TESTING THE ABILITY OF RF-AMIDE NEUROPEPTIDES TO PROMOTE

AMYLOIDOSIS OF RECOMBINANT HUMAN PRION PROTEIN

by

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ABSTRACT

Protein folding is a process in which polypeptides assume their correct conformational tertiary structure from a random coil to produce a functional protein. When the protein does not fold correctly, complications can occur that can cause major problems. This process is known as protein misfolding and is observed in amyloid related diseases. Progression of protein misfolding often involves the structural conversion of normal cellular protein into amyloid oligomers rich in β-sheet structure. These oligomers form plaques of insoluble fibrils that accumulate in tissues and organs, causing cytotoxicity and are detrimental to neuronal cells.

The conformational changes in protein structure that are associated with protein misfolding can occur through binding interactions with other molecules. Previously, it was shown that a small peptide ligand containing the sequence lysine-phenylalanine-alanine-lysine-phenylalanine (KFAKF) could promote amyloid conversion of recombinant purified human prion protein (hPrP) under physiological-like conditions. Of note, the sequence of this small peptide was homologous with a class of mammalian neuropeptides that are called RF-amide neuropeptides. These endocrine signaling peptides have the consensus sequence of arginine-phenylalanine-methionine-arginine-phenylalanine (RFMRF) and end with a carboxy-terminus capped with an amide group. To investigate that observation, we have tested the ability of synthetic peptides with sequences identical to naturally occurring RF-amide neuropeptides to promote amyloid

conversion of natively-folded recombinant hPrP to amyloid particles under physiological conditions. Results presented here demonstrated that synthetic RF-amide homologs were able to convert native recombinant hPrP to amyloid particles, suggesting that RF-amide neuropeptides may play a role in diseases that are associated with amyloid misfolding of the prion protein.

CHAPTER I

INTRODUCTION

The conversion of cellular protein into insoluble amyloid fibrils is an identifying

factor associated with several mammalian diseases. The mechanism of the disease is

thought to progress through the misfolding of specific proteins to β-rich amyloid fibrils.

Structurally, amyloid fibrils are filamentous nanostructures that resist protease digestion

and cellular clearance[1-3]. Amyloid diseases are often characterized by the accumulation

of plaques of amyloid material in affected tissue and organs, which are thought to cause

cytotoxic effects[1]. The severity of cytotoxicity is of growing concern in

neurodegenerative amyloid related diseases where plaque formation leads to synaptic

dysfunction and neuronal cell death. Symptoms of dementia and decreased psychomotor

skills accompany neurodegenerative diseases and progressively lead to the death of the

host[4]. Most common forms of amyloid diseases include Alzheimer's, type II diabetes and

Creutzfeldt-Jakob disease (CJD)[6]. This is a concern for aging demographics in

industrialized regions where cases are reported in higher frequencies[16]. For this reason,

studying compounds that interact with amyloid related proteins can lead to better

methods of blocking the progression of the amyloid formation. Previously, the Whitten

lab developed a recombinant system for studying amyloid-related structural changes by

identifying small peptide ligands that interact with the human prion protein and promote

its conversion to amyloid particles. This recombinant system provides a strategy to

initiate the structural conversion of the protein for direct study under defined solution

conditions, identify molecular interactions that promote amyloidosis, and test potential physiological cofactors of prion diseases.

Using this system, a peptide was tested that included the sequence arginine-phenylalanine-methionine-arginine-phenylalanine (RFMRF) for amyloid-converting activity against recombinant human prion protein (hPrP)[16]. This peptide was tested because it was observed that peptides containing the sequence lysine-phenylalanine-alanine-lysine-phenylalanine (KFAKF) could convert natively folded recombinant human prion protein to amyloid particles. The KFAKF sequence is homologous to the consensus sequence of a class of mammalian neuropeptides that play a role in neuroendocrine function. These peptides are called RF-amide neuropeptides owing to a C-terminal consensus sequence of RFMRF-amide, or slight variations of that motif, and are found in several mammalian species, including humans[28-30]. The goal of this thesis project is to test a set of known RF-amide neuropeptides for their ability to convert natively folded hPrP to amyloid fibrils under physiological-like conditions.

1.1 Amyloid disorders

The etiology of amyloid disorders has been established as protein misfolding diseases (PMDs), whereby protein aggregates change the biological function of cells and affect tissue or organ function. The symptoms and outcomes of the disease are dependent on particular proteins found in the fibrilar deposits. Some of the most common PMDs include Alzheimer's, Parkinson's, Huntington disease, and diabetes mellitus type II. While these amyloid disorders tend to be linked to mutations in genes coding or sporadic onset, some forms of PMD are transmissible and include TSE, Kuru and Creutzfeldt-

Jakob disease (CJD)[6]. Transmissible and neurodegenerative forms of amyloidosis are of grave concern due to the accumulation of insoluble deposits in the brain that lead to neuronal cell death, brain degeneration and inflammation[7]. For a list of proteins associated with their related amyloid disorders, please refer to Table 1.1. This project has investigated only the human prion protein.

Table 1.1 Clinical Pathologies of Amyloid Proteins[23].

Amyloid Protein	Clinical Syndrome
Beta Amyloid (1-40, 1-42)	Alzheimer's Disease
	Inclusion Body myositis
Amylin or Islet Amyloid protein (IAPP)	Type II Diabetes
β 2-Microglobulin	Dialysis-Related Amyloidosis
α-Synuclein	Parkinson's Disease
Prion Protein	Transmissible Spongiform Encephalopathies
	Fatal Familial Insomnia (FFI)
	Gerstmann-Straussler-Scheinker (GSS)
Tau	Front-Temporal Dementias
Huntingtin (Poly Q Expansion)	Huntington Disease
Superoxide Dismutase	Amyotrophic Lateral Sclerosis
Transthyretin	Senile Systemic Amyloidosis
	Familial Amyloidotic Polyneuropathy
γ-Crystallin	Cataract
Lysozyme	Lysozyme Systemic Amyloidosis
Ig Light Chains	Primary Systemic Amyloidosis
Serum Amyloid A	Secondary Systemic Amyloidosis

1.2 Structure of Amyloid

Beta (β) sheet structures are a secondary structure in proteins and are not as common as alpha (α) helical structures, yet this structure is heavily involved in protein misfolding. The β-sheet structure contains an anti-parallel polypeptide conformation that is stabilized by hydrogen bonds in the backbone of peptides in amyloid fibrils[33]. This often causes a twist in the structure of the protein backbone. Amyloid fibrils are organized into β-sheets arranged in polymeric stacks known as cross-β structures, which are stabilized by intermolecular interactions allowing for larger polymers to form. Growth of the fibrils leads to the formation of insoluble protein aggregates[11]. These fibrils are often rigid oligomers and range in length from 100 to 1600 nm. Additionally, fibrils are often straight and unbranched structures that have a high affinity for Congo red and thioflavin dyes[34]. Thioflavin is a fluorescent dye that binds to oligomers and amyloid structures rich in β-sheet content. Congo red shows birefringent properties when bound to fibrils which can be measured using spectrophotometric techniques. Both dyes bind to amyloid structures and undergo spectral changes that are not detected in the presence of monomers or amorphous aggregates of the protein[34]. Arrangement into β-sheet structures allows for the most favorable organization for aggregates and produces oligomers ranging from dimers to large fibrilar structures[9,10]. The structural similarities of misfolded proteins associated with the various amyloid diseases suggest that there may be a common or shared misfolding mechanism that initiates the disease condition.

In respect to amyloid formation, protein misfolding seems to be initiated by several means. Gene mutations affect the thermodynamic stability of a protein by amino acid substitutions, promoting unfolding and population of aggregation-prone states[1-3].

Errors in transcription or translation can lead to incorrectly folded structures as well as mistakes by post-translational modifications and the chaperone machinery[2]. Environmental factors can also cause modifications to proteins that induce conformational changes within the protein[2]. Protein misfolding may also occur through seeding and cross-seeding, where protein oligomers and fibrils follow a mechanistic pathway of aggregation through the seeding nucleation model[1, 3]. In this model, initiation takes place through a thermodynamically unfavorable protein misfolding step. As oligomers are formed and stabilized, exponential growth occurs as fibril formation begins[8]. It is our hypothesis that the synthetic peptides used in our experiments promote amyloidosis through interactions with hPrP in a manner to thermodynamically stabilize amyloid-prone states, allowing rapid conversion of protein to amyloid fibrils.

1.3 Ligand Induced Amyloidosis

The initiation of protein misfolding can result from the binding of peptide ligands that induce conformational changes to the protein structure. The misfolded proteins may serve as templates that facilitate amyloid formation. This led us to test small peptide ligands that could bind to recombinant prion protein and cause misfolding[16]. Studying peptide binding interactions with the prion protein allows us to investigate the molecular interactions that are important to amyloid formation within prion related diseases. The first series of peptides tested were based off the sequence-motif KFAKF. This peptide sequence was chosen owing to a chemical complementarity to a binding site on the C-terminal helix of the protein that was identified using structure-based methods[16]. The native structure of hPrP and the targeted binding site can be seen in Figure 1.1. The initial tests were conducted by Melody Adam (Texas State University, San Marcos TX). The

peptides that were tested were KFAKF, cyclo-CGKFAKFGC and cyclo-

CGGKFAKFGGC. Of these three peptides, only cyclo-CGGKFAKFGGC induced

amyloid-misfolding of recombinant hPrP[16]. To determine if peptides showing sequence

homology could likewise induce prion amyloidosis, peptides with the sequences of

RFMRF, cyclo-CGRFMRFGC, and cyclo-CGGRFMRFGGC were also tested. Of these

three peptides, cyclo-CGGRFMRFGGC demonstrated amyloid-converting activity

against hPrP[16].
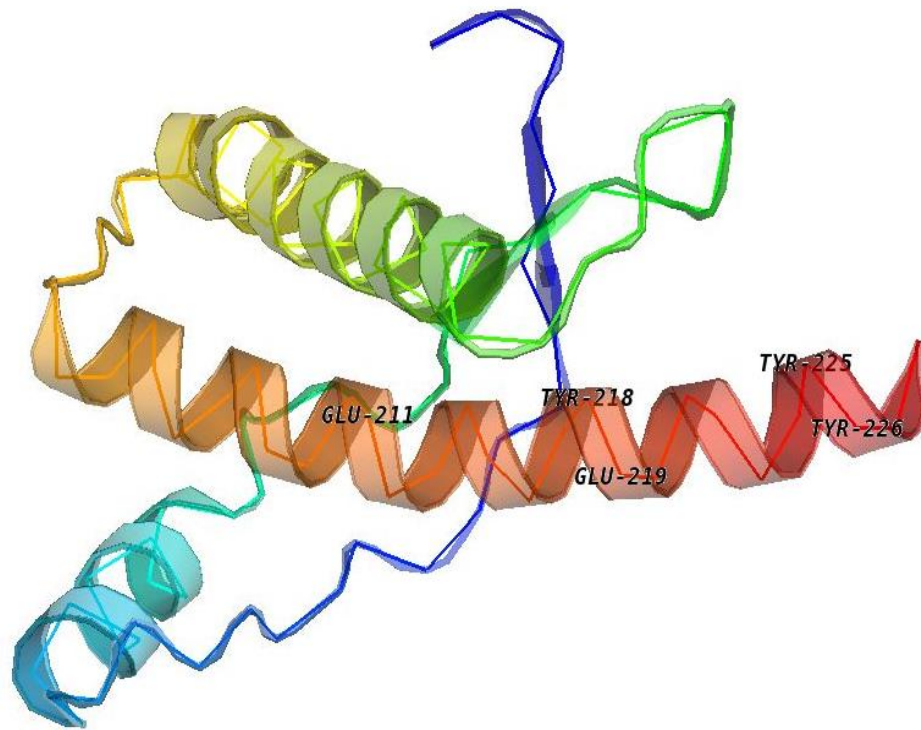
Figure 1.1 Native structure of the human prion protein. The image displays a molecular model of cellular hPrP. The two glutamate residues (211, 219) and three tyrosine residues (218, 225, 226) labeled are the binding site for the synthetic peptide motif KFAKF. This image was created using PyMol software.

