FINDING UNIVERSAL INHIBITOR OF AMYLOID AGGREGATION

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BO13M1003

A Dissertation Submitted to
Dr. BASANT KUMAR PATEL,
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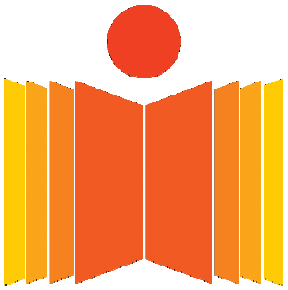
In Partial Fulfillment of the Requirements for
The Degree of Master of Technology



भारतीय प्रौद्योगिकी संस्थान हैदराबाद
Indian Institute of Technology Hyderabad

M.Tech Biotechnology Department

Medical Biotechnology stream

JUNE, 2015

Declaration

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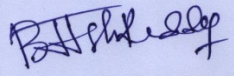

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Approval Sheet

This thesis entitled **"FINDING UNIVERSAL INHIBITOR OF AMYLOID AGGREGATION"** by B TIRUMALESHWAR REDDY is Approved for the degree of Master of Technology from IIT Hyderabad.

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ACKNOWLEDGEMENT

I express profound gratitude to my supervisor, Dr.Basant kumar patel for extending his valuable guidance throughout the span of the project and for its accomplishment. I also acknowledge my sincere regards to Faculty members of the Thesis Committee: Dr. Basant Kumar Patel, Dr.T.Rathinavelan , Dr. Parag Pawar and Dr. Rajakumara Eerappa for their insightful comments and suggestions.

I would like to thank my lab members vishwanath shivalingam, archana prasad, neetu Sharma, amandeep and kiruthika for the support provided whenever it was necessary.

I also extend a special note of thanks to my Parents and Brother for the moral support that they have incessantly bestowed

ABSTRACT:

Protein misfolding and aggregation results in many human diseases and some diseases are caused when protein aggregation leads to amyloid formation (Chiti and Dobson, 2006). Amyloids are rigid, insoluble, unbranched, fibrous and well organized proteinaceous materials having cross-β core structure (Chiti and Dobson, 2006) (Nelson et al., 2005). They have characteristic "cross β-sheet" structure, revealed by X-ray diffraction studies. Detection of amyloid can be done by both congo red binding and thioflavin-T (Th-T) assay.Thermodynamic properties of complexes of Congo Red (CR) dye with amyloid β (Aβ) peptides were studied by absorption spectroscopy and thioflavin-T were studied by fluorescence spectroscopy. Inhibition of pathogenic protein aggregation may be an important and straight forward therapeutic strategy for curing amyloid diseases.inhibitory effect of 3-aminophenol , GPS-1, GPS-2 on bovine serum albumin , lysozyme, rnq1 prion protein amyloid aggregates were studied. 3 aminophenol shows significant inhibitory action on lysozyme amyloid aggregates.

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1. INTRODUCTION:

1.1 AMYLOID AGGREGATION:

Peptides or proteins convert under some conditions from their soluble forms into highly ordered fibrillar aggregates. Such transitions can give rise to pathological conditions . these pathological conditions are generally referred to as protein misfolding desease(Chiti and Dobson, 2006). Some of these diseases are caused due to protein aggregation in an amyloid fashion. Amyloids are unbranched, fibrillar, rigid, insoluble, highly organized and protease-resistant aggregates of

proteins(Chiti and Dobson, 2006) (Nelson et al., 2005).. These are approximately 75-100 Å in

diameter. They have characteristic “cross β-sheet” structure, revealed by X-ray diffraction studies.

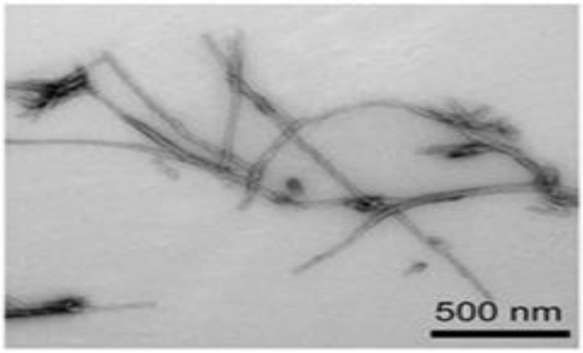


Figure 1: Typical morphology of amyloid fibrils observed under electron microscope (Sunde and Blake, 1997).

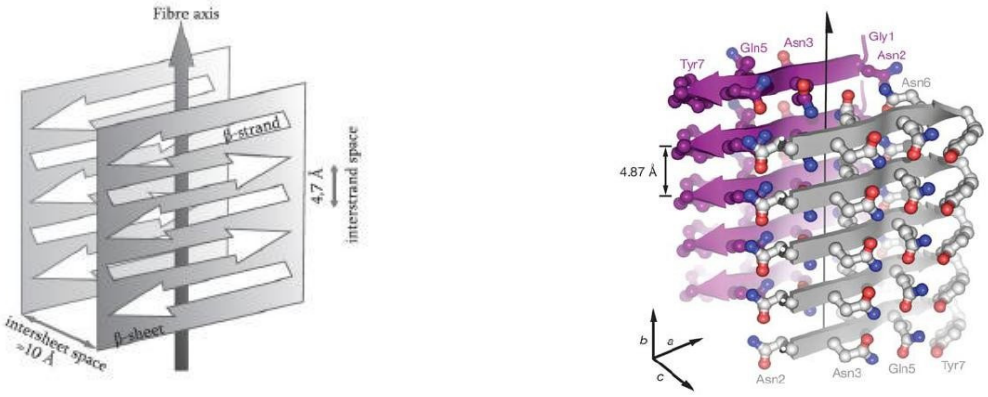


Figure 2: Cross beta structure present in amyloids (Nelson et al., 2005).

Amyloids differ from other fibrillar structures like collagen or silk fibroin in their ability to bind the dye Congo red and exhibit bright apple green birefringence when viewed under polarized light, and the dye Thioflavin T exhibiting bright fluorescence (LeVine, 1993). Amyloid aggregates are partially stable against protease (proteinase K) and detergents like SDS and sodium lauroyl sarcosinate at room temperature.

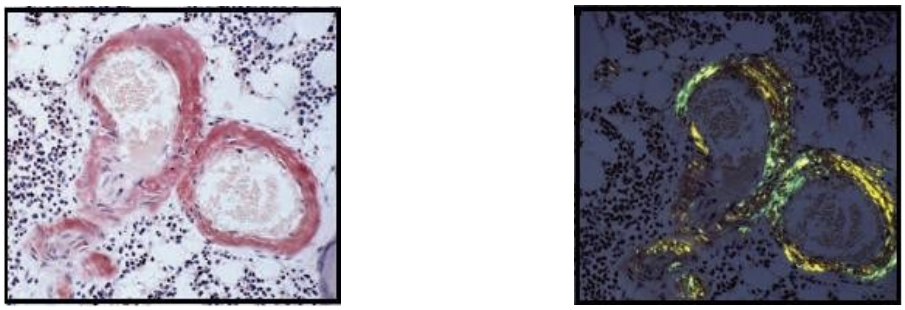
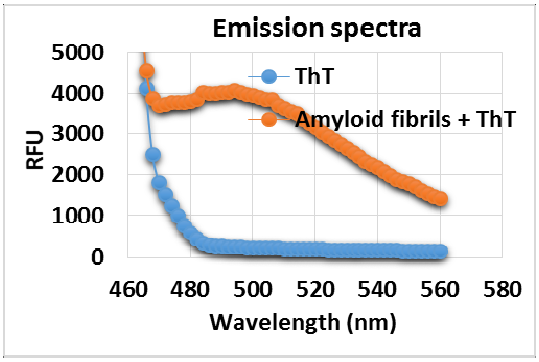


Figure 3: (a) Red in Bright field microscopy (b) Bright apple green birefringence in Polarized light microscopy (Merlini and Bellotti, 2003).



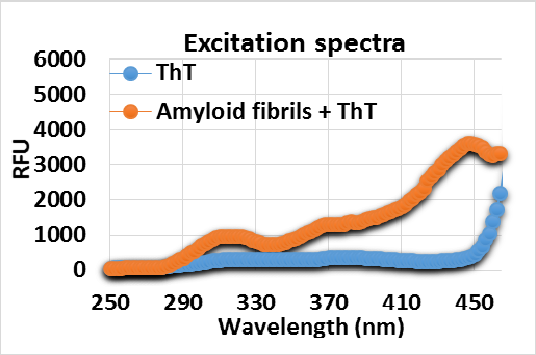
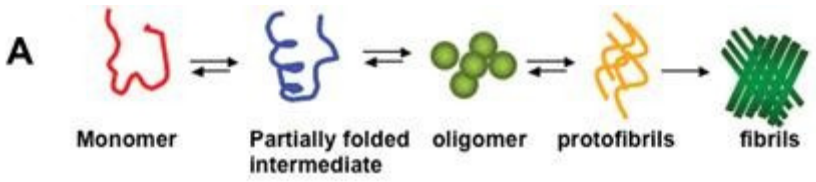


figure 4: Characteristic increase in ThT fluorescence upon binding to amyloid fibrils.

Amyloid formation is a nucleation dependent process. The rate of growth of amyloid fibres is increased by the presence of pre-formed aggregates i.e. seed. This process is known as seeding.



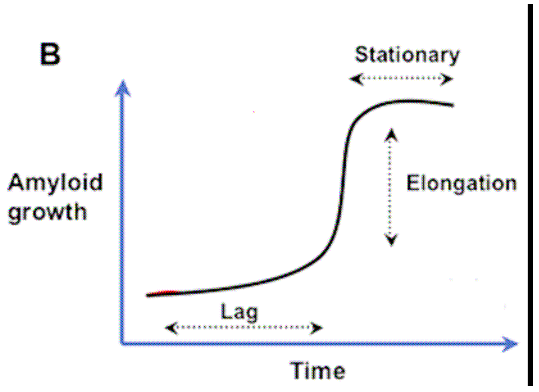


Figure 5: (A) Schematic of Amyloid aggregation. (B) Amyloid formation by nucleation dependent polymerization mechanism (Sunde and Blake, 1997).

