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**Identification and Characterization of an Insulin
Based Inhibitor of Amylin and A β 40 Amyloid
Formation**

A Thesis Presented

by

Trisha Barua

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Chemistry

Stony Brook University

August 2010

Stony Brook University

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Abstract of Thesis

**Identification and Characterization of an Insulin Based Inhibitor of Amylin and
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2010

The formation of amyloid fibrils plays a role in a wide range of diseases collectively known as amyloidosis. Amyloid fibrils are formed when peptides or proteins misfold and deposit in tissues and organs. These deposits are a characteristic feature of a number of diseases including Type II diabetes, Alzheimer's disease, and Parkinson's disease. Our lab studies the amyloidogenic peptides Islet Amyloid Polypeptide (IAPP) from type II diabetes and the A β 40 and A β 42 peptides from Alzheimer's disease. There is intriguing, albeit indirect, evidence that suggests that inhibitors of IAPP amyloid formation may also inhibit amyloid formation by A β . If true, this suggests a new strategy for designing inhibitors; specifically inhibitors of amyloid formation by one peptide can be used against the other.

Insulin is known to be an inhibitor of IAPP. It is made up of an A and B chain and the B chain displays potent inhibitory effects. In addition, a short segment located in the B chain has been showed to bind IAPP and thus might inhibit amyloid formation. Based on these findings, experiments were conducted to test the possible inhibitory effects of insulin, the insulin B chain, and a short fragment from the insulin B chain on A β 40 and IAPP.

Solid phase peptide synthesis was used to synthesize the A β 40 peptide and a 12-residue fragment of the insulin B chain (IBSF). A new protocol was developed and employed to obtain the kinetic curve of A β 40 amyloid formation using Thioflavin-T binding fluorescence assays. A β 40 was incubated with insulin, the insulin B chain, and IBSF. Transmission electron microscopy was used to observe what had occurred during the incubation and confirm the results of the fluorescence assays. Similar experiments with IAPP were done as well, however, the rate of fibrillization was found to be much slower. This suggested that there is indeed an interaction between these two peptides. Thus, concentration dependent studies and further experimentation were done to better observe and assess these interactions.

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List of Abbreviations

μ L, microliter; μ M, micromolar, A β 40, Amyloid beta 1-40; A β , Amyloid beta 1-42; APP, Amyloid Precursor Protein; Cys, Cysteine; Da, Dalton; DDI, distilled deionized water; DMSO, dimethyl sulfoxide; *et al.*, et alii; Fmoc, fluorenylmethyloxycarbonyl; Glu, Glutamic acid; HCl, hydrochloric acid; HFIP, hexafluoroisopropanol; His, Histidine; IAPP, Islet Amyloid Polypeptide; IBSF, corresponding to residues 9 to 20 of the insulin B chain with Cys19 mutated to Ser; MALDI-TOF, Matrix-assisted laser desorption/ionization-time of flight; mM, millimolar; nm, nanometer; PAL-PEG; p-cyanoPhe, p-cyanoPhenylalanine; 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid polyethylene glycol; TEM, Transmission electron microscopy; TFA, trifluoroacetic acid; Tyr, Tyrosine; UV-VIS, Ultraviolet/Visible; v/v, volume by volume

Chapter 1: Introduction to Amyloid Formation

1.1 Amyloid

Amyloidosis is a pathological state where there is a formation of extracellular amyloid deposits, caused by the conversion of peptides or proteins from their soluble functional states to highly organized fibrillar structures. These insoluble, toxic structures then deposit onto cells and tissues giving rise to diseases (1). There are a number of diseases that fall under the category of amyloidosis. These make up the largest group of misfolding protein diseases. Each consists of their own characteristic protein that forms amyloid fibrils or plaques. Examples of these diseases include Type II Diabetes, Alzheimer's disease, Parkinson's disease, and Huntington's disease.

These amyloid forming proteins do not share any sequence or structural homology. However, the structure of their amyloid fibrils is similar, forming a characteristic canonical crossed β -sheet structure (2). All amyloidogenic peptides form oligomers, which form pre-fibrillar structures called protofilaments that twist to form ropelike fibrils. Using transmission electron microscopy and atomic force microscopy, these fibrils are assessed to be around 7-13 nm wide. (Fig. 1.1) The term "crossed β -sheet" refers to the position of the protein or peptide arrangement. It runs perpendicular to the axis of the fibril in a polypeptide, β - sheet form. (Fig. 1.2)

Amyloid fibril formation has been well established as following a nucleated growth mechanism. The time course of fibril formation comprises of a lag phase, where the nucleus is supposed to form. This is followed by an exponential growth

phase, which is thought to be due to the rapid formation of fibrils from the association of monomers and oligomers with the nucleus. The end of the fibril forming process is known as the plateau phase where most or all possible fibrils have formed and is the state where the fibrils are in equilibrium with soluble protein. (2)

1.2 Type 2 Diabetes Mellitus and Amylin

In Type II diabetes, the body builds a resistance to insulin and thus does not respond when insulin is present in the blood. Islet Amyloid Polypeptide (IAPP), also called Amylin, is the amyloid forming peptide that deposits in the pancreas in Type II diabetes. The deposits or the process of their formation are toxic to the β -cells and are believed to contribute to the later stages of type II diabetes. There is also growing evidence that islet amyloid contributes to graft failure in islet transplantation. Amylin co-localizes with insulin in the β -cell secretory granules of the human pancreatic islets. It is thought to play some regulatory roles in blood glucose level (3). The structure of Amylin consists of 37 amino acid residues with a disulfide bond and an amidated C-terminus. (Fig. 1.3)

Amylin is, obviously, not the cause of type II diabetes, but it is present in fibrillar form in pancreatic β -cells. There is much research into understanding what induces the aggregation of this peptide as well as how this affects patients. Thus far, a common hypothesis is that Amylin forms an intermediate helical, secondary structure that ends up forming β sheet structure. There are regions within the Amylin sequence that have been characterized as major players in forming these organized fibrillar structures (4-7). Residues 20 to 29 has been acknowledged as one of the

amyloidogenic regions within Amylin and is believed to play a key role in amyloid formation. However, the sequence between residues 20 to 29 cannot be the only factor, which determines the ability to form amyloid. For example, our lab has shown that substitutions outside of the core 20 to 20 region can prevent amyloid formation (8), while it is known that dog and cat Amylin have an identical sequence but one species forms islet amyloid and the other does not.

1.3 Alzheimer's Disease A β

The amyloid β -protein (A β) is the amyloid forming peptide in Alzheimer's disease. There are isoforms of this peptide that are the most common. One is A β 40 and is 40 residues in length (Fig. 1.4) and the other is A β 42 and thus is 42 residues in length. These peptides form extracellular fibrillar structures and deposits in neuritic plaques. A β 42 is the more aggressive type since it is the slightly longer and more hydrophobic. Although more of A β 40 is produced, neuritic plaques contain more of fibrillar A β 42.

Both of these peptides are products of a sequential proteolytic cleavage of amyloid precursor protein (APP) (9). APP is a transmembrane glycoprotein whose function is not yet fully understood. APP can be cleaved by α -, β - and γ -secretases. β secretase and γ secretase cleaves APP and can form a number of isoforms of 39-43 amino acid residues in length. (10)

1.4 Figures

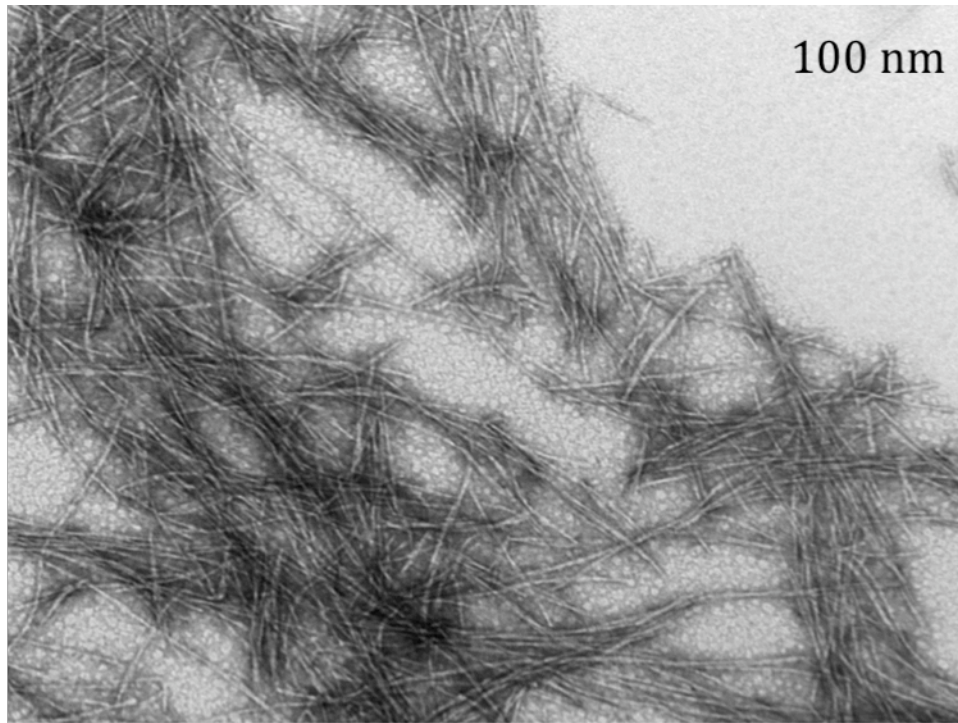


Figure 1.1: Transmission Electron Microscopy image of A β 40 amyloid fibrils formed *in vitro*.



Figure 1.2: A schematic representation of a cross β sheet structure. Each line represents a β strand that is lining up perpendicular to the fibril axis. The intrastrand hydrogen bonds are parallel to the fiber axis (Chiti, F., & Dobson, C. M. (2006) *Annu Rev Biochem* 75:333-366)

1	10	20	30
KCNTATCAT	QRLANFLVHS	SNNFGAILSS	TNVGSNTY

Figure 1.3: Primary sequence of IAPP (Amylin). There is a disulfide bond between Cys 2 and Cys 7.

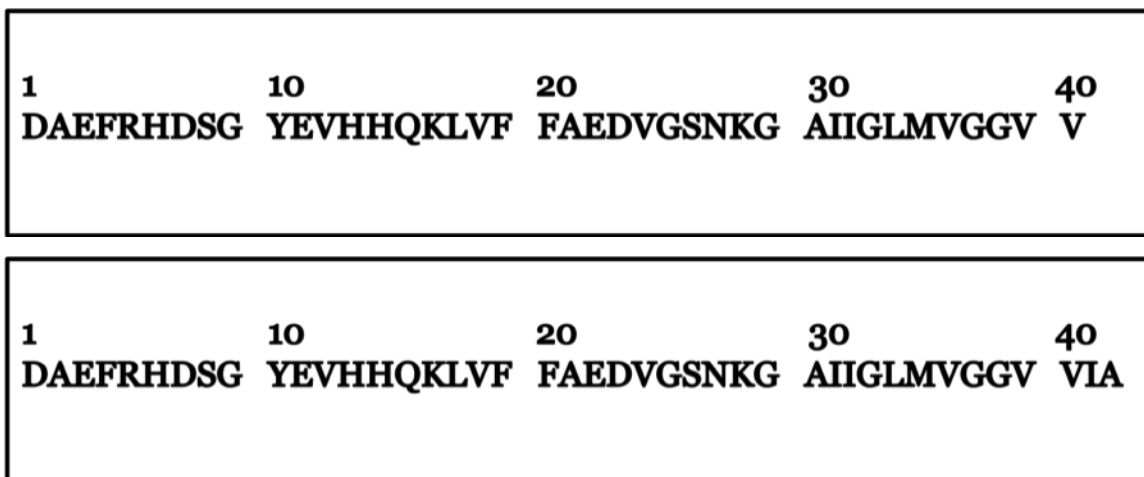


Figure 1.4: Primary sequence of A β 40 (top) and A β 42 (bottom).

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Chapter 2: The Inhibitory Effects of Insulin, the Insulin B Chain and the Insulin B Small Fragment on A β 40 Amyloid Formation

2.1 Introduction

Our lab studies the amyloidogenic properties of certain peptides, including Amylin and A β , and tests different types of potential inhibitors of fibril formation. Studies of one of these peptides can provide insight about how to approach studying the others. Since most of these peptides form the same amyloid structure, it can be helpful to use similar experiments to study multiple amyloidogenic proteins or peptides. That is the approach we took towards A β .

Both Amylin and A β share at least one common inhibitor. In addition, their sequences are 25% identical and 50% similar (1). This led to us to propose that inhibitors of IAPP fibril formation might be possible inhibitors of A β fibril formation. On top of this, there are recent reports that insulin protects neurons from the effects of the A β peptide, suggesting an interaction between A β and IAPP. Knowing this and knowing that insulin is a good inhibitor of IAPP fibril formation, we hypothesized that insulin might also inhibit amyloid formation by A β .

I also studied a 12- residue fragment derived from the insulin B-chain. The complete B-chain of insulin, which is 30 residues, has been shown to inhibit amyloid formation by IAPP. Studies from other labs have shown that the major region of interaction involves the region near residues 9 through 20 of the B-chain. It is thought to interact with residue 7 to 19 of Amylin (2). I prepared a peptide corresponding to residues 9 to 20 of the B chain with a Ser in place of the Cys in position 19 and tested

its ability to inhibit amyloid. The goal of this work is to develop a smaller inhibitor of amyloid formation, which targets the N-terminal half of Amylin and which might also inhibit A β amyloid formation. The peptide is denoted IBSF.