1.4 Project Goals

Building upon those prion results, that demonstrated a peptide containing the sequence RFMRF could convert natively folded hPrP into insoluble particles that have amyloid character[16], the goal of this project was to test if synthetic peptides with sequences that are identical to naturally occurring RF-amide neuropeptides could also promote prion amyloidosis. Originally found in clams, neuropeptides containing the sequence RFMRF-NH$_2$ are found among all mammalian species. Many other neuropeptides that are homologous to the RFMRF-amide motif are now classified as RF-amides and play a variety of roles in mammalian brains including hormone secretion in the hypothalamus[29, 31, 32]. Examples of small, naturally expressed neuropeptides that contain the RF-amide motif are: LPLRF-amide, FLFQPQRF-amide, KGGFSFRF-amide, and FMRF-amide.

To test if RF-amide neuropeptides can convert recombinant hPrP to amyloid particles, the following was done: 1) isolate recombinant hPrP from *E. coli* lysates and demonstrate that it is correctly folded, 2) test the ability of synthetic peptides with the sequences of LPLRF-amide, FLFQPQRF-amide, KGGFSFRF-amide and FMRF-amide to cause amyloid misfolding of natively folded recombinant hPrP, and 3) test the ability of the peptide-induced misfolded protein to self-replicated its misfolded structure through seeding reactions, a key property of amyloid particles. From the initial data presented in the following chapters, each synthetic peptide that was tested seemed to show an ability to misfold recombinant hPrP to self-replicating amyloid oligomers under physiological-like conditions.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

All chemicals and reagents used for this project were Molecular Grade or higher. Synthetic peptides were synthesized commercially to 98% purity by GenScript (Piscataway, NJ) and Peptide 2.0 (Chantilly, VA). Water used in sample preparation was filtered and deionized by a Millipore Milli-Q purification unit (Billerica, MA). Glassware, pipette tips and other basic equipment were sterilized with a HICLAVE HV-50 autoclave vessel by Hirayama (Westbury, NY).

2.2 Expression and Purification of Recombinant Prion Protein

2.2.1 Cloning and Transformation

The amino acid sequence used in the studies was taken from a wild-type consensus of hPrP and is shown in Figure 2.1. The gene sequence contains residues 23-230 of hPrP and was cloned into a plasmid expression vector by DNA 2.0 (Menlo Park, California)[17]. Protein expression was carried out in *E. coli* cells using a pJexpress bacterial plasmid vector (pJexpress 404) containing a T5 promoter sequence for isopropyl β-D-1-thiogalactopyranoside (IPTG) induced expression[18]. The plasmids were used for their high-copy-number pUC origin of replication (~150-200 copies/cell) and contained an ampicillin resistance gene (*ampR*). Plasmids were solubilized in DNA grade sterile water at a concentration of 1 ng/μL and stored at -80 $^{\circ}$C.

BL21 (DE3) pLysS competent cells by Novagen (Darmstadt, Germany) were transformed using 7 μg of plasmid in 50 μL of BL21 (DE3) pLysS competent cells suspended in 60 mM calcium chloride. The solution was gently vortexed and placed on ice for 5 minutes, heated for 30 seconds at 42 °C and iced again for 2 minutes. The mixture was brought to room temperature and 250 μL of Super Optimal Broth (SOC) was added. Cells were then spread on Lysogeny Broth (LB) agarose plates containing 100 μg/mL of ampicillin. Cells were mixed with 20% v/v glycerol stocks for long term storage as outlined in previous works[16].