1.2 AMYLOIDOSIS:

Amyloidosis is the pathological state associated with the deposition of conformationally altered proteins in the amyloid state, generally in the extracellular spaces of organs or tissues (called plaques or amyloid fibrils) or sometimes as intracellular "inclusions bodies". The most well known neurodegenerative disease caused due to amyloid deposits is the Alzheimer's disease caused by the deposition of Aβ 1-40 peptide fragments. Parkinson's and Huntington's diseases are caused by the aggregation of α-synuclein and huntingtin protein, respectively, however they lack amyloid characteristics.

Amyloid deposition can be systemic or localized. When the amyloid is restricted to a particular organ or tissue, it is called localized amyloidosis. Organs generally affected by amyloid depositions are the kidneys, spleen, cardiac muscles and brain. Example- Injection-localized amyloidosis due to repeated insulin injections occurs in the kidneys and the muscles. When the depositions are found in any or all of the body regions, in the blood and blood vessel walls, it is called systemic amyloidosis. Example- immunoglobin light chain amyloidosis (AL amyloidosis) occurs in most tissues.

1.3 AMYLOID DETECTION:

Detection of amyloid can be done by both congo red binding and thioflavin-T (Th-T) assay.Thermodynamic properties of complexes of Congo Red (CR) dye with amyloid β (Aβ) peptides were studied by absorption spectroscopy and thioflavin-T were studied by fluorescence spectroscopy. This fibrillar material possesses extensive β-sheet structure as revealed by far-UV CD and IR spectroscopy. Furthermore, the fibrils exhibit Congo red birefringence, increased fluorescence with thioflavine T and cause a red-shift of the Congo red absorption spectrum. All of these characteristics are typical of amyloid fibrils. The results indicate that formation of amyloid occurs when the native fold of a protein is destabilized under conditions in which

noncovalent interactions, and in particular hydrogen bonding, within the polypeptide chain remain favorable.

1.4 FUNCTIONAL AMYLOID AGGREGATES:

Formation of amyloid structure is an inherent or generic property of polypeptide chains . Amyloids are not only cytotoxic but amyloid like structures are also present naturally which do not cause diseases, are called "functional amyloids" having a beneficial role, for example *E.coli* have a protein called curlin which forms amyloid structures and helps the organism to colonize on inert surfaces and mediate binding to host proteins. The fungus *Neurospora crassa* secrete hydrophobins which are amyloid forming proteins that lower the water surface tension and allow the development of aerial hyphae.

Other functional amyloids include chaplins (*Streptomyces coelicolor*), hydrophobin EAS (*Neurospora crassa*), proteins of chorion of the eggshell (*Bombyx mori*), intralumenal domain of Pmel17 (*Homo sapiens*), Ure2p (*Saccharomyces cerevisiae*), Sup35p (*Saccharomyces cerevisiae*), Rnq1p (*Saccharomyces cerevisiae*), HET-s (*Podospora anserina*) and neuron-specific isoform of CPBE (*Aplisia californica*) (Chiti and Dobson, 2006).

1.5 Amyloid aggregate forming proteins:

Amyloid formation is an intrinsic property of most proteins not just of those involved in disease states. The SH3 domain found in many proteins involved in the rapid formation of amyloid fibrils. It is 60–85 amino acid long and forms a compact β-barrel motif.

Amyloid aggregate forming proteins either associated with diseases for e.g. Huntingtin, Alpha- synuclein, Aβ peptide and Lysozyme etc or no association with diseases for e.g. BSA, HSA, Sup35 and Curlin etc. Non-disease amyloid forming proteins are used as a model system to understand: General mechanism of formation of amyloid (Bhattacharya et al.,

2011); cross-seeding e.g. study of crossing of species barrier by : (a) sup35 prion protein between *S. cerevisiae* and *C. albicans* (Vishveshwara and Liebman, 2009). (b) cow prions are infectious to humans causing vCJD disease (Zou and Gambetti, 2009); potential application as structural nano-materials (Cherny and Gazit,

2008); to understand the propagation of amyloid and prion e.g. sup 35 is used to study th e infection mechanism of prion (Tanaka et al., 2005) and any other applications e.g. drug delivery agent. Insulin amyloid aggregates can be used for diabetes treatment (Gupta et al., 2010).

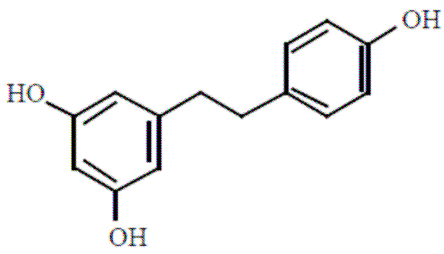
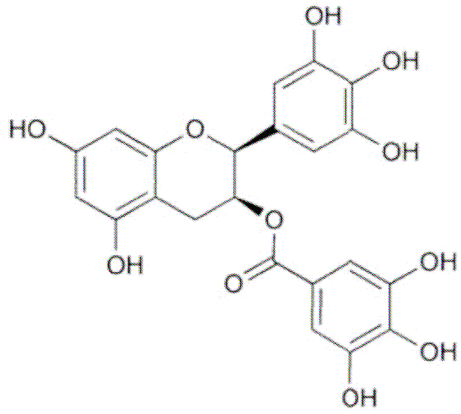
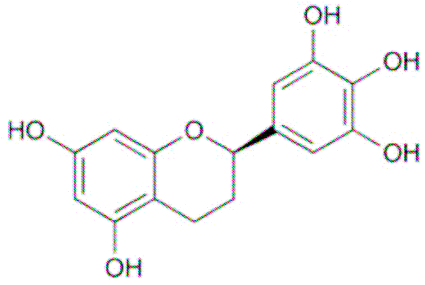
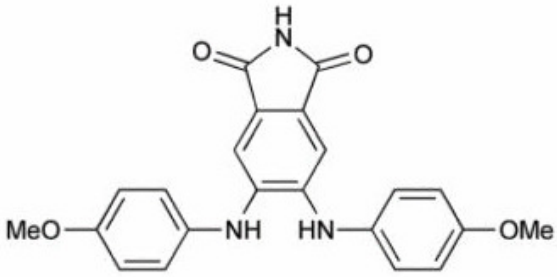
1.6 Bovine serum albumin:

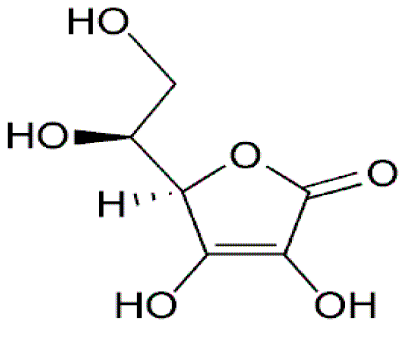
BSA is a serum albumin found in a biological subfamily Bovinae (domestic cattle, African buffalo, the water buffalo, the yak, and the four-horned antelopes etc). This protein is acidic, all alpha, highly soluble, multi-domain and characterized by high structural stability. Molecular weight of BSA is 66 KD. BSA is the single peptide chain of 583 amino acids. The secondary structure of BSA molecule is composed of 67% a l p h a -helix, no beta-sheet, 10% turn, and 23% extended chain.

1.7 INHIBITORS:

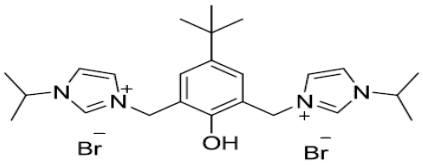
Inhibition of pathogenic protein aggregation may be an important and straight forward therapeutic strategy for curing amyloid diseases. Numerous phenolic compounds have been reported to have an inhibitory role on amyloid formation of proteins. No inhibitor known for [psi^{+}]

s.no.	Name of the compound	Structure

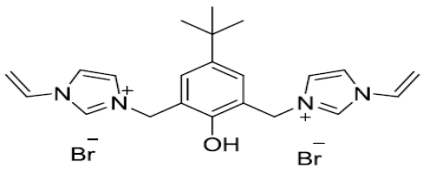
1	RESVERATROL	
2	EGCG	
3	EGC	
4	DAPH-12	

5	VITAMIN-C		

GPS-1 &GPS-2



Chemical Formula: $C_{24}H_{36}Br_2N_4O$
Molecular Weight: 556.3768



Chemical Formula: $C_{22}H_{28}Br_2N_4O$
Molecular Weight: 524.2919

2 MATERIALS AND METHODS:

2.1 MATERIALS:

bovine serum albumin (BSA),lysozyme,3-aminophenol,GPS-1,Congo red (CR), Thioflavin T (ThT), sodium salt, PBS(Ph-7.4), p1510 , 10mM Hcl, SDS, distilled water , Ni-NTA

2.2METHODS

2.2.1 PREPERATION OF BSA PROTEIN AND LYSOZYME AMYLOID AGGREGATES:

BSA and 50mM NaCl were dissolved in sodium phosphate buffer, pH 7.4 to a final concentration of 10-20 mg/ml and incubated at 65°C in a water bath for 6 hrs and 10 hrs respectively without agitation.

Incubate 10 mg/ml lysozyme in 10 mM HCl solution (pH 2.0) at 65 °C in a water bath for 24 hrs.

The growth of amyloid fibrils was monitored and characterized by ThT fluorescence and transmission electron microscopy.

2.2.2 AMYLOID CONFIRMATORY ASSAYS:

2.2.2(A) Congo red dye binding assay

BSA was dissolved with Congo red dye in phosphate buffered saline, pH 7.4 in 1:1 ratio and the mixture was incubated for 30 min at RT. The absorbance spectrum was measured from 250nm to 700nm in Molecular Devices M5e Spectra Max Multimode microplate reader (Holm et al., 2007) (Hawe et al., 2008).