2.2 Materials and Methods

2.2.1 Protein Synthesis and Purification

Human A β 40, and IBSF was synthesized on a 0.25 mmol scale using 9-fluornylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 433A. A valine coupled Wang resin was used for A β 40 to generate a free C terminus and PAL-Peg-PS resins were used for IBSF to generate an amidated C terminus. Pseudoproline dipeptide derivatives were used in the synthesis of A β 40 for better technique (3). The pseudoprolines Ser-Gly and Ser-Asp were used to replace residues 25-26 and 7-8, respectively. Fmoc-protected pseudoproline (oxazolidine) dipeptide derivatives were purchased from Novabiochem. All the other reagents were purchased from Advanced Chemtech, Fischer Scientific, PE Biosystems, and Sigma Aldrich. All solvents used were of A.C.S. grade. Standard Fmoc reaction cycles were used. The first residue added to the resin, pseudoproline dipeptide derivatives, all β -branched residues and all residues following the β -branched residues were double coupled. The peptides were cleaved from the resins using standard trifluoroacetic acid (TFA) methods.

The crude peptides was dissolved in 20% (v/v) acetic acid and lyophilized. The peptides were then dissolved in DMSO and purified using reverse-phase HPLC using Vydac C-18 preparative column. A two-buffered system using HCl as an ion-

pairing agent was used. Buffer A contained 0.045% (v/v) HCl in distilled de-ionized (DDI) water. Buffer B contained 80% (v/v) acetonitrile, 20% (v/v) DDI water and 0.045% (v/v) HCl. The gradient used was 0-90% buffer B in 90 minutes with a flow rate of 10 ml/min. The A β 40 peptide eluted near 42 % buffer B and the IBSF peptide near 33% buffer B. The purified peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy. The expected molecular mass of IBSF is 1287.4 Da and A β 40 is 4329.8 Da. The observed mass for IBSF was 1309.886 and A β 40 was 4332.003. The extra 22.5 Da on the IBSF observed mass is due to Na⁺, which is 23 Da.

2.2.2 Thioflavin-T binding Fluorescence Assays

The protocol for A β 40 fibril formation was developed by Dr. Ruchi Gupta. The dry A β 40 was weighed and 0.4-0.5 mg was added to 200 μ l of DDI water. The sample was then vortexed for 20 sec. and 200 μ l of 200 mM of Tris buffer at pH 7.4 was added. The solution was vortexed for 20 sec. more and then centrifuged at 17,200 g for 3.5 min. The supernatant was removed and its concentration was calculated using UV-Vis at 280 nm. Then, the supernatant was dissolved in 100 mM Tris buffer at pH 7.4 to give a final concentration of between 130-150 μ M. The solution was poured into a cuvette and stirred by a stir bar. Aliquots were withdrawn every 1-1.5 hour and diluted into 100 μ M Thio-T in 100 mM Tris buffer at pH 7.4 to give a final concentration of 15 μ M. The emission scan of the aliquot was recorded from 462 nm to 600 nm with an Applied Photon Technologies fluorimeter. The intensity of thioflavin-T fluorescence at 485 nm was plotted vs time to obtain kinetic curves. For experiments with insulin, insulin B chain, and IBSF, peptides were weighed and

dissolved in 100 mM Tris buffer and added to the solution mixture at the same concentration as A β 40. Concentrations were measured by UV-Vis at 280 nm.

2.2.3 Transmission Electron Microscopy

TEM was performed at the Microscopy imaging center at the State University of New York at Stony Brook. Samples were prepared by placing 15 μ l of the mixtures onto formvar coated 300 mesh copper grids and counterstained with 2% aqueous uranyl acetate. The grids were viewed with a FEI Technai12 BioTwinG² transmission electron microscope at 80 kV. Digital images were recorded with an AMT XR-60 CD Digital Camera System.

2.3 Results

There have been many claims in the literature about a possible role for insulin in Alzheimer's disease and there have been suggestions that insulin may physically interact with the A β peptide (4). One way we were able to test this was to test the possible inhibition of A β amyloid formation by insulin.

Initial experiments consisted of synthesizing the necessary peptides, IBSF and A β 40. The IBSF peptide correspond to residues 9 to 20 of the insulin B chain with Cys19 replaced by Ser. Figure 2.1 shows the HPLC trace of the crude peptide after synthesis and cleavage. It was relatively easy to separate the major peak, which confirmed to be the correct peptide (Fig. 2.2). The purity of the peptide collected can be judged by examining the analytical HPLC trace of the purified product (Fig. 2.3).

The synthesis of 40-residue peptide, A β 40 was conducted. The HPLC trace (Fig. 2.4) of the crude peptide showed good results. The major peak was collected

and mass spectra confirmed that it has the correct molecular weight (Fig. 2.5). The purity of the peptide was confirmed by analytical HPLC of the purified material (Fig. 2.6). Thioflavin-T fluorescence experiments on A β 40 were conducted using a new protocol developed by my mentor, Dr. Ruchi Gupta. This protocol shortened the time it took for A β 40 to form fibrils *in vitro*. Typically, it took about 5-11 hours for full fibril formation. The time varied based on how careful the preliminary steps were conducted. Too much time used or too much to prepare the mixture for thioflavin-T scans initiate aggregation early. Therefore, sometimes, the fibrils would be formed by only 5 hours or by 11 hours. All the experiments were, however, monitored for about 25-30 hours.

Thioflavin-T is a dye that is able to bind to fibrils (5). The more fibrils that are formed, the more thioflavin-T that binds. In a normal kinetic experiment scan, there is a lag phase, a growth phase, and a plateau. During the lag phase, there is an initiation of fibril formation. The growth phase involves increased fibril formation. When fibrils are formed the curve comes to a plateau and becomes gradually constant. In this stage insoluble fibers are in equilibrium with soluble peptide. TEM images were taken to confirm the results of the kinetic assays. This is important because thioflavin-T binding studies can sometimes give misleading results. (6)

Figure 2.7 shows a kinetic curve for A β 40 in the presence and absence of IBSF. Both curves are very similar and the results show that IBSF does not bind inhibit A β 40 amyloid formation. The results were confirmed by TEM (Fig. 2.8). Dense mats of fibrils were observed in the image of the A β 40 sample. In the presence of insulin, the same results were observed (Fig. 2.9). Insulin did not bind A β 40. This

was confirmed by TEM (Fig 2.10). Figure 2.11 shows the kinetic curve for A β 40 with and without insulin B chain. There was no inhibition of amyloid formation. The results are confirmed with TEM images (Fig. 2.12).

2.4 Discussion

The results indicate that if there are interactions between insulin and A β 40, then they do not inhibit amyloid formation. This argues against a direct interaction of insulin and A β 40 *in vivo*. The experiments also showed that not all inhibitors of Amylin inhibit A β . Although both Amylin and A β share some common inhibitors, it did not prove to be true in this case.

2.5 Figures

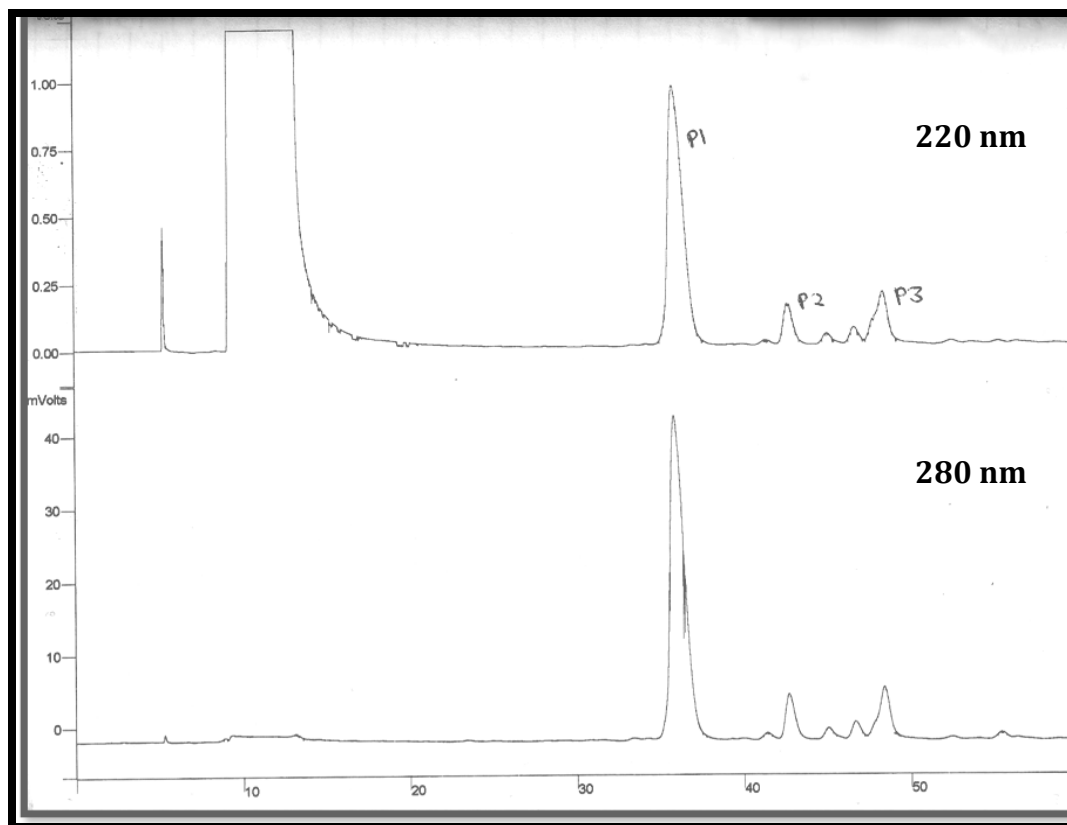


Figure 2.1: HPLC trace of crude IBSF using a gradient of 0-90% buffer B in 90 minutes with a C18 preparative column.

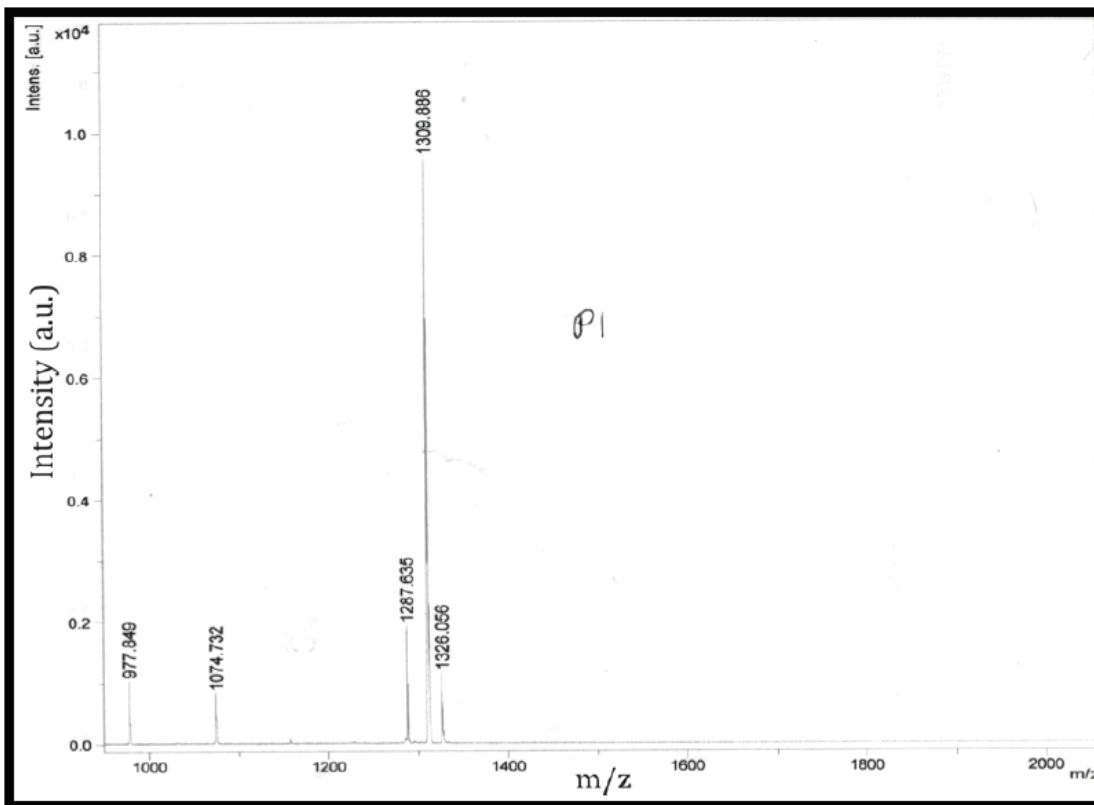


Figure 2.2: MALDI-TOF of purified IBSF. The observed molecular weight is 1310.03 Daltons. The theoretical weight is 1287.4 Daltons. The additional 22.6 Daltons is due to Na^+ (23 Da).