200 mL of LB and 100 μg/mL of ampicillin was aseptically inoculated with a single colony of *E. coli* cells transformed with plasmid containing the hPrP gene as described above. The 200 mL culture was incubated overnight in a rotary incubator (Max*Q, MIDSCI, St. Louis, MO) at 30°C to slow bacterial growth. The next morning, 5 mL of cell culture was transferred to 200 mL of fresh LB with 100 μg/mL ampicillin and incubated with orbital rotation at 37°C to an optical density (OD_{600}) of 0.6. At this point, 800 μL of cell culture was mixed with 200 μL of sterile 80% v/v glycerol. Glycerol stocks containing transformed cell cultures were stored in sterile cryovial tubes at -80°C

```
1 manlgcwmlv lfvatwsdlg lckkrpkpgg wntggsrypg qgspggnryp pqggggwgqp
61 hggggwgqphg 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. Full-length amino acid sequence of hPrP. Residues 1-23 constitute a signal peptide that directs the prion protein for synthesis via the secretory pathway and is removed during translation.

2.2.2 Expression of Recombinant hPrP

Cells from glycerol stocks were aseptically spread onto an LB/Amp plate containing 100 µg/mL of ampicillin. The plate was incubated at 37 $^{\circ}$C overnight until sufficient colonies were detected. Next, an overnight culture was setup using 25 mL of LB broth with 100 µg/mL of ampicillin and grown overnight at 30 $^{\circ}$C on an orbital shaker (Max*Q, MIDSCI, St. Louis, MO). The next day, 5 mL aliquots were divided into five flasks containing 200 mL LB/amp broth. Cell growth was continued for 3.5-4 hrs at 37 $^{\circ}$C until an OD_{600} of 0.6 was obtained. At this time 10 mM IPTG was added to induce expression of hPrP. Induction continued for 4 hrs, and then the cells were centrifuged at 7,000 x g at 4 $^{\circ}$C for 20 min using a Beckman J2-21 centrifuge with a JA-14 rotor (Beckman-Coulter, Brea, CA). The supernatant was discarded and cell pellets stored at -20 $^{\circ}$C for up to one month.

2.2.3 Pufication of recombinant hPrP

Cell lysis was carried out by re-suspending a frozen cell pellet in 20 mL lysis buffer (10 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, 100 µg/mL lysozyme, pH 7.5). The solution was incubated for 30 minutes at 37 $^{\circ}$C to increase lysozyme activity. Cells were sonicated on ice using a Bronson Sonifier S-450A (Danbury, CT) set to an output control of 5 and duty cycle of 80%. Sonication was carried out with 1 minute pulses followed by 1 minute of rest on ice for 3 cycles. Ten percent triton X-100 was added to the mixture at a final concentration of 1 % v/v and the sample was centrifuged at 25,000 x g, 4 $^{\circ}$C for 1 hr. Following centrifugation, the supernatant was discarded and the cell pellet resuspended in 10 mL resuspension buffer (8 M urea, 20 mM Tris-HCl, 100 mM

NaCl, pH 8.0) and incubated overnight at 4 $^{\circ}$C. This step was needed due to the

accumulation of protein in inclusion bodies. The following morning, the solution was

prepped for column chromatography by centrifuging at 10,000 x g, 4 $^{\circ}$C for 20 minutes.

The supernatant was loaded onto DE52 media to remove bacterial nucleic acids and then

loaded onto a nickel-charged affinity (Ni-NTA) column for purification by affinity

chromatography. Recombinant hPrP has a natural affinity for Ni-NTA media (GE

Healthcare, New Orleans, LA) and does not require an engineered 6x-his tag.

Protein purification was conducted on a Biologic LP system from Bio-Rad

Laboratories (Hercules, CA). Protein solution was first passed through DE52 media (GE

healthcare, New Orleans, LA) to remove any nucleic acids and proteases from the

sample. This was achieved by equilibrating the media with 20 mL of equilibration buffer

(8 M urea, 20 mM Tris-HCl, 100 mM NaCl, pH 8.0). Prior to equilibrating, the media

beads were allowed to swell in 20 mM Tris-HCl, 100 mM NaCl solution at a pH of 3.5

and degassed for 20 minutes. After reaching equilibration, the protein sample was passed

through the column and all peaks collected with 20 mL of equilibration buffer added to

flush protein off the column. The Ni-NTA media was equilibrated using 20 mL of

equilibration buffer and protein sample loaded onto the column, controlling bed volume.

Additional equilibration buffer was added to the column resin at this point. Next, wash

buffers were added to the sample to assist in protein folding of hPrP on the column. The

first buffer solution was composed of 20 mM Tris-HCl, 100 mM NaCl, pH 8.0 and 20 to

30 mL of solution added. The following buffer was added in the same column and

contained 20 mM Na_2HPO_4, 100 mM NaCl, pH 8.0. Eluting the protein results from a pH

change on the column as the protein of interest elutes out with imidazole. This was done

by added 30 mL of elution buffer (20 mM Na_2HPO_4, 100 mM NaCl, 500 mM imidazole,

pH 4.5) and collecting the corresponding peak off the column (Figure 2.2).
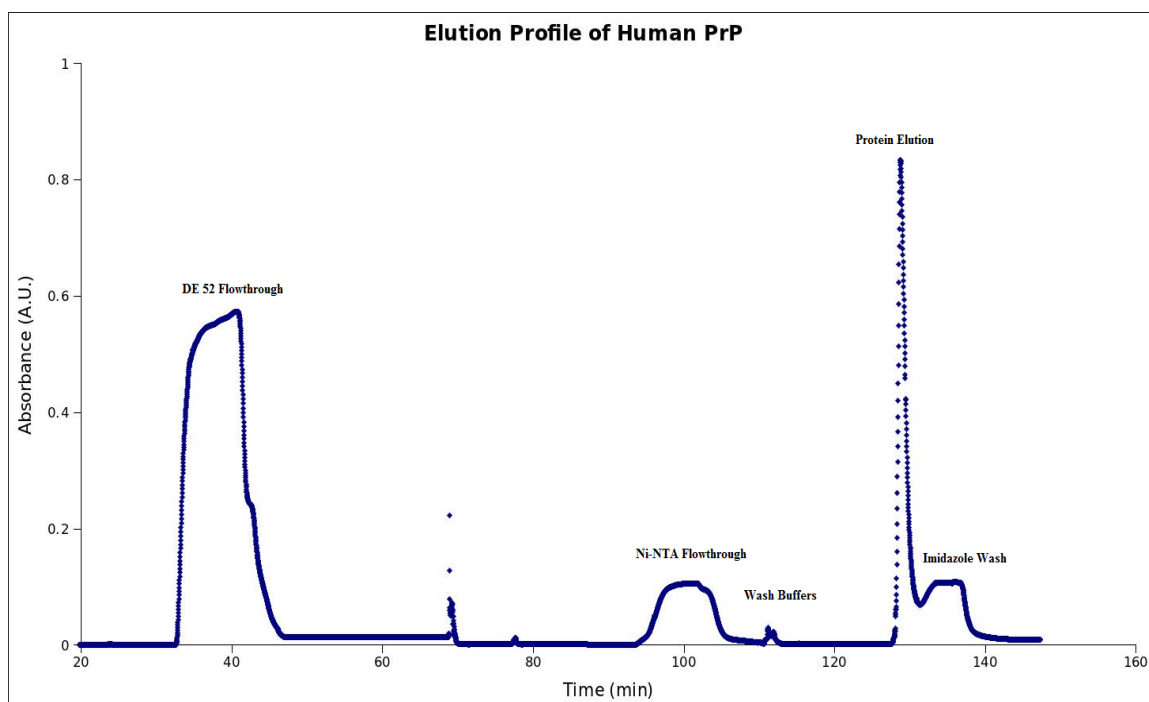
Figure 2.2 Chromatogram of the liquid chromatography steps that were used to isolate recombinant human prion protein from bacterial lysate. Purification of hPrP used Ni-NTA media owing to the intrinsic affinity of this protein for cationic nickel. Elution of hPrP occurs from high imidazole concentrations and low pH, causing a sharp elution peak at ~130 min followed by a small peak associated with the natural absorbance of imidazole. Other peaks in the chromatogram are from bacterial proteins.

Protein was then dialyzed against two different buffers for several hours. The first dialysis was done in a buffer with (10 mM Na_2HPO_4, pH 5.8) for at least 12 hours. Secondly, the protein was dialyzed against 10 mM Tris-HCl, pH 8.5. Dialysis solutions were pre-chilled and kept at 4 °C. The protein was then filtered through a 0.45 micron filter to remove pre-formed aggregates. Protein concentration was determined using an UV-Vis spectrophotometer (Bio-Rad, company town) at A_{280} with an extinction coefficient of 58780 $M^{-1}cm^{-1}$. The Beer-Lambert law ($A = \varepsilon c l$) was used to convert measured absorbance's to μM concentration of protein.

2.2.4 Circular Dichroism Spectroscopy of Native hPrP

