[4]. The propagation of amyloid disease is caused by the binding of native protein to one in the amyloid state[5]. The amyloid state of a protein is characterized as being β sheet rich, protease resistance, and able form large insoluble amyloid fibrils[5]. In humans, almost 25 amyloid forming proteins have been identified to date and due to broad tissue distribution and lack of sequence homology it is thought that these polypeptide sequences have intrinsic amyloid forming propensities[1]. Interestingly, the presence of amyloid proteins is not always associated with a disease and can even have a beneficial role in a cell, or organism. For example, in melanin producing cells there is a pH dependent conversion of the amyloid forming fragments of Pmel17 into soluble particles which are able to promote eumelanin formation[6]. Other beneficial uses include

the mounting of cellular immune response to a viral infection. For example, a Sendai virus specific immune response pathway is triggered by the formation of amyloid on the surface of the cytosolic mitochondrial membrane.[7] Another beneficial use of amyloid is found in yeast where it plays a role in the non-DNA based transmission of heritable traits. In yeast, the inheritance of phenotypic traits without changing the DNA is achieved through the sequestration of translation factors such as Sup53 into amyloid structures[4].

I.2 Prion Protein

The prion protein (PrP) is thought to be the causative agent of amyloid diseases such as the transmissible encephalopathies (TSEs), which effect many mammalian species[1-2, 5]. However, it is only after the conversion of the cellular (PrP^{c}) or native state into the amyloid state (PrP^{sc}) that PrP can act as an infection agent[5, 8]. This model of conversion is termed the "protein only hypothesis" which was pioneered by Stanley B. Prusiner, and lead to him being awarded the 1997 Nobel Prize in Physiology or Medicine. In the protein only hypothesis, the conversion of PrP^{c} to PrP^{sc} is achieved solely by a self-replicating mechanism, where one PrP^{sc} acts as a template that converts PrP^{c} molecules[8]. Evidence in support of this hypothesis are that common techniques known to degrade nucleic acids failed to neutralize TSE infectivity, and that PrP^{c} knockout mice are immune to infectivity[5, 8].

The human variant of the prion protein (hPrP) has been shown to be converted into an amyloid state both *in vitro* and *vivo.* Currently our lab is focused on understanding the protein-ligand interactions that drive the amyloid conversion of PrP^{c}. To gain insight into the amyloid conversion of PrP we developed an *in vitro* system where ligands are mixed with recombinantly purified ($rPrP^{c}$) in a conversion buffer and with mild agitation

are able to produce $rPrP^{sc}$. The ligands are synthetic cyclic-peptides, whose sequences are either derived from naturally occurring neuropeptides or have been designed based on computational predictions of functional binding sites on the surface of PrP^{c}. Prior to the work presented here, we had three primary assays at our disposal to validate the presence of $rPrP^{sc}$. These were the Protease K, the Thioflavin-T and the turbidity assays.

I.3 Current Methods of Amyloid Detection

The first assay, the Proteinase K (PK) assay is a classical standard for the detection PrPsc[2]. In this assay PrP^{c} is readily digested by PK, but PrP^{sc} is resistant to digestion and the product is the β sheet rich core of the amyloid particles[2]. PK resistant products are then typically resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting where an amyloid specific antibody recognizes the PK resistance core. PK is able to function in a large variety of buffer conditions including those that contain detergents and chaotropic agents such as urea or guanidinium HCl. This broad compatibility allows it to digest PrP^{sc} samples from both *in vitro* and *vivo* system. A limitation of the PK assay is that PK is a non-specific serine protease, PK can generate a collection of digestion products of difference molecular weights if the digestion reaction experiences experimental variability. For example, in our hands small errors in PK or PrP^{Sc} concentration can lead to the formation of abnormal digestion patterns[2]. These alternative patterns add to the difficulty of interpreting the effect that a novel ligand may have had on the size or rate of formation of the amyloid core.

The second assay available was the Thioflavin-T (ThT) assay. ThT is a small fluorescent molecule that binds to the β sheet rich core of amyloid particles[9]. Upon intercalating into the β sheet rich environment, ThT experiences a red shift in its excitation (385 nm to 450 nm) and emission (445 nm to 482 nm) maxima[9]. A typical ThT assay involves suspending a PrP^{sc} sample into a buffer containing 10 μM ThT solution, prior to performing fluorescence intensity measurements. In our lab we find that a major limitation of the ThT assay is inconsistent intensity measurements, even when repeating a florescence scan with the same sample. This inconsistency could be attributed to the insolubility of amyloid samples. Upon settling in the cuvette, amyloid will not interact with the light beam and therefore, while present in the sample, this fraction of the PrP^{sc} sample is not contributing fluorescent signal.

The final available assay was the turbidity assay. Turbidity, is the result of the scattering of light by large particles in solution. As monomeric PrPc undergoes the conversion to PrPsc it forms polydisperse amyloid particles causing the turbidity of the sample to increase, which is observed by measuring the absorbance at 400 nm. A major drawback of this method however, is that it isn't amyloid specific and detects the formation of amorphous aggregates as well. The tendency of rPrPc to spontaneously form amorphous aggregates is a concern due to the protein's poor stability. The predicted instability index (II) of our rPrPc construct is computed to be 45.39, and an II >40 predicts that the protein may be unstable[10]. The II is a statistical metric of a protein's stability based on the known dipeptide sequences of stable and instable proteins and the measured half-lives of these proteins. The amino acid sequence of a protein can therefore be used predict stability based on the presence of destabilizing dipeptides.

