COMPARING THE EFFECTS OF MICROWAVE AND CONVENTIONAL HEATING ON THE FORMATION AND COMPOSITION OF HEN EGG WHITE LYSOZYME NANOFIBRILS

by

ANA JAWORSKI

(Under the Direction of Derek Dee)

ABSTRACT

Amyloid fibrils are stable protein aggregates that have a highly ordered beta-sheet secondary structure. There is a growing interest in using protein amyloid fibrils (termed 'nanofibrils') for use in nanomaterials and food applications. Nanofibrils can be formed from many different proteins and under a variety of conditions, although the mechanism of their assembly is unclear. This study focused on comparing the effects of microwave and dry-bath heating to induce the formation of nanofibrils using hen egg-white lysozyme as a model for human lysozyme. In this study, it was found that using microwave versus conventional heating affected the kinetics, morphology, and peptide composition of hen egg white lysozyme nanofibrils. These results further highlight the sensitivity of nanofibril formation to environmental conditions and indicate that microwave heating might be used to engineer nanofibrils with unique properties.

INDEX WORDS: Amyloid Fibril, Microwave, Hen Egg White Lysozyme, Protein Aggregation

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

1.1 Protein Folding and Fibrils

Understanding the structure of amyloid fibrils requires understanding basic features of protein structure and folding. There are four levels of protein structure, primary through to quaternary. The primary structure is the sequence of amino acids that comprise the protein. There are 21 amino acids, each containing an amide group, alpha carbon, carboxylic acid, and a unique "R group". Amino acids are covalently connected by amide bonds to create peptides. Secondary structure refers to the local folding properties and includes features such as alpha-helices and beta-sheets. Alpha helices are largely stabilized by local hydrogen bonding along the polypeptide, while beta-sheets employ hydrogen bonding between backbones, as these stack linearly side-by-side. The tertiary structure of a protein is its folded form including interactions between other small molecules or water. Packing motifs can be seen here as well, for example with hydrophobic cores. Quaternary structure of proteins is the overall organization of the protein including its many subunits. (1)

Protein folding is largely determined by the primary sequence of each specific protein. A protein will naturally fold to its most stable form, or the form with the least amount of free energy. Protein fibrils are forms of aggregation that are extremely stable and ordered.

Although fibrils can form from many different proteins, there are some conditions and structures that are similar amongst all the aggregates. Fibrils share a similar basic structure,

predominantly crossed beta sheets aligned perpendicular to the long axis. These are grouped together into longer ribbon like strands. (2)

Fibrils can be formed through many different pathways although there are a few common generic modes. Some of these models start with natively disordered proteins, some change conformation locally, or the proteins must unfold and refold. With the natively disordered model, proteins in their native state have regions that easily form cross beta structures due to a lack of stability in its native state (3). Proteins that have local conformational change may align and bond with another protein due to increased and new surface area, forming a fibril (3). These fibrils start off small when they are first formed and as they mature there is an increase in length as well as aggregation.

The most common method of fibril formation is when a protein unfolds and then refolds, incorrectly, into a fibril. The intermediate state in this process is vitally important because the protein retains partial structure, leading to intermolecular interactions that drive fibril formation (2). Although this process is complicated, a simple explanation is that the fibrils grow through the addition of monomers to the fibril and when incubated will grow to a mature state. During maturation these fibrils begin to aggregate with each other, touching at points, stacking and twisting. Most fibrils are a left handed spiral (2).

Fibrils are protein aggregates on the nm-length scale and therefore cannot be seen by the naked human eye. Methods of detection are used to first verify the presence of fibrils and then to quantify them. These methods range from simple to complex. The more complex methods include electron microscope, atomic force microscope, x-ray diffraction, and gel electrophoresis. Some of the simpler methods utilize measurement of absorbance and fluorescence.

The first step in detection of fibrils often starts with thioflavin T (ThT) and/or congo red. These are common dyes that are used because as they bind a change in intensity is measured (4). ThT specifically binds to the beta sheet components of fibrils and a corresponding change in fluorescence is detected. However this method can sometimes yield false results due to the inability to bind when fibrils are tightly packed (false negative) or to bind to structures that are not fibrils, such as bacteria (false positive) (5). Despite its limitations, ThT fluorescence has been used extensively to study amyloid fibril formation (5). The congo red assay is similar to ThT but it measures a change in absorbance once the congo red dye binds to the fibrils in solution. This method should always be used in conjunction with others to accurately detect amyloid fibrils. The Congo Red assay is suitable for a first step to detect the presence of fibrils, but should be combined with other methods for confirmation (5).

It is suggested that methods such as the ThT or Congo Red assay be used as a tentative marker, followed by more complex steps such as transmission electron microscope or x-ray diffraction (5). Transmission electron microscopy can be used to study overall fibril length and form as well as finer details with magnification up to 25,000x (5).

1.2 Hen Egg White Lysozyme

Hen egg white lysozyme (HEWL) is a relatively small protein containing areas of both beta-sheets and alpha-helices, as well as stabilization by four disulfide bridges (Figure 1). HEWL has a molar mass of 14.3 kDa and is highly soluble in water. HEWL shares a highly similar tertiary structure with human lysozyme, although the two proteins are only 40% identical in sequence (6). Due to this similarity, HEWL is often used as a model for human lysozyme, which can form amyloids in the body and cause disease.

Hen egg white lysozyme has been studied extensively in respect to amyloid fibril formation (Table 1). There are many conditions that can be manipulated while forming HEWL fibrils, including protein concentration, temperature, incubation, salt concentration, shaking or stirring (7).