Circular dichroism (CD) spectroscopy is used extensively in protein structural studies because of its ability to distinguish between α helical and β sheet conformation. The native, cellular form of the prion protein (PrP^C) is predominantly α helical in structure, consisting of 3 large helices that span residues 144-154, 173-194, and 200-228, and a very small β sheet that maps to residues 128-131 and 161-164. This allows us to confirm that our purified recombinant protein is correctly folded[16]. Protein samples were measured using a Jasco J-710 spectropolarimeter (Easton, MD) while purging the optical housing with N_2 gas at a flow rate of 5 L/min. Each sample was measured in a 1 mm quartz cuvette at a scan rate of 50 nm per minute in 5 nm steps and averaged over 12 scans per spectrum to cancel out effects of background noise. The CD was blanked against 20 mM sodium phosphate at pH 7.0. The raw output data was converted from ellipicity, which represents the rotation of plane polarized light in millidegrees, to the normalized mean molar ellipicity (ϑ_{MRE}) using the following equation:

$$\theta_{MRE} = \theta \frac{M}{1 \times C \times l \times n} \left[\frac{\text{deg} \times cm^2}{dmol \times residue} \right]$$

Where *M* is the molecular weight of hPrP (22.9 kDa), *C* is the molarity of the protein, *l* is the path length of the cuvette and *n* is the number of residues.

2.3 Protein Detection Methods

2.3.1 SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Protein samples were subjected to SDS-PAGE, a common method for detecting proteins based on their molecular weights. The denaturing condition of sodium dodecyl sulfate (SDS) allows proteins to separate according to the length of the polypeptide chain in correspondence of the molecular weight marker. Purity of protein was checked by gel electrophoresis to confirm that monomeric hPrP was present at an apparent molecular weight of approximately 23 kDa. All gels were run on pre-cast polyacrylamide gels (4-20% Tris-HCl) from Bio-Rad (Hercules, CA) using a Bio-Rad criterion cell gel rig at 200 V for 45 min in 1X TGS (25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS, pH 8.3) running buffer (Figure 2.3). Samples were prepared using Laemmli Sample Load Buffer (62.5 mM Tris-HCl, 25 % glycerol, 2% SDS, 0.01 % bromophenol blue, pH 6.8) and boiled for 10 minutes prior to loading in the gel. Following electrophoresis, gels were either used for Western blot analysis or stained with commassie blue dye for 1 hr and allowed to destain overnight.
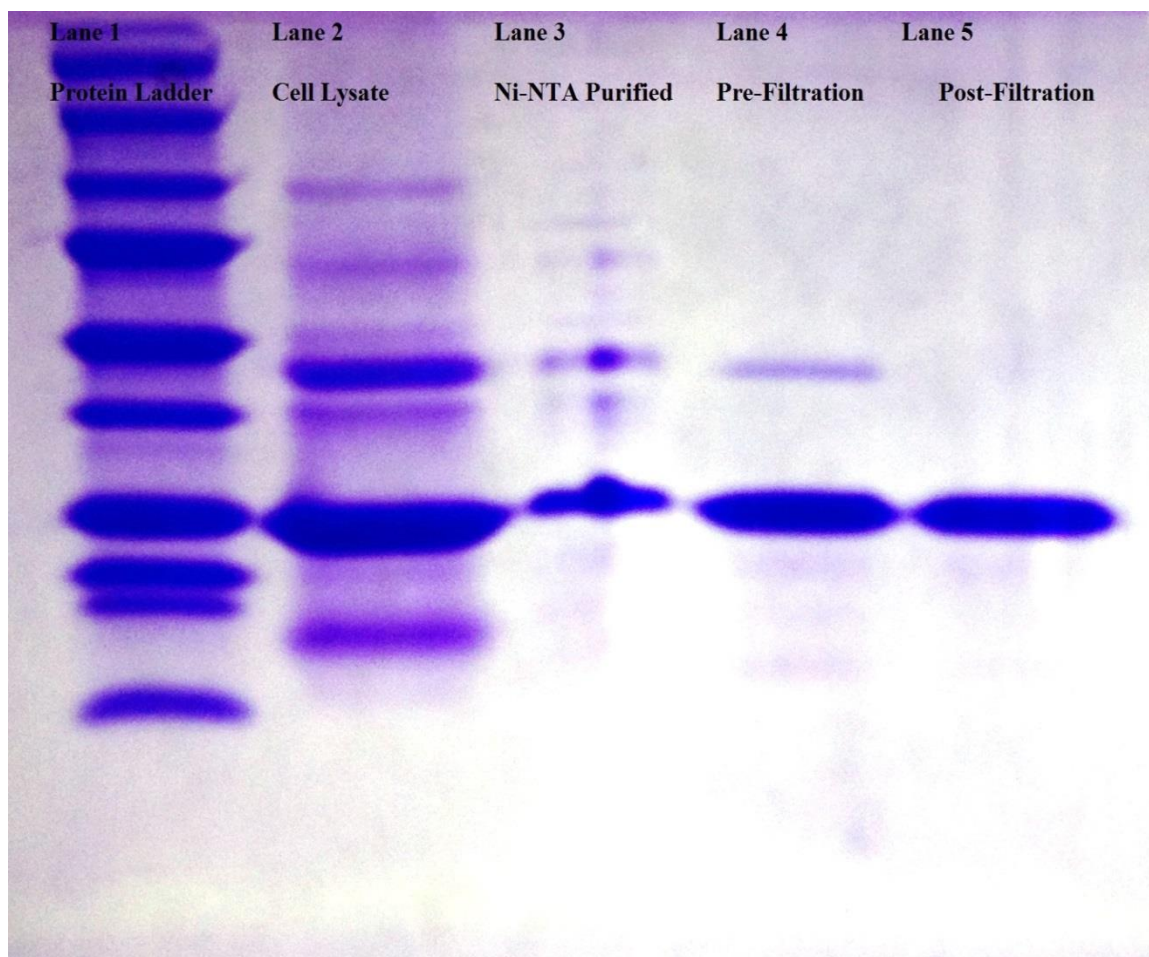
Figure 2.3 SDS-PAGE of recombinant human prion protein isolate from bacterial lysate. The gel above shows the purification scheme for hPrP. Lanes 2-4 show the steps of purification to the point of filtration of the protein through a 0.45 micron filter. Lane 5 is recombinant human prion protein, estimated from the gel image to be > 98 % purity.
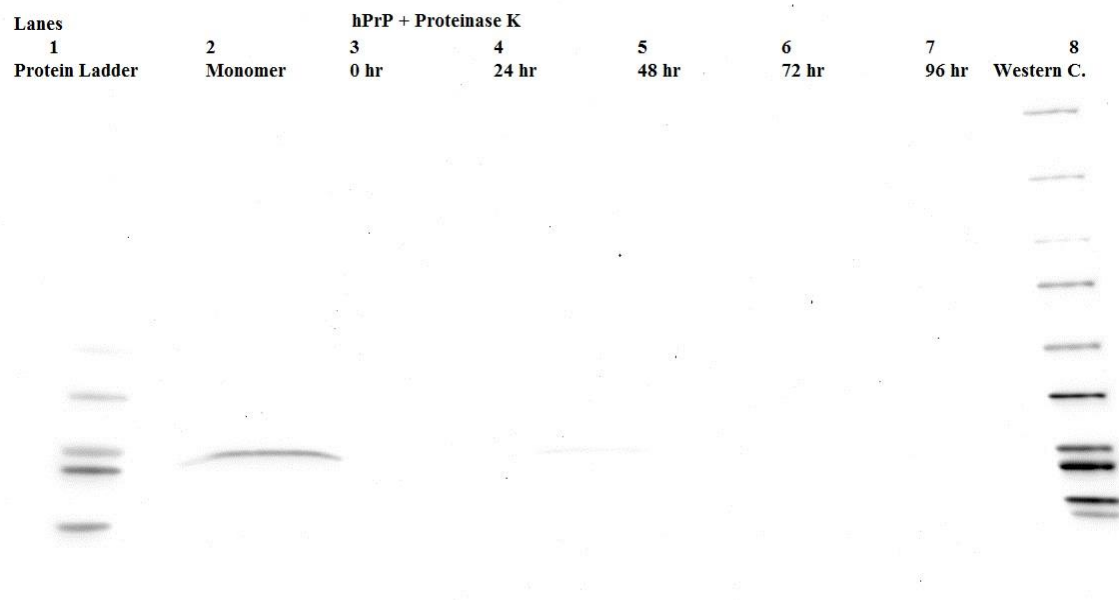
2.3.2 Western Blot Analysis

The presence of hPrP in a sample was detected using the mouse monoclonal antibody 3F4 (Covance, Princeton, NJ) and a standard Western blot technique. Western blots were used in studying results from Proteinase K digestion assays (2.3.3) and SDD-AGE (2.3.4) assays. However, the following pertains specifically for methods used for the proteinase K assay. Initially, pre-cast gels were removed and soaked in towbin transfer buffer (25 mM Tris, 192 mM glycine, 20 % w/v methanol, pH 8.3) for 15 minutes. This allowed for the gel to equilibrate and pre-shrink prior to transferring. Additionally, 0.45 micron nitrocellulose, purchased from Bio-Rad (Hercules, CA), was also equilibrated for 15 minutes. The gel, nitrocellulose and pads were assembled and placed in a criterion blotter from Bio-Rad Laboratories (Hercules, CA) and transferred for 1 hr at 33 V while keeping the towbin running solution at a constant temperature of 4 $^{\circ}$C. The nitrocellulose membrane was then removed and incubated overnight at 4 $^{\circ}$C with constant shaking in a solution of 5 % w/v dry milk powder in a Tris-tween buffer saline (20 mM Tris, 0.1 M NaCl, 0.1 % w/v Tween-20, pH 7.5). The following day, the membrane was washed several times with Tris-tween buffer saline (T-TBS) and soaked for 1 hr in a solution containing dissolved dry milk powder, Tris-tween and primary antibody on an orbital shaker. The primary antibody chosen for these trials was the 3F4 antibody (Covance, Princeton, NJ) which has been shown to bind to residues 109-112 of hPrP. The primary antibody was diluted from the original stock to a 1:100,000 working concentration. After incubation, the primary was removed and stored for multiple uses. The nitrocellulose 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 + dry milk powder for one hour. The nitrocellulose was then washed multiple times and allowed to dry. To image the membrane, a chemiluminsence solution was applied to the nitrocellulose for 5 minutes and incubated 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).

2.3.3 Proteinase K Digestion Assay

Previous studies have shown that proteinase K (PK) has the ability to hydrolyze monomeric cellular prion proteins. Due to their solubility in native conditions, the protein is completely degraded (Figure 2.4). Scrapie prions form insoluble particles that have been shown to resist PK digestion[17-19]. This causes fragmentation of scrapie that is often seen at around 16 kDa but can also produce smaller fragments (Figure 2.5). The 16 kDa fragment is produced by cleavage of the N-terminal end of the misfolded prion protein. The PK resistant core contains residues 97-230 of hPrP[20]. To digest samples, samples were mixed with PK to a final concentration of 7.5 µg/mL proteinase K. Samples were placed in a thermomixer for 45 minutes at 37 $^\circ$C at 700 rpm. To terminate digestion, samples were mixed with Laemmli sample load buffer (20:30 ratio) and boiled for 10 minutes to deactivate proteinase K. PK samples were either kept at -20 $^\circ$C until analysis or immediately run on an SDS gel. Following the completion of SDS-PAGE, the gel was either analyzed using commassie blue or exposed to Western blot transfer.

Lanes			hPrP + Proteinase K				
1	2	3	4	5	6	7	8
Protein Ladder	Monomer	0 hr	24 hr	48 hr	72 hr	96 hr	Western C.