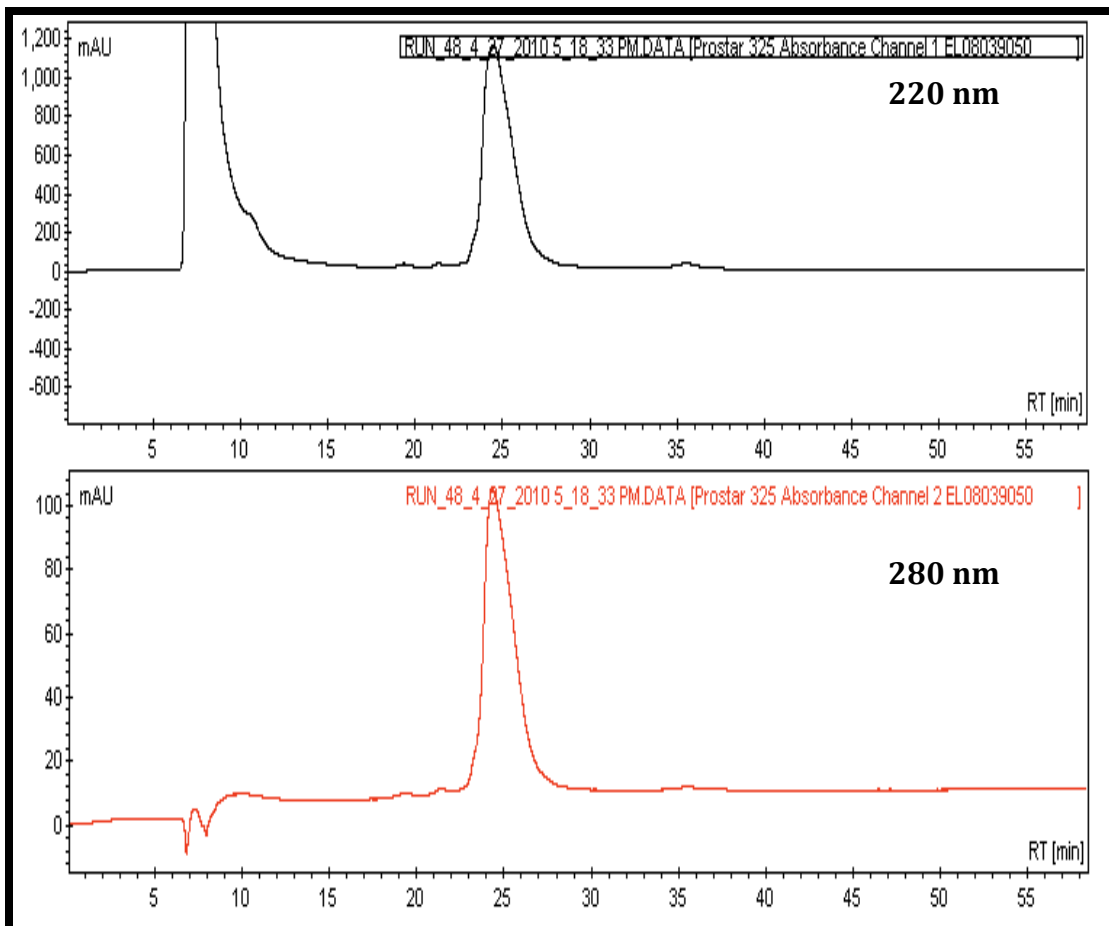


Figure 2.3: Analytical HPLC trace of purified IBSF using a gradient of 25-75% B in 50 minutes with a C18 analytical column.

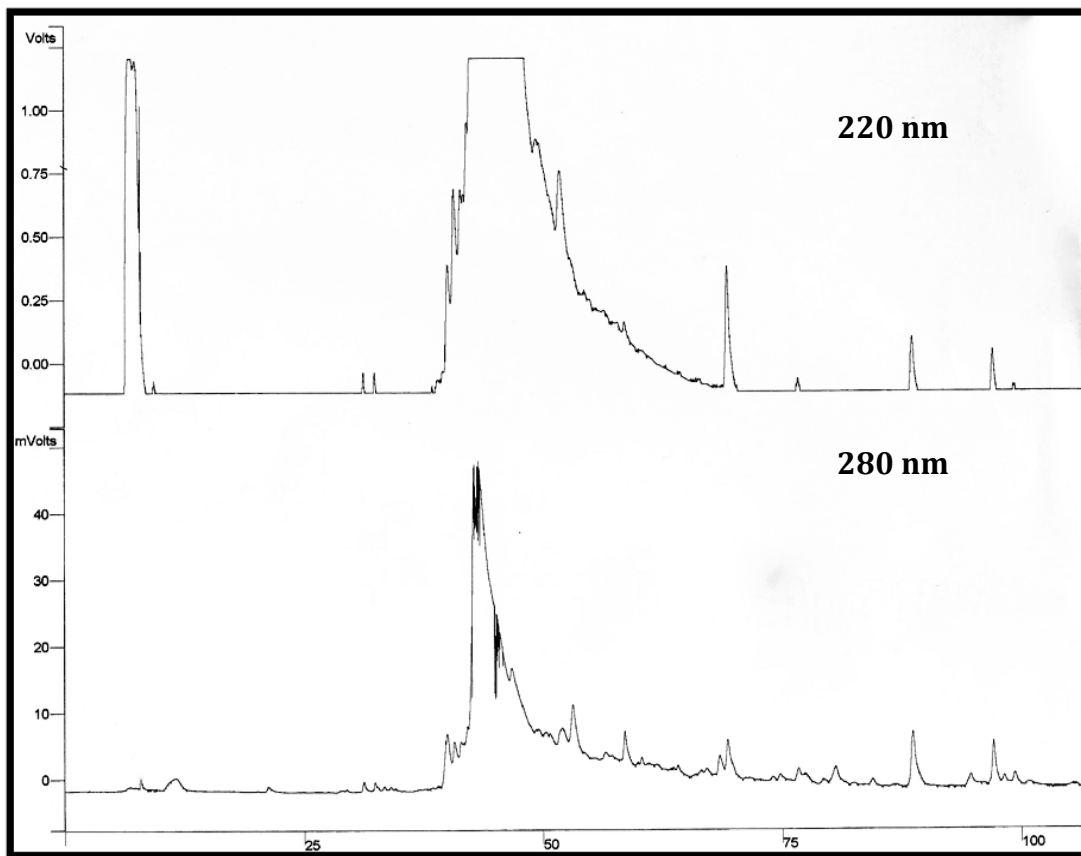


Figure 2.4: HPLC trace of crude Aβ40 using a gradient of 0-90% buffer B in 90 minutes, with a C18 preparative column.

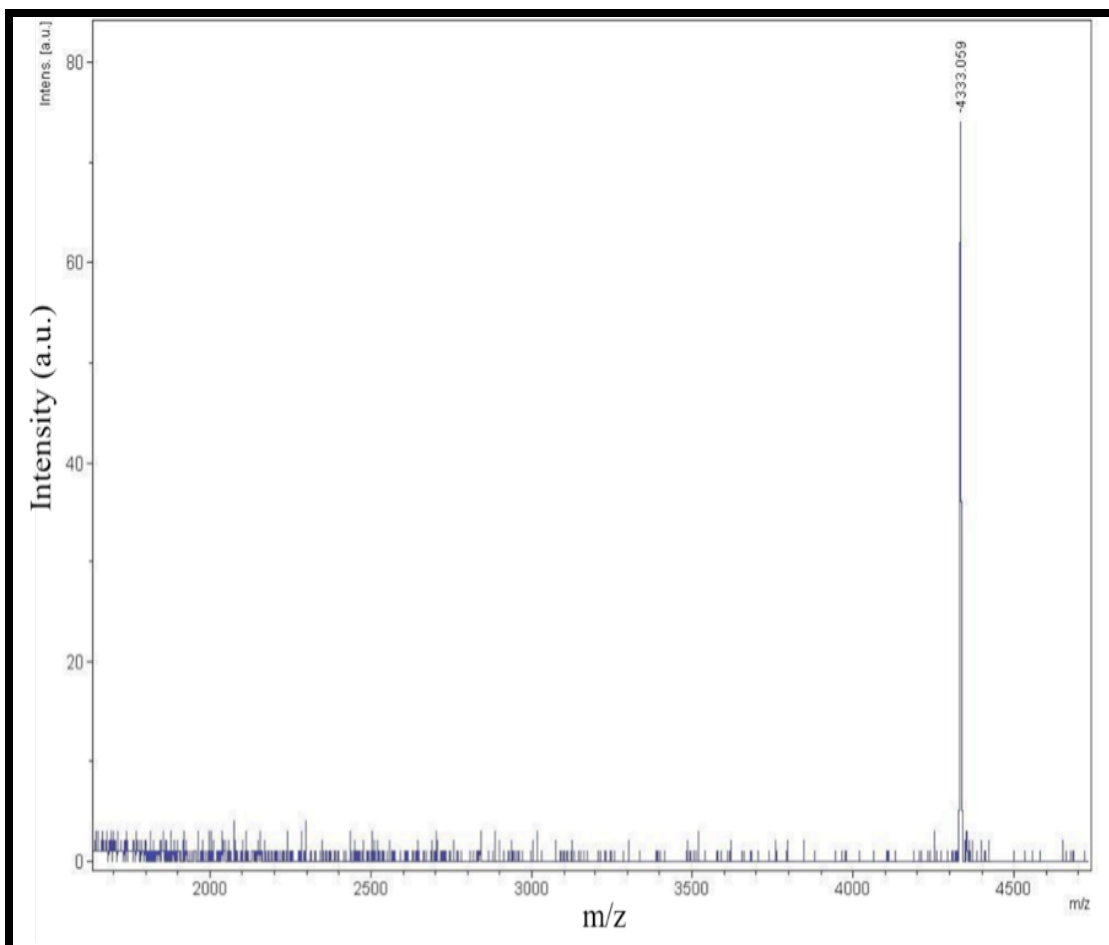


Figure 2.5: MALDI-TOF of purified A β 40 showing an observed molecular weight of 4333.1 Daltons. The theoretical weight is 4329.8 Daltons.

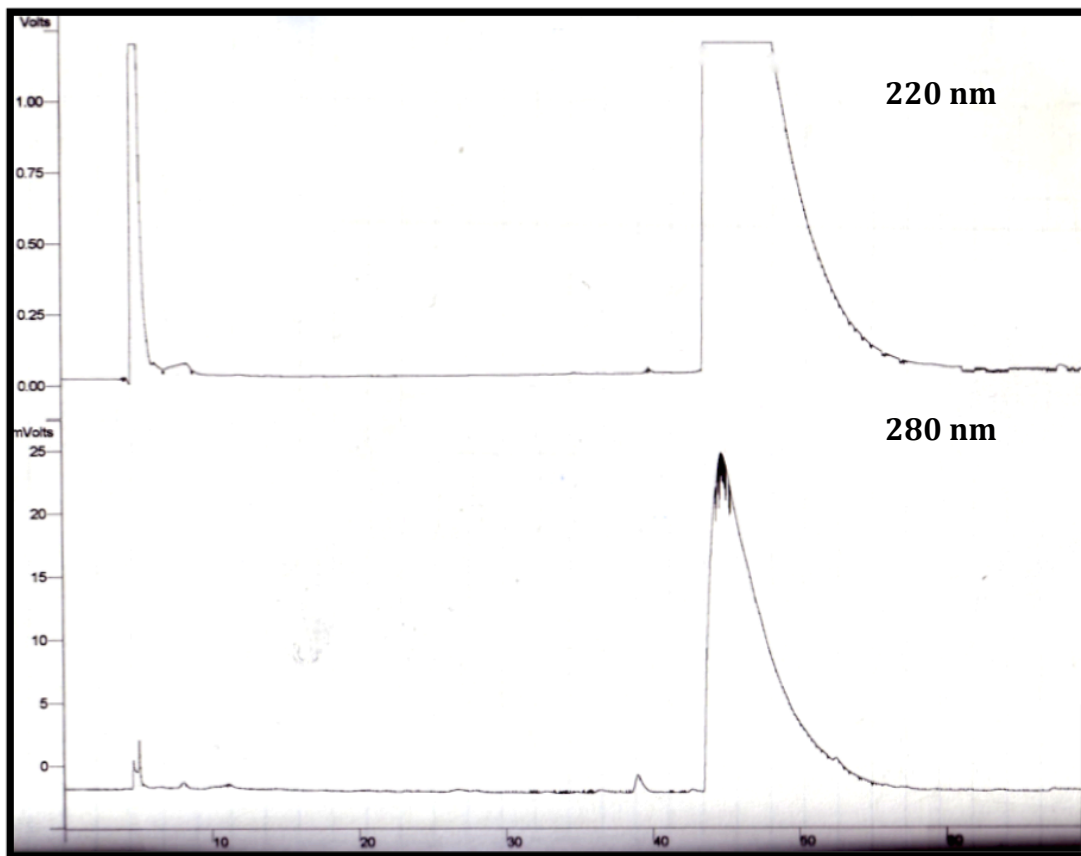


Figure 2.6: HPLC trace of purified A β 40 with a gradient of 0-90% buffer B in 90 minutes, using a C18 analytical column.

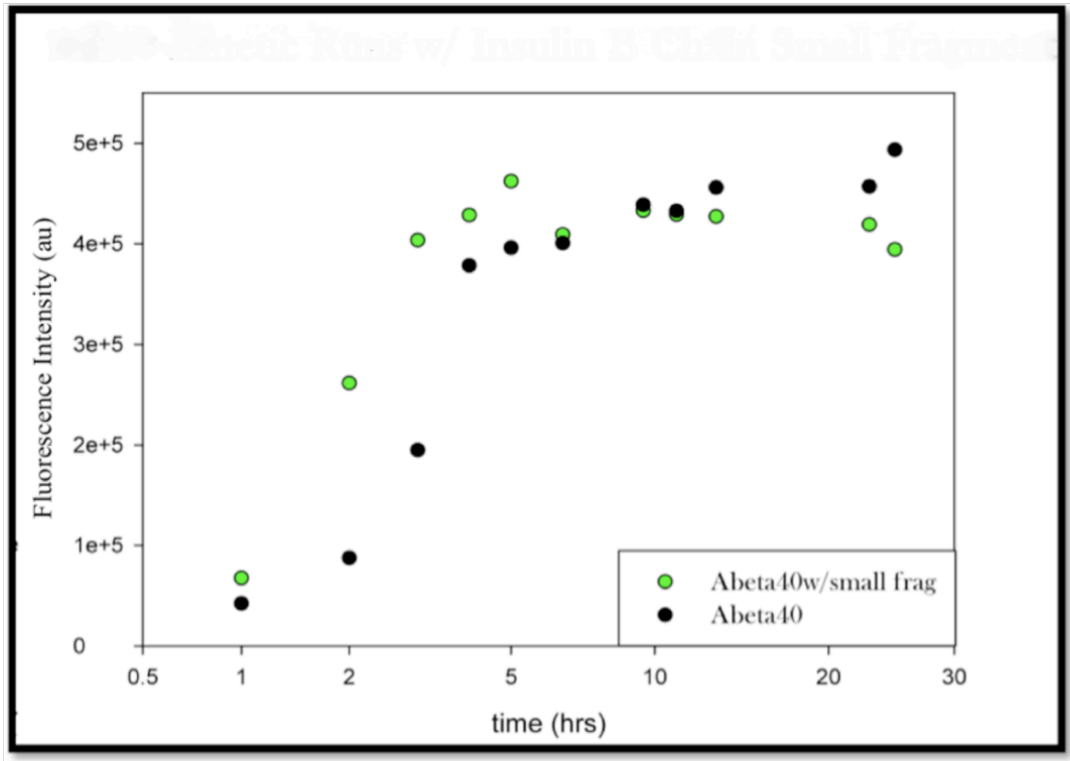


Figure 2.7: Thioflavin-T binding fluorescence assay of amyloid formation by A β 40 (black) and A β 40 in the presence of insulin B small fragment (green). Full fibril formation is seen by 5 hours for A β 40, based on where the points plateau. The concentration of A β 40 was 15 μ M and the insulin B small fragment concentration was also 15 μ M. Experiments were conducted at pH 7.4 using 100 mM Tris buffer with 2% HFIP.