Figure 1. Crystal structure of HEWL (PDB ID: 1HEW). The image is colored by secondary structure, alpha helices in pink and beta sheets in yellow.

Ow and Dunsten (7) studied various conditions under which HEWL forms fibrils, temperature being one of them. 65°C is the temperature at which ThT fluorescence intensity is highest for the HEWL solution. Ow and Dunsten studied temperature verses peak fluorescence intensity and found the most intense point was between 60°C and 75°C. They proposed that between these two temperatures there are a couple factors that impact the fibril formation; the collision frequency of unfolded proteins and the balance of disordered aggregation with unfolded protein refolding into fibril structures.

It has been suggested by Hill *et al*.(8) that HEWL fibrils form and lengthen through nucleation- limited growth. Oligomers are the basic nucleation and growth unit in hen egg white lysozyme, based on their experimental data. Oligomers aggregate linearly and as they grow they start to curve and spiral.

Frare *et al* (6) analyzed the peptide residues after fibrils were formed under acidic conditions and found they were made up almost entirely of peptides from residues 49-101. After further experimentation, they determined that there was a certain region of the protein that more readily form fibrils and regions that were less likely. The protein was broken into fragments and studied. Fragment 57-107 is the region containing the beta domain, the amino acids that form an alpha helix, and two intramolecular disulfide bridges. This region has been called the amyloid core of HEWL and is thought to be the possible starting point of the protein aggregation. This is in contrast to fragments 1-38 or 108-129, which contain covalent disulfide brides and alpha helices but do not readily form fibrils. (6)

Table 1 Summary of HEWL fibril formation experiments at acidic pH.

Protein Concentration	Temperature	Incubation Time	Stirring Speed (if applicable)	Solution	pH	Other Notes & Procedures	Reference
1 mM	37°C	56 days			pH 2	Also performed at pH 7.4	(9)
1 mM	65°C	48 hours			pH 2	Also performed at pH 7.4	(9)
0.5 mg/mL	55°C	24 hours- 7 days	30 rpm		pH 2		(10)
0.2, 0.5, 1, 2.5 mg/mL	65 °C ideal at 0.2 mg/mL	40 hours	550 and 840 rpm	HCl	pH 1.6	Samples contained 50 μM ThT, tested temperatures between 55°C and 80°C	(7)
1 mM	65°C	up to 10 days		10 mM HCl	pH 2		(6)
10 mg/mL	57°C	2 days		40 mM Phosphate Buffer, 0.2% sodium azide	pH 2.1	Performed with and without hydroxyproline	(11)
10 mg/mL	58°C	300 hours		10 mM Glycine HCl, 130 mM NaCl	pH 2.0		(12)

10 μM	65°C	120 minutes	1200 rpm	70 mM Glycine Buffer, 80 mM NaCl	pH 2.7		(13)
2 mg/mL	55°C	2 weeks		50 mM Glycine HCl buffer	pH 2		(14)
17 mg/mL	50°C	4-5 days		175 mM NaCl	pH 2		(8)

1.3 Food Protein Fibrils

Although it may seem that protein aggregation is detrimental to food products, fibrils have the unique potential to be useful in processing. Due to the length and strength of fibrils, they can be used to help with gelling and foaming abilities. Many different food proteins have been studied under conditions favorable to fibril formation, including beta-lactoglobulin, alpha-lactalbumin, lysozyme, kappa-casein, whey protein, and soy protein.

Lassé *et al* (15) formed and tested food fibrils from many protein sources to determine the possible toxicity in the human body. Fibrils from kidney bean, soy, egg white and whey were successfully created and tested against human cell lines for toxicity. It was seen that these fibrils did not show toxicity however they were mostly resistant to proteolysis at 3 hours, suggesting that they can survive partial digestion (15).

Soy protein isolate was studied in an attempt to compare it to whey protein isolate, which has been studied extensively. The results of this experiment showed that soy protein isolate forms fibrils similar to those of whey protein isolate. Akkermans *et al* states that due to the shear thinning behavior and high viscosity, the fibrils from soy protein isolate are highly likely to be used in food products. (16)

Beta-lactoglobulin and whey protein isolate aggregates have been studied in regards to gelation, foaming and emulsification (17). One such experiment used long BLG aggregates formed at pH 2 and 80°C for 10 hours. The pH was then raised and the solution was crosslinked using $CaCl_2$. The cold set gels that were formed using this method were shown to create gel networks at a lower concentration than is usually required (18).

Although most of the knowledge on aggregates and their functionality in foams is based more on small pieces, it can still be relevant to long fibril like structures. Long, fully formed

fibrils can be broken down into smaller, seed like aggregates. It is possible that these have the same functional value as small aggregates formed specifically for functional testing. Jung *et al* tested processed long fibers and smaller aggregates against shear stress. While the long fibers produced the highest interfacial yield stress response, the small aggregates showed a very small one. Essentially the longer fibers produced a more rigid system verses the samples that contained smaller aggregates. (19)

Alpha lactalbumin (ALA) is a common globular protein found in milk and is important to the biosynthetic production of lactose in mammary glands. Alpha lactalbumin is also a protein with strong calcium binding sites and multiple stable intermediate states (20). Alpha lactalbumin can form fibrils in a way similar to that of hen egg white lysozyme due to its similarity in folded structure. De Laureto *et al* studied the various states at which ALA would form fibrils and the data indicated that a more stable form than the globular native state was needed to form fibrils, and that proteolysis is a causative force in fibrillation of alpha lactalbumin. (21)

Bolder *et al* (22) studied alpha lactalbumin, bovine serum albumin, and beta lactoglobulin in respect to gelling and fibril formation. They found that alpha lactalbumin and bovine serum albumin increased gelling capabilities but did not readily form fibrils unless beta lactoglobulin was present in the solution. In solutions where BLG formed fibrils, both ALA and BSA helped the solutions gel at lower concentrations of BLG fibrils. (22)

Ghahghaei *et al* (23) studied the effects of added components on solutions of ALA during fibril formation. The addition of dextran to the fibril forming solution increased the kinetics of ThT binding (23). It was speculated that the dextran has a destabilizing effect on the protein which allows for more aggregation, and when the UV spectra was studied there was a large change in tertiary structure in the protein when dextran was added (23).