Figure 2.4 Proteinase K digestion of recombinant human prion protein. The Western blot gives results from prion protein in the presence of PK in lanes 3-7 from time points of 0-72 hours. All samples were incubated at 37 ^{o}C in a thermomixer as described above. Lane 2 shows recombinant human prion protein without PK present in the sample. The molecular weight of recombinant human prion protein is 23 kDa.

Lane							
1	2	3	4	5	6	7	8
Protein Ladder	0 hr	24 hr	48 hr	72 hr	96 hr	Monomer	Western C
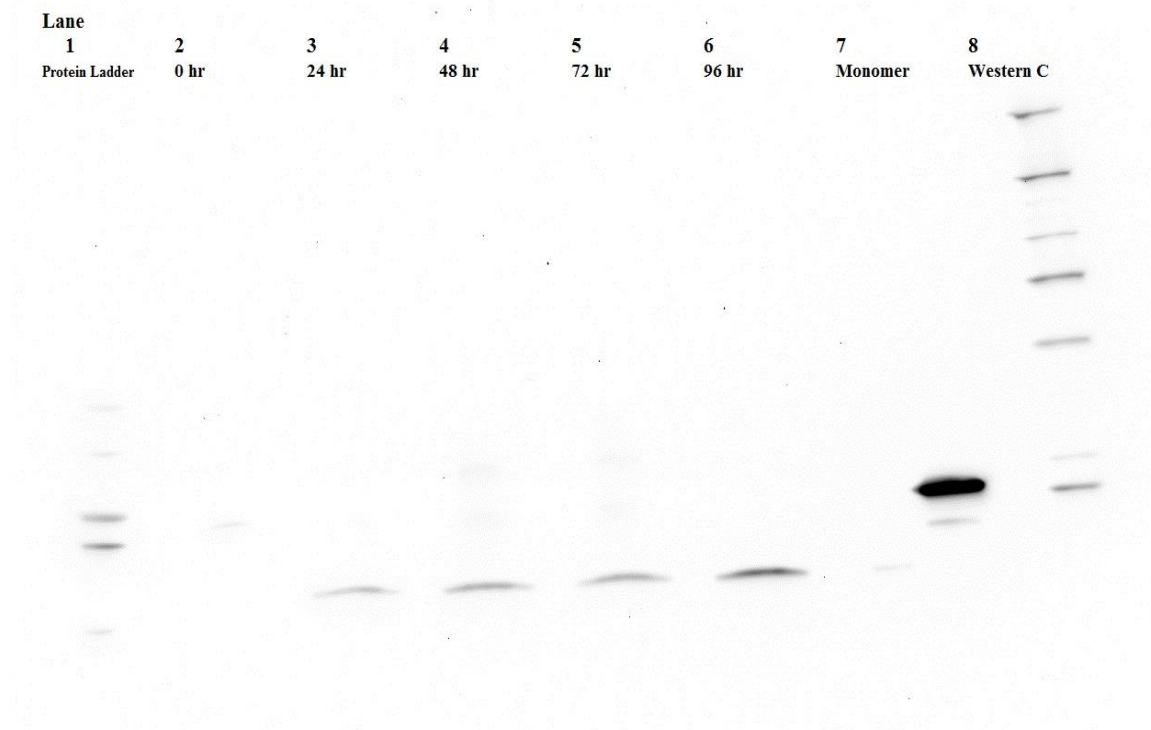
Figure 2.5 Proteinase K digestion of amyloid-misfolded human recombinant prion protein. Using the protocols described in Melody Adam's thesis[16], hPrP was misfolded to amyloid particles by incubating the protein with a cyclic peptide that had the sequence cyclo-CGGKFAKFGGC. Initially (lane 2, 0 hour time point), hPrP that was mixed with the peptide was digested by PK, but peptide-induced conversion of natively-folded hPrP to amyloid produced PK resistant fragments that are visible in lanes 3-6. Lane 7 shows hPrP without PK in the sample.

2.3.4 Semi-Denaturing Detergent Agarose Gel Electrophoresis

The method of Semi-Denaturing Detergent Agarose Gel Electrophoresis (SDD-AGE) was used to detect amyloid particles by exploiting differences in size and detergent solubility. Amyloid oligomers are much larger in size than the monomeric protein subunits that they are composed of. Also, while most protein aggregates are soluble in 2% SDS, amyloid oligomers are structurally very stable and resist detergent dissolution[37].

Because of the size of amyloid oligomers, this method uses agarose which has larger pores than polyacrylamide, and, consequently, provides better separation[21, 22]. In short, as a sample migrates through a gel, monomeric proteins migrate faster than large amyloid oligomers. Amyloid samples typically contain a range of oligomeric sizes (i.e., amyloid are polydisperse in subunit length) that causes streaking of high molecular weight species. An example of an SDD-AGE assay is provided in figure 2.6 where the SDS-resistant oligomers streak through the polyacrylamide gel.
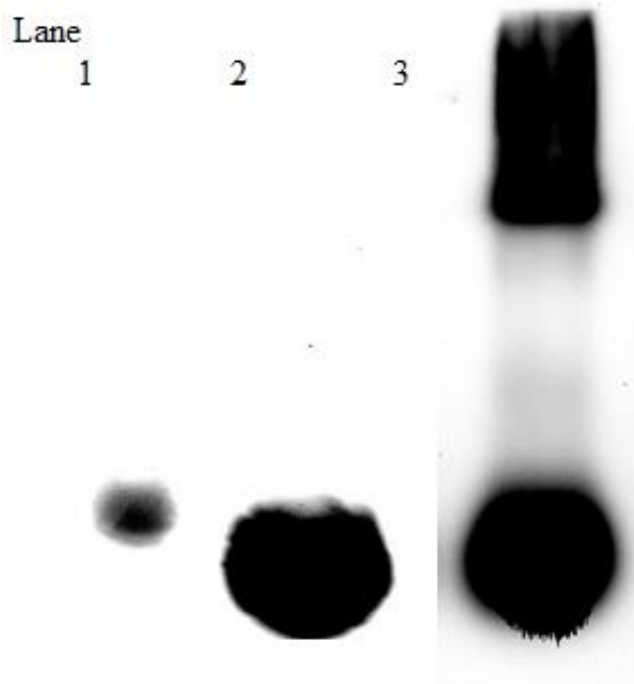
Figure 2.6 SDD-AGE detection methods of hPrP. SDD-AGE identifies amyloid oligomers from their low solubility in SDS and slow migration through agarose gels. Lane 1 is a weight standard that transferred at a slightly higher molecular weight than monomeric hPrP (23 kDa). Lane 2 contained native hPrP. Lane 3 shows hPrP that has been misfolded to amyloid, recognized by the streaking of high-molecular weight oligomers that are resistant to SDS dissolution.

Heat aggregates of purified hPrP were created by heating samples above 90 $^{\circ}$C in a buffer (1 X PBS, 0.1 % SDS, 0.1 % Triton X-100, pH 7.0) with no peptides present. The denatured sample was analyzed by SDD-AGE to confirm that hPrP does not produce sporadic amyloid-like structures in the recombinant system after the protein has become unfolded and cooled to RT. These results can be seen in Figure 2.5 and are often referred to as non-amyloid amorphous aggregates.
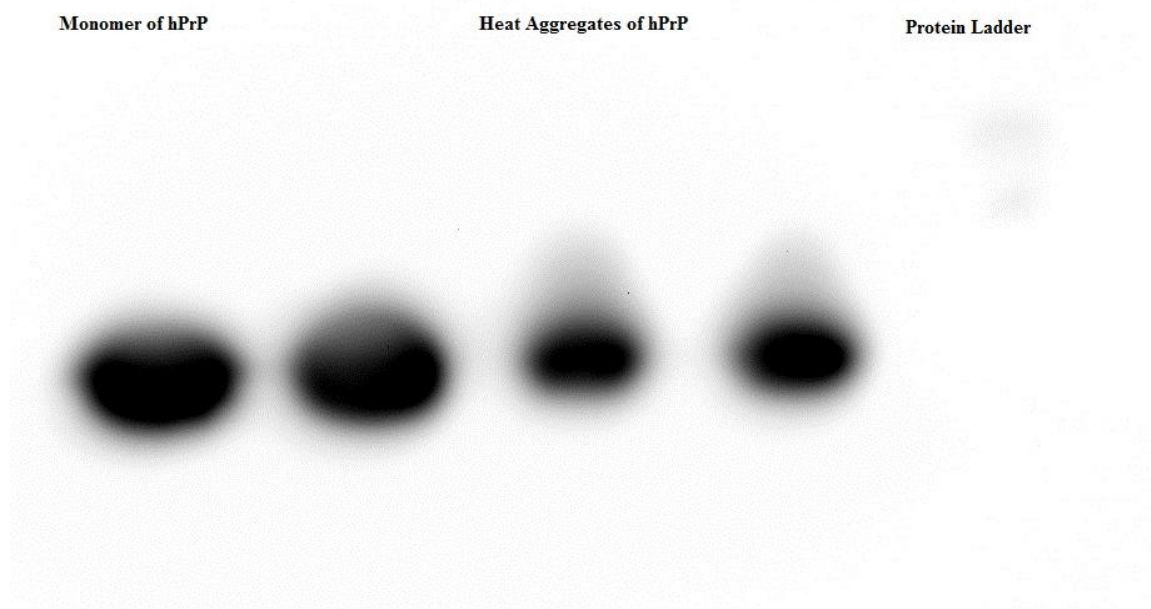
Figure 2.7 Heat denaturation of recombinant human prion protein. The recombinant hPrP samples were prepared in a 1 X PBS, 0.1 % SDS, 0.1 % Triton, X-100, pH 7.0 buffer solution. Heat aggregates were created by incubating the samples above 90 ^{o}C for 10 min to cause the protein to unfold. The SDD-AGE shows that denaturing the protein produces no SDS-resistant oligomers.

Preparation of the gel was done on a 20 cm x 24 cm casting tray with an 18 well comb. Agarose gels were made at 1.8 % by heating 2.16 g of agarose in 120 mL of 1 X TAE (10 mM Tris-HCl, 5 mM acetic acid, 5 mM EDTA . The agarose was heated for a total of 3 minutes with gentle swirling every 30 seconds. The solution was cooled for several minutes and 120 µL of 10 % SDS was added to the liquid agarose. Gels were poured onto the casting tray and any air bubbles removed prior to setting the comb. The gel was allowed to solidify for up to 1 hr. Samples were prepared using SDD-AGE loading buffer (2 X TAE, 20 % glycerol, 8 % SDS, 1 % Bromophenol blue) in a 1:1 ratio and incubated at room temperature for 7 minutes. The agarose gel was then placed in a 1 X TAE running buffer containing 0.1 % SDS and ran in a Bio-Rad Sub Cell GT (Hercules, CA) for 1.2 hr at 120 V. Transferring proteins from the gel for Western blot analysis was done by capillary electrophoresis . To employ this method, the agarose gel was placed on fiber pads with nitrocellulose directly on top of the gel. Additional pads were placed on top of the nitrocellulose in order to assist with capillary action of the transfer buffer. Transfer trays were placed higher than the gel and membrane, and contained wicks allowing for transfer buffer to produce capillary action for western blot transfer. Transfer took place for 12-16 hrs and was done in a solution of Tris buffer saline (20 mM Tris, 0.1 M NaCl, pH 7.5). Following the transfer onto the nitrocellulose membrane, the western blot analysis was performed as described above (section 2.3.2).

2.4 In vitro Amyloidosis Induced by Peptide: hPrP Interactions

Preparation of samples began by mixing synthetic peptides with purified recombinant hPrP. This was done in 100 µL aliquots that contained 1 mM peptide and 4.3 µM hPrP in a 1 X Quic buffer (1 x PBS, 0.1 % SDS, 0.1 % TritonX-100, pH 7.0).

Samples were incubated in a thermomixer set at 1500 rpm with 1 minute agitation and 1 minute rest cycles. The temperature in the thermomixer was held constant at 37 ^{o}C. Time trials per experiment were tested up to 96 hrs with samples pulled every 24 hrs and stored at -20 ^{o}C for up to 1 month.

CHAPTER III

AMYLOID MISFOLDING OF NATIVE RECOMBINANT HUMAN PRION PROTEIN PROMOTED BY RF-AMIDE NEUROPEPTIDES

3.1 Introduction