2.2.2(B) Thioflavin T dye binding assay

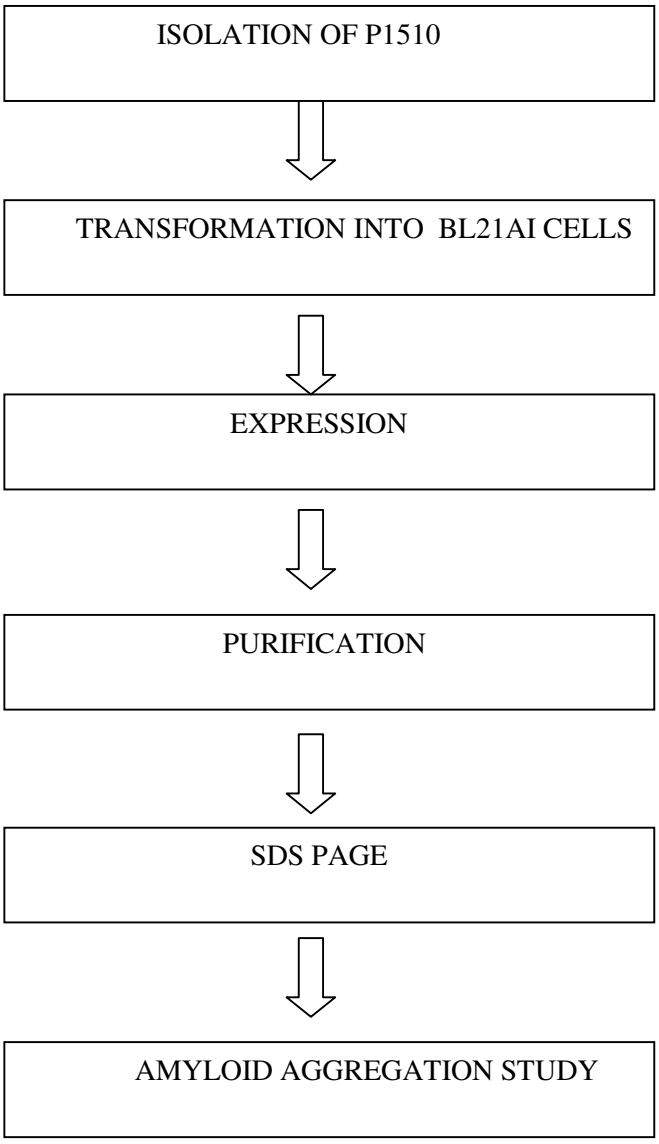
45µl BSA aggregate sample was mixed with 5µl ThT of 30 mM stock. Fluorescence was measured in a Molecular Devices M5e Spectra Max Multimode microplate reader with excitation and emission wavelengths of 450 and 485nm, respectively for endpoint reading. Excitation spectrum was measured by scanning the excitation wavelength from 250 nm to 470 nm and detecting emission at 495 nm. For emission spectrum, excitation wavelength is 442nm and scanned emission wavelength from 460nm to 560nm(LeVine, 1993).

2.3 INHIBITORY ASSAY:

To study inhibitory effect on BSA amyloid fibrillization triplicates of following samples were taken. 1) BSA aggregate sample with inhibitor 2) BSA aggregate sample without inhibitor 3)blank with inhibitor 4)blank without inhibitor

And add 5µl ThT of 30 mM stock mix with water to makeup volume. Fluorescence was measured in a Molecular Devices M5e Spectra Max Multimode microplate reader

2.4 procedure to find the inhibitor for p1510 rnq1 protein



2.4.1. ISOLATION OF P1510 (Rnq+ prion domain):

Alkaline lysis is a method used in molecular biology , to isolate plasmid DNA or other cell components such as proteins by breaking the cells open. Bacteria containing the plasmid of interest is first grown, and then allowed to lyse with an alkaline lysis buffer consisting of a detergent sodium dodecyl sulfate (SDS) and a strong base sodium hydroxide. The detergent

cleaves the phospholipid bilayer of membrane and the alkali denatures the proteins which are involved in maintaining the structure of the cell membrane. Through a series of steps involving agitation, precipitation, centrifugation, and the removal of supernatant, cellular debris is removed and the plasmid is isolated and purified.

procedure:

1. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
2. Add 250 µl Buffer P2 and gently invert the tube 4–6 times to mix.
3. Add 350 µl Buffer N3 and invert the tube immediately but gently 4–6 times.
4. Centrifuge for 10 min at 13,000 rpm in microcentrifuge.
5. Apply the supernatants to the QIAprep Spin Column by decanting or pipetting.
6. Centrifuge for 30–60 s. Discard the flow-through.
7. Wash the QIAprep Spin Column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.
8. Wash QIAprep Spin Column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.
9. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer
10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep Spin Column, let stand for 1 min, and centrifuge for 1 min.

2.4.2. TRANSORMATION INTO BL21AI CELLS:

Thaw one vial of BL21-AI cells on ice per transformation. Add 5–10 ng of DNA, in a volume of 1–5 µL to the cells and mix. Incubate the vial on ice for 30 minutes. Heat shock the cells by incubating the vial for 30 seconds – 1 minute in the 42°C water bath. Again incubate the vial on ice for 15 minutes then add 200 µL LB Broth. shake the vial at 37°C for 1 hour at 225 rpm. Centrifuge at 5000 rpm for 5 minutes. remove the supernatant and add 100 µL of distilled water and gently vortex. Plate two different volumes of the transformation reaction onto LB amp plates and spread with L-rod. and incubate at 37°C overnight. Select transformants from the plates and culture.

2.4.3. PROTEIN EXPRESSION:

The positive clone was transformed into BL21 (DE3) pLys cells in LB Agar media, with ampicillin (50µg/ul) and chloramphenicol (30µg/µl) and incubated at 37^0C at 250rpm for 13-14hrs.Picked 3 colonies from BL21 (DE3) pLysS transformed plate and inoculated into 3ml culture media by adding 50µg/ul of ampicillin, incubated at 37^0C at 250rpm for overnight which serve as a pre-inoculum. Took 5% preinoculum into fresh 3ml culture tubes with 50µg/ul of ampicillin and grow the bacterial culture till it reaches 0.5-0.6 OD at 600nm.Induce with 1mM IPTG and incubated at 37^0C at 250rpm for 4hrs. Loaded the samples in 12% SDS PolyAcrylamideGel with reference to protein ladder .

2.4.4. PROTEIN PURIFICATION:

Added 1% of pre-inoculum into 125ml LB media by adding 50µg/ul of ampicillin and grown the bacterial culture till OD reaches 0.5-0.6. Induced with 1mM IPTG and incubated at 37°C at 250rpm. Protein purification was performed by using Ni NTA column, in which cells were lysed in lysis buffer(50mMTris-Cl (pH8), 150mM NaCl, 20mM imidazole,1mM BME, 0.5% Triton-X100,1mM PMSF) followed by sonication and centrifuged at 14.8k rpm for 10 min. Added cell lysate to Ni NTA column and collected Flow through followed by adding wash buffer (50mMTris-cl (pH8), 150mM NaCl, 20mM imidazole) and collected wash which then followed by elution buffer and collected eluted fractions(50mMTris-cl (pH8), 150mM NaCl, 400mM imidazole) loaded on in 12% SDS Poly Acryl amide Gel with reference to protein ladder.

2.4.4. AMYLOID AGGREGATION STUDY:

Check the amyloid aggregation of above obtained protein by performing congo red dye binding assay and Th-T dye binding assay as mentioned above .

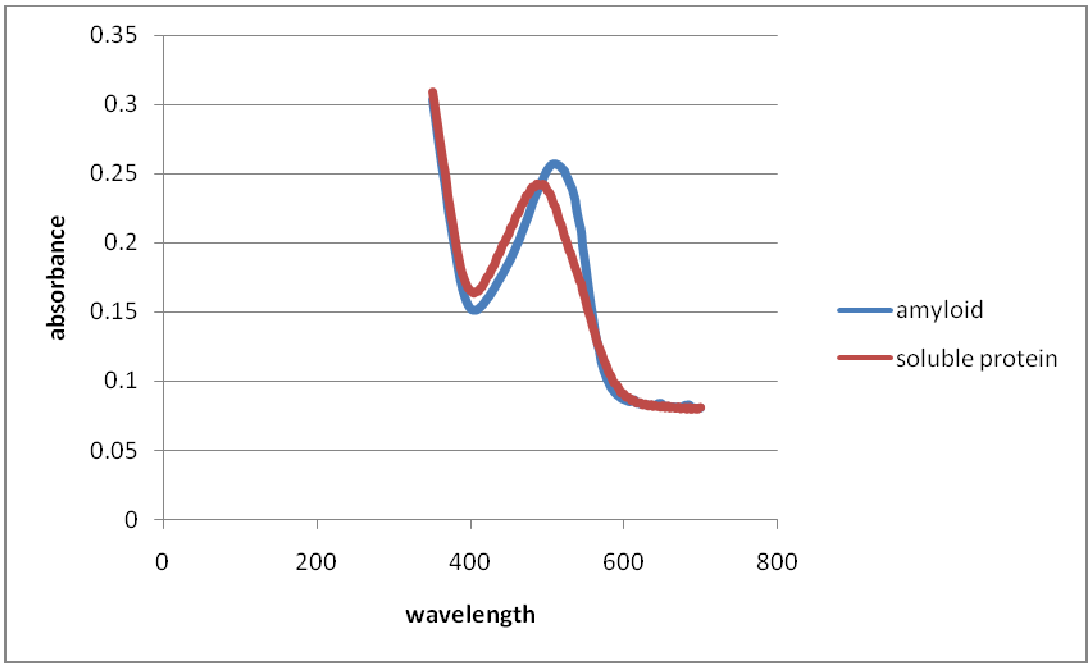
3 RESULTS AND DISCUSSION:

3.1 In vitro formation of BSA protein amyloid aggregates

BSA and 50mM NaCl were dissolved in 10mM sodium phosphate buffer pH 7.4 and the concentration of protein was 20 mg/ml. Fibrillization was induced at 65°C for 6 hrs and 10 hrs without shaking. After fibrillization some confirmatory assays were performed to confirm the formation of amyloid aggregates(Holm et al., 2007, Juarez et al., 2009).

3.2 BSA amyloid aggregation detected by Congo-Red dye binding Assay:

CR with amyloid shows a little red-shift in its absorption maximum (from around 490 to 540 nm) and high absorption at 540 nm . The CR+BSA amyloid aggregates shows red shift from 490 to 540 nm against only CR and high absorption near 540nm. These results are consistent with the presence of cross-beta structures in the BSA amyloid aggregates.