I.4 Semi Detergent Denaturing-Agarose Gel Electrophoresis

Semi Detergent Denaturing-Agarose Gel Electrophoresis (SDD-AGE) is a method of amyloid detection predominantly used for yeast prions. Yeast prions, while named after the PrP share little sequence homology, but have been termed prions due to their ability to self-replicate and form amyloid fibrils[11]. Yeast prions have been studied for their physiological roles and as analogs to gain insight into amyloid conversion[5, 12]. SDD-AGE is typically performed on cell lysate from yeast strains that have had amyloid formation induced. Amyloid samples are then incubated with 2% Sodium Dodecyl Sulfate (SDS), this concentration of detergent is sufficient to dissolve amorphous aggregates and protein complexes. Amyloid particles however, are resistant to denaturation by 2% SDS[3]. SDS resistance is thought to be a result of the β sheet rich core. After incubation, the sample is ran on an agarose gel which due to its large pore size allows separation, but not resolution, of different molecular weight amyloid particles. After separation, western blotting allows for the detection of proteins present in the SDS resistant amyloid particles, which appear as smearing on the blot.

I.5 Project Goals

Upon a review of the literature we realized that there was room for improvement in the currently accepted SDD-AGE experimental design. We believed that through optimization we could achieve better separation and the ability to partially resolve SDS resistant amyloid particles. Our optimization involved modifying the percent agarose in the gel and the type of electrophoretic buffer, as well as changing the voltage, runtime, gel thickness and temperature. We also sought to, for the first time, show that SDD-AGE can be used to detect $hPrP^{sc}$ derived from a recombinant system.

CHAPTER II

Materials and Methods

II.1 Materials

All chemicals and reagents used for this project were Molecular Biology grade or higher. Synthetic peptide, 98% pure, was purchased from GenScript (Piscataway, NJ) and Peptide 2.0 (Chantilly, VA). Precision Plus Protein™ All Blue Standard was obtained from BioRad Laboratories (Hercules, CA.) Water used in sample preparation was filtered and deionized by a Millipore Milli-Q purification unit (Billerica, MA). To ensure sterility, all glassware, pipette tips, and micro-centrifuge tubes were autoclaved in a HICLAVE HV-50 autoclave vessel (Hirayama, Westbury, NY).

II.2 Cloning, Over-Expression, and Purification of Recombinant hPrP

II.2.1 Cloning and Heat Shock Transformation of *E.coli*

A high expression hPrP plasmid was created using DNA2.0's design services (Menlo Park, California). The pJexpress plasmid was selected for the insertion of the hPrP sequence. The pJexpress plasmid contains a Lac inducible T5 promoter upstream of the insertion site. pJexpress also has a high copy origin of replication. pJexpress allows for the positive selection of bacteria containing the plasmid through the expression of beta lactamase, which cleaves the beta lactam moiety of ampicillin. The pJexpress had the consensus hPrP amino acid sequence for residues 23-230 inserted into it, the DNA sequence was codon optimized for bacterial expression. In mammalian cells, residues 1-22 are responsible for PrP's sorting and trafficking, and residues 231-246 are removed during protein maturation[13]. These sorting and maturation sequences were therefore

removed for bacterial expression. Figure 2.1 shows the amino acid sequence used in our studies, and represents the consensus wild type hPrP sequence. Lyophilized pJexpress, with hPrP inserted, DNA was solubilized in DNA grade sterile water to a concentration of 1 ng/µL and stored in sterile cryovial tubes at -80 °C.

1 MANLGCWMLV LFVATWSDLG LCKKRPKPGG WNTGGSRYPG QGSPGGNRYP PQGGGGWGQP

61 HGGGWGQPHG GGWGQPHGGG WGQPHGGGWG QGGGTHSQWN KPSKPKTNMK HMAGAAAAGA

121 VVGGLGGYVL GSAMSRPIIH FGSDYEDRYY RENMHRYPNQ VYYRPMDEYS NQNNFVHDCV

181 NITIKQHTVT TTTKGENFTE TDVKMMERVV EQMCITQYER ESQAYYKRGS SMVLFSSPPV

241 ILLISFLIFL IVG

Figure 2.1. Consensus amino acid sequence of full length hPrP. Residues 1-22 are a signaling motif that are needed for protein trafficking, and residues 231-246 are removed during protein maturation. The sorting and maturation sequences have been removed and the studies presented here have been performed with residues 23-230.

Heat shock transformations of BL21 (DE3) pLysS competent cells by Novagen (Darmstadt, Germany) were performed by adding of plasmid stock to 50 µL of competent cells. The mixture was incubated on ice for 5 minutes, then heat shocked in a heat bath (42°C) for 30 seconds, and then on ice again for 2 minutes. Next, 250 µL of Room Temperature (RT) Super Optimal Broth with Catabolite repression (SOC) was added. At this point, 150 µL of the cell culture was then transferred to Lysogeny Broth (LB) agar plates containing 100 µg/mL of ampicillin and 15 mg/mL agar. The plates were incubated overnight at 37°C.

II.2.2 hPrP Glycerol Cell Stocks For long Term -80°C Storage.

An Erlenmeyer flask containing 200 mL of 100 µg/mL ampicillin and LB was aseptically inoculated with a single colony of *E. coli* cells transformed with the pJexpress hPrP plasmid. The 200 mL cell culture was incubated overnight in a rotary incubator (Max*Q, MIDSCI, St. Louis, MO) at 30°C with orbital rotation. 5 mL of the overnight cell culture was transferred to 200 mL of 100 µg/mL ampicillin and LB and incubated with orbital rotation at 37°C to an Optical Density at 600 nm (OD_{600}) of 0.6. Glycerol stocks were created by mixing 800 µL of cell culture with sterile 80% glycerol to a final concentration of 16%, glycerol stocks were stored in sterile cryovial tubes at -80°C.