Conversion of cellular prion protein to the scrapie isoform (PrP^{sc}) is associated with several terminal human disorders including fatal familial insomnia (FFI) and transmissible spongiform encephalopathy's (TSE's). To continue the studies of protein: peptide interactions that may facilitate amyloid diseases, we tested naturally occurring RF-amide neuropeptides that shared sequence homology with a KFAKF motif. Previous work has shown that peptides with this sequence have the ability to promote amyloidosis of recombinant human prion protein (hPrP) under native conditions[16]. The physiological relevance of peptide-induced amyloidosis of the prion protein is unclear. This finding is noteworthy, however, for two reasons: 1) a recombinant test system was developed that can be used to experimentally determine the molecular interactions that promote amyloid misfolding of prion protein, and 2) the KFAKF sequence motif shares homology with a class of mammalian neuropeptides that are known as RF-amide neuropeptides. Consequently, those previous results suggest that some RF-amide neuropeptides may be physiological cofactors in the prion disorders.

As discussed in Chapter I, the goal of this project was to test if synthetic peptides with sequences that are identical to naturally occurring RF-amide neuropeptides could also promote amyloidosis of recombinant hPrP. In the study presented here, the ability of synthetic peptides with the sequences of LPLRF-amide, FLFQPQRF-amide, KGGFSFRF-amide and FMRF-amide to convert natively-folded hPrP to amyloid was tested. The four sequences that were tested are identical to naturally expressed mammalian neuropeptides that contain the RF-amide motif.

3.2 Results and Discussion

3.2.1 Purification and Native Folding of Recombinant hPrP

Recombinant hPrP was isolated from bacterial lysate following the protocols described in Chapter II. The purity of the isolated recombinant protein was judged to be >98 % by SDS-PAGE (see Figure 2.3). Circular dichroism (CD) spectroscopy confirmed that this protein was correctly folded into its normal native structure (Figure 3.1). The measured CD spectrum was identical to previously published spectra for this protein[16, 36], and showed the two local minima at ~210 nm and ~220 nm that are characteristic of proteins folded into predominately α-helical conformations[27].

The stability of the native hPrP structure was tested by heat denaturation. Heat induced unfolding of hPrP was monitored by the temperature sensitivity of its CD spectrum. Shown in Figure 3.2, hPrP is folded at temperatures below ~ 50 $^{\circ}$C. All amyloid conversion reactions that are presented here were performed at 37 $^{\circ}$C. Consequently, all tests for peptide-induced misfolding of hPrP occurred under native conditions.
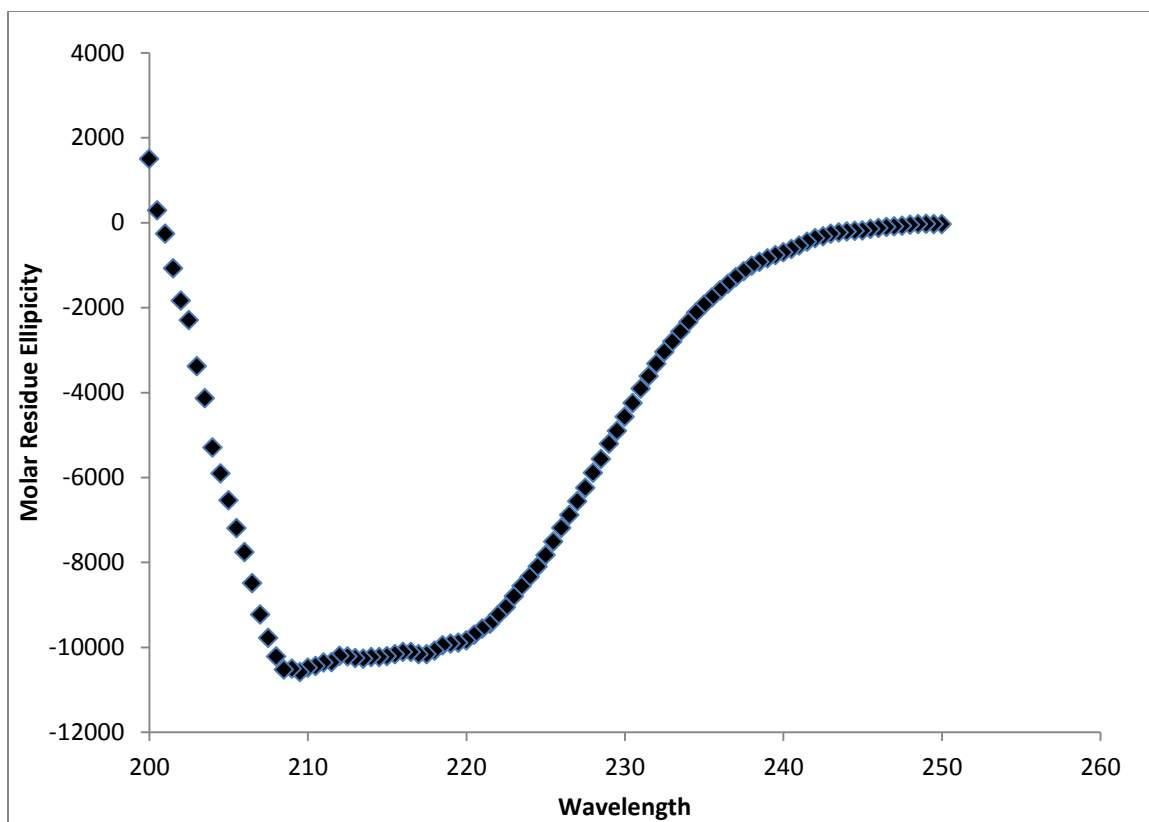
Figure 3.1 Circular dichroism spectrum of recombinant human prion protein. The CD spectrum for hPrP was measured in 100 mM Tris-HCl at room temperature. The CD values are reported in units of deg $cm^2 dmol^{-1} res^{-1}$. The concentration of hPrP in the sample was ~14 mM. The two local minima in the spectrum at ~210 and ~220 nm indicate that hPrP was folded into a structure that is primarily α-helical with minimal β content.
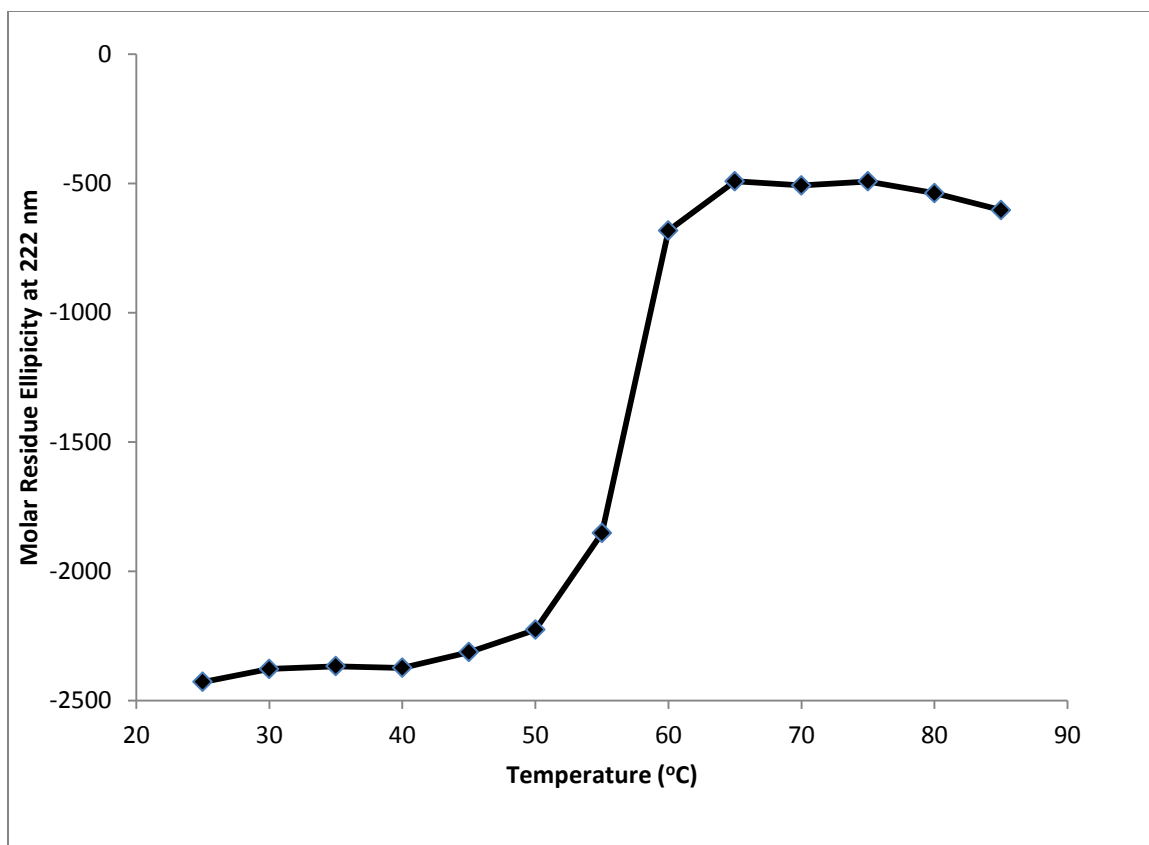
Figure 3.2 Thermal stability of recombinant human prion protein. Heat-induced unfolding of recombinant hPrP was monitored by the temperature sensitivity of its CD spectrum (see Figure 3.1) at 222 nm, which tracks with α-helical content. These data indicate that the native structure of hPrP is stable at 37 ^{o}C, the temperature that peptide-induced amyloid conversion reactions were performed.

3.2.2 Neuropeptide-Induced Amyloidosis of Recombinant hPrP

Synthetic peptides containing the sequence KFAKF can cause amyloid conversion of natively folded recombinant hPrP[16]. Neuropeptides that are naturally expressed in mammals called RF-amide neuropeptide contain similar sequences. Originally discovered in clams, a neuropeptide with the sequence of FMRF was shown to have mammalian homologs. Numerous other neuropeptides have since been discovered with similar sequences and are now referred to as the RF-amides[29]. Expression of RF-amide neuropeptides was found to play important roles in regulating neuroendocrine function in mammalian brains[30]. The neuropeptides chosen for our studies are as follows: LPLRF-amide (Leucine-Proline-Leucine-Arginine-Phenylalanine), FLFQPQRF-amide (Phenylalanine-Leucine-Phenylalanine-Glutamine-Proline-Glutamine-Arginine-Phenylalanine), KGGFSFRF-amide (Lysine-Glycine-Glycine-Phenylalanine-Serine-Phenylalanine-Arginine-Phenylalanine), and FMRF-amide (Phenylalanine-Methionine-Arginine-Phenylalanine).

To test the ability of synthetic RF-amide peptides to convert recombinant hPrP into amyloid particles, samples were made according to the protocol outlined in Chapter II. In short, following removal from dialysis, recombinant hPrP was diluted to a final concentration of 4.3 μM in the presence of 1 mM peptide and a buffer solution (1 x PBS, 0.1 % SDS, 0.1 % Triton X-100, pH 7.0). Samples were incubated for 96 hr in a thermomixer set to 37 °C with periodic agitation. The Proteinase K (PK) digestion assay (section 2.3.3) was used to detect amyloid formation. The significance of using PK is its inability to digest a region of hPrP that forms the core of the amyloid oligomers when the protein misfolds. The fragment that remains after digestion was visible by SDS-PAGE

and Western blot analysis as a 16 kDa band. SDD-AGE was used to confirm the results of the PK assays.

The neuropeptide FLFQPQRF-amide has the ability to modulate morphine-like activity in the brain and can inhibit the effects of opiates[28]. In the presence of purified hPrP, this neuropeptide seems to induce amyloid formation. This can be seen by the presence of a PK resistant core as shown in Figure 3.3. The image shows formation of a PK resistant core forming almost immediately when the prion protein and peptide are mixed in the reaction buffer. The protein levels increase over the time trials up to 96 hours and are visible in lanes 2-6. The ability of this peptide to produce amyloid-like structures was further confirmed using SDD-AGE analysis. Unlike the PK digestion assay, no amyloid can be detected at the initial 0 hour time point. The remaining time trials up to 96 hrs show formation of amyloid as indicated in Figure 3.4. Interactions of hPrP and the neuropeptide also produce a defined intermediate in the SDD-AGE image that increases in band intensity up to 96 hrs. Results from these trials indicate that the peptide sequence can promote amyloid formation of purified recombinant hPrP under physiological conditions.
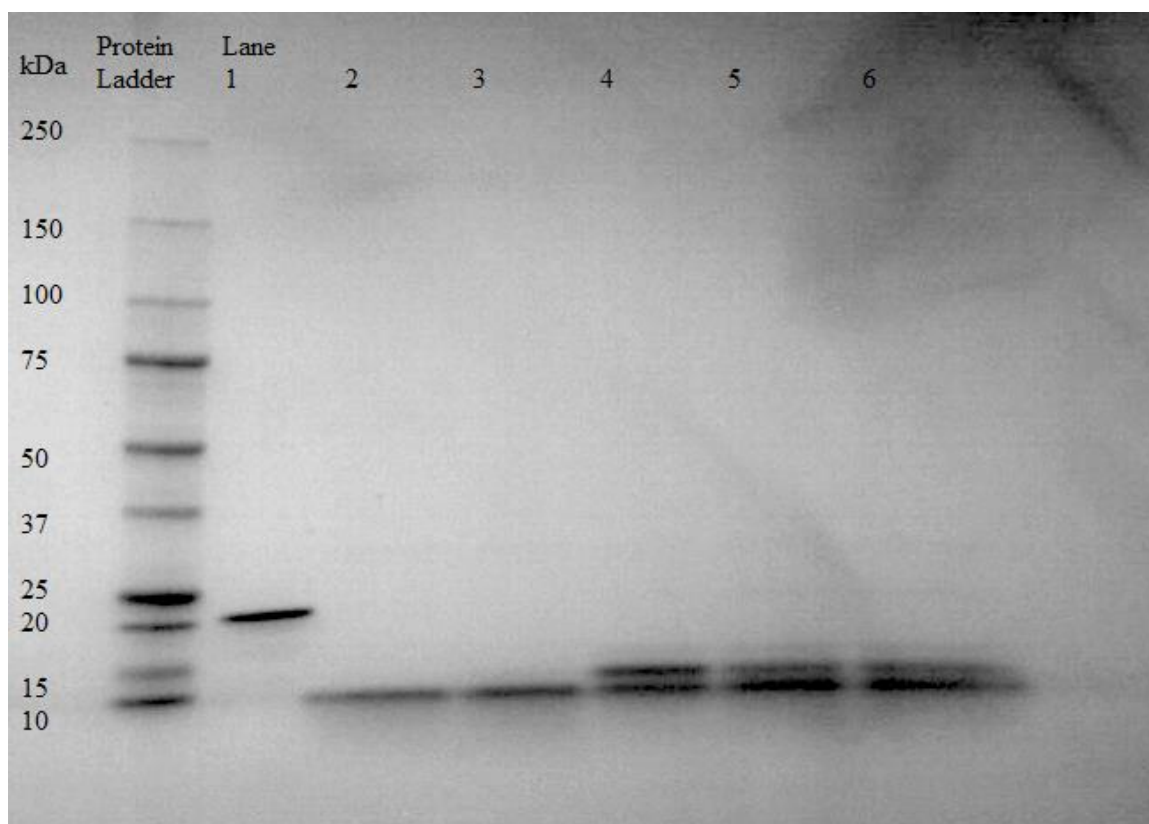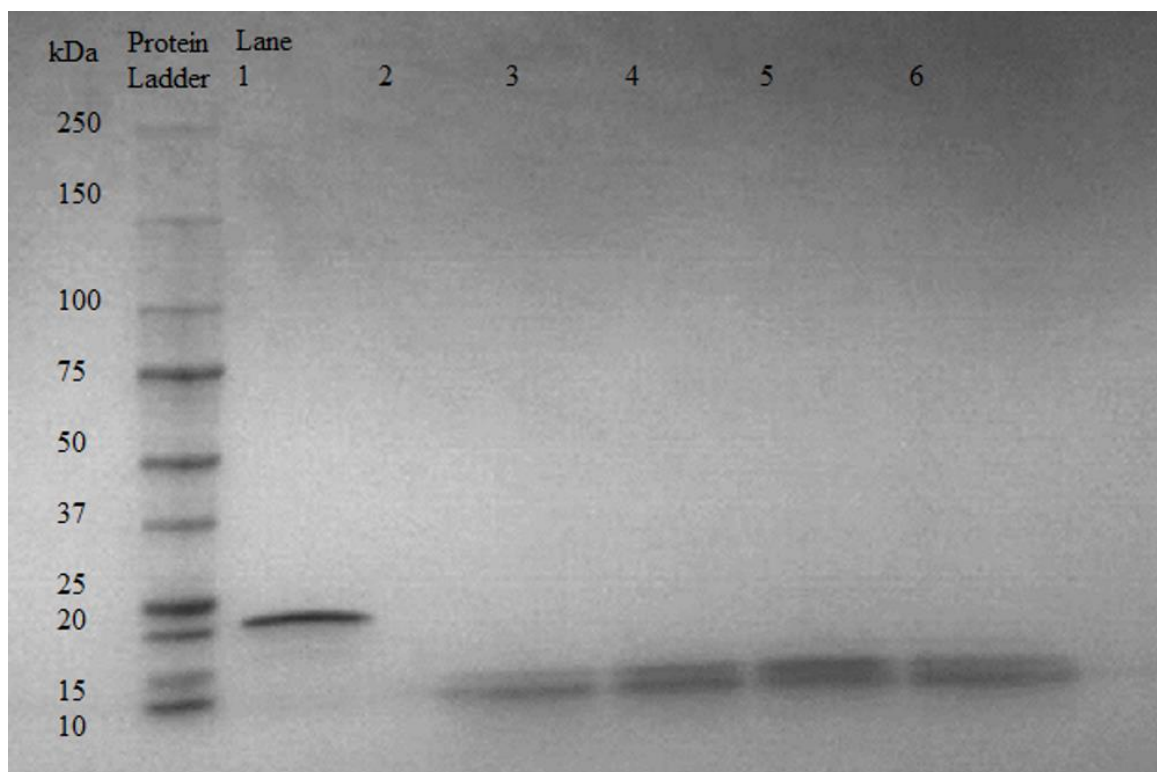