1.4 Nanofibrils and Technology

There is growing interest in developing functional protein nanofibrils, and although this filed is only a few years old, already there are several examples of applications of protein nanofibrils (24). Both natural and artificial amyloid fibrils are utilized in many roles under a variety of categories and are formed from multiple proteins.

So far, roles that natural fibrils are known to play include controlling gene transcription/translation, hormone storage, and adhesion (24). For example, curli fibrils composed of CsgA are integral to the structure of E. coli biofilms (25) and human peptide hormone fibrils control release within the human body (26). Common functions of artificial nanofibrils include adding structure or strength. One major area of research involving nanofibrils and technology is health and the drug industry. Targeted drug action along with diagnostics and imaging are all areas of this research that are being explored with fibrils.

Bolisetty *et al* focused on transfer of metal particles into cells. They demonstrated enhanced transfer of these nanoparticles into dendric and MCF7 cancer cells when coupled with fibrils (27). The fibril solution was fixed with metal particles beforehand and then exposed to the cells and allowed to interact. The fibrils fixed with metal particles were found in greater number inside the cell than those that were not fixed (27).

1.5 Microwave and Food Protein Fibrils

Within the past couple of decades microwave heating has gained popularity. Not only are scientists employing and researching microwaves but they are commonly found in homes across the world. The relationship between microwave radiation and the sample can fall under two encompassing categories, thermal and non-thermal effects (28). One of the most important points

about microwave heating is that it heats up the entire sample directly as opposed to a conductive type heating method that will heat water, or a surrounding area, which transfers the heat to the sample. The importance of this is that a protein in solution can be heated directly and rapidly and it is possible that fibrils will form easier.

Although microwave heating has many variables such as its dependence on dielectric constants, overheating of polar substances, and hot spots, it has been shown to increase the rate of reactions in organic synthesis (28) and fibril formation of beta-lactoglobulin (29; 30). Limited research has been conducted on food proteins that form fibrils under microwave conditions. Two studies of beta-lactoglobulin used microwave heat to form fibrils in a significantly shortened amount of time compared to conventional heating (29; 30).

It was speculated by Lee *et al* that the synthesis of fibrils with microwave is different than with classical heating methods due to the distribution of heat (29). With microwave heat the water molecules in the solution are rotated whereas classical heating methods use an increase in air temperature which transfers to the solution. Beta-lactoglobulin fibrils were formed with different characteristics based on the irradiation time period ranging from short (<80 seconds), to mid (=80 seconds), and long (>80 seconds). Raising the temperature quickly combined with a short irradiation time interval led to rapid unfolding and denaturation of the protein and formation fo small aggregates. With a gradual increase in temperature; and an irradiation time period of 80 seconds, thin fibrils were formed. The diameter of the fibrils was also found to depend on the time interval (29).

Hettiarachchi *et al* (30)used and compared both microwave and conventional heating (conductive heat) methods in respect to kinetics of fibril formation. When studying a solution of Beta Lactoglobulin, the 2 hour microwave sample is where the highest Thioflavin T fluorescence

was seen and fibril formation was thought to reach its plateau. This is similar to the 8 or 10 hour sample heated under conventional heating methods. The 16 hour microwave heated sample resulted in shorter aggregates than that of the 2 hour microwave sample. Hettiarachchi *et al* (30)speculates that microwave heating irreversibly alters the fibrils where after 2 hours they no longer grow in length. This could be due to the more rapid unfolding of Beta Lactoglobulin in the microwave verses conventional heating methods at the same temperature.

Hettiarachchi *et al* (30)studied the peptide composition of microwave formed fibrils and conventionally heated fibrils as well as the kinetics. Microwaved samples contained not only intact protein but large peptides, both of which were not present in conventionally heated samples. It was proposed that a non-thermal effect of microwave is the decoupling of hydrolysis from other processes, which in turn changes the denaturation of the protein. This would result in peptides of different lengths then have been previously reported.

1.6 Mechanism and Peptide Composition

Proteins first undergo destabilization. It is supported that the process of fibril formation is most likely to begin with a partially unfolded protein. This partial unfolding can be induced from many different means including low pH and high heat, however this isn't always necessary because fibrils can start to form through local folding changes as well. Fibrils then form through a method called nucleation- growth, a nucleus of monomeric structures is first created and then more monomers are then added upon that nucleus. These structures eventually form protofilaments which twist upon each other to create mature fibrils. (2)

The compositional aspects in the formation of fibrils between microwave heat and common conventional methods could stem from changes to the aggregation process in any stage.

Four main stages in fibril formation when microwave could affect the process include during unfolding, hydrolysis, initial fibril formation, and growth (30). When microwaving solutions there is rapid unfolding which may allow for quicker fibril formation.