II.2.3 Over-Expression of Recombinant hPrP Into Bacterial Inclusion Bodies

To initiate bacterial growth an aseptic dab of *E. coli* from a glycerol stock was spread onto an LB agar plate containing 100 µg/mL of ampicillin. The plate was then incubated at 37°C overnight. The next day a single colony grown on the agar plate was used to inoculate 10 mL of sterile 100 µg/mL ampicillin and LB. The inoculated sample was incubated with orbital rotation at 37°C until visibly turbid (~ 3-4 hours). Then, a hundred fold dilution of the inoculated sample was performed into each of 4 flasks containing 250 mL of sterile 100 µg/mL of ampicillin and LB. The 4 flasks were incubated with orbital rotation at 37°C until an OD_{600} of 0.6 was measured. At this point isopropyl β-D-1thiogalactopyranoside (IPTG) was added to a concentration of 0.5 mM to induce hPrP expression. IPTG is a lactose analog and allows transcription to occur at the T5 promoter, which is lactose inducible. The cell cultures were incubated for an additional 4 hours at 37°C with orbital rotation, cell pellets were formed by

centrifugation at 7,000 RPM, 4°C, for 20 minutes, using a Beckman J2-21 centrifuge with a JA-14 rotor. The supernatant was decanted and cell pellets were stored at -20°C.

II.2.4 Purification of Recombinant hPrP From Bacterial Inclusion Bodies

Cell pellets containing hPrP were thawed and suspended in 20 mL of lysis buffer (10 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, pH 7.5), lysozyme was added to a final concentration of 100 µg/mL. The suspension was then incubated for 30 minutes at 37°C to allow lysozyme to degrade the peptidoglycans of the bacterial cell wall, which hinder cell lysis. The cell suspension was then sonicated using a Bronson Sonifier S-450A (Danbury, CT). The sonication procedure consisted of three, one minute pulses separated by one minute rest periods, all while on ice. The sonifier's power settings were set to a duty cycle of 80% and half-maximum output (control set to 5).

After sonication, TritonX-100 was added to a final concentration of 1% and the sample was centrifuged at 25,000xg, 4°C, for 45 minutes. Under these expression conditions hPrP is localized to insoluble inclusions bodies by *E.coli*. Following centrifugation of the cell lysate, the supernatant was discarded. The insoluble pellet of cellular fragments and protein was dissolved in 10 mL of resuspension buffer (8 M urea, 20 mM Tris-HCl, 100 mM NaCl , pH 8.0) and incubated overnight at 4°C. The following morning, the protein solution was cleared of urea insoluble biomolecules by centrifuging at 10,000xg, 4°C, for 20 minutes.

The supernatant was loaded onto a column containing DE52 media (GE healthcare, New Orleans, LA), DE52 is an anion exchange resin and is used to remove nucleic acids from the sample. The DE52 media was first hydrated with a 20 mM Tris-HCl, 100 mM NaCl solution at pH of 3.5 and degassed for 20 minutes with constant

stirring. The media was then equilibrated with 20 mL of equilibration buffer (8 M urea, 20 mM Tris-HCl, 100 mM NaCl, pH 8.0). The protein sample was loaded onto the DE52 column and washed with 20 mL of equilibration buffer, the flow through fraction was collected.

Next, the flow through fraction was loaded onto a nickel (II)-nitriloacetate (Ni-NTA) agarose column for purification by Immobilized Metal Affinity Chromatography (IMAC). In both the native and urea denatured state, hPrP has a natural affinity for Ni-NTA agarose resin and doesn't require the commonly used 6x-Histidine tag for recombinant protein purification. IMAC purification of hPrP was performed using a BioLogic LP system from BioRad Laboratories (Hercules, CA). A packed Ni-NTA resin column was rinsed with 30 mL of dH2O to wash out the storage solution (20% ethanol). The column was equilibrated with 30 mL equilibration buffer (8 M urea, 20 mM Tris-HCl, 100 mM NaCl, pH 8.0), after which the hPrP protein sample was carefully loaded onto the column. The column was then washed with 30 mL of the following three buffers, wash buffer A (20 mM Tris-HCl, 100 mM NaCl, 8 M urea, pH 8.0), wash buffer B (20 mM Tris-HCl, 100 mM NaCl, pH 8.0), and wash buffer C (20 mM Na2 HPO4, 100 mM NaCl, pH 8.0). After the column wash steps, hPrP was eluted using a combination of low solution pH and imidazole. The elution buffer consisted of 20 mM Na2HPO4, 100 mM NaCl, 500 mM imidazole, pH 4.5.

Sample from the eluted protein peak was consecutively dialyzed against 4 L each of pre-chilled dialysis buffer A (10 mM Na2HPO4, pH 5.8) and dialysis buffer B (5 mM Tris-HCl, pH 8.5) at 4°C for 12 hours (buffer A) and then a minimum of 4 hours (buffer B). The protein was then filtered through a 0.22 micron filter to remove pre-formed

aggregates. hPrP purified in this manner has been previously shown to be 99% pure by silver staining [14]**.** Furthermore, biophysical studies using circular dichroism spectroscopy to determine the secondary structure and thermal unfolding characteristics have shown that hPrP has been properly refolded on the column during this purification[15].