Figure 3.3 Proteinase K digestion of human recombinant prion protein in the presence of 1 mM FLFQPQRF-amide. The image shows PK digestion of hPrP in the presence of 1 mM neuropeptide FLFQPQRF-amide. Methods for the assay are as described above. The unlabeled lane is the protein ladder with the according molecular weights labeled. Lanes 1 contains monomeric protein only with no PK or peptide in the sample. Lanes 2-6 contain samples of hPrP mixed with synthetic peptide from 0 to 96 hrs. In these lanes, the PK resistant core is slightly lower than the expected 16 kDa band. This is caused by the formation of smaller PK resistant fragments. Lanes 4-6 show both bands producing the 16 kDa fragment and slightly smaller fragment.
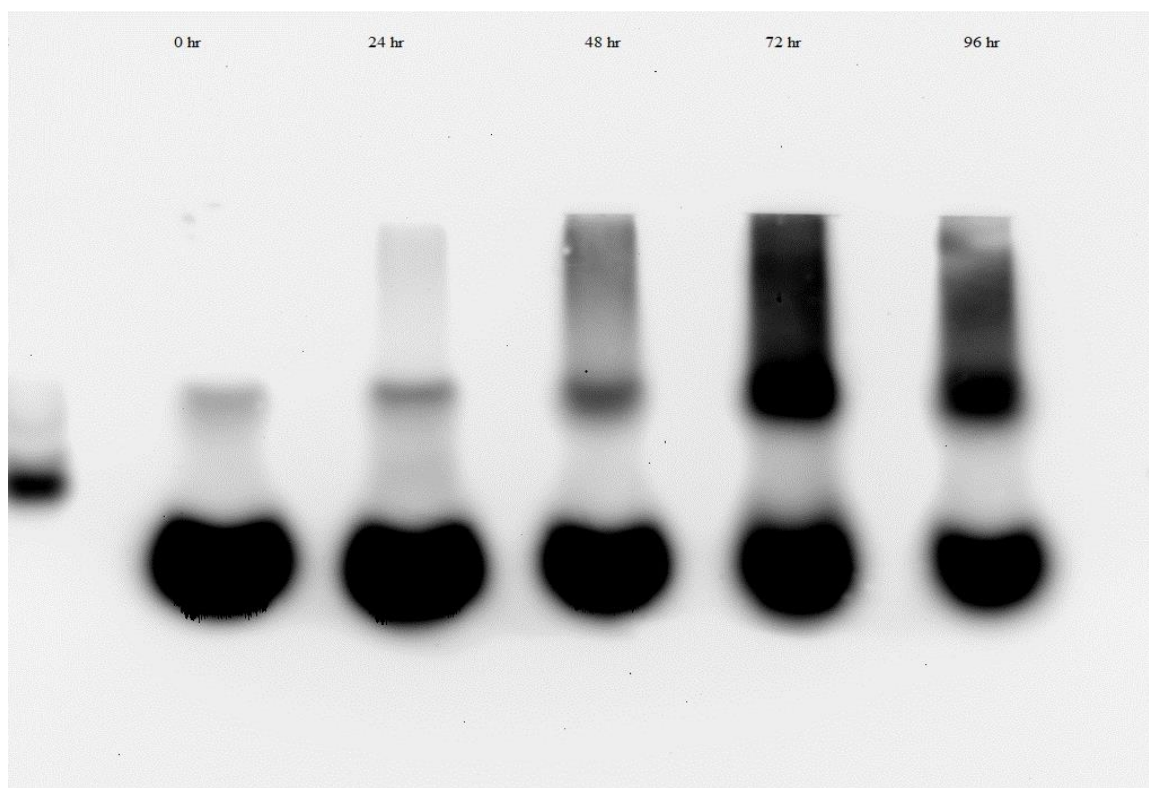
Figure 3.4 Conversion of monomeric hPrP to SDS-resistant oligomers by the peptide FLFQPQRF-amide. Samples containing 4.3 μM of natively-folded hPrP were incubated with 1 mM FLFQPQRF-amide in a buffer solution (1 x PBS, 0.1 % SDS, 0.1 % Triton X-100, pH 7.0) at 37 °C for the times indicated in the figure. The SDD-AGE technique (see Methods section 2.3.4) was used to detect detergent-resistant oligomers, a characteristic trait of amyloid particles. The hPrP in the gel was visualized using a standard Western blot protocol (see Methods section 2.3.2).

LPLRF-amide is a neuropeptide that is conserved across vertebrate species.

Expression of this neuropeptide is thought to be involved in control of pituitary hormone

secretion[29]. Upon mixing a peptide with this sequence with purified, recombinant hPrP,

oligomers with amyloid-like characteristics were detected. This is demonstrated in Figure

3.5 using a standard PK assay. At the initial time point (0 hr), there were no PK resistant

cores detected. At later time points (24-96 hrs), however, PK resistant fragments were

seen. The ability of LPLRF-amide to convert monomeric hPrP to SDS-resistant

oligomers was tested using SDD-AGE. As seen in Figure 3.6, no SDS-resistant oligomers

were initially detected. However, at the later time points (24-96 hrs), oligomers that resist

SDS dissolution were visible in this assay.

Figure 3.5 Proteinase K digestion of recombinant hPrP mixed with 1 mM LPLRF-amide. Lane 1 shows human recombinant prion protein with no PK or LPLRF-amide present in the sample. Lane 2 is the 0 hr time point that contained 4.3 μM hPrP and 1 mM LPLRF-amide in 1 X PBS, 0.1 % SDS, 0.1 % Triton X-100, pH 7.0 at 37 $^{\circ}$C. Similarly, lanes 3-6 show samples identical to the sample in lane 2, but incubated at 37 $^{\circ}$C for 24, 48, 72, and 96 hrs (increasing incubation time with increased lane number).
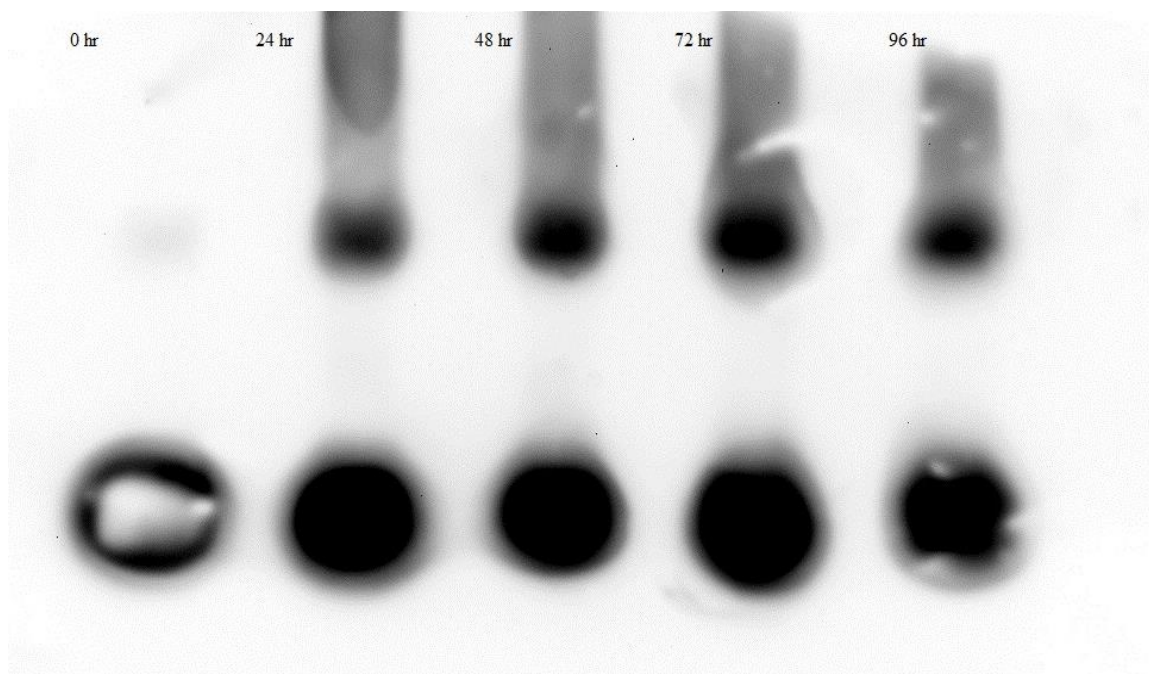
Figure 3.6 Conversion of monomeric hPrP to SDS-resistant oligomers by the peptide LPLRF-amide. Samples containing 4.3 μM of natively-folded hPrP were incubated with 1 mM of LPLRF-amide in 1 X PBS, 0.1 % SDS, 0.1 Triton X-100, pH 7.0 at 37 $^{\circ}$C for the times indicated in the figure.

The peptide sequence KGGFSFRF-amide is found to be part of a larger 26 amino acid neuropeptide found in the hypothalamus and spinal cord region in humans. Studies are still being conducted on the role of the neuropeptide but it's thought to contribute to sexual behavior, transmission of nonciceptive stimuli and feeding[30]. After mixing the KGGFSFRF-amide with purified, recombinant hPrP, oligomers with amyloid-like characteristics were detected. The results of the PK assay are presented in Figure 3.7. At the initial time point (0 hr), no PK resistant core was detected. At later time points (24-96 hrs), however, PK resistant fragments were detected. In these samples, two bands are present in the PK assay. This was thought to be caused by reduced proteinase K activity and the incomplete digestion of monomeric hPrP (bands at ~23 kDa). The bands in the lower part of the PK assay are the fragments at a molecular weight of ~16 kDa. SDD-AGE was then used to test the ability of KGGFSFRF-amide to convert monomeric hPrP to SDS-resistant oligomers. Figure 3.8 shows that no SDS-resistant oligomers were initially detected. However, later time points (24-96 hrs) demonstrate evidence of oligomers that resist SDS dissolution in this assay.
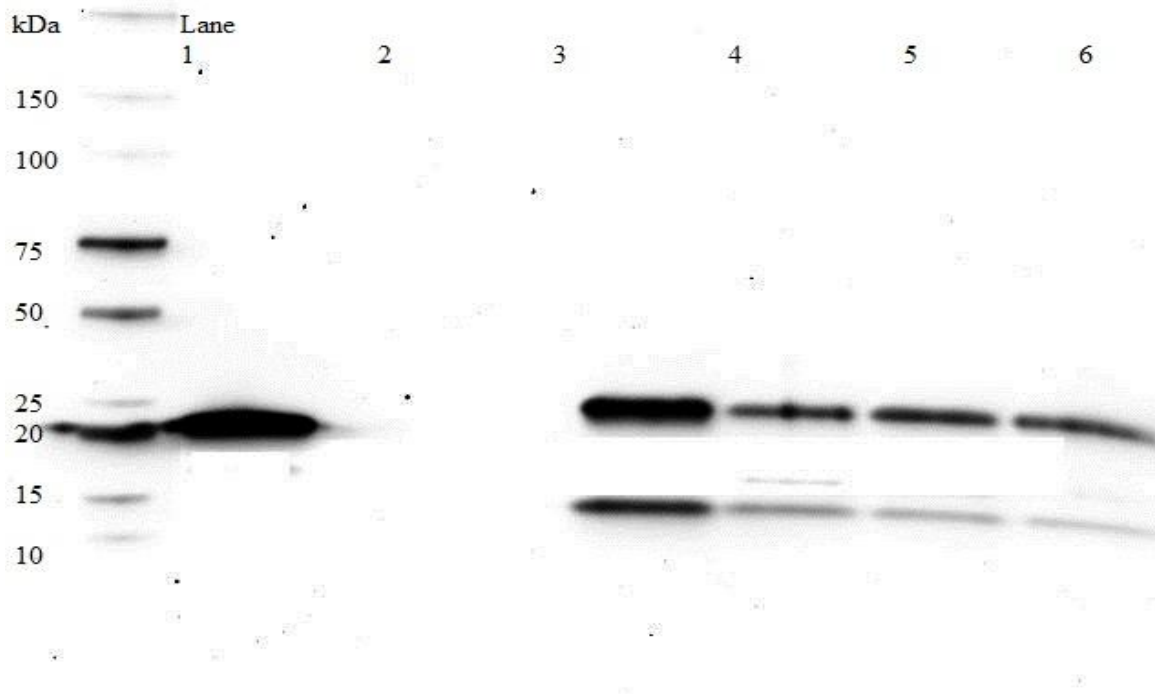
Figure 3.7 Proteinase K digestion of recombinant hPrP mixed with 1 mM KGGFSFRF-amide. Lane 1 shows the control of purified recombinant prion protein with no PK or KGGFSFRF-amide present. Lane 2 is the 0 hr time point of 4.3 μM hPrP and 1 mM KGGFSFRF-amide in 1X PBS, 0.1 % SDS, 0.1 % Triton X-100, pH 7.0 at 37 ^{o}C. This sample was completely digested by PK. Lanes 3-6 show samples identical to lane 2, but incubated for longer time points (24, 48. 72 and 96 hrs) at 37 ^{o}C. In these samples, the PK resistant core can be detected.
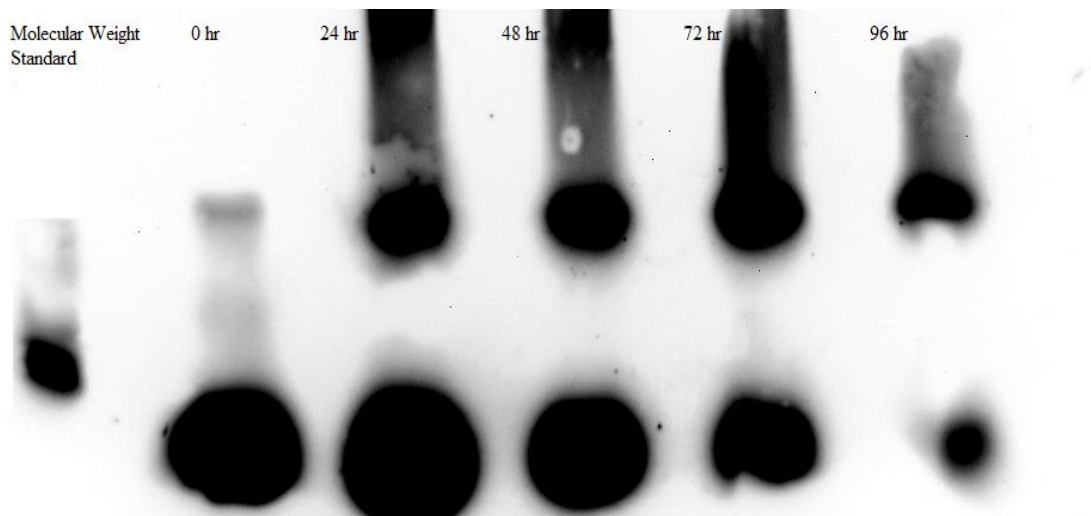
Figure 3. 8 Conversion of monomeric human prion protein to SDS-resistant oligomers by the peptide KGGFSFRF-amide. All samples in the SDD-AGE above contained 4.3 µM of natively-folded hPrP incubated with 1 mM KGGFSFRF-amide in a 1 X PBS, 0.1 % SDS, 0.1 % Triton X-100, pH 7.0 buffer at 37 °C for the times indicated in the figure.

The neuropeptide containing the sequence FMRF-amide, is related to a class of neuropeptides all sharing a similar sequence at their C-terminus. In the body, it is thought that the neuropeptide affects both the force and frequency of the heartbeat by increasing cytoplasmic cAMP in the ventricular region[31, 32]. Upon mixing the FMRF-amide peptide with purified, recombinant hPrP, oligomers with amyloid-like characteristics were detected. This is demonstrated again using the standard PK assay as seen in Figure 3.9. This shows that no PK resistant core is formed in the initial 0 and 24 hr time points. In the later time points (48-96 hrs), however, PK resistant fragments were seen. The ability of FMRF-amide to convert monomeric hPrP to SDS-resistant oligomers was tested using SDD-AGE. These SDS-resistant oligomers are not detected, however, until later time points (72- 96 hrs) as seen in Figure 3.10. The earlier time points (0- 48 hrs) show no formation of SDS-resistant oligomers.
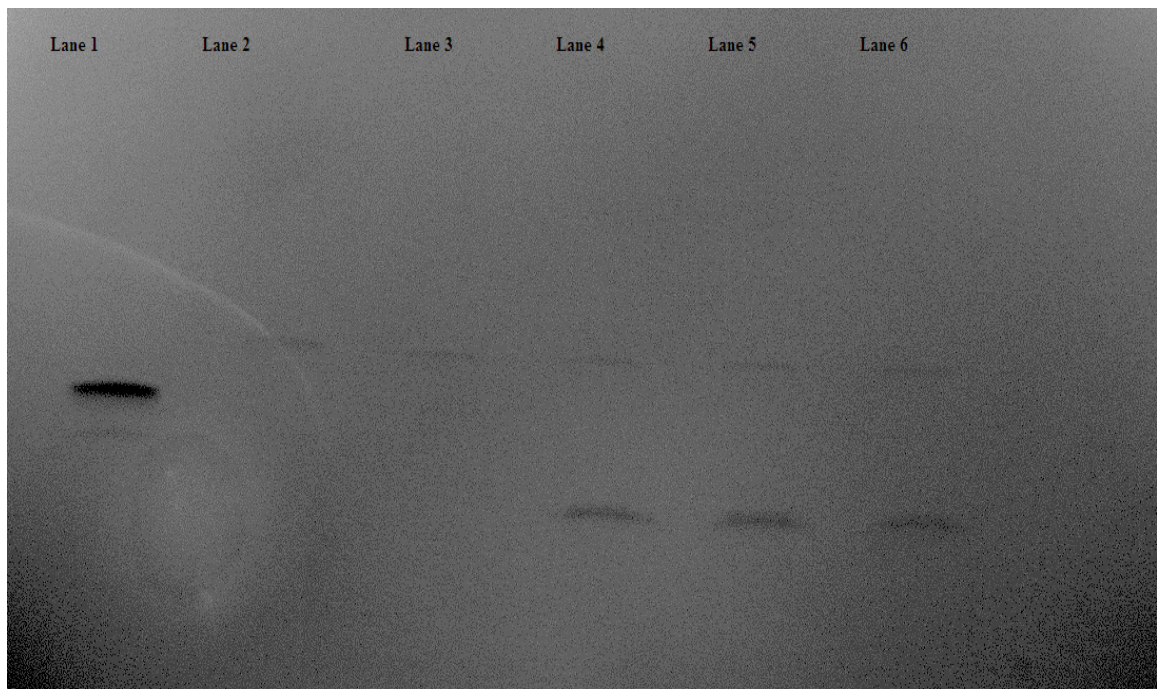