It has been suggested that proteins fold through units of secondary structure (31). It is then logical to look at the effects of microwave on secondary structure of proteins and how it is altered through this method of heating. Calabrò *et al* studied the secondary structure of HEWL in respect to microwave and found that it has an effect verses conductive heating. They stated that there was an increase in the intensity of amide 1 vibration after microwave, showing a change in the proteins confirmation, as well as an increase in beta sheet and beta turns in the amide 1 mode, corresponding to increased disorder and unfolding. Both increases were seen greater in samples treated with microwave heat verses other forms of heating. (32) Gomaa *et al* tested the effects of microwave on beta lactoglobulin. Substantial thermal unfolding and decrease in all secondary structure was seen in this experiment as well as accelerated changes in tertiary structures (33).

As previously stated, Hettiarachchi *et al* studied the peptide composition of fibrils formed via microwave with those produced under other conventional heating methods. The difference in these fibrils stemming from size of peptides. Microwaved fibrils produced more sites accessible for binding verses conventional heating methods as well as increased availability of hydrophobic sites. These microwaved fibrils are larger and therefore have more parts extending out from the core, leading to these increases in accessibility. Regardless of size, fibrils heated through both methods stacked in a similar manner. Hettiarachchi *et al* also suggested that the faster kinetics of fibrils formed through microwave heating could be due to a more flexible backbone due to rapid unfolding of the protein. (30)

Studies have shown that the first step in fibril formation begins with a defined starting point, whether this is rapid unfolding via hydrolysis, local conformation change, or a change in the equilibration of protein (2). When taking into account information from previous microwave studies on secondary structure (32; 33), it is reasonable to assume that fibrils can vary compositionally based on the starting point.

1.7 Objective

Although research on fibril formation and kinetics is widespread and varied, the amount of knowledge on fibrils being used specifically for food applications is limited.

The main question of this work is does microwave effect hen egg white lysozyme fibril formation kinetics and morphology? Two previous papers on microwave effecting beta-lactoglobulin fibril formation are the basis of this work. Microwave alters kinetics of beta-lactoglobulin fibrils to form in hours, can control the morphology, and changes the peptide composition (29), (30).

If similar results are also seen with hen egg white lysozyme, are these effects related to changes in hydrolysis? The second half of this research focuses on these changes due to microwave and discusses the role hydrolysis plays.

Beta Lactoglobulin solutions heated under microwaves were considered preliminary results for this research and can be viewed in Appendix A.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental Design

The flow chart below shows the set-up of this research, the measurable results in circles.

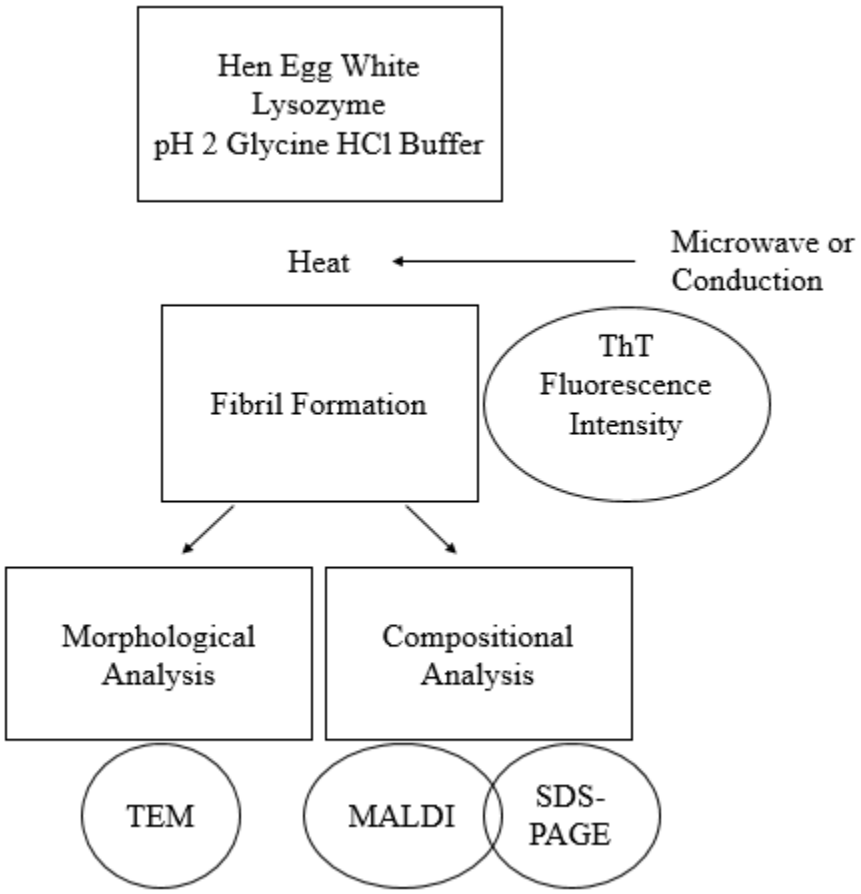

Figure 2. Flow chart of experimental design

2.2 Materials

Ninety percent purity hen egg white lysozyme was purchased from Sigma Aldrich (St. Louis, Missouri). Beta-lactoglobulin was obtained from DavisCo Foods International Inc (Le Sueur, Minnesota), product ID JE 003-6-922. All other chemicals were of ACS reagent grade or higher.

2.3 Sample Preparation

Twenty-five milliliter stock solutions of hen egg white lysozyme were prepared at 13 mg/mL. HEWL stock solution was prepared using a Glycine-HCl buffer at pH 2 and filtered with PES 0.22 μm filters. Stock solutions of BLG were prepared at 11 mg/mL, protein was first dissolved in H_2O on ice while stirring for 10 minutes. This was then filtered using PES 0.22 μm filters and titrated to pH 2 using 100% HCl. Final concentrations were determined using a ThermoScientific Nanodrop One.