II.3 Seeded Amyloid Samples from Peptide Induced Amyloid Conversions

Synthetic peptides were mixed with freshly purified and filtered recombinant hPrP. The total reaction volume was 100 µL. Each reaction contained 1 mM cyclic CGGKFAKFGGC (cystine-glycine-gylcine-lysine-phenylalanine-alanine-lysine-phenylalanine-glycine-gylcine-cystine) peptide and 4.3 µM hPrP in Quic buffer (138 mM NaCl, 2.7 mM KCl, 0.1 % SDS, 0.1 % TritonX-100, pH 7.0). Samples were incubated in a 37 °C thermomixer set at 1500 rpm with one minute agitation and one minute rest cycles. Samples were removed after 24 hours for the formation of seed stocks or frozen at intervals for time course experiments. To create seed stocks the insoluble portion of amyloid samples was pelleted by centrifugation at 16,000xg, RT, using a Beckman Coulter Benchtop Microfuge (Fullerton, California). After centrifugation, the supernatant was decanted and the fibrils were suspended in 100 µL of molecular biology grade sterile Phosphate Buffered Saline (PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4). Next, the suspended fibrils were gently sonicated using a Bronson Sonifier S-450A (Danbury, CT) to create a homogenous amyloid sample. The sonifier was set to a duty cycle of 20% and an output control of 1. Sonication was performed in three, one minute pulses separated by one minute rest periods, all while on ice. Without this step the polydisperse amyloid in the sample cannot be accurately dispensed due the challenge of aliquoting small volumes (10 µL) of the insoluble sample. The insoluble fraction contains fibrils of different

molecular weights which, when dispensed at a fixed volume prevents the accurate addition of amyloid protein. The inability to precisely add fixed amounts of amyloid greatly affects experimental reproducibility. Now a homogenous amyloid solution, 10 µL of this sample was added to a solution of freshly purified and filtered hPrP to make a 100 µL sample of 4.3 µM hPrP, 1X PBS, 0.1% SDS, 0.1% TritonX-100. The sample of fresh hPrP and amyloid seed was then incubated for up to 72 hours at 37°C in an Eppendorf Thermomixer R (Hauppauge, NY) with one minute pulses of agitation separated by one minute periods of rest. Agitation consisted of rapid shaking of the sample at 1500 RPM. Samples were frozen for up to a month prior to SDD-AGE analysis.

II.4 Semi Denaturing Detergent-Agarose Gel Electrophoresis

SDD-AGE gels were cast in a 10 cm x 15 cm casting tray with an 18 well comb. 1.8 % agarose gels were made by melting 2.16 g of agarose in 120 mL of TAE (10 mM Tris-HCl, 5 mM acetic acid, 5 mM EDTA). The agarose slurry was heated for a total of three minutes in 30 seconds intervals with gentle swirling at each interval. The liquid agarose was cooled for several minutes. Slowly 120 µL of 10 % SDS was added to avoid localized solidifying. Care was taken to avoid the formation of air bubbles during casting and any formed were removed prior to setting the comb. The gel was left to cool for at least an hour at RT. Protein samples were mixed with 4X SDD-AGE loading buffer (2 X TAE, 20 % glycerol, 8 % SDS, 1 % Bromophenol blue) in a 4:1 ratio and incubated at room temperature for 7 minutes, 24 µL of sample was loaded into every other well of the gel. The gel was placed in a Bio-Rad Sub Cell GT (Hercules, CA) with pre-chilled TAE running buffer containing 0.1 % SDS. Electrophoresis was performed for 2 hours at 12 V/cm at 4 °C. Protein was transferred from the gel to a nitrocellulose membrane for

western blot analysis using capillary action. This method as originally described, involved the agarose gel being placed on top of nitrocellulose, with fiber pads directly on top and below the membrane:gel stack[3]. However, we find that this method only works when performed using thick, large area, 20 cm x 24 cm gels which are heavy enough to displace trapped air bubbles at the membrane:gel interface. The protocol as described, suggests making the gel as thick as possible to maximize detection[3]. With our thin, small area, 10 cm x 15 cm gels we found it necessary to apply the nitrocellulose to the gel in order to avoid bubbles. This method allows for proper contact and prevents dead spots on the blots. Additional pads were placed on top of the nitrocellulose in order to assist with capillary action of the transfer buffer. The top of the entire stack had a 1 kg weight to promote both membrane:gel contact and capillary transfer. Transfer trays were filled with Tris Buffer Saline (TBS, 20 mM Tris, 0.1 M NaCl, pH 7.5) and were placed on either side of the transfer stack. Transfer trays contained wicks allowing for transfer buffer to flow and produce capillary action. Capillary transfer proceeded for 12-16 hrs. Following the transfer onto the nitrocellulose membrane, western blot analysis was performed. During optimization some of these conditions were modified and this will be described in the Results sections. All optimization trials were performed at least in duplicate.

II.5 Western Blot Analysis

The nitrocellulose membrane was removed from the transfer stack and incubated for 1 hour with constant shaking in a solution of 5 % dry milk in a Tris-Tween Buffer Saline (T-TBS, 20 mM Tris, 0.1 M NaCl, 0.1 % Tween-20, pH 7.5). Next, the membrane was washed several times with T-TBS and soaked for 1 hr in a solution containing dry milk, T-TBS and primary antibody. The primary antibody used was 3F4 antibody

(Covance, Princeton, NJ) which recognizes residues 109-112 of hPrP as an epitope. The 3F4 stock was diluted to a 1:100,000 working concentration. After 3F4 incubation, the nitrocellulose membrane was then washed several times with T-TBS and placed in a secondary antibody that consisted of anti-mouse IgG conjugated to horseradish peroxidase (GE Healthcare, Piscataway, NJ) diluted to 1:40,000 in T-TBS and dry milk for one hour. The nitrocellulose was then washed multiple times. To image the membrane, a chemiluminsence solution was applied to the nitrocellulose for 5 minutes while incubating in the dark. The ECL chemiluminescence kit was purchased from GE Healthcare. After the incubation period, the membrane was imaged using a FOTO/Analyst FX imager from Fotodyne (Hartland, WI).