Figure 3.9 Proteinase K digestion of recombinant hPrP mixed with 1 mM FMRF-amide. Lane 1 shows human recombinant prion protein with no PK or FMRF-amide peptide present in the sample. Lane 2 was the initial 0 hr time point that contained 4.3 μM hPrP and 1 mM FMRF-amide in 1 X PBS, 0.1 % SDS, 0.1 % Triton X-100, pH 7.0 at 37 °C. Lanes 3-6 contain samples identical to lane 2, but incubated at 37 °C for 24, 48, 72 and 96 hrs. The formation of a PK resistant core is not detected before 48 hrs.
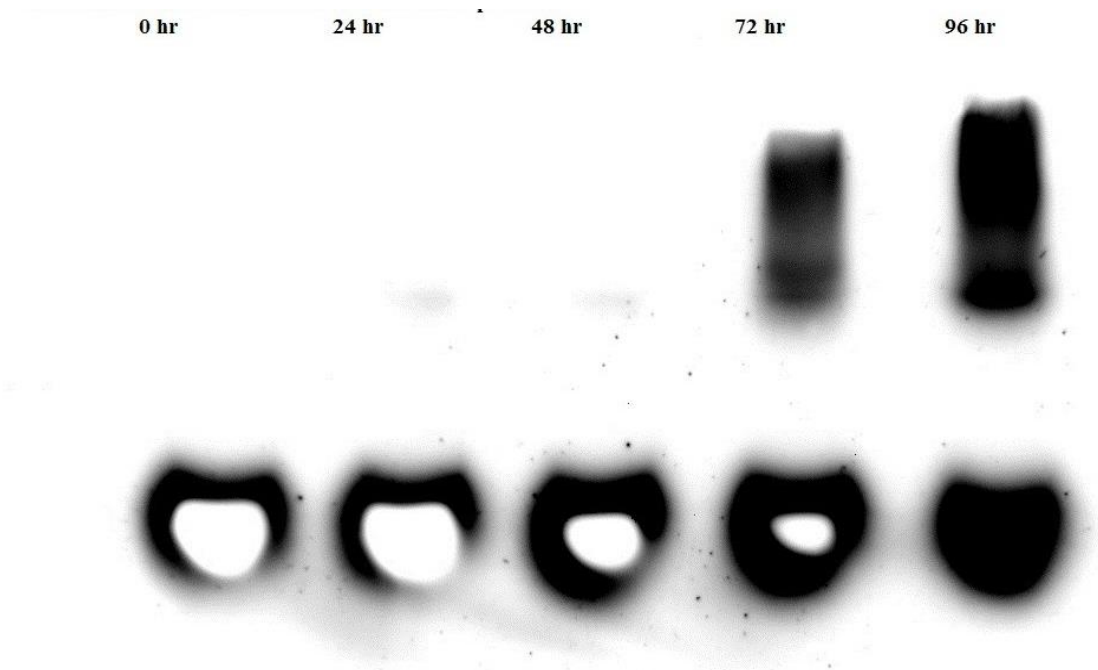
Figure 3.10 Conversion of monomeric hPrP to SDS-resistant oligomers by FMRF-amide. Samples containing 4.3 µM of natively-folded hPrP were incubated with 1 mM FMRF-amide in 1 X PBS, 0.1 % SDS, 0.1 % Triton X-100 at 37 °C for the times indicated in the figure.

3.3 Propagation of Amyloid States in the Absence of Neuropeptides

3.3.1 Amyloid Formation in the Absence of the Neuropeptide LPLRF-amide

The synthetic peptide sequences were further studied to determine the ability to propagate amyloid formation by seeding further reactions. This was previously demonstrated by Melody Adam, who showed that amyloid particles formed from the incubation of synthetic peptide with recombinant hPrP, has the ability to continue producing amyloid material after the peptide has been removed[16]. This amyloid is used to seed further conversion reactions when mixed with freshly purified recombinant hPrP. Particulates formed from the amyloid conversion reactions, and used for seed reactions, were created by incubating samples with 1 mM synthetic peptide for 24 hrs. After the 24 hr time point, samples were centrifuged at high speed for 45 minutes and re-suspended in fresh 1 X phosphate-buffered saline (PBS). The seed samples were either frozen or immediately incubated with freshly purified recombinant hPrP in 1 X PBS, 0.1 % SDS, 0.1 % Triton X-100, pH 7.0 buffer at 37 $^{\circ}$C for time points of 0, 24, 48, 72 and 96 hrs.

Seeds produced from the neuropeptide LPLRF-amide have shown the ability to propagate amyloid formation as indicated by SDD-AGE in Figure 3.11. The initial time point (0 hr) indicates that amyloid-like structures are not detected. However, increasing time points (24, 48, 72 and 96 hrs) show formation of SDS-resistant oligomers. These results are significant in the notion that this peptide sequence has potential in propagating amyloid states in the absence of the synthetic peptide. This will be further tested by the PK digestion assay and will be a focus of future studies.
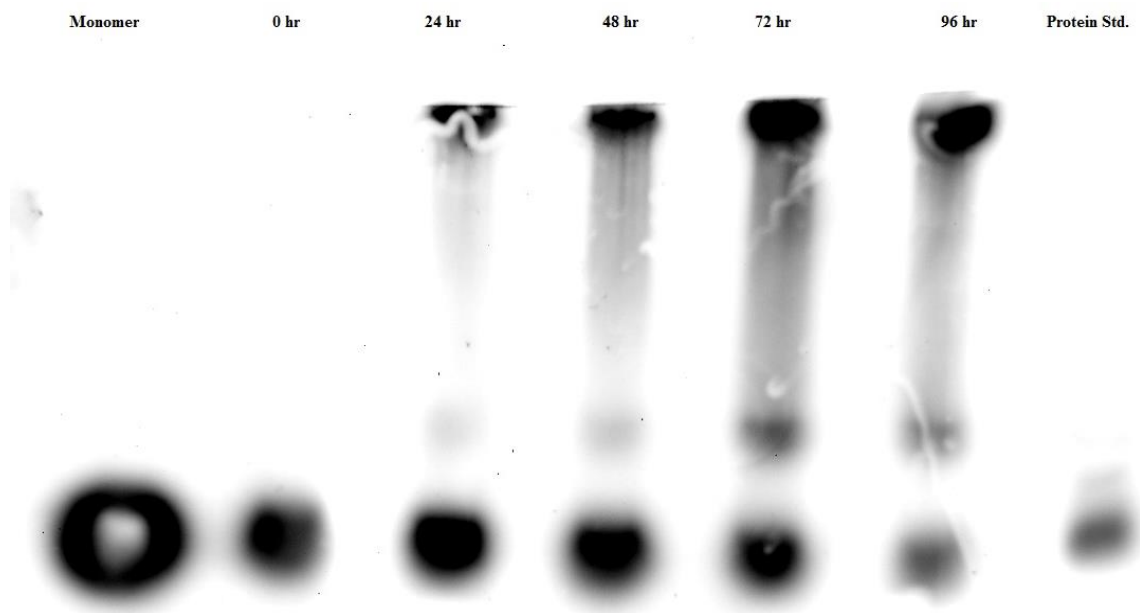
Figure 3.11 Conversion of purified recombinant human prion protein in the absence of LPLRF-amide. The Western blot image shows the results of the seed reactions initially produced from the RF-amide. This image confirms the ability of the neuropeptide to propagate amyloid formation as shown beginning at 24 hrs. The formation of SDS-resistant oligomers can be detected as the time points increase (48, 72 and 96 hrs).

3.3.2 Amyloid Formation in the Absence of the Neuropeptide FLFQPQRF-amide

Seeding recombinant hPrP using the peptide sequence FLFQPQRF-amide showed the ability to propagate amyloid formation in the absence of the peptide. Seed reactions from this peptide were prepared as previously described and were incubated in a thermomixer at 37 ^{o}C for up to 96 hrs. Reactions were then analyzed by SDD-AGE. According to results given in Figure 3.12, amyloid formation can be detected beginning after 24 hrs of incubation. Formation of an intermediate is present in time points excluding the initial 0 hr time point. This indicates the possibility that polymerization of the amyloid-like structures continued to occur after the peptide is diluted out of solution and using amyloid seeds to propagate amyloid formation. Results from seeding with this peptide sequence will be further studied using PK digestion and other methods of amyloid detection in future studies.
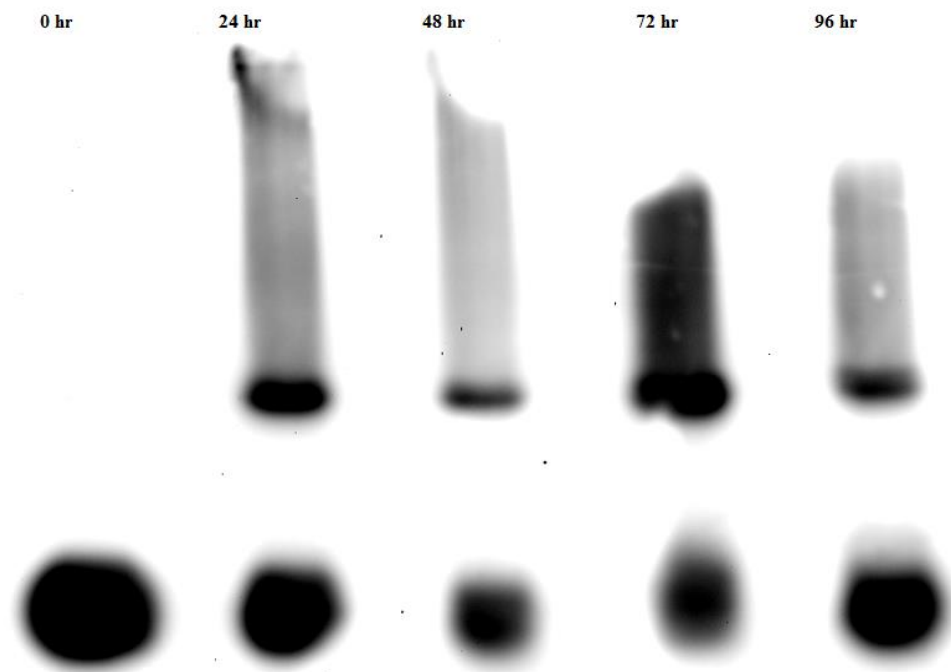
Figure 3.12 The conversion of recombinant hPrP in the absence of FLFQPQRF-amide. The SDD-AGE image shows the potential of amyloid seeds to continue forming amyloid when the neuropeptide is diluted from solution. The seeds used in this reaction were made from the FLFQPQRF-amide synthetic peptide. Formation of intermediates can be seen after 24 hrs and show an increase in band intensity up to 96 hrs. The bands indicate the presence of amyloid-like structure.

3.4 Conclusions

The synthetic peptide sequences FLFQPQRF and LPLRF show the formation of a PK resistant core by the PK digestion assay and SDS-resistant oligomers using SDD-AGE. Additionally, these two sequences have shown the ability to continue producing amyloid particles by seeding freshly purified recombinant hPrP with the amyloid material they produce. The peptides FMRF-amide and KGGFSFRF-amide form a PK resistant core and SDS-resistant oligomers when tested in their respective assays, but seed reactions were not conducted on these peptide sequences. These results will be the focus of future graduate studies. The synthetic peptides in this thesis will need to be further investigated for their ability to produce and seed synthetic amyloid. Additional studies will include: 1) good reproducibility of all neuropeptide sequences, 2) dilution studies of the peptides to find a potential minimum concentration of amyloid conversion and 3) focused time points to better isolate an intermediate in the formation of amyloid-like structures.

Additionally, future studies could also be conducted to test the binding ability of modified neuropeptide sequences. The RF-amides tested in this thesis were linear and contained an NH_2-end cap. Future trials could determine if removal of the amide group would have an effect on the peptide to bind to hPrP. Alternatively, if the peptides are cyclized, as in the KFAKF trials, would cyclic peptides affect binding?

For the purpose of this thesis, it shows a further interest in studying the ability of the synthetic neuropeptide sequences to produce amyloid-like structures when mixed with purified recombinant human prion protein. These peptide sequences can help us

understand specific binding interactions needed in protein misfolding and identify

possible cofactors responsible for prion diseases.

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