Absorption spectra of BSA fibrillization

3.3 BSA amyloid aggregation detected by Thioflavin T dye binding Assay:

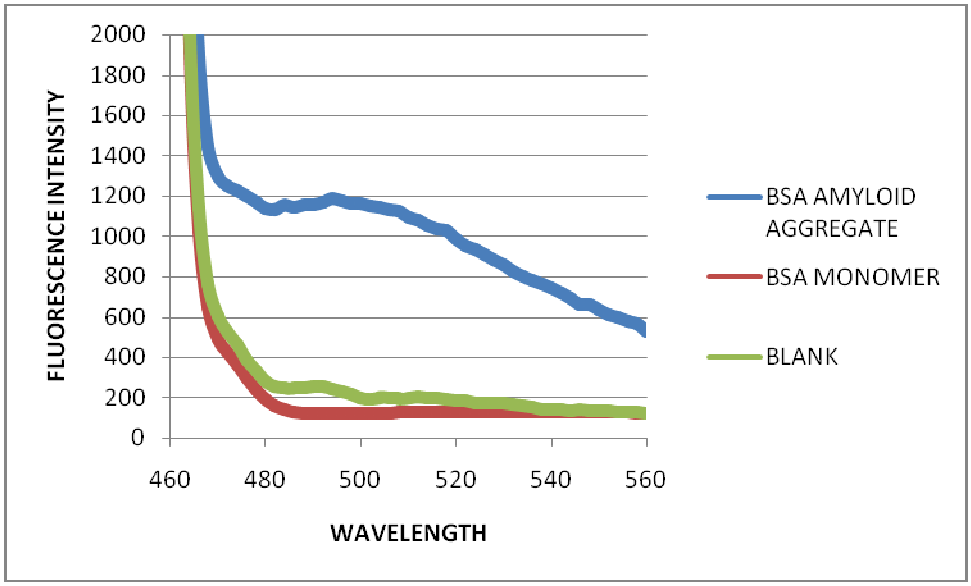
Fluorescence endpoint reading of BSA was measured at 450 nm excitation & 485 nm emission wavelength. The endpoint reading of BSA amyloid aggregates at 65°C showed 16.3 fold Thioflavin T fluorescence increase . This much fold increase in Th-T fluorescence is an indication of amyloid formation.

Thioflavin-T fluorescence end point reading at 450nm excitation and 485 nm emission

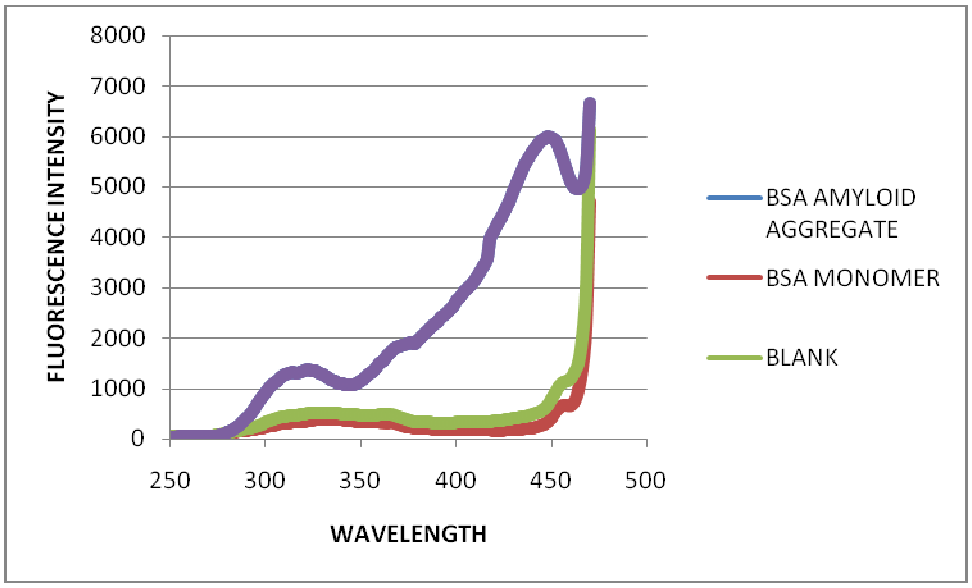
Blank (pH 7.4 buffer)	BSA amyloid aggregates	Fold increase
155.7	2537.9	16.3

3.3.1 Emission and Excitation spectra of BSA fibrillization:

Thioflavin-T binds with amyloid aggregates, and giving rise to excitation maximum at 450 nm and enhanced emission at 482nm while for free dye excitation maximum at 385 nm and emission maximum at 445 nm. BSA showed excitation maxima λmax at 442 nm and emission maxima λmax at 495 nm. This indicated the amyloid nature of the aggregated BSA .



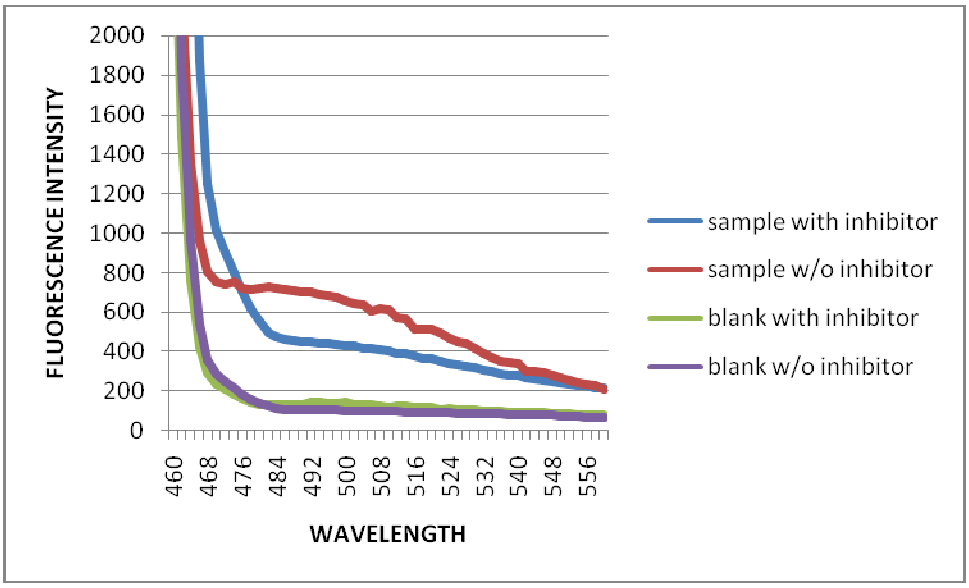
Emission spectra of BSA fibrillization



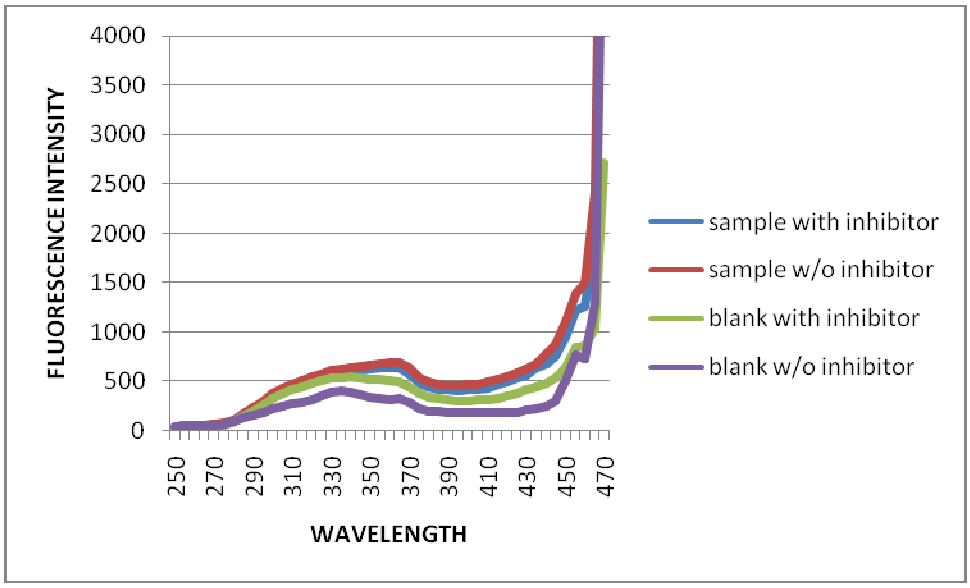
Excitation spectra of BSA fibrillization

3.4. The inhibitory effect of 3-aminophenol on lysozyme fibrillization:

Incubation of lysozyme under conditions of low pH and elevated temperature resulted in formation of amyloid fibrils. Increased temperature, ionic strength or lysozyme concentration significantly shortened the lag time of fibril growth. In the present study, we incubated 10 mg/ml lysozyme in 10 mM HCl solution (pH 2.0) at 65 °C in the presence or absence of an inhibitor(lysozyme:3-aminophenol 1:4). The growth of amyloid fibrils was monitored and characterized by ThT fluorescence and transmission electron microscopy.ThT is a fluorescent probe for monitoring the formation and growth of amyloid fibrils. The fluorescence intensity of ThT increased significantly when the compound specifically binding to the highly ordered β-sheet structure of amyloid fibrils . As depicted, an increase in ThT fluorescence emission was observed after incubating the control sample . The intensity of ThT fluorescence increased gradually as the amyloid formation progressed.