2.4 Heating

Using a CEM Discover Proteomics instrument, the samples were heated under microwaves while being held at a constant temperature. Two mL of the protein solution was transferred to CEM 10 mL glass vials sealed with septa caps and a stir bar added if needed. The instrument was set at a 4 W cut off, programed to the desired temperature, with no pressure cut off. Continuous 60-minute runs were performed, with the stir setting on low (440 rpm) if needed. The 60-minute runs were repeated until the desired time period was reached.

The conventional heating method included a Pierce Reacti-Therm III Heating/Stirring module. For these conventionally heated a glass vial and stir bar were used in a heating block

designed to fit the Reacti-Therm instrument. Aliquots were taken at time intervals and held on ice until read under fluorescence spectrophotometry at 25°C.

Using either heating method, beta lactoglobulin was heated at 80°C and hen egg white lysozyme was heated at 65°C.

2.5 Fluorescence Spectrophotometry

Fluorescence spectra were recorded using an Agilent Technologies Cary Eclipse (Santa Clara, California) fluorescence spectrophotometer with a 10 mm quartz cuvette cell and the following settings: excitation at 440 nm, emission scan from 465-510 nm, a 5nm slit width for the excitation and emission light, a 30 nm/min scan speed, and a PMT detection filter voltage of 700 V. The sample volume was 2.5 mL and consisted of a mixture of 25 µl protein sample and 2475 µl 50 mM ThT solution.

2.6 Transmission Electron Microscopy

Transmission electron microscopy was performed on a JOEL JEM1011 transmission electron microscope (Peabody, Massachusetts) using 150 grid copper mesh grids coated with formvar and carbon. After grids were formvar and carbon coated they were prepared with the samples to be viewed. An aliquot of the sample was diluted and approximately 2 µL of this dilution was loaded onto the grid. This was set to dry for 5-10 minutes, but was not allowed to completely dry onto the grid. The grid was then washed with a drop of H_2O and the excess water was wicked off. After the washing step was completed, a 2% uranyl acetate solution was used to negatively stain the sample. A drop of the 2% uranyl acetate solution was placed on the grid, just

enough to cover it entirely, and allowed to sit for approximately 30 seconds. The excess was then wicked off. At this point the grid was prepared and ready to view

Images of the fibrils were analyzed using both Image J (34) and Easyworm (35). Fibril widths were measured in Image J- and the persistence length determined using the contour function in the Easyworm software. After loading the images into Image J, under the analyze tab the set scale and measure functions were applied. First the "straight" tool measured the scale bar from the original images and was used to set the scale, and second the length output of the measure tool was used to find the average. Two images from each sample were used and at least 100 widths were measured on each image. Images need to be square when loaded into Easyworm therefore the first step was to crop the images to be analyzed. After loading the images into Easyworm1, 10 fibrils in each image were highlighted by hand and the data was exported through the software (see Figure 3). This data was then opened in Easyworm2 and under the "contour/end-to-end" tab, launch fit was pressed and the bins and upper limit were adjusted until the R value was adequate. The value in the "contour/end-to-end" tab is the average persistence length of the fibrils in that TEM image.