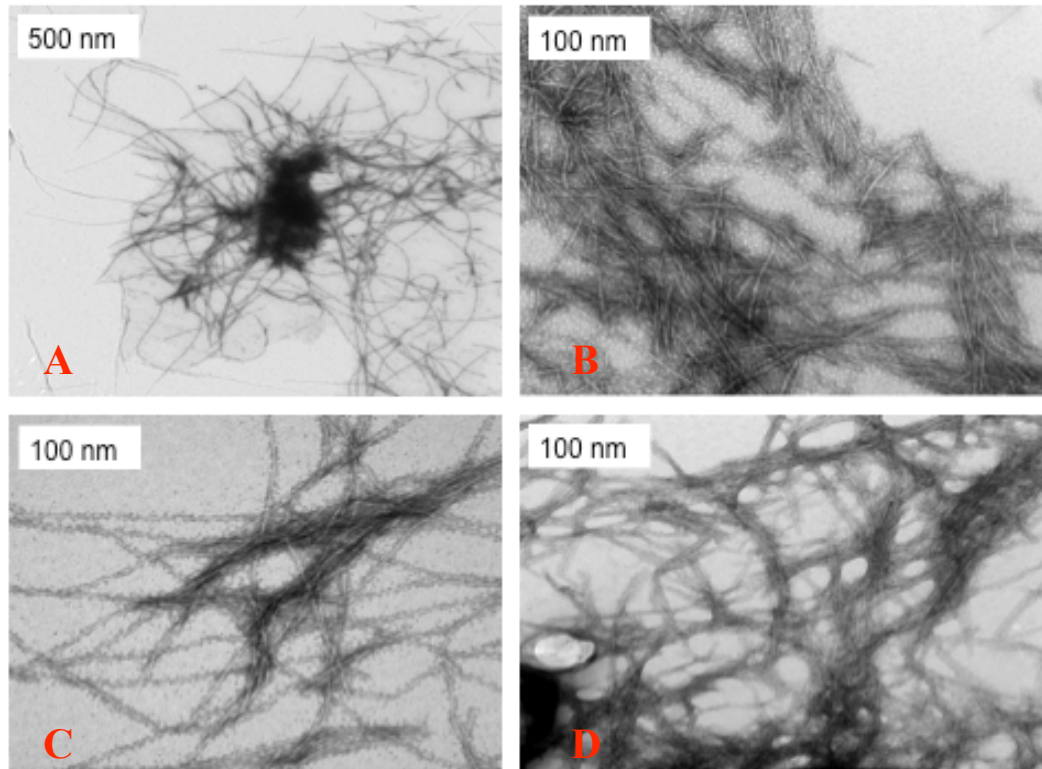


Figure 2.8: TEM images of A β 40 and A β 40 incubated with insulin B small fragment at the time the fluorescence assays showed the plateau at 5 hours. A) A β 40 alone. B) A β 40 with insulin B small fragment. C) A β 40 alone. D) A β 40 with insulin B small fragment.

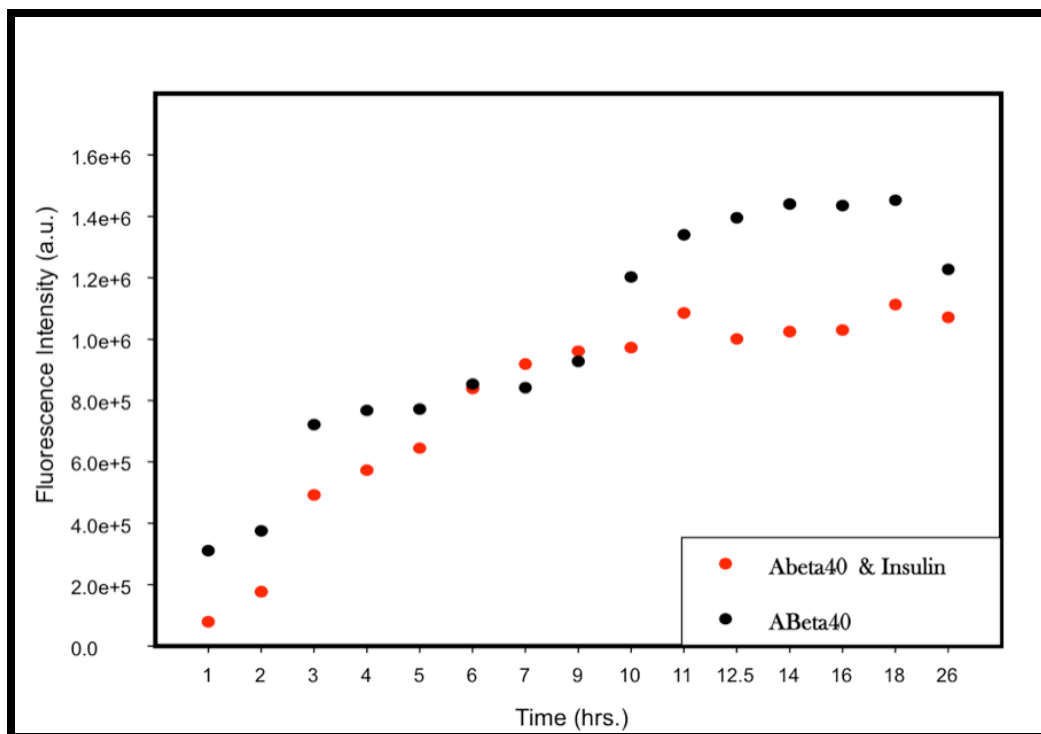


Figure 2.9: Thioflavin-T binding fluorescence assay of amyloid formation by A β 40 (black) and A β 40 in the presence of insulin (red). Full fibril formation is seen by 11 hours, based on where the points plateau. The concentration of A β 40 was 15 μ M and the insulin concentration was also 15 μ M. Experiments were conducted at pH 7.4 using 100 mM Tris buffer with 2% HFIP.

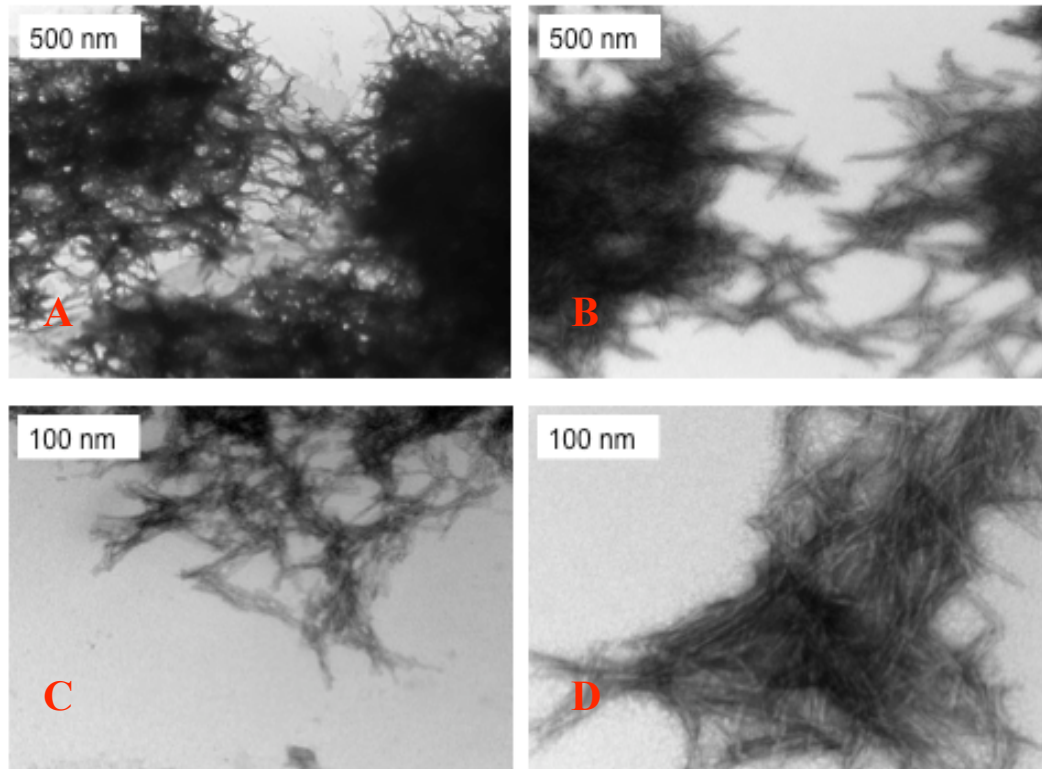


Figure 2.10: TEM images of A β 40 and A β 40 incubated with insulin at the time the fluorescence assays showed the plateau, 11 hours. A) A β 40 alone. B) A β 40 with insulin. C) A β 40 alone. D) A β 40 with insulin.

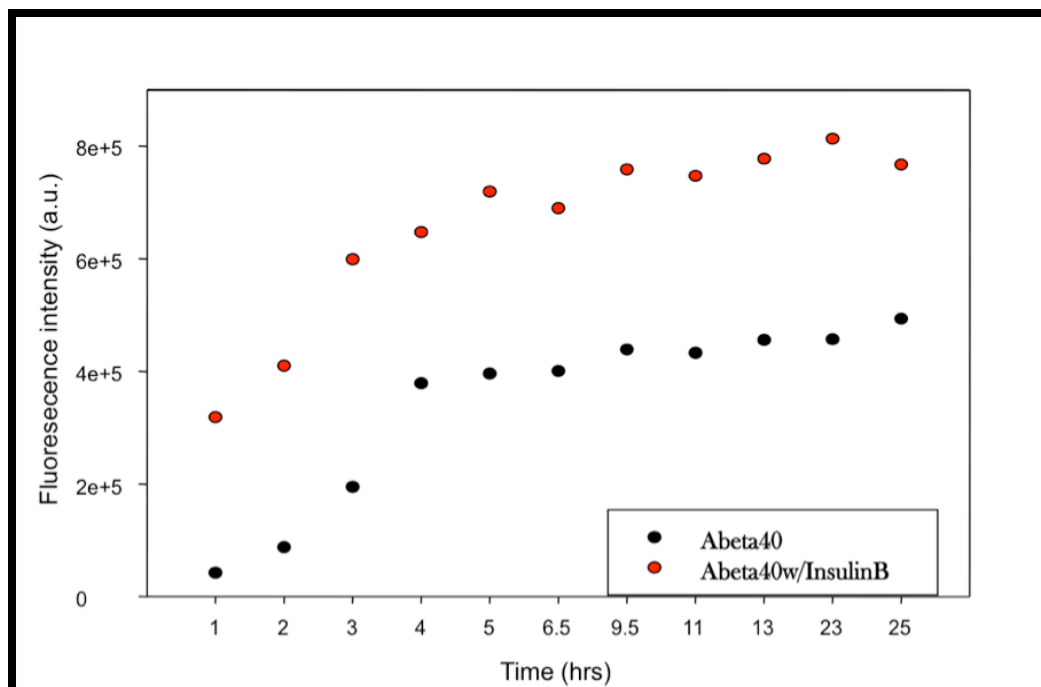


Figure 2.11: Thioflavin-T binding fluorescence assay of amyloid formation by A β 40 (black) and A β 40 in the presence of insulin B chain (red). Full fibril formation is seen by 5 hours, based on where the points plateau. The concentration of A β 40 was 15 μ M and the insulin B chain concentration was also 15 μ M. Experiments were conducted at pH 7.4 using 100 mM Tris buffer with 2% HFIP.