CHAPTER III

Results and Discussion

III.1 SDD-AGE Voltage Optimization

The optimization of SDD-AGE of recombinant PrP^{sc} involved changing the electrophoretic conditions. The original protocol calls for a voltage ≤ 3 V/cm of gel length and a continuation of the run until the dye front is 1 cm from the bottom[3]. Under these conditions a SDD-AGE run would take 8 hours during electrophoresis alone. During this time, the sample's signal would be suffering from the effects of diffusion which causes the signal to become weaker and more spread out. However, this low voltage is required to avoid the generation of heat, which also causes the protein signal to become more diffuse. Diffuse signal is due to increased macromolecular diffusion caused by the combination of the introduction of added kinetic energy and the large pores in agarose gels. The large pores in agarose have a poor sieving effect on proteins when compared to acrylamide based gels. The resistance that a thick, large area gel produces greatly limits the voltage that a run can be performed at without the production of a significant amount of heat. With our system using a thin, small area gel we saw the potential to greatly increase the voltage of the run, which would decrease the overall run time. This faster run would have the effect of minimizing the constant effect of macromolecular diffusion by the monomeric protein. Our first attempt at a faster SDD-AGE was performed at 12 V/cm, RT, for 2 hours. The transfer stack was assembled as described by Lindquist[3]. As apparent in Figure 3.1.1, the monomer and amyloid signals greatly overlap using these experimental conditions. It is obvious though, that the time

course of peptide induced amyloid conversion can be observed using SDD-AGE, agreeing with what has previously been observed using the PK, ThT, and turbidity assays.[14-15] Also of note, is the many dead spots created by poor membrane:gel contact.

To further explore the effects of decreasing the run time we moved all future runs to 4 °C to minimize the production of heat cause by increasing the voltage. From this point on we also chose to use our modified transfer stack to diminish the appearance of dead spots on the blot. During the rest of our optimization we chose to use seeded amyloid samples that gave consistent and heavy smearing to simplify the interpretation of experiments. Our molecular weight ladder or Molecular Marker (MM) was used as a metric for how the experimental changes altered resolution. Next, we tried a series of voltage conditions at 4 °C. 12 V/cm for 2 hours (Figure 3.1.2), 24 V/cm for an hour (Figure 3.1.3) and 30 V/cm for 30 minutes (Figure 3.1.4) were attempted. As voltage was increased a significant loss in the MM's signal was observed as well. Also the 24 and 30 V/cm runs generated a great deal of heat even though performed in a refrigerator. Based on these findings 12 V/cm for 2 hours was selected.

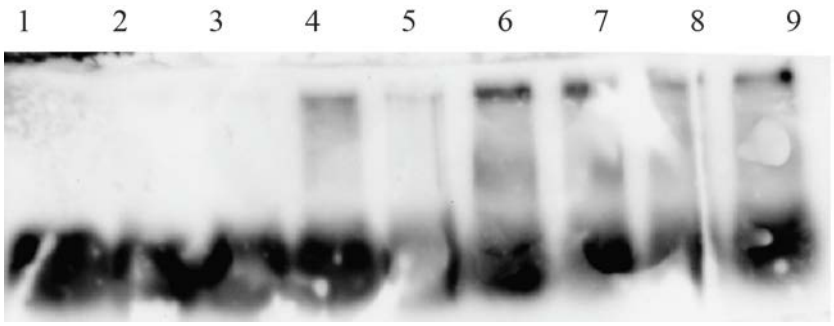

Figure 3.1.1 SDD-AGE of peptide induced amyloid conversion. First attempt at SDD-AGE using Lindquist's transfer stack and 12 V/cm, RT. Lanes: 1 = PrPc, 2 = 0 hours. 3 = 12 hours, 4 = 18 hours, 5 = 24 hours, 6 = 30 hours, 7 = 36 hours, 8 = 42 hours, 9 = 48 hours.

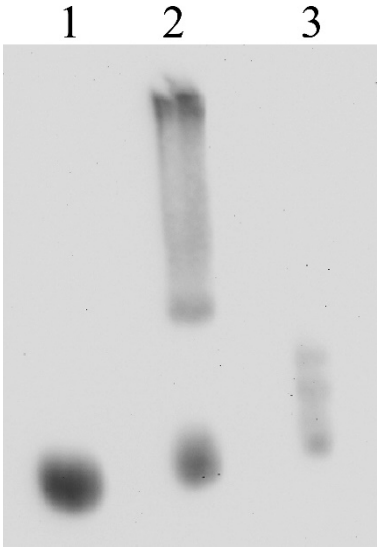

Figure 3.1.2 SDD-AGE performed at 12 V/cm for 2 hours at 4 °C. Voltage optimization of SDD-AGE. Lane 1 = $rPrP^{c}$, Lane 2 = $rPrP^{sc}$, Lane 3 = MM.

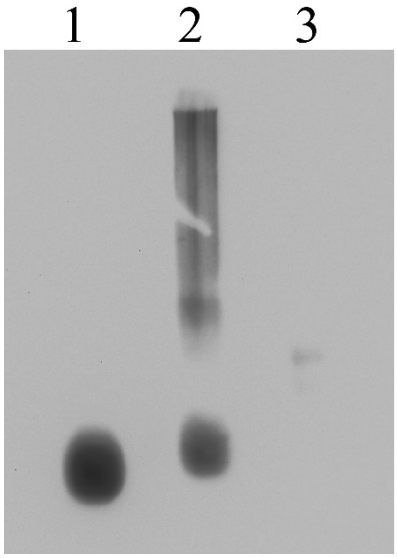

Figure 3.1.3 SDD-AGE performed at 24 V/cm for 1 hours at 4 °C. Voltage optimization of SDD-AGE. Lane 1 = $rPrP^{c}$, Lane 2 = $rPrP^{sc}$, Lane 3 = MM.