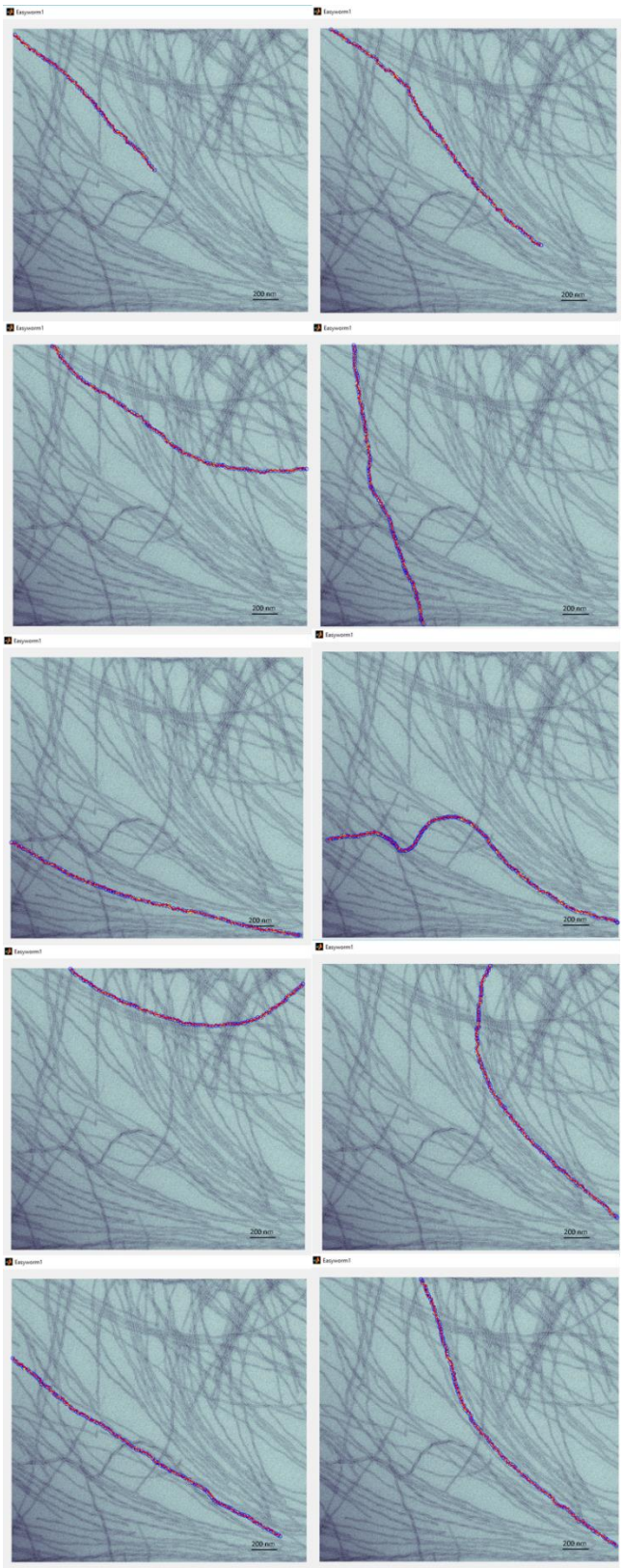

Figure 3. Easyworm1 example of fitting fibrils from 15 hour microwave

2.7 SDS-PAGE

16.5% Tricine gels were purchased from BioRad, along with Tricine running buffer and sample buffer. All samples were prepared in sample buffer containing 0.02% beta-mercaptoethanol solution (reducing conditions), heated for >10 min at 70 C and loaded into the gel along with a Thermo Scientific Spectra Multicolor Low Range Protein Ladder pre-stained standard. Running conditions were executed under 100 V for approximately 100 minutes, or until the dye front reached the bottom of the gel. Gels were then fixed with a solution of 50% methanol, 40% H_2O, and 10% acetic acid, stained using coomassie brilliant blue R250 dye solution, and de-stained with a solution of 87.5% H_20, 7% acetic acid, and 5 % methanol.

2.8 MALDI-TOF

Samples were sent to the University of Georgia Proteomics and Mass Spectrometry Core Facility where they were prepared and analyzed with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) using a Bruker Autoflex mass spectrometer (Billerica, Massachusetts). The instrument in this facility has been used extensively to look at peptide fingerprints. The resulting data were analyzed using Origin, calculating the area under the curve for the peak corresponding to intact HEWL.

CHAPTER 3

RESULTS

3.1 Kinetics

Thioflavin T (ThT) fluorescence was used as an indicator of fibril formation. ThT fluorescence intensity correlated to the amount of fibril formation seen in each sample, the greater the intensity the more beta sheet structures in the sample (and fibrils). While ThT fluorescence was not used to quantify the absolute amount of fibril formation, following the intensity increase was used to measure the kinetics of fibril formation.

The kinetics of HEWL fibril formation via microwave heating include a lag and growth phase, ending in a plateau (Figure 4). The lag phase ends around 7 hours with the growth phase the next 4 to 5 hours before tapering off to a plateau. Measurements were limited to a 15-hour scale due to instrumental limitations.

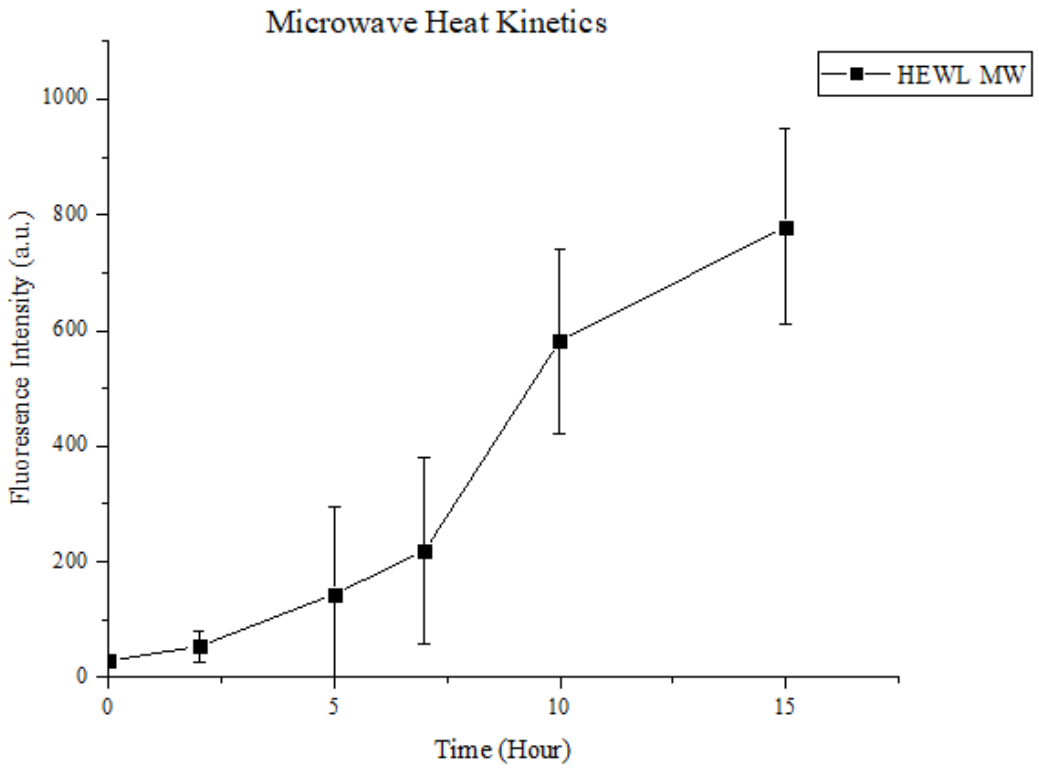

Figure 4. HEWL fibril formation via microwave heating. Samples were maintained at 65°C, pH 2, with stirring at 440 rpm. Data points correspond to the average ± standard deviation. For hours 7 and 10, n = 3; hours 5 and 15, n = 4; hour 2, n = 5; and time zero, n = 7.