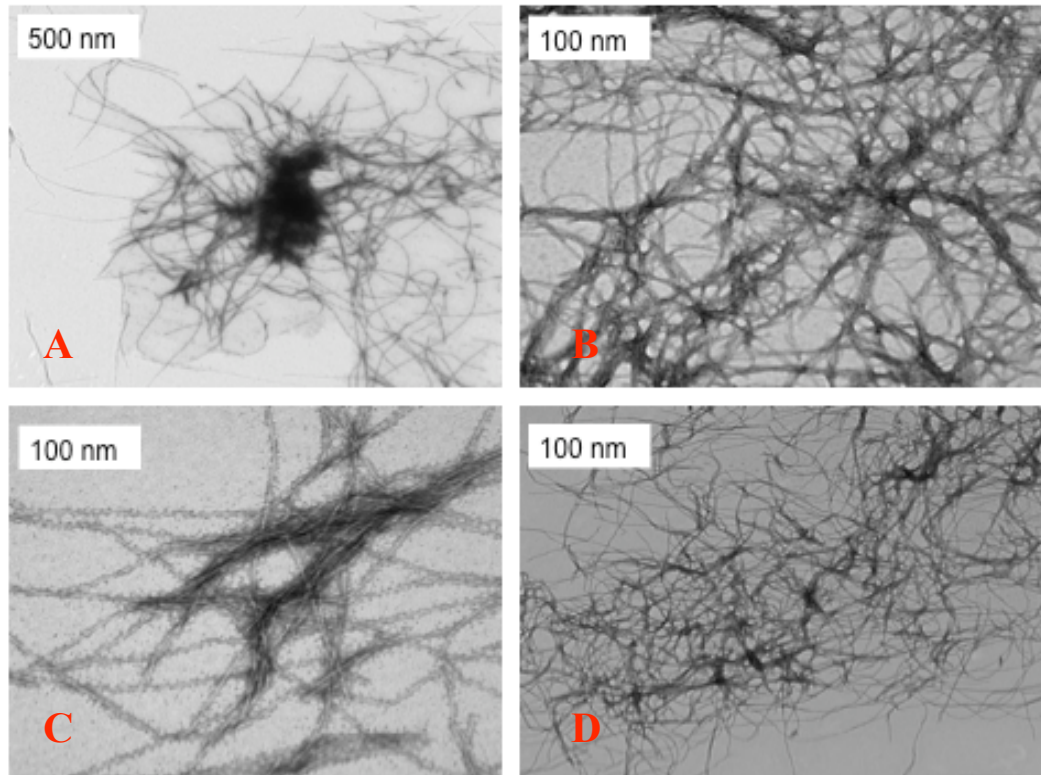


Figure 2.12: TEM images of A β 40 and A β 40 incubated with insulin B chain at the time the fluorescence assays showed the plateau, 5 hours. A) A β 40 alone. B) A β 40 with insulin B chain. C) A β 40 alone. D) A β 40 with insulin B chain.

2.6 References

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Chapter 3: The Inhibitory Effects of Insulin, Insulin B and Insulin B Small Fragment on Amylin Amyloid Formation

3.1 Introduction

The ability of IBSF was also tested on Amylin. The sequence of this peptide was chosen based on theories of the interaction between Amylin and insulin (1-4). Insulin is the most potent inhibitor of Amylin. It is also co-localised and co-secreted with Amylin. Interestingly, insulin itself can form fibrils under certain conditions. When both Amylin and insulin are secreted from the pancreas in Type II diabetes patients, Amylin aggregates and deposits on cells. Although insulin is an inhibitor of Amylin, aggregation still occurs. Whether insulin plays any role in the system that keeps Amylin from aggregating is still unclear. Understanding how insulin inhibits Amylin fibril formation can allow for more knowledge to be gained. Our IBSF peptide can aid in understanding the interaction between insulin and Amylin.

3.2 Materials and Methods

3.2.1 Protein Synthesis and Purification

Human Amylin and IBSF was synthesized and purified as described in section 2.2.1.

3.2.2 Thioflavin-T binding Fluorescence Assays

Amylin was dissolved in HFIP to attain a concentration of 1.58 mM. Experiments were performed by diluting this stock solution of peptide in 20 mM Tris

buffer at pH 7.4 with thioflavin-T to a final concentration of 32 μ M Amylin (2% HFIP), 100 μ M Thioflavin-T, and 20 mM Tris buffer at pH 7.4. A time-based scan was done on the sample using an excitation wavelength of 450 nm and an emission wavelength of 485 nm with an Applied Photon Technologies fluorimeter. If insulin, insulin B chain, or IBSF were added, they would be dissolved in 20 mM Tris buffer at pH 7.4 and adjusted to concentrations in relation to Amylin. The total volume of the mixture was always kept at 1666 μ l. The curves were attained and plotted.

3.2.3 Transmission Electron Microscopy

The Amylin grids were made and analyzed as described in section 2.2.3.

3.3 Results

Insulin and the insulin B chain were tested on Amylin fibril formation (Fig. 3.1). Complete inhibition of fibril formation was observed. The curves containing the inhibitors showed no growth phase. Results were confirmed with TEM images (Fig. 3.2), which did not show any signs of fibrils.

Concentration dependent experiments were done with IBSF due to its promising inhibitory effect on Amylin fibril formation. Consistent results were observed (Fig. 3.3). As the concentration of IBSF increased in ratio to Amylin, more inhibition was observed. The corresponding TEM images (Fig. 3.4-3.6), showed a consistency with the results from the kinetic curves. The fibrils became less and less dense as more IBSF was added. This showed a nice trend in the ability of IBSF to inhibit amyloid.

3.4 Discussion

It is accepted that insulin and the insulin B chain have potent inhibitory effects on Amylin fibril formation. If the recognized region within the insulin B chain showed strong interactions with Amylin, then it is logical that the 12-residue peptide, IBSF, which was synthesized from this region would have some kind of effect on Amylin fibril formation

Through the use of IBSF, it was confirmed that there is a strong interaction between a region of insulin and Amylin. This region seems to be located towards the N-terminal side of IAPP. Most inhibitors of Amylin interact towards the C-terminal side. From this, it can be stated that IBSF is unique as an inhibitor. In addition, since IBSF is a small peptide, it provides easier ways to experiment the insulin Amylin interaction.

3.5 Figures

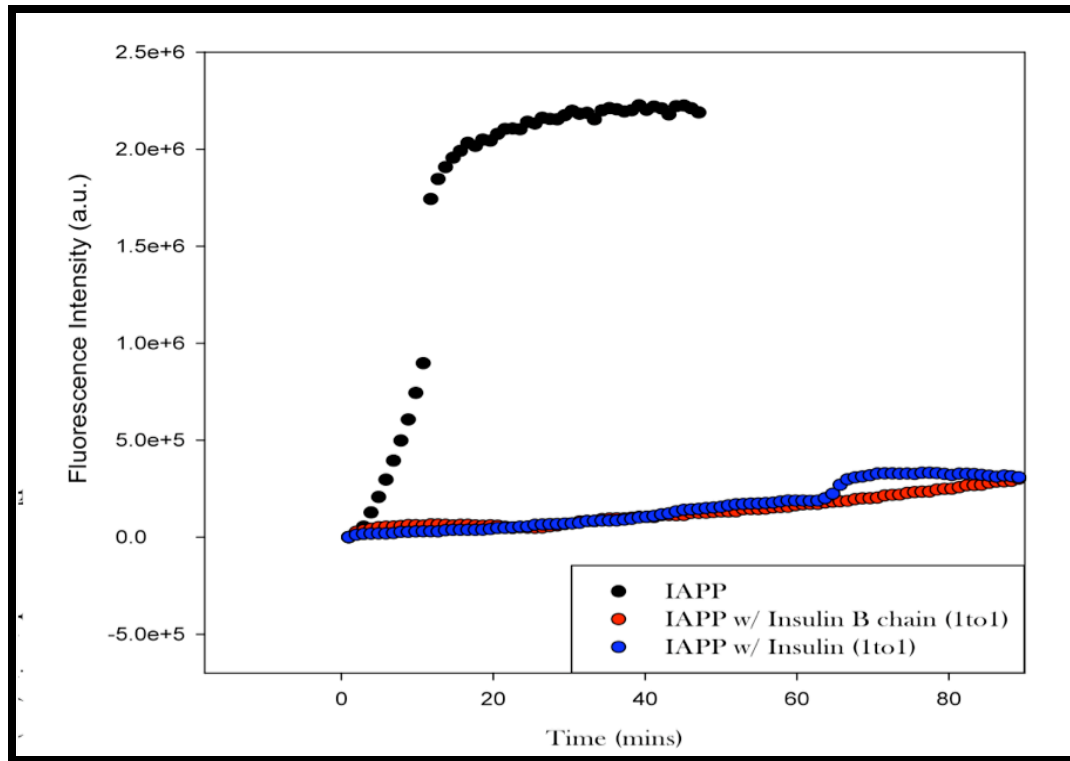


Figure 3.1: Thioflavin-T binding fluorescence kinetic curves of IAPP (black) fibril formation, IAPP incubated with insulin (blue) and insulin B chain (red). All the curves are shifted down to start at 0 a.u. (unshifted is in the appendix). Experiments were carried out in 20 mM Tris buffer at pH 7.4 with 2% HFIP. The concentrations of all three peptides were kept consistently 32 μ M.

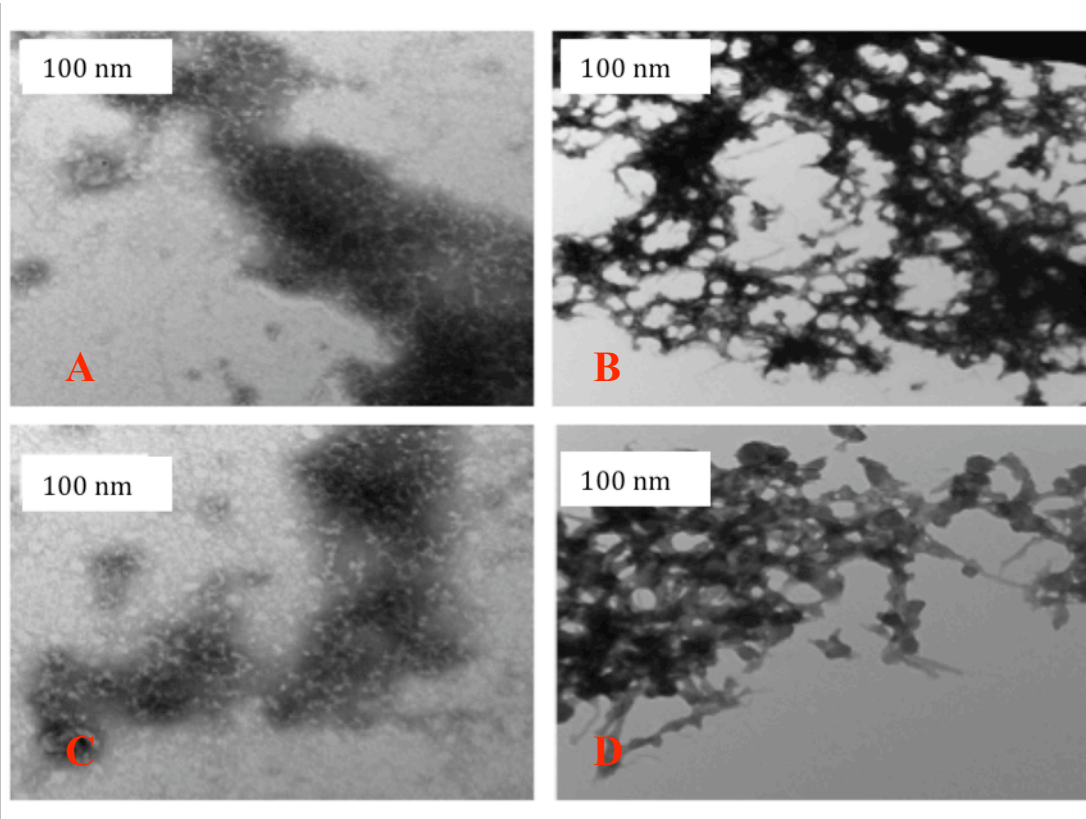


Figure 3.2: TEM images of IAPP with insulin and insulin B chain taken at the end of the kinetic experiment. A) IAPP with insulin. B) IAPP with insulin B chain. C) IAPP with insulin. D) IAPP with insulin B chain.

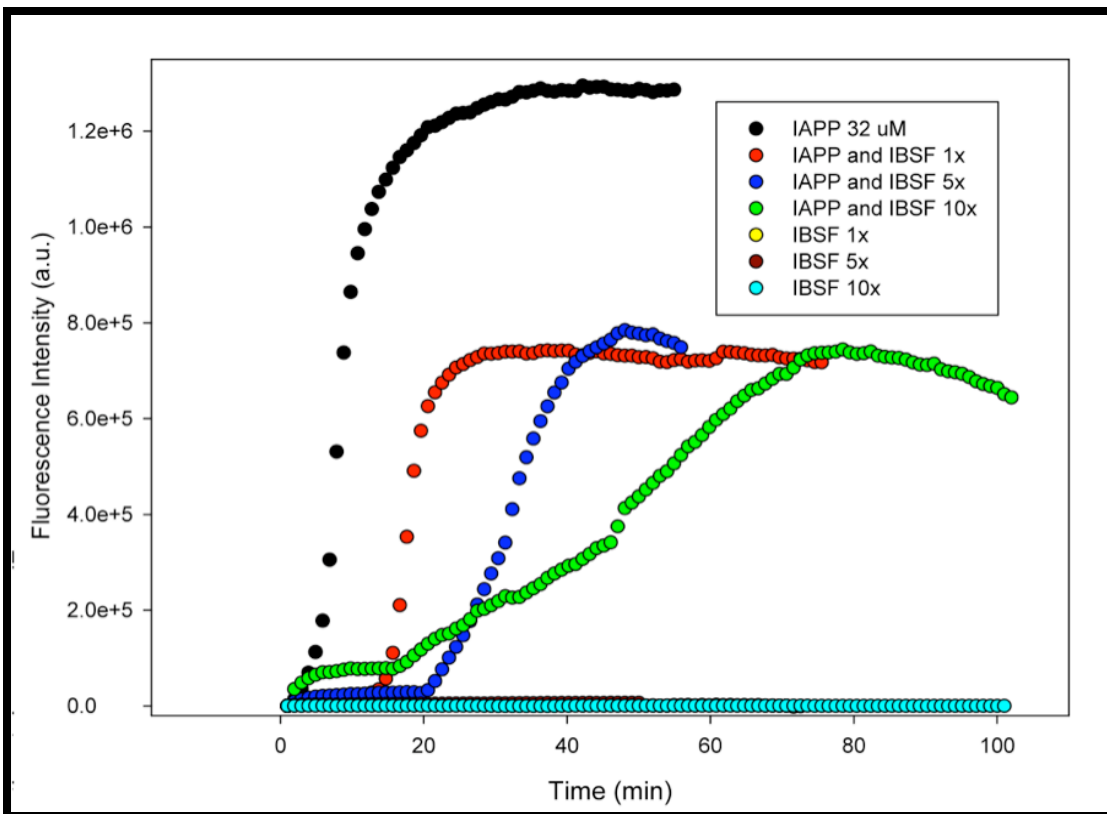


Figure 3.3: Thioflavin-T binding fluorescence of IAPP (black) and IAPP incubated with Insulin B chain small fragment with different concentrations. All the curves are shifted down to start at 0 a.u. (unshifted is in the appendix); One-to-one concentration of IAPP and IBSF (red), one-to-five concentration of IAPP and IBSF (blue), and one-to-ten concentration of IAPP and IBSF (green). Experiments were carried out in 20 mM Tris buffer at pH 7.4 with 2% HFIP. The concentration of IAPP is 32 μM and IBSF for 1 to 1 is 32 μM , for 1 to 5 is 160 μM , and for 1 to 10 is 320 μM .