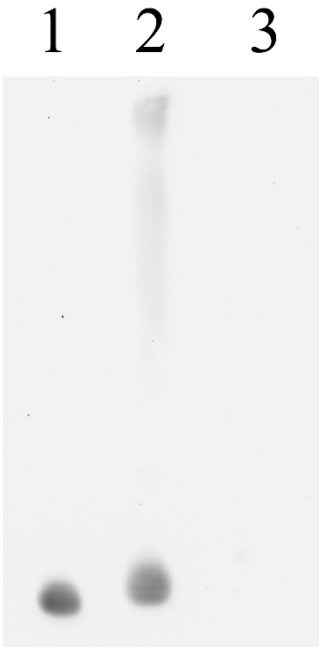

Figure 3.1.4 SDD-AGE performed at 30 V/cm for 30 minutes at 4 °C. Voltage optimization of SDD-AGE. Lane 1 = $rPrP^{c}$, Lane 2 = $rPrP^{sc}$, Lane 3 = MM.

III.2 SDD-AGE Electrophoretic Buffer Optimization

Our next step in optimization involved determining if changing the electrophoretic buffer could improve the resolution of SDD-AGE. We chose to examine the following common electrophoretic buffer systems, Tris-Glycine-SDS (TGS, 25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3) and Tris-Borate-EDTA (TBE, 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3.) Note that 0.1% SDS was added to the TBE, which like TAE does not normally contain SDS. Interestingly, TGS created very tight bands reminiscent of what was seen in SDS-PAGE gels Figure 3.2.1. However, we know that the MM in lane represents a set of proteins with molecular weight ranging from 10-250 kDa. Since the MM was compressed into a single band it is clear that this buffer system has created a loss in resolution. The fact that the amyloid smearing is barely discernable due to the compression effects, further rules out this system. It should be noted that the relative migration of the TGS and the TAE systems at 12 V/cm for 2 hours, 4 °C are very similar (data not shown). TBE on the other hand didn't show compression effects, and migration under the same conditions was only about 30% of TAE's (data not shown). The TBE buffer system showed a greater degree of diffusion then TAE due to this lack of electrophoretic mobility. TBE also failed to produce the smearing effect. Due to these findings we chose to continue to use TAE as our buffer system.

Figure 3.2.1 SDD-AGE performed at 12 V/cm for 2 hours at 4 °C in the TGS buffer system. Electrophoretic buffer optimization of SDD-AGE. Lane 1 = $rPrP^{c}$, Lane 2 = $rPrP^{sc}$, Lane 3 = MM.

Figure 3.2.2 SDD-AGE performed at 12 V/cm for 2 hours at 4 °C in the TBE buffer system. Electrophoretic buffer optimization of SDD-AGE. Lane 1 = $rPrP^{c}$, Lane 2 = $rPrP^{sc}$, Lane 3 = MM.

III.3 SDD-AGE Agarose Concentration Optimization

We next sought to optimize the agarose concentration present in the gels used for SDD-AGE. Pore size can be altered by varying concentration of agarose in a gel. This parameter has two experimental limits, with 1.8% agarose in the middle of them. The lower limit is imposed by the fact that the SDD-AGE gel's structural integrity comes from the agarose itself. Due to this there are limits to how little agarose can be used when making a gel that can withstand the capillary transfer method. A result of this lack of structural integrity is seen as the reemergence of large dead spots that were seen when using Lindquist's transfer stack and are visible in Lane 3 of Figure 3.3.1. The decreased pore size has also caused diffusion to increase and the protein signals overlap laterally. Another interesting phenomena is the lack smearing. With the small pore size it is possible that the amyloid fraction of the sample has been spread so diffusely during both electrophoresis and transfer that it is no longer detectable. The upper limit of agarose concentration is created by the pore size being so small that it impairs electrophoretic mobility and/or the capillary transfer method. Figures 3.3.2 and 3.3.3 clearly illustrate that at the upper limit of agarose concentrations SDD-AGE is rendered useless. Transfer of the pre-stained MM standard onto the nitrocellulose membrane was not evident before the western blotting protocol, and the chemiluminescent signal is not seen for either concentration once complete. Because of the practical limitations imposed by the agarose, the 1.8% agarose SDD-AGE gels remained unchanged from the published method.

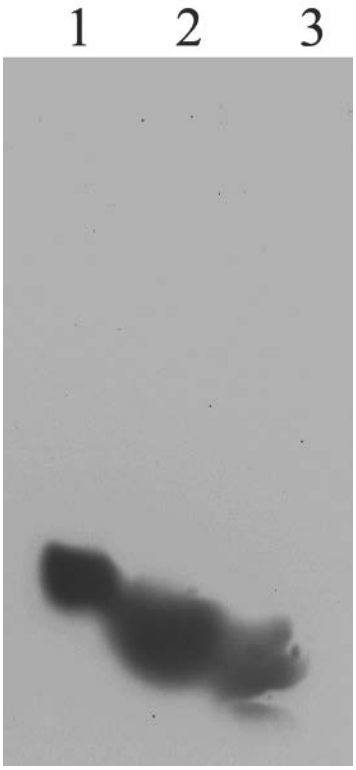

Figure 3.3.1 SDD-AGE performed at 12 V/cm for 2 hours at 4 °C with a 0.8% agarose gel. Electrophoretic buffer optimization of SDD-AGE. Lane 1 = $rPrP^{c}$, Lane 2 = $rPrP^{sc}$, Lane 3 = MM.