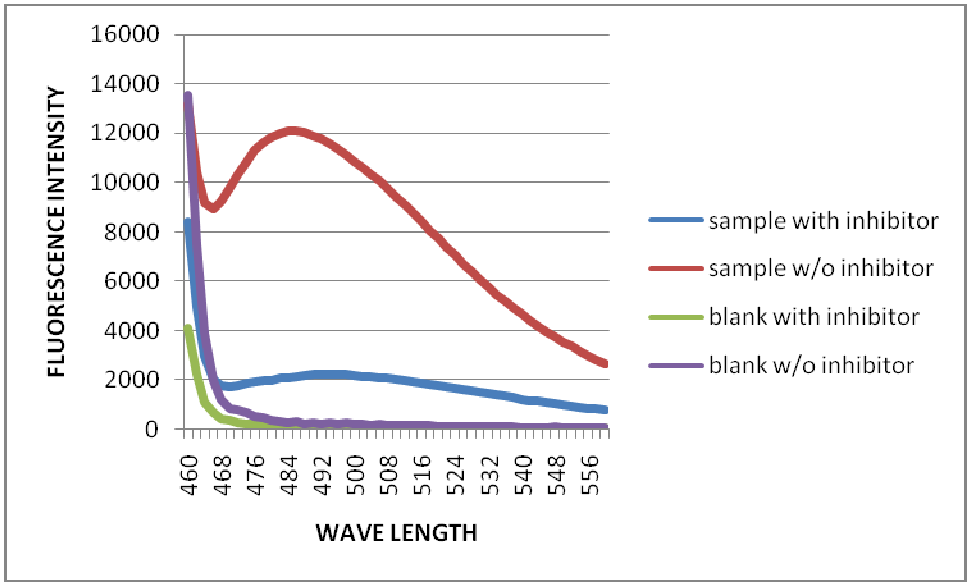


Emission spectrum at 0 hrs incubation

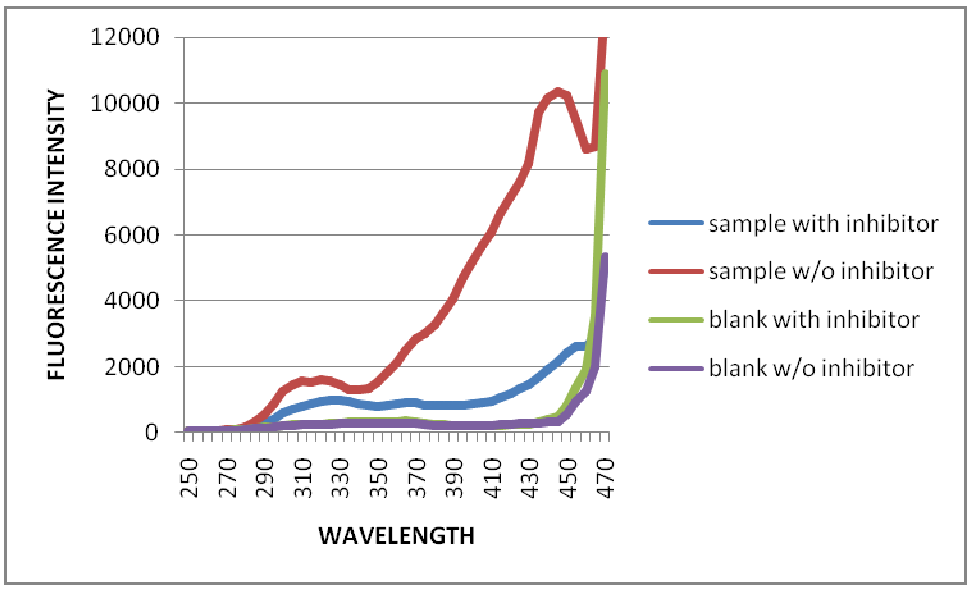


Excitation spectrum at 0 hrs incubation

AFTER 24 HRS INCUBATION:



Emission spectrum



Excitation spectrum

3-aminophenol showed significant inhibitory effect on lysozyme amyloid formation

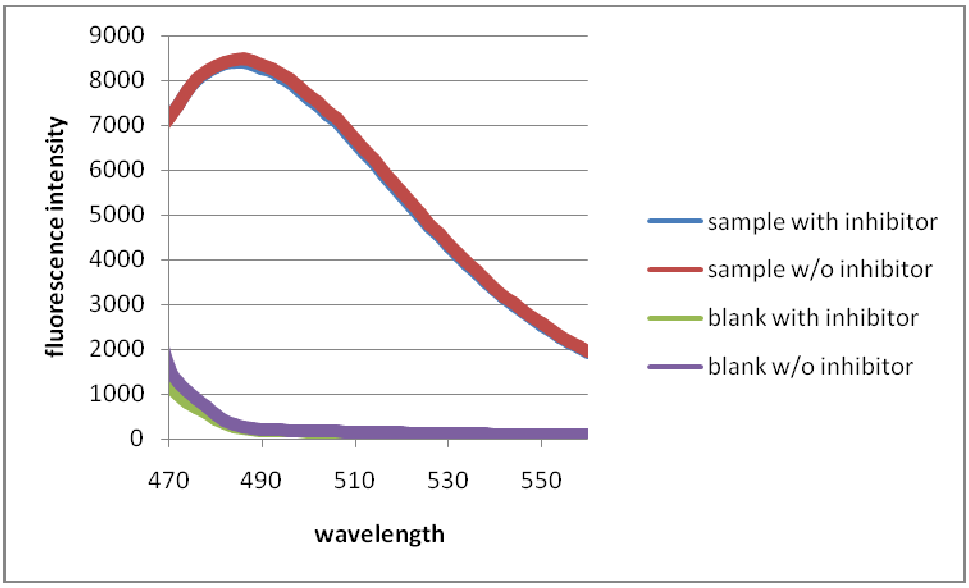
3.5 The inhibitory effect of 3-aminophenol on BSA fibrillization:

Various small molecule inhibitors of amyloid fibrillogenesis have been introduced. 3-aminophenol was studied class of such inhibitors, have been shown to inhibit lysozyme amyloid-forming systems. To determine whether that compound affect the process of BSA amyloid formation or not.

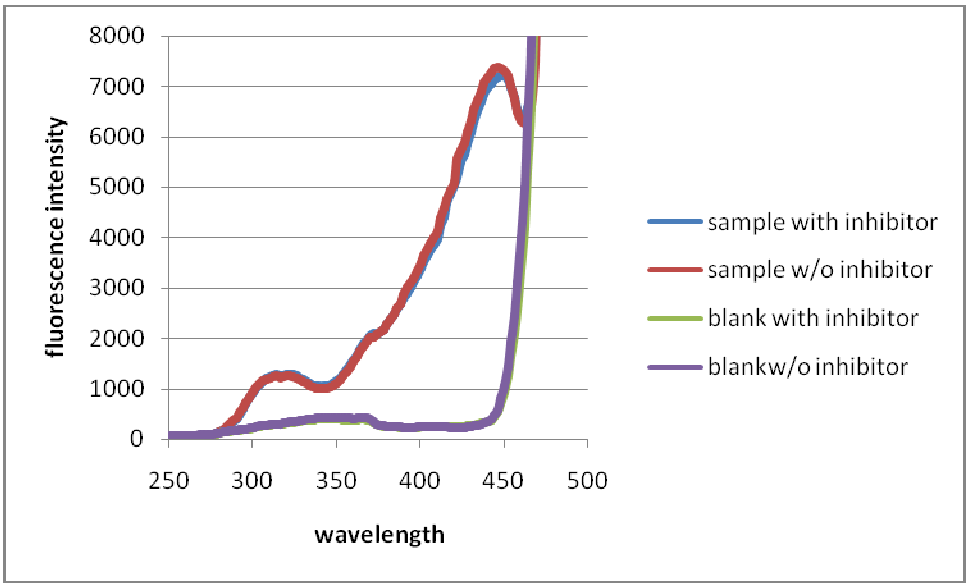
Incubation of BSA under conditions of pH 7.4 and elevated temperature resulted in formation of amyloid fibrils. Increased temperature, ionic strength or BSA concentration significantly shortened the lag time of fibril growth . In the present study, we incubated 10 mg/ml BSA in 10 mM PBS solution (pH 7.4) at 65 °C in the presence and absence of an inhibitor. The effective molar concentration ratio BSA to 3 aminophenol is 1:4. The growth of amyloid fibrils was monitored and characterized by ThT fluorescence. In the case of lysozyme, 3-aminophenol prevent the formation of lysozyme fibrillization

ThT is a fluorescent probe for monitoring the formation and growth of amyloid fibrils. The fluorescence intensity of ThT increased significantly when the compound specifically binding to the highly ordered β-sheet structure of amyloid fibrils. ThT fluorescence emission was observed after incubating the sample for 10 hrs.

ThT fluorescence emission and excitation specta profiles showed similar pattern in the cases of with and without inhibitor(3-aminophenol),indication that 3-aminophenol had no effect on BSA fibrillization.