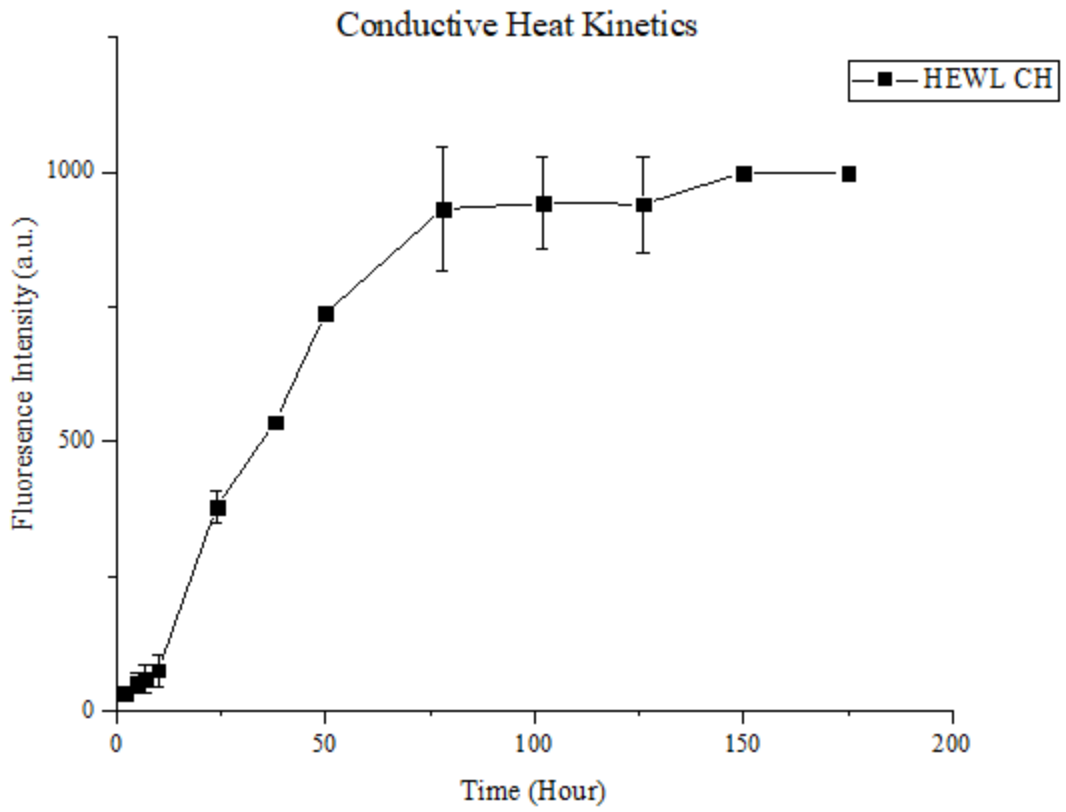

Figure 5. HEWL fibril formation via conductive heat. Samples were heated at 65°C, pH 2, using a ReactiTherm at medium level stirring. Data points correspond to the average ± standard deviation, n = 3.