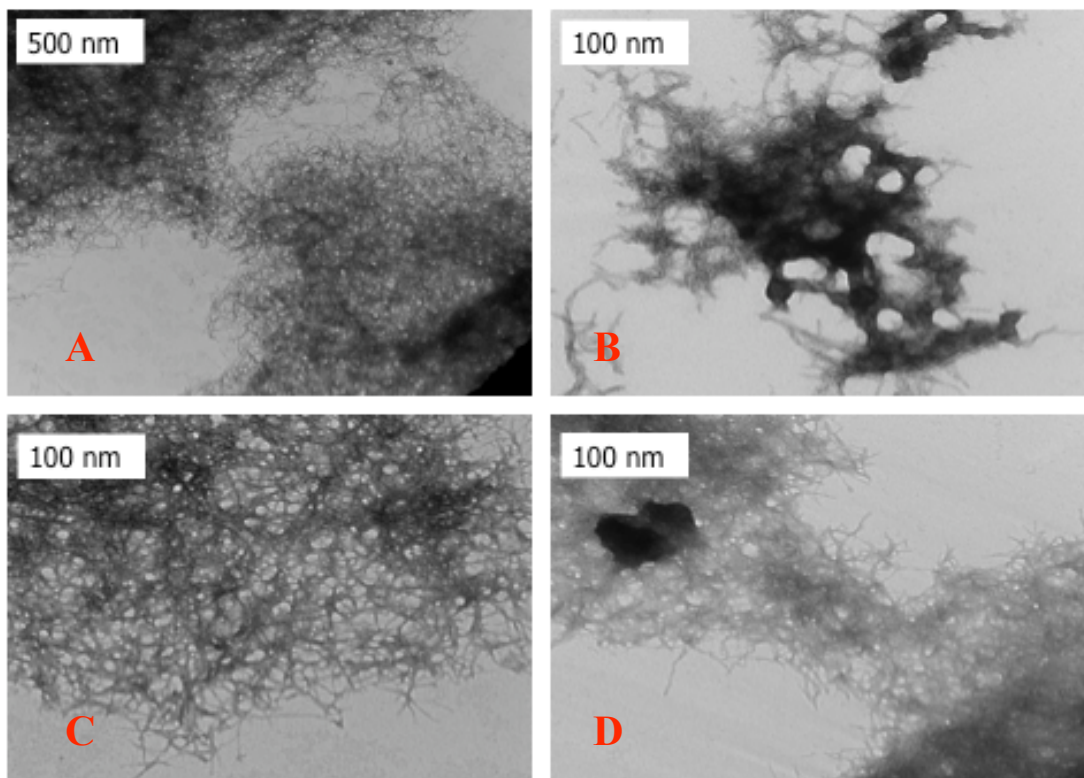


Figure 3.4: TEM images of IAPP alone and with IBSF at a one-to-one concentration taken at the time when the fluorescent curve reached a plateau. A) IAPP alone. B) IAPP with IBSF 1:1. C) IAPP alone. D) IAPP with IBSF 1:1.

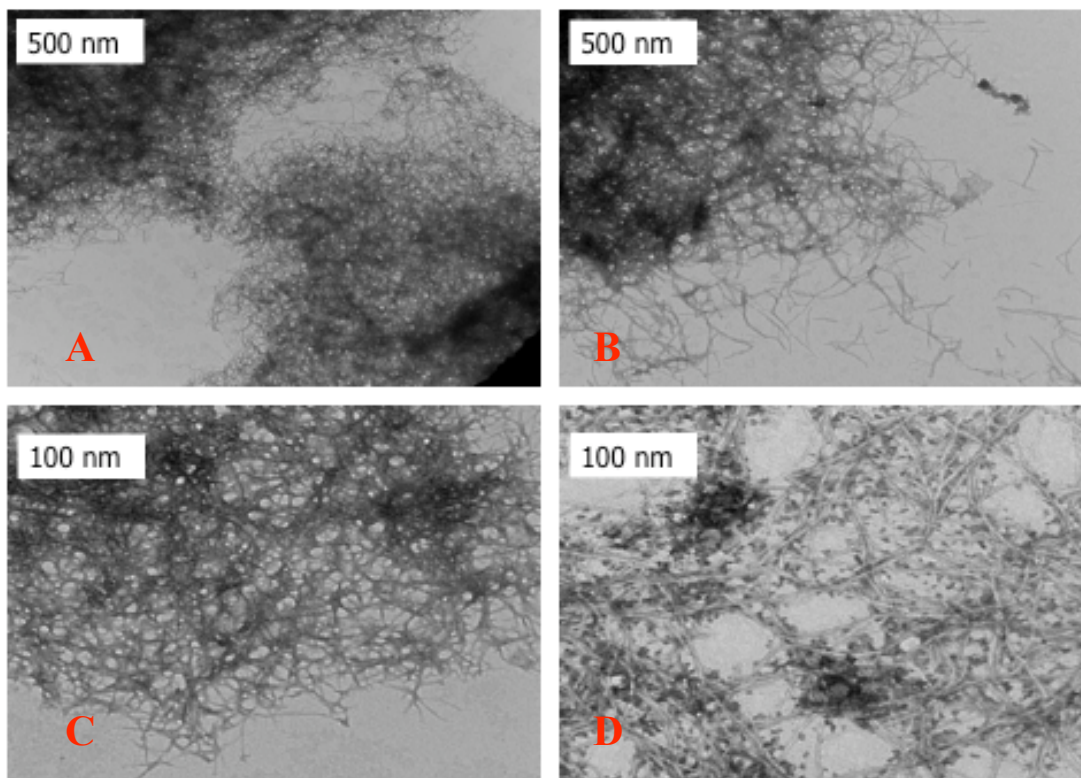


Figure 3.5: TEM images of IAPP alone and with IBSF at a one-to-five concentration taken at the time when the fluorescent curve reached a plateau. A) IAPP alone. B) IAPP with IBSF 1:5. C) IAPP alone. D) IAPP with IBSF 1:5.

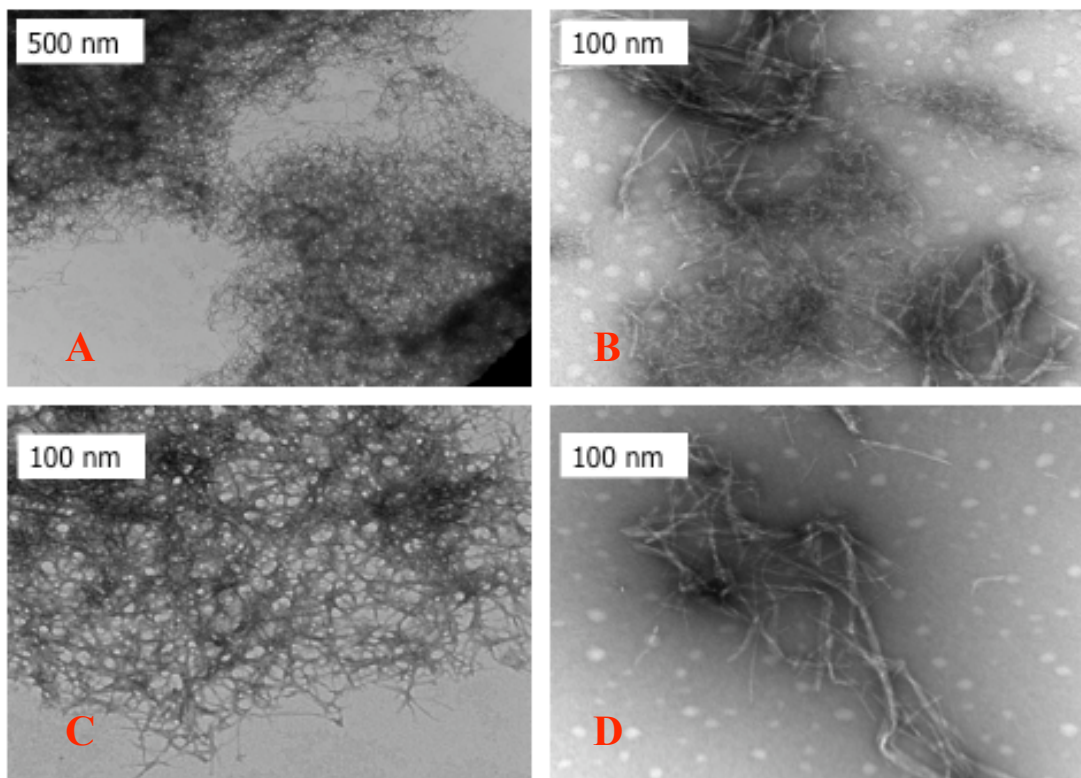


Figure 3.6: TEM images of IAPP alone and with IBSF at a one-to-ten concentration taken at the time when the fluorescent curve reached a plateau. A) IAPP alone. B) IAPP with IBSF 1:10. C) IAPP alone. D) IAPP with IBSF 1:10.

3.6 References

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Chapter 4: Characterization of the Insulin B Small Fragment as an Amylin Amyloid Inhibitor

4.1 Introduction

Over the years, there have been a few regions of Amylin that have been identified as highly amyloidogenic. The Amylin 20-29 region has been considered to be the central region that plays a key role in amyloid formation (1-3). However, the Amylin 8-20 region has also been acknowledged to be important (4-6). This region also contains a segment that plays a role in the interaction of Amylin and insulin (7-9). Based on these observations, we had synthesized the small fragment from insulin for use as a tool that can be used to study the Amylin-insulin interaction.

One theory in Amylin amyloid formation is that π -stacking, aromatic stacking interactions, plays a key role in fibril assembly (10). Gazit et al. and Eisenberg et al. have suggested that interactions of Phe15 in Amylin with Tyr16 in insulin are important for Amylin insulin interaction (7-8). Since both are aromatic residues, their interaction may be aiding in disrupting the π -stacking of Amylin fibril assembly and hence inhibiting fibril formation. However, there is another theory that suggests that the inhibition of Amylin fibril formation by insulin could be due to a salt bridge formation between both peptides, which would form between His 18 in Amylin, a basic residue and Glu13 in insulin, an acidic residue (9). IBSF is consists of residues 9-20 of insulin and hence contains the regions that are discussed.

Therefore, conducting experiments using IBSF can produce data that can provide a better understanding this interaction.

p-cyanoPhenylalanine (p-cyanoPhe), an unnatural amino acid, can be used as a fluorescence probe to observe the burial of the residue during fibril formation.

p-cyanoPhe fluorescence can also be quenched via FRET to tyrosine. Using this unnatural amino acid, our lab synthesized Amylin derivatives, including one that replaced Phe15 to p-cyanoPhe (10). Since IAPP-F15 might interact with insulin-Y16 or IBSF-Y8, FRET effects might be observed. In the complex, Thioflavin-T and p-cyanoPhe kinetics can be measured simultaneously (Fig 4.1). By using these derivatives we might be able to observe interactions between IAPP-F15_{FCN} and IBSF.

To test the possible role of the Glu13-His18 salt bridge, a mutant of the IBSF peptide was prepared in which a glutamine was substituted in place of glutamic acid. Observation of a decreased ability of the IBSF-E5Q mutant to inhibit fibril formation would provide evidence in support of the salt bridge theory.

4.2 Materials and Methods

4.2.1 Protein Synthesis and Purification

Human IAPP, IBSF, and IBSF-E5Q were synthesized as described in section 2.2.1. Human IAPP-F5_{Fcn} was synthesized by Peter Marek, a graduate student in our lab.

4.2.2 Thioflavin-T binding Fluorescence Assays

The protocol for IAPP-F15_{FCN} is based on experiments that were previously done (10). The conditions were performed as described in section 3.2.2 with the

exception of the final concentration of IAPP-F15F_{CN}, 6.5 μM. Also, in addition to thioflavin-T fluorescence, p-cyanoPhe fluorescence was observed when the sample was excited at 240 nm and detected at 296 nm on an Applied Photon Technologies fluorimeter. Experiments with Amylin with IBSF and IBSF-E5Q were performed as described in section 3.2.2.

4.3 Results

IAPP- F15F_{CN} kinetics was monitored under the presence of IBSF. If there is a close enough interaction between IAPP-F15F_{CN} and IBSF, quenching of the p-cyanoPhe by the tyrosine located on the inhibitor would be observed. Thus, compared to a control system with no inhibitor the fluorescence intensity would decrease much faster. At the same time, thioflavin-T interaction can be monitored, which will confirm if there is inhibition. Figure 4.2 shows both the p-cyanPhe and thioflavin-T curves separately. It contains IAPP- F15F_{CN} by itself (black) and with IBSF (red). Figure 4.3 contains the corresponding p-cyanoPhe and thioflavin-T curves combined. The results show that there is no significant quenching.

The mutated version of IBSF, which lacks the potential salt bridge-forming residue of IBSF, glutamic acid, was tested on the kinetics of Amylin fibril formation. Figure 4.4 shows the thioflavin-T kinetics of Amylin with a 10 times concentration of IBSF and its mutant. The kinetics of the mutant on Amylin showed a less inhibitory effect on amyloid formation compared to IBSF.