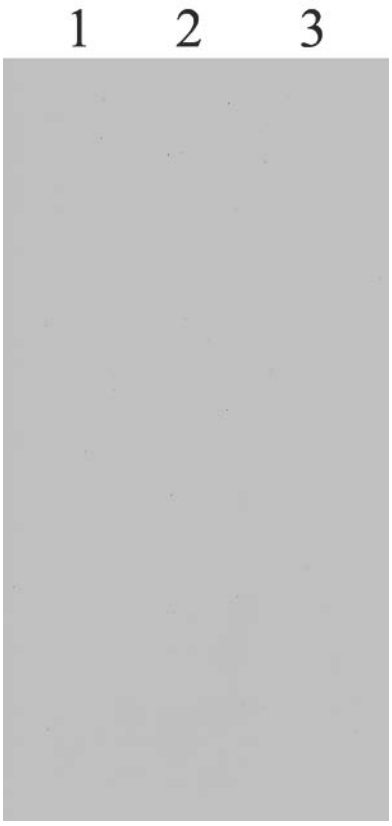

Figure 3.3.2 SDD-AGE performed at 12 V/cm for 2 hours at 4 °C with a 2.8% agarose gel. Electrophoretic buffer optimization of SDD-AGE. Lane 1 = $rPrP^{c}$, Lane 2 = $rPrP^{sc}$, Lane 3 = MM.

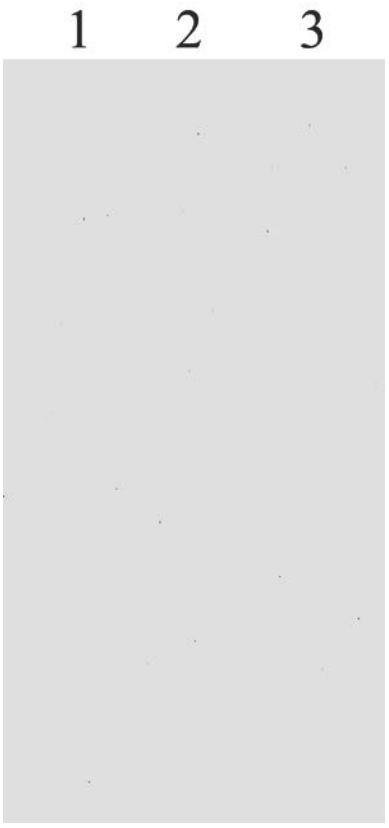

Figure 3.3.3 SDD-AGE performed at 12 V/cm for 2 hours at 4 °C with a 3.8% agarose gel. Electrophoretic buffer optimization of SDD-AGE. Lane 1 = $rPrP^{c}$, Lane 2 = $rPrP^{sc}$, Lane 3 = MM.

III.4 SDD-AGE Buffer Incubation Time Optimization

The motivation of the work presented here has been to validate the use of SDD-AGE on a peptide induced amyloid system using recombinant hPrP. Therefore, we wanted to verify that the amount of time that the amyloid sample incubated in SDD-AGE buffer was sufficient to generate a strong amyloid signal. Lindquist's method, which is designed to screen for yeast prions calls for a 5 minute incubation. We tested whether this

was an appropriate incubation time for PrPsc samples in our system. Figure 3.4.1 shows a series of time incubation time points from zero to 7 minutes. It is clear that a 7 minute incubation time is needed to observe the expected smearing band.

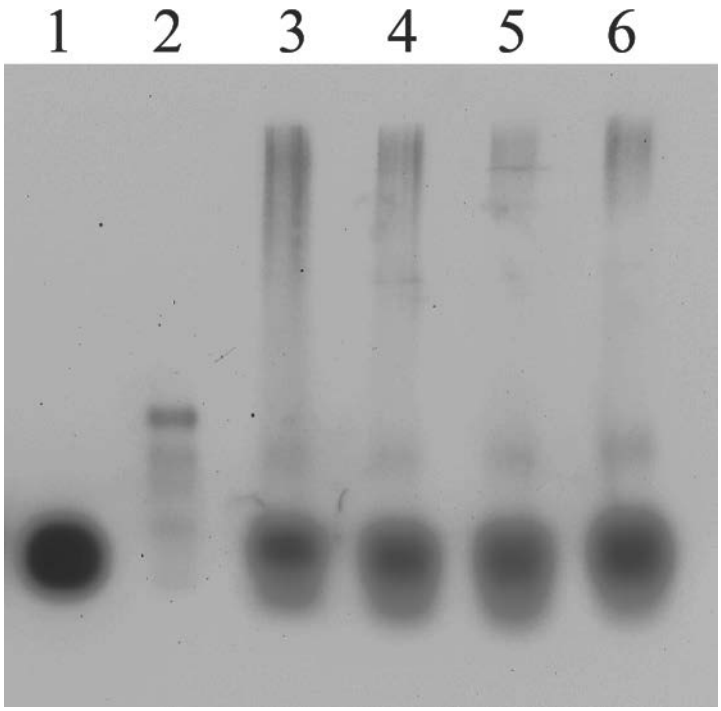

Figure 3.4.1 SDD-AGE performed at 12 V/cm for 2 hours at 4 °C, SDD-AGE buffer incubation time optimization. Electrophoretic buffer optimization of SDD-AGE. Lane 1 = $rPrP^{c}$, Lane 2 = MM, Lane 3 = $rPrP^{sc}$ with 7 minute incubation, Lane 4 = $rPrP^{sc}$ with 5 minute incubation, Lane 5 = $rPrP^{sc}$ with 2 minute incubation, Lane 6 = $rPrP^{sc}$ with no incubation.