Emission spectrum



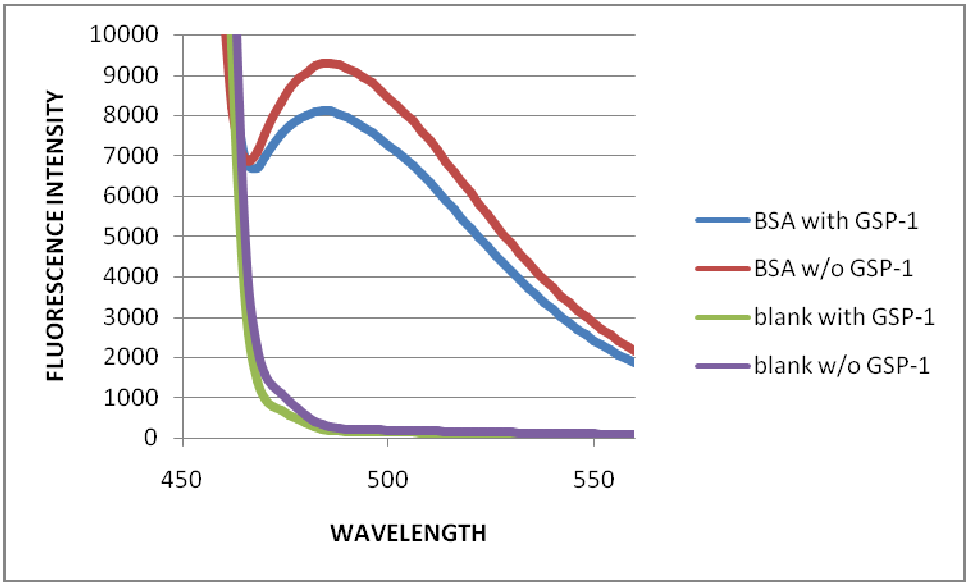
Excitation spectrum

3.6 The inhibitory effect of GPS-1 on BSA fibrillization:

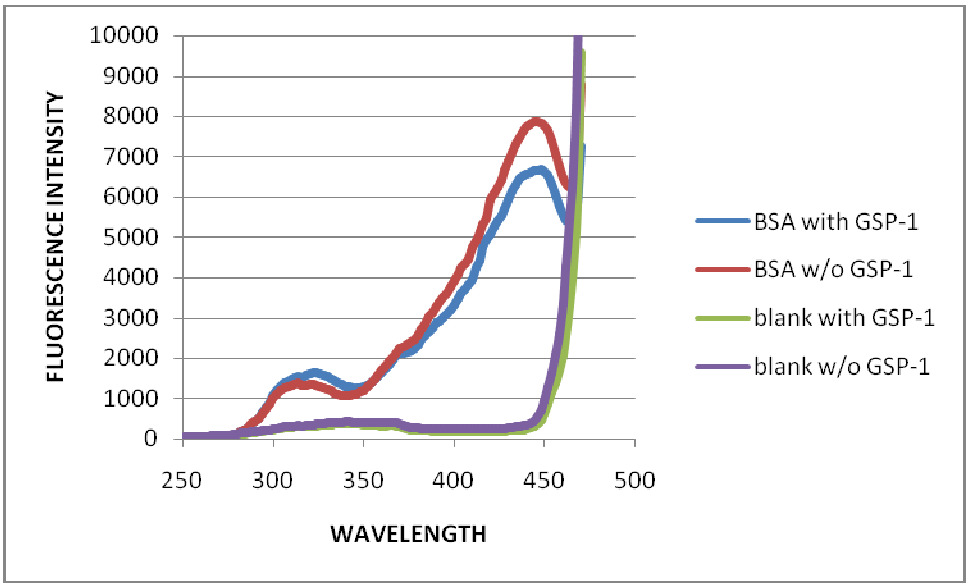
Incubation of BSA under conditions of pH 7.4 and at 65^{o}c temperature for 10 hrs resulted in formation of amyloid fibrils. In the present study, we incubated 10 mg/ml BSA in 10 mM PBS solution (pH 7.4) at 65 °C in the presence and absence of an inhibitor. The effective molar concentration ratio BSA to GPS-1 is 1:4. The growth of amyloid fibrils was monitored and

characterized by ThT fluorescence. ThT fluorescence spectra was observed after incubating the sample for 10 hrs.

ThT fluorescence emission and excitation profiles showed different pattern in the cases of with and without inhibitor unlike 3 aminophenol ,indication that GPS-1 has effect on BSA fibrillization.



Emission spectrum

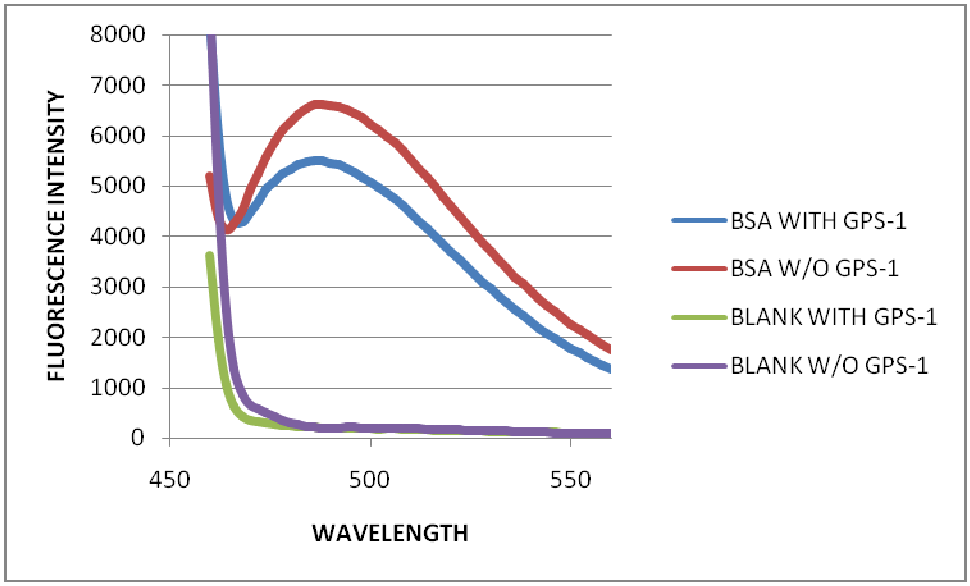


Excitation spectrum

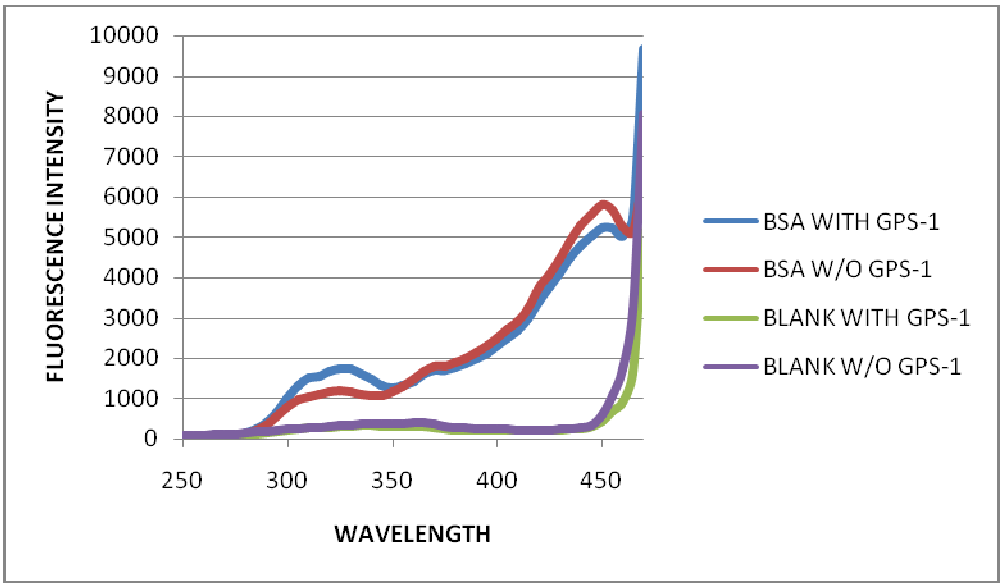
GPS-1 showed little significant inhibitory effect on BSA amyloid at dose 4:1 (inhibitor:BSA). So that if we can increase the molar concentration ratio , it may show inhibitory more effect on BSA amyloid fibrillization.

The molar concentrations has to be taken 1:10 , 1:20 , 1:50 , 1:100 (BSA:GPS-1) .

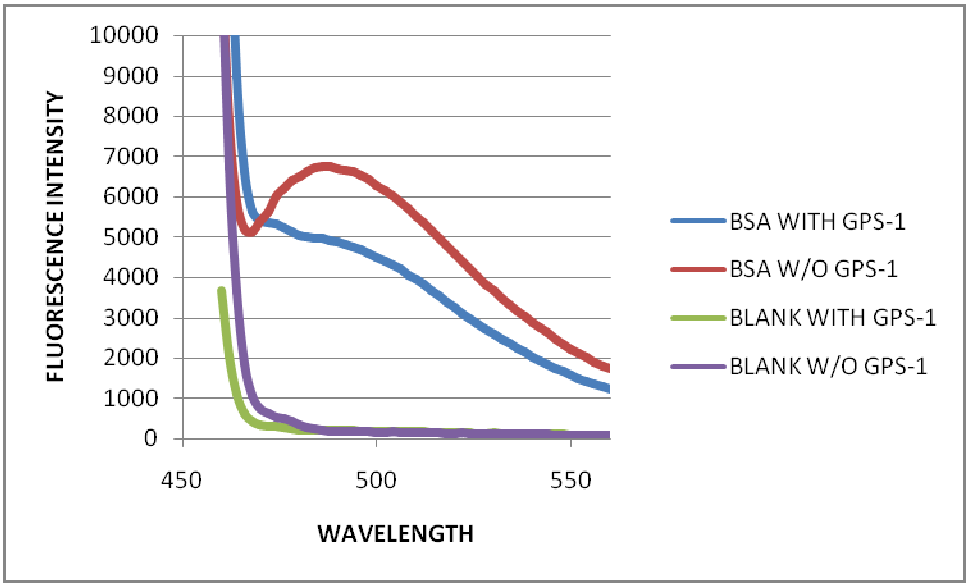
1:10



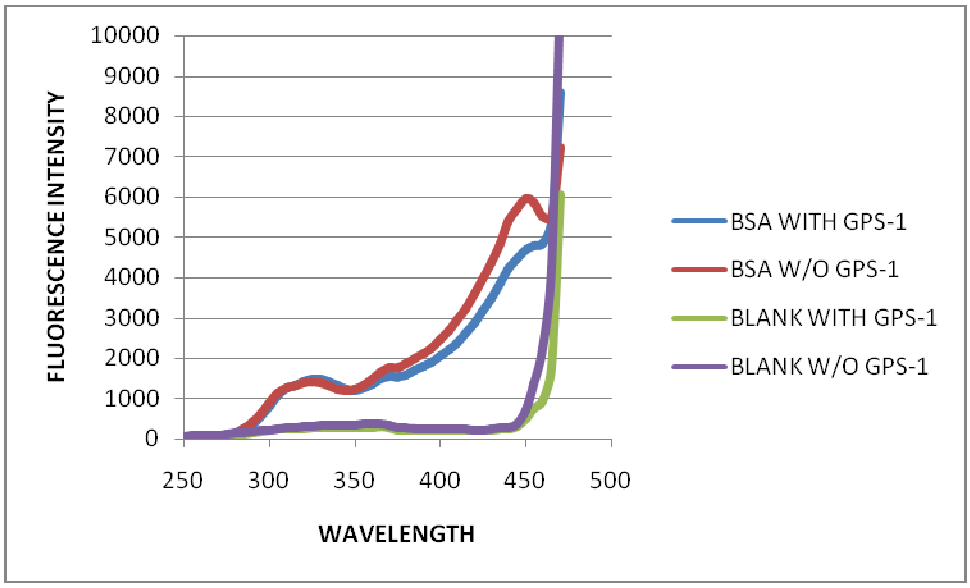
Emission spectrum (BSA:GPS-1=1:10)