4.4 Discussion

IAPP-F15Fcn fibril formation was tested with IBSF at 10 times the concentration. At this ratio, there is a strong evidence of inhibition of fibril formation based on the thioflavin-T fluorescence data. However, there was no observation of quenching on the p-cyanoPhe kinetics. This suggests that the interaction between IAPP-F15 and insulin-Y16 is not close enough to show quenching and that the interaction between the two peptides is not close enough to observe FRET. Another explanation is that there is no interaction between the residues. The two alternatives cannot be distinguished on the basis of our studies.

The IBSF mutant had a different effect on the kinetics of Amylin fibril formation. Although, it does not have a stronger inhibiting effect compared to IBSF, it does have enough to extend the lag phase. This implies that a salt bridge formation may be important for the interaction between these two peptides and further studies should be done to compare results at different concentrations.

4.5 Figures

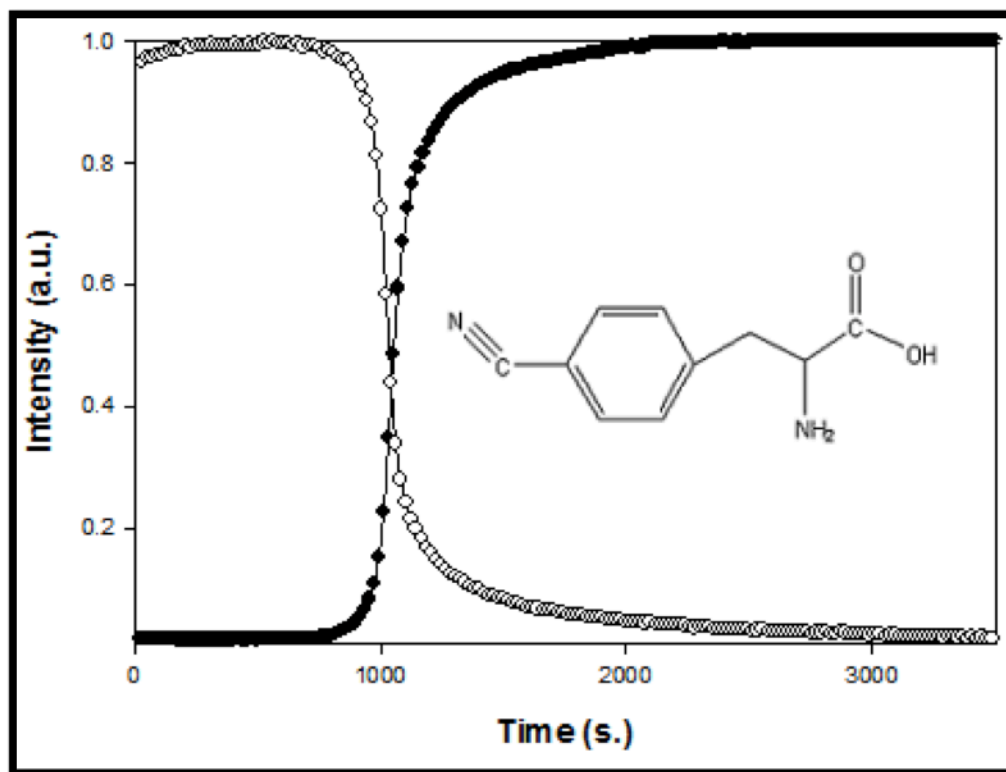


Figure 4.1: Shows the thioflavin-T (black) and p-cyanoPhe (white) kinetic curves that intersect at the midpoint. The molecule on the right is p-Cyanophenylalanine. (Marek, P., Gupta, R., Raleigh, D. P.) *ChemBioChem*. 2008, 9, 9, 1372-1374.

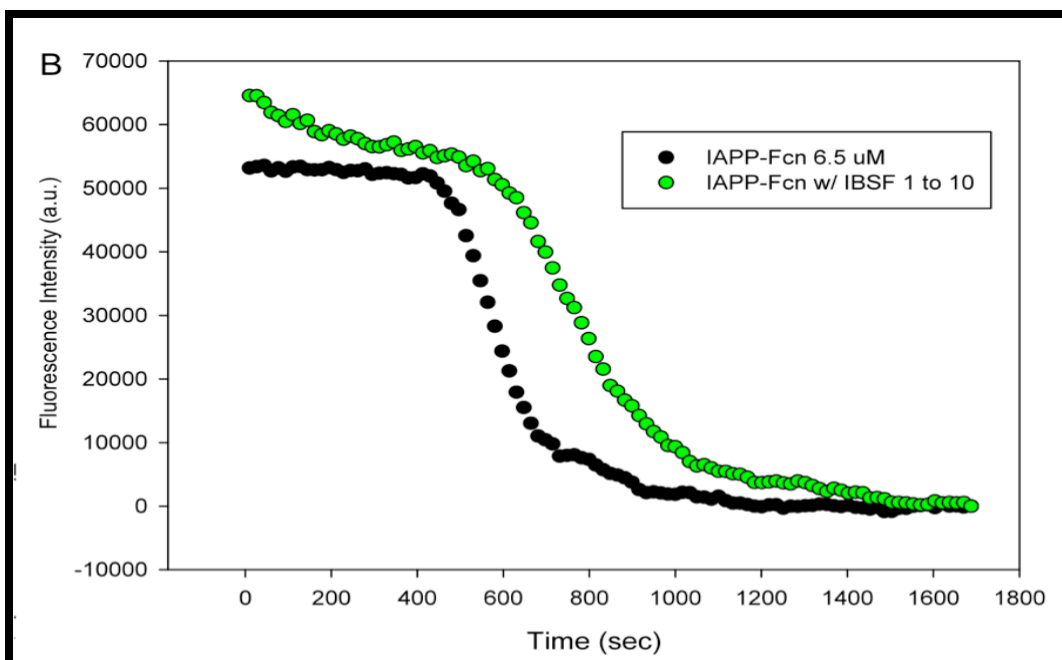
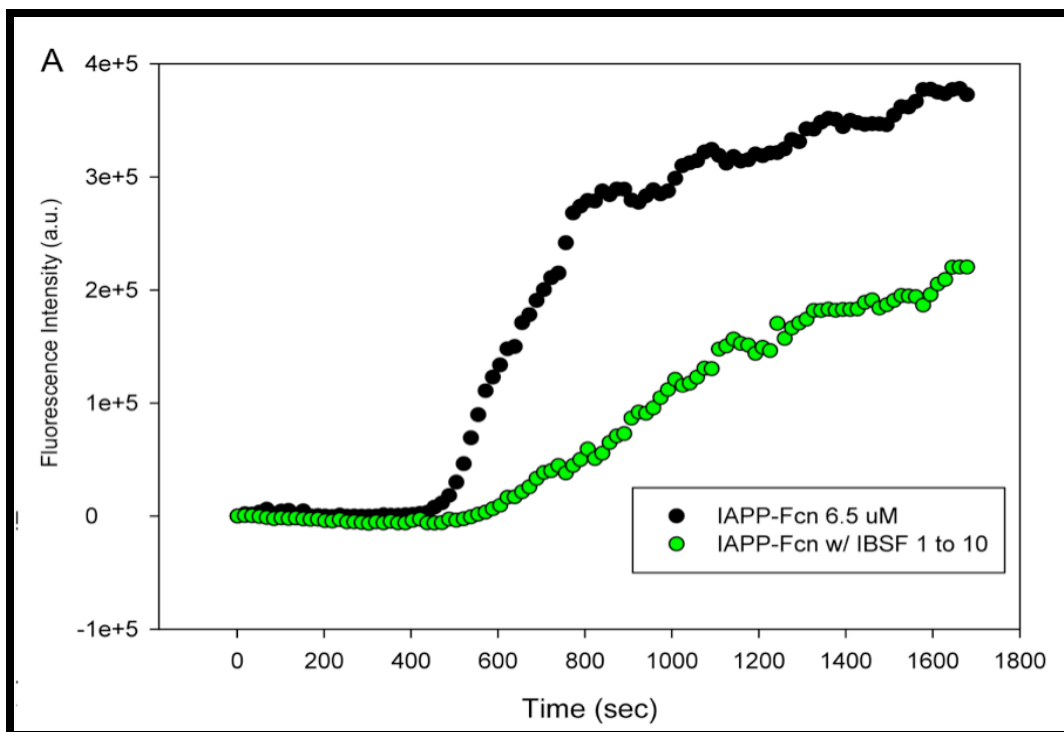


Figure 4.2: Thioflavin-T and p-cyanoPhe fluorescent kinetic curves, normalized, in the absence (black) and presence of IBSF (green). A is the thioflavin-T kinetic curves. B is the p-cyanoPhe curves. Experiments were carried out in 20 mM Tris buffer at pH 7.4 with 2% HFIP. The concentration of IAPP is 6.5 μM and IBSF is 65 μM .

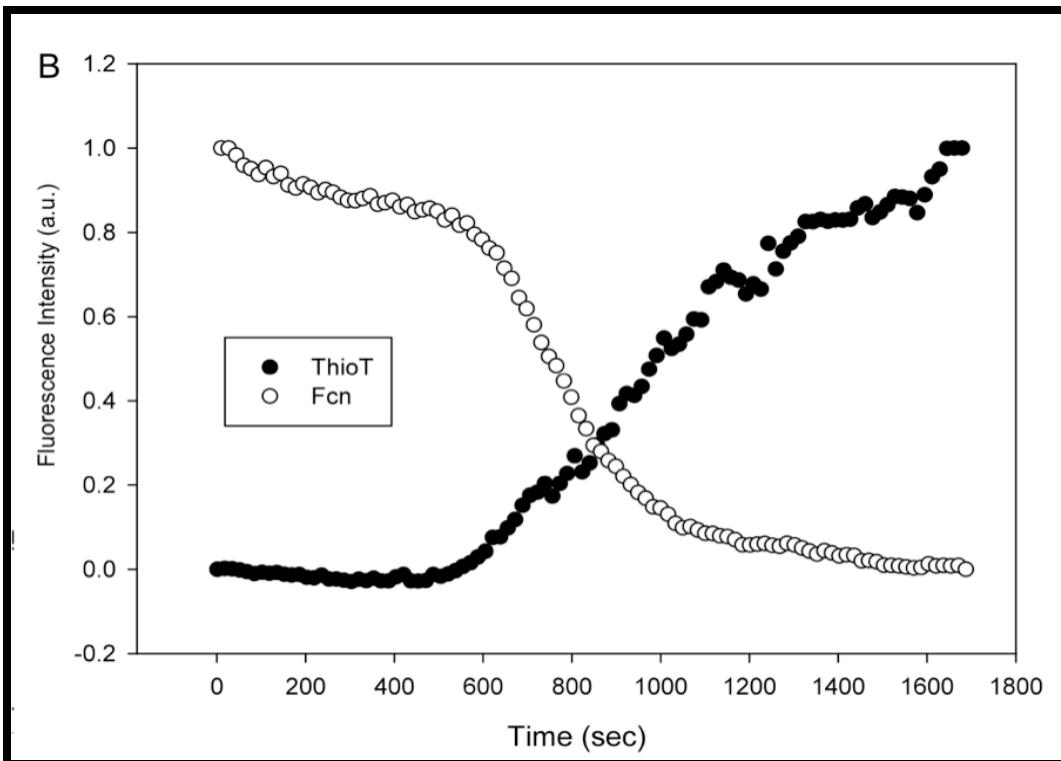
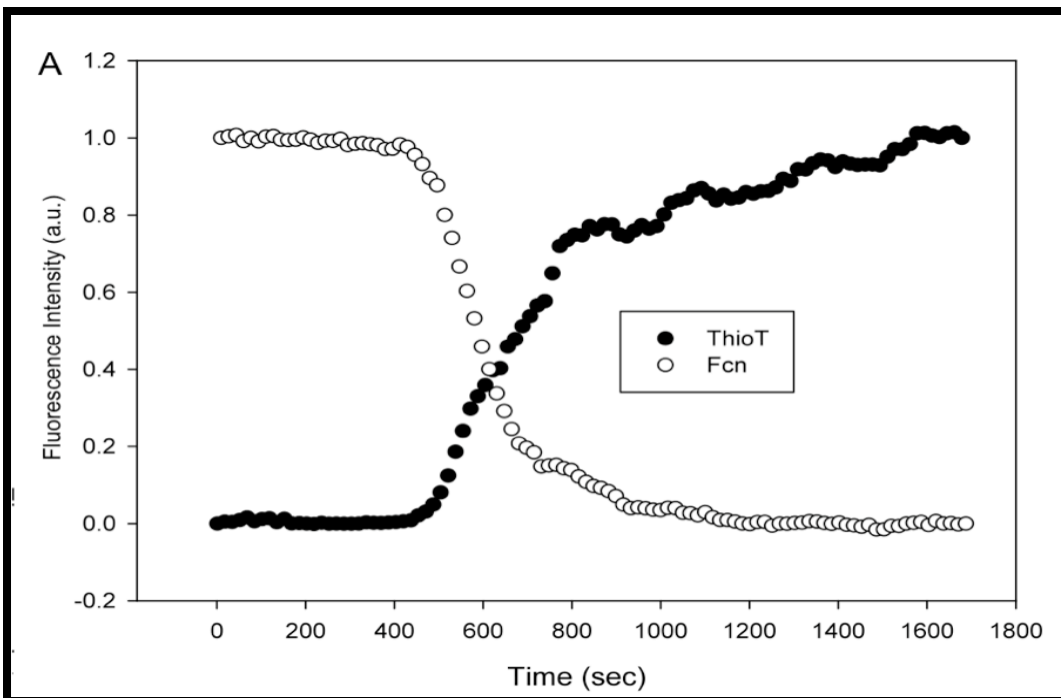


Figure 4.3: Thioflavin-T (black) and p-cyanoPhe (white) fluorescent kinetic curves, normalized, combined. A is IAPP alone. B is IAPP with IBSF. Experiments were carried out in 20 mM Tris buffer at pH 7.4 with 2% HFIP. The concentration of IAPP is 6.5 μ M and IBSF is 65 μ M.