III.5 SDD-AGE Gel Thickness Optimization

The final parameter to be varied was the thickness of the gel. We only examined thinner gels as this would minimize the diffusion that occurs during the overnight transfer. Gel volume was decreased in 10 mL increments and then cast, the well volume capacity using an 18- well comb was then measured. The gel thickness of these gels was estimated as shown in Table 2. At 60 mL the well volume is approximately 30 μL, which is large enough to allow the pipetting of 24 μL volumes of sample into a well without spill over. A 60 mL, 1.8% agarose gel was cast and run for 2 hours at 12 V/cm in the TAE buffer system. The SDD-AGE in figure 3.4.1 shows the time course of peptide induced amyloid conversion in a thin gel under the described conditions. Striking improvement is observed from what is seen in Figure 3.1.1. Note the clear separation of the monomer and amyloid signal in wells 3-8. Also greatly improved is the resolution of the individual proteins in the MM, Lane 9. We have determined that a 0.4 cm thick gel gives us the best resolution.

III.6 Conclusions

The conclusions of our SDD-AGE optimization are that the TAE buffer system and 1.8% agarose gel conditions are ideal for SDD-AGE, and should remain unchanged. Variation of these two conditions greatly diminished the resolution of SDD-AGE. Through our voltage optimization procedure we discovered that 12 V/cm offered us the best tradeoff between decreased run time and a loss of resolution due to heat generated during the run. This represents a 4 fold decrease in run time from what published protocols call for. We also found that performing the electrophoresis at 4 °C, helps to minimize lost resolution due to the heat generated by the higher voltage. We have tested

the pre-electrophoresis incubation time with the 2% SDS SDD-AGE buffer. We discovered that unlike the Lindquist method, we require a 7 minute incubation to achieve a strong amyloid signal. We were able to further improve the resolution of SDD-AGE by decreasing agarose gel thickness, from 0.8 cm to 0.4 cm. Overall we found that with a higher voltage (12 V/cm) run at 4 °C in a thin gel (0.4 cm), gives us the best SSD-AGE resolution. We also found that a 7 minute pre-electrophoresis incubation with SDD-AGE buffer maximizes the amyloid signal.

With these improvements we believe that SDD-AGE will be a powerful tool in our efforts to understand amyloid conversions. Using this improved method has already yielded new mechanistic insights into the amyloid conversion of rPrP. The peptide induced conversion seems to involve an 8-mer intermediate, with a molecular weight of ~200 kDa. In the future this improved SDD-AGE protocol will be used to probe the thermal and chemical stability of amyloid, as well as the molecular weight distribution of PK digestion products.

Table 2. Agarose gel thickness estimates. Gel thickness was estimated for gels cast in a 10 X 15 cm casting tray.* Does not account for volume displacement of 18 well comb.

Gel Volume (mL)	Gel Thickness* (cm)
120	0.8
110	0.733333
100	0.666667
90	0.6
80	0.533333
70	0.466667
60	0.4

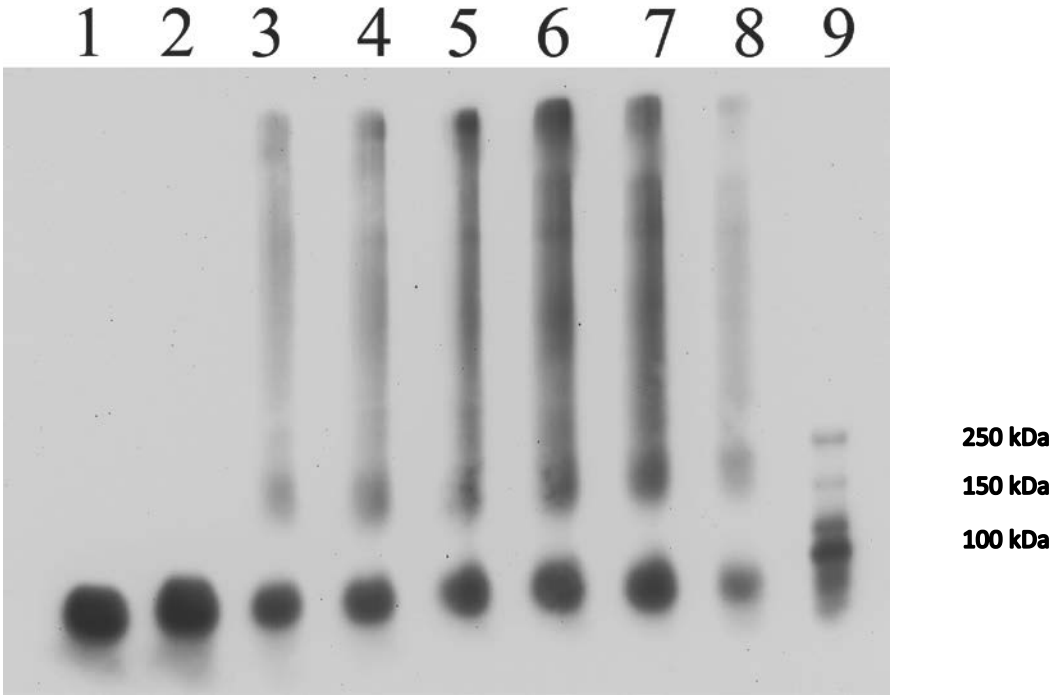

Figure 3.5.1 SDD-AGE performed on a peptide induced amyloid conversion at 12 V/cm for 2 hours at 4 °C in with a 0.4 cm gel. Electrophoretic buffer optimization of SDD-AGE. Lane 1 = $rPrP^{c}$, Lane 2 = 0 hours, Lane 3 = 4 hours, Lane 4 = 12 hours, Lane 5 = 24 hours, Lane 6 = 36 hours, Lane 7 = 48 hours, Lane 8 = 72 hours, Lane 9 = MM

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