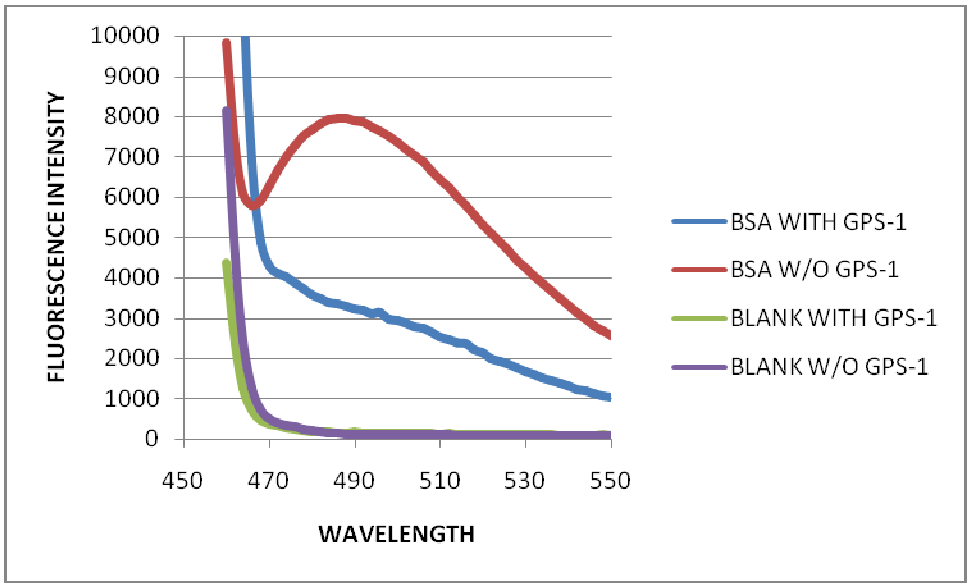
Excitation spectrum (BSA:GPS-1=1:10)



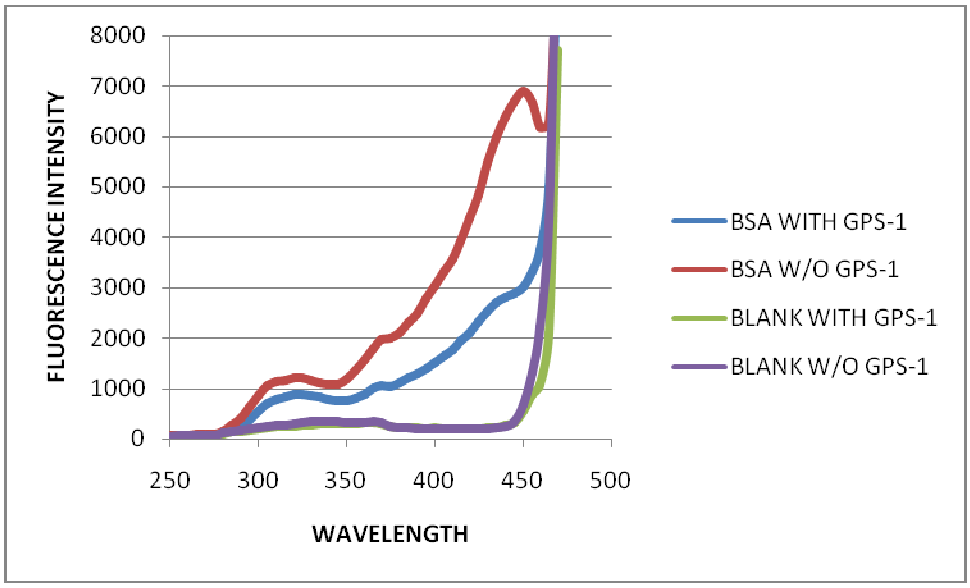
Emission spectrum (BSA:GPS-1=1:20)



Excitation spectrum (BSA:GPS-1=1:20)

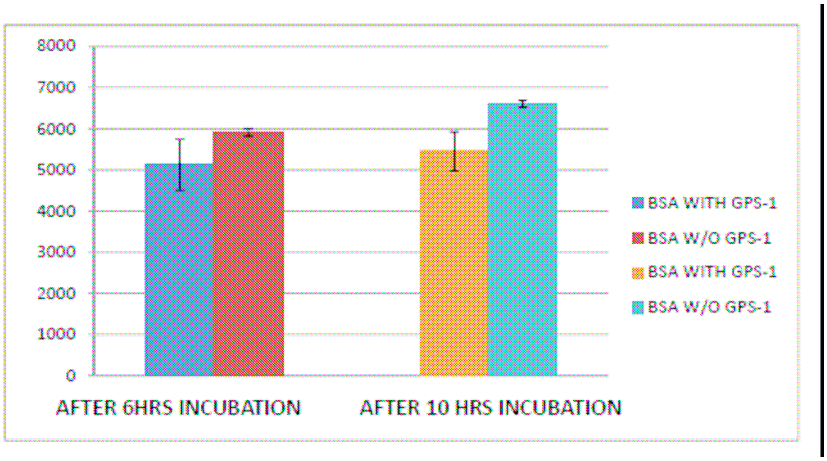


Emission spectrum (BSA:GPS-1=1:50)

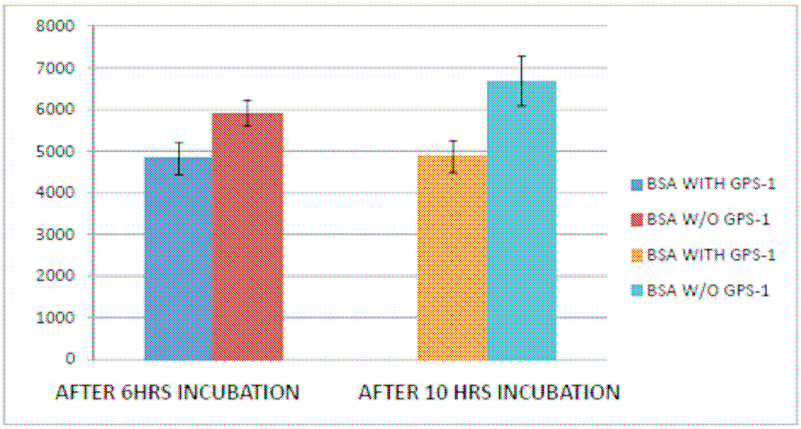


Excitation spectrum (BSA:GPS-1=1:50)

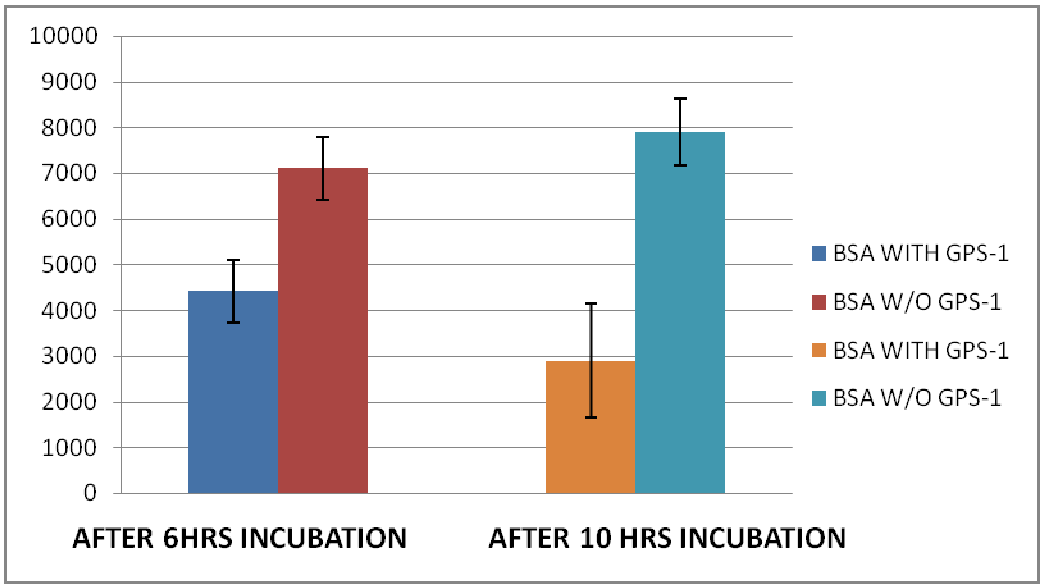
BSA:GPS-1=1:10



BSA:GPS-1=1:20



BSA:GPS-1=1:50

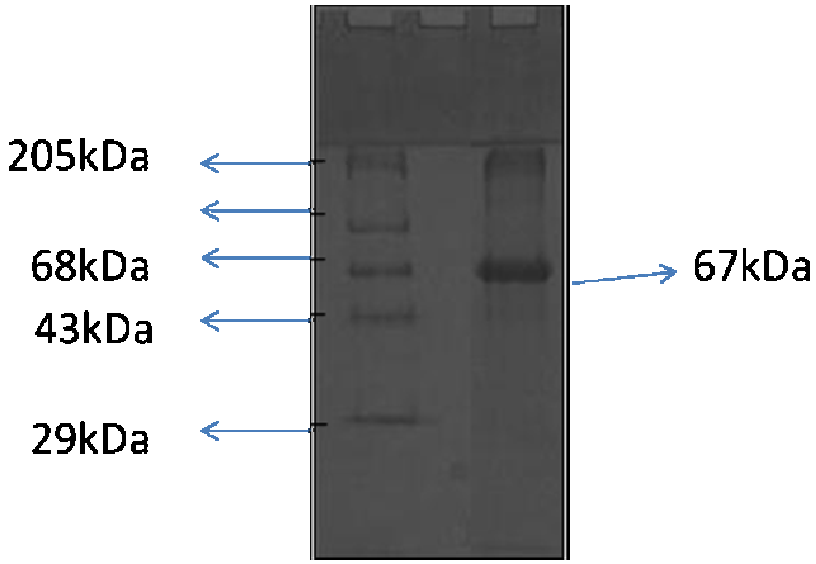


GPS-1 showed more significant inhibitory effect on BSA amyloid at dose 50:1 (inhibitor:BSA) compare to all other effective molar concentration ratios.