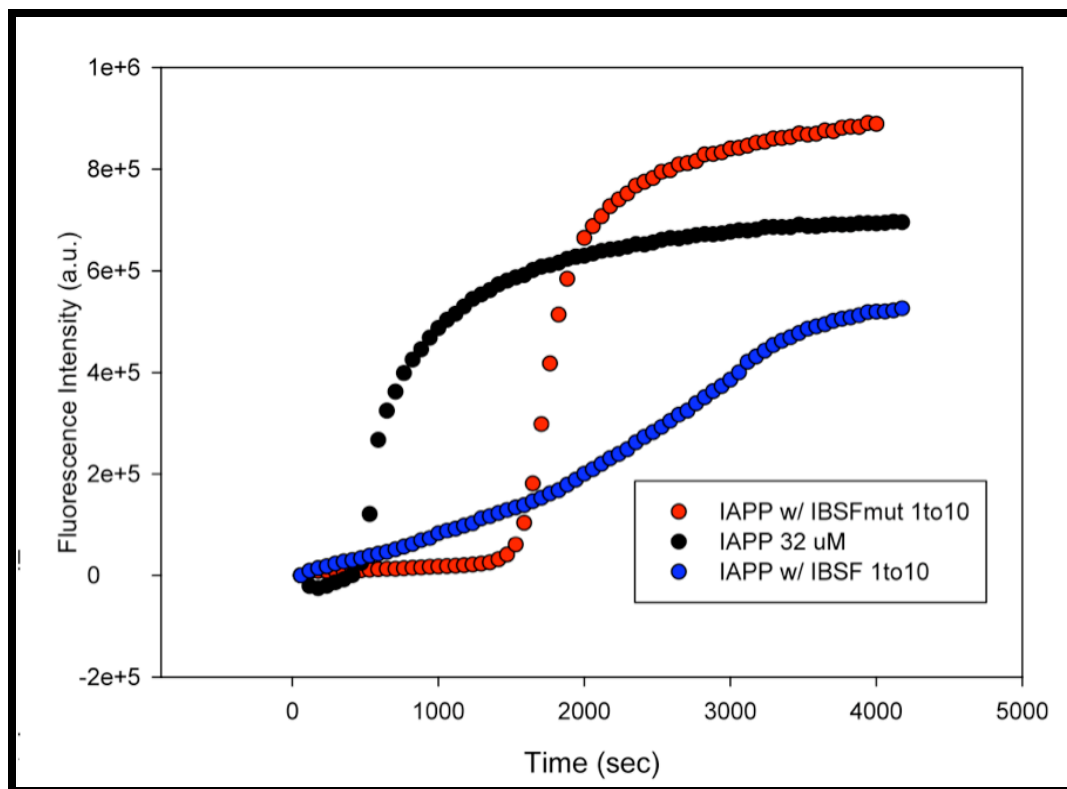


Figure 4.4: Thioflavin-T fluorescent curves of IAPP alone (black), IAPP with IBSF (blue) and IAPP with IBSF mutant (red). All the curves are shifted down to start at 0 a.u. (unshifted is in the appendix). Experiments were carried out in 20 mM Tris buffer at pH 7.4 with 2% HFIP. The concentration of IAPP is 32 μM , and IBSF and IBSF mutant is 320 μM .

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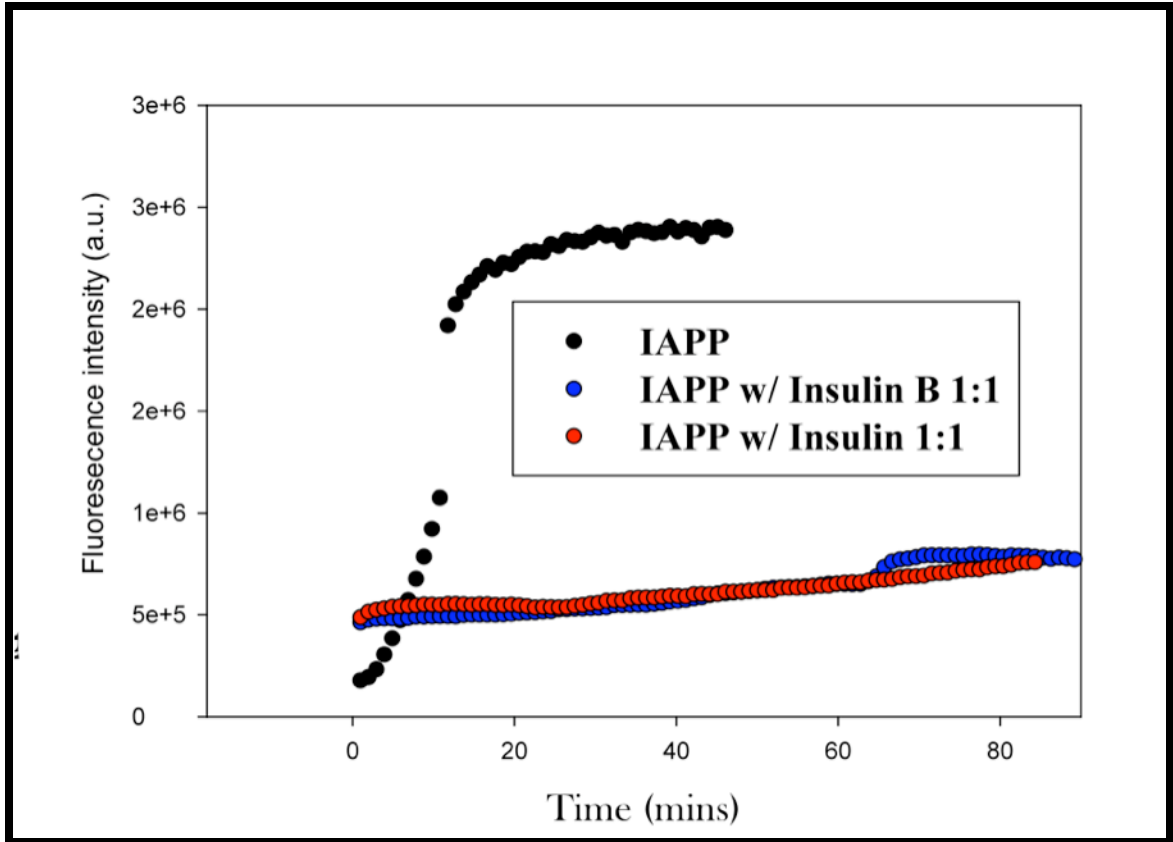
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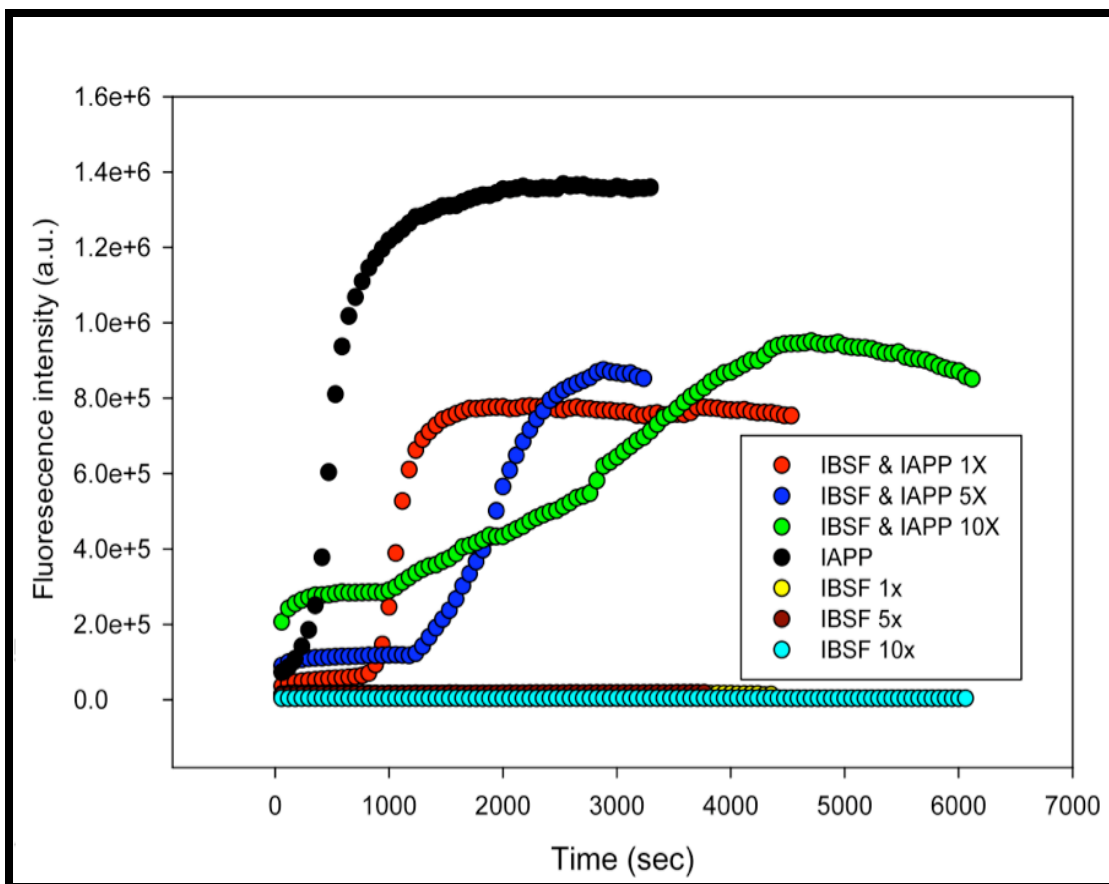
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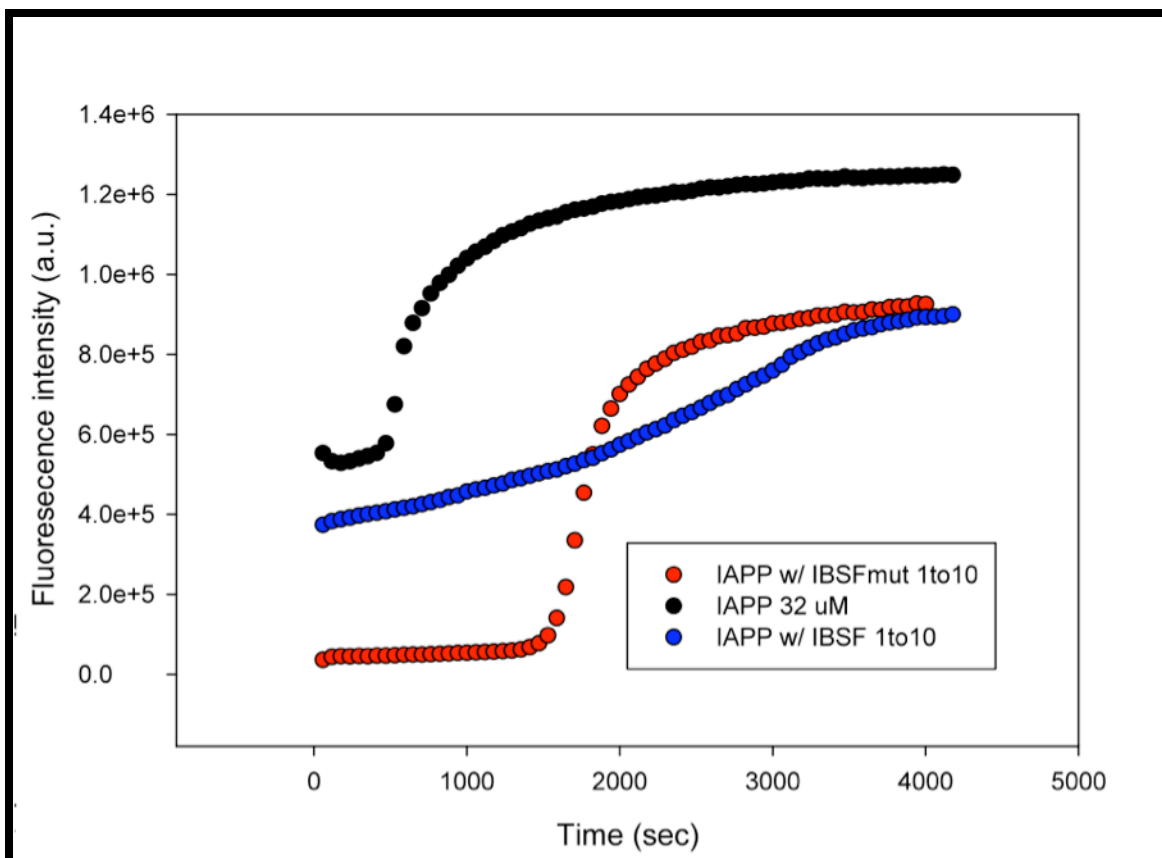
Appendix



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Thioflavin-T binding fluorescence of IAPP (black) and IAPP incubated with Insulin B chain small fragment with different concentrations, unshifted; One-to-one concentration of IAPP and IBSF (red), one-to-five concentration of IAPP and IBSF (blue), and one-to-ten concentration of IAPP and IBSF (green). Experiments were carried out in 20 mM Tris buffer at pH 7.4 with 2% HFIP. The concentration of IAPP is 32 μM and IBSF for 1 to 1 is 32 μM , for 1 to 5 is 160 μM , and for 1 to 10 is 320 μM .



Thioflavin-T fluorescent curves of IAPP alone (black), IAPP with IBSF (blue) and IAPP with IBSF mutant (red), unshifted. Experiments were carried out in 20 mM Tris buffer at pH 7.4 with 2% HFIP. The concentration of IAPP is 32 μ M, and IBSF and IBSF mutant is 320 μ M.