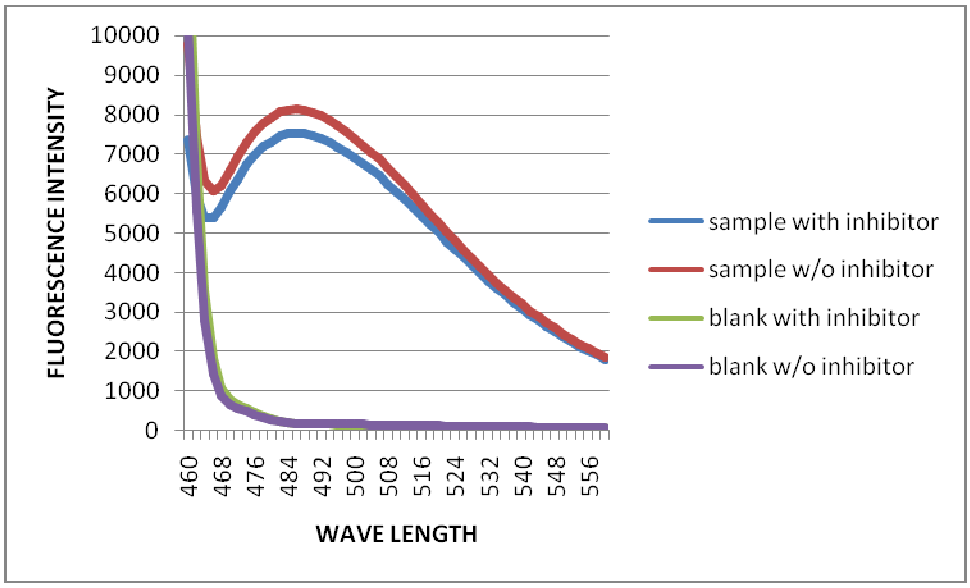
3.7. Finding inhibitor for p1510 rnq 1 prion protein:

After isolation of p1510 1 prion protein by mini-prep , which is transformed in to BL21AI cells. Protein expression was performed as mentioned in methods. Protein purification was performed

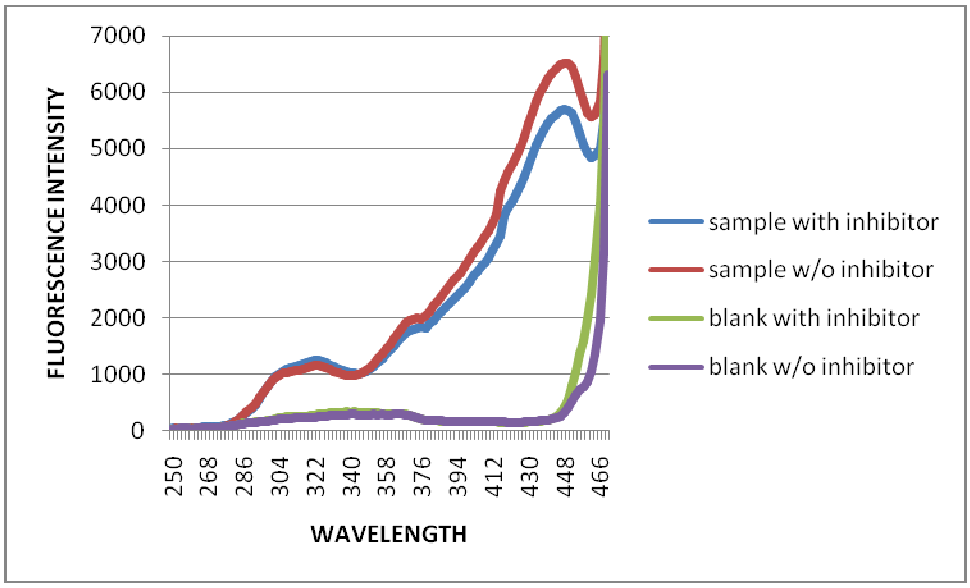
by using Ni NTA column in which cells were lysed in lysis buffer(50mMTris-Cl (pH8), 150mM NaCl, 20mM imidazole,1mM BME, 0.5% Triton-X100,1mM PMSF) followed by sonication and centrifuged at 14.8k rpm for 10 min. Added cell lysate to Ni NTA column and collected Flow through followed by adding wash buffer (50mMTris-cl (pH8), 150mM NaCl, 20mM imidazole) and collected wash which then followed by elution buffer and collected eluted fractions(50mMTris-cl (pH8), 150mM NaCl, 400mM imidazole) loaded on in 12% SDS Poly Acryl amide Gel with reference to protein ladder.



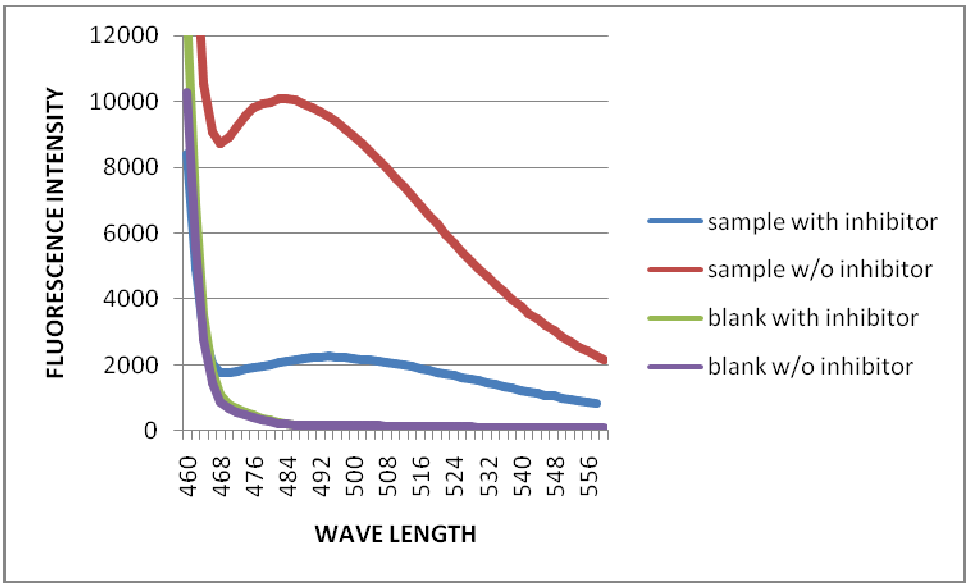
The molecular weight of the obtained protein is 67kDa. Inhibitory assay was performed after obtaining of protein. GPS-1 is the inhibitor which shows singnificant inhibitory action on p1510 rnq1 prion protein aggregates. The molar ratio of protein and GPS-1 is 1:10. mixed with 5µl ThT. Fluorescence was measured in a Molecular Devices M5e Spectra Max Multimode microplate reader with excitation and emission wavelengths of 450 and 485nm, respectively for endpoint reading. Excitation spectrum was measured by scanning the excitation wavelength from 250 nm to 470 nm and detecting emission at 495 nm. For emission spectrum, excitation wavelength is 442nm and scanned emission wavelength from 460nm to 560nm(LeVine, 1993).



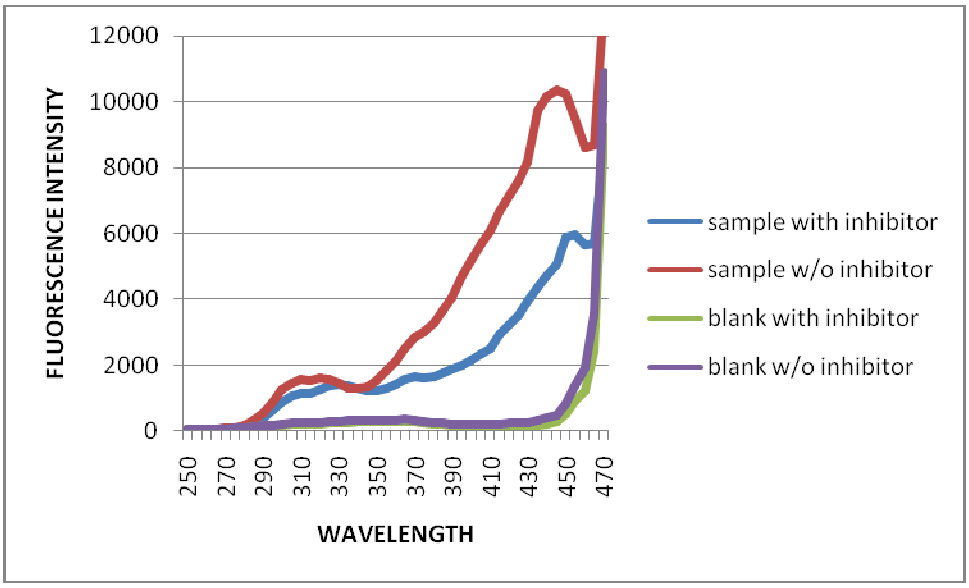
Emission spectrum after 6hrs of incubation



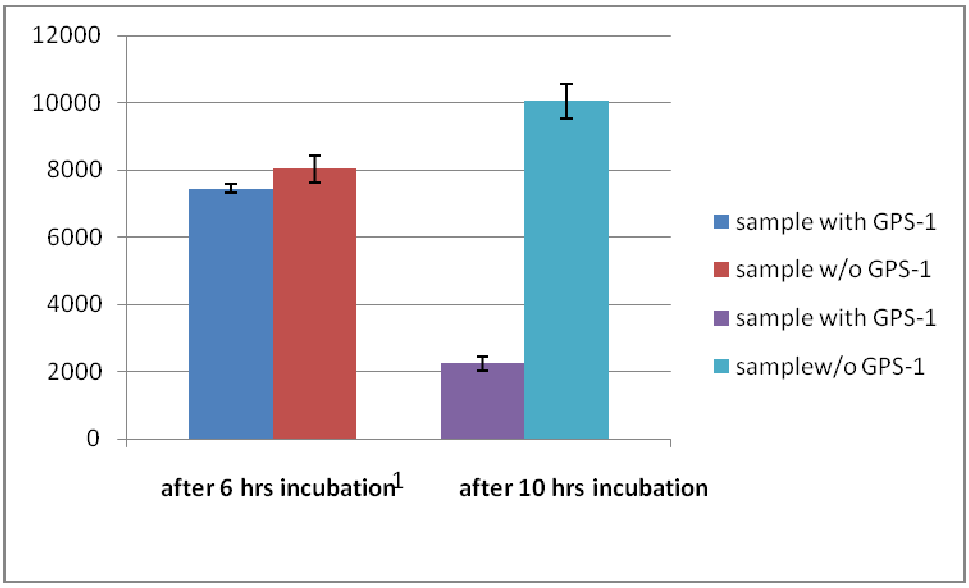
Excitation spectrum after 6 hrs of incubation



Emission spectrum after 10 hrs of incubation



Excitation spectrum after 10 hrs of incubation


12000
10000
8000
6000
4000
2000
0
after 6 hrs incubation1
after 10 hrs incubation
sample with GPS-1
sample w/o GPS-1
sample with GPS-1
samplew/o GPS-1

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