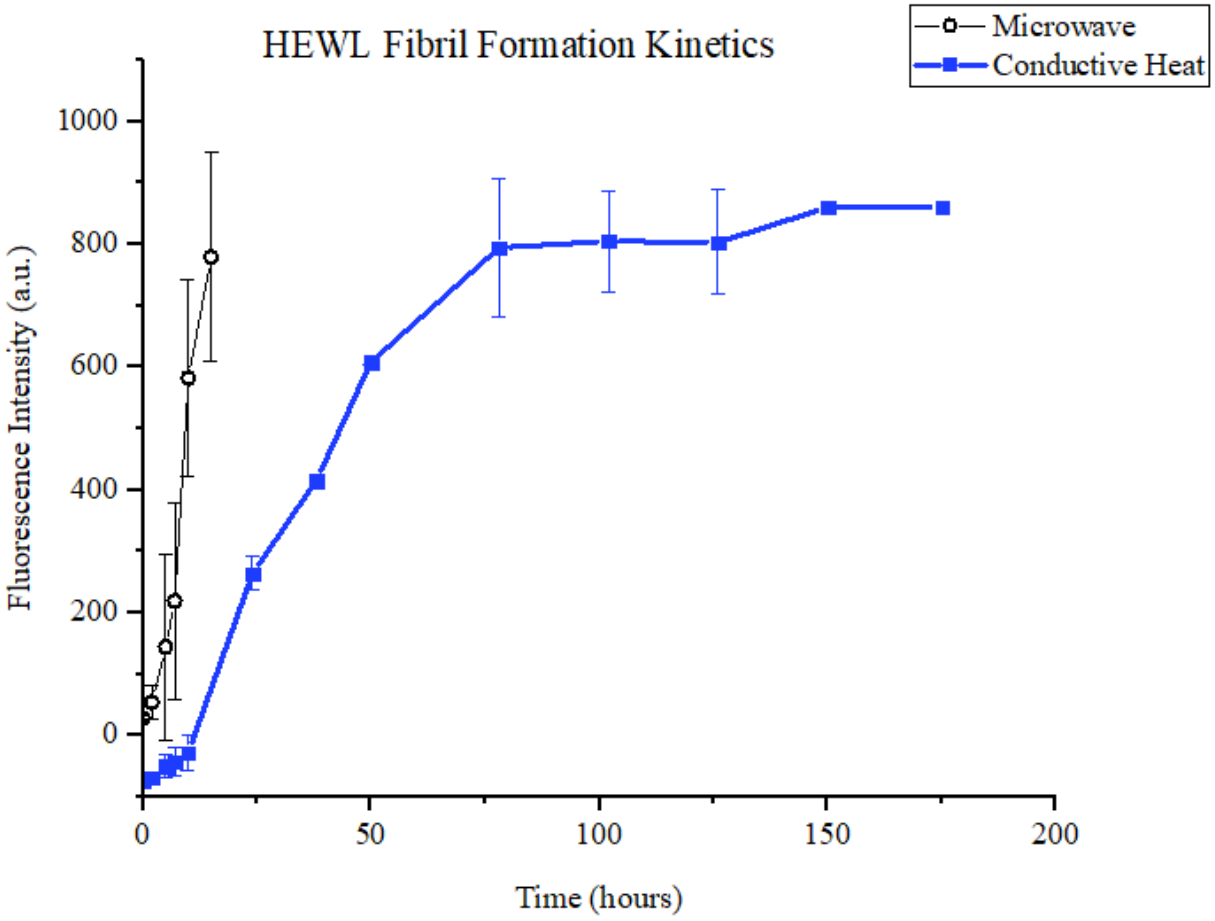

Figure 6. Comparing fibril formation induced by conductive (ReactiTherm) and microwave heating. The full time-scale of samples heated under microwaves (black line) and conductive heat using the ReactiTherm instrument (blue line) is shown. All samples were maintained at 65°C, pH 2, with stirring.

3.2 Morphological Analysis

Transmission electron microscopy images were taken using the JEOL instrument in the Georgia Electron Microscopy center (Figure 7). Samples were diluted appropriately and then

loaded onto grids and negatively stained to see contrast and view the protein aggregates. The images were loaded into software for further processing.

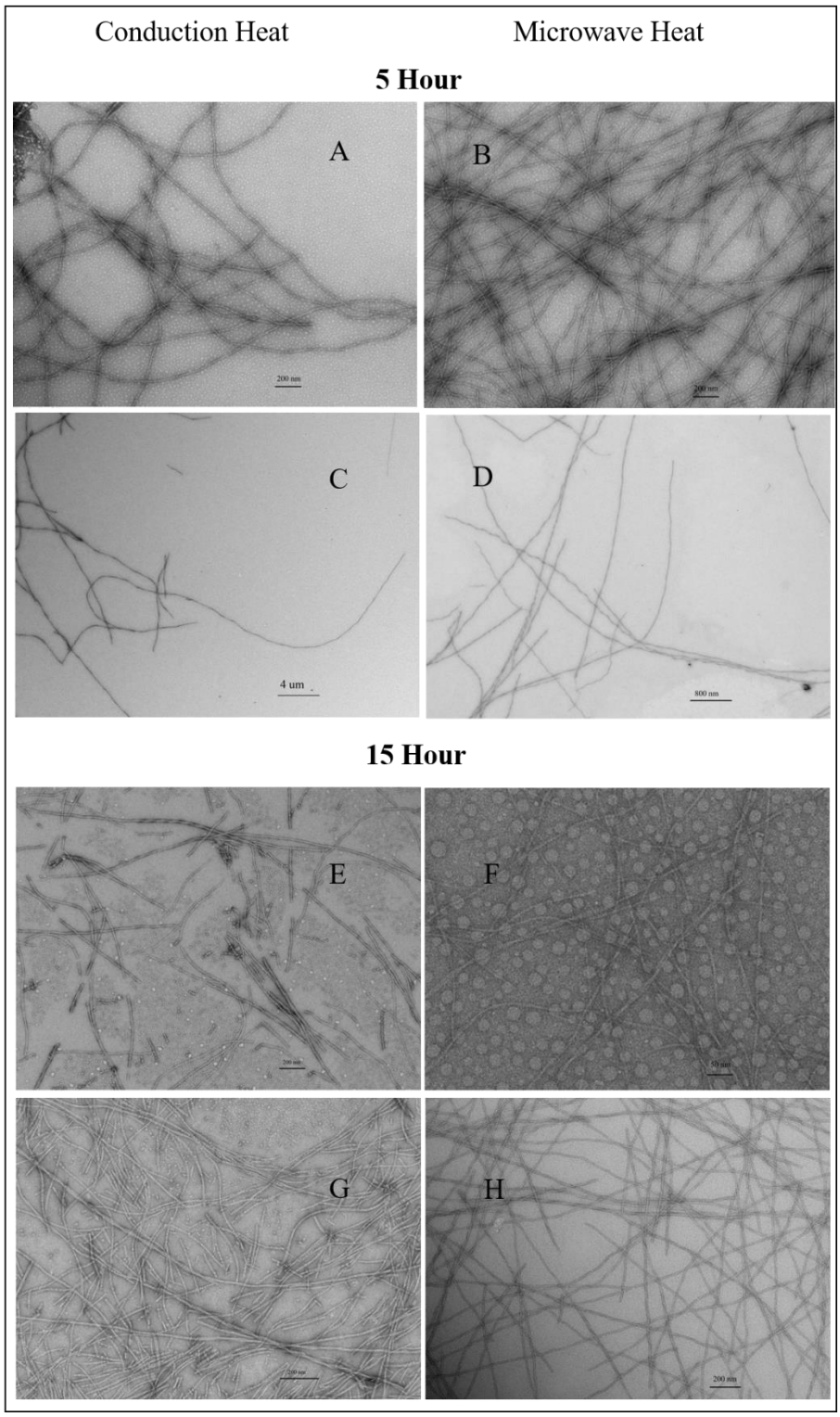

Figure 7. Transmission Electron Microscopy Images. Images A, B, E, G & H include a 200 nm scale bar, Image C a 4 μm scale bar, Image D a 800 nm scale bar, Image F a 50 nm scale bar.

ImageJ was used to measure the width of fibrils. Two images of each sample were chosen and at least 100 points were measured on each image. The widths of the fibrils at 5 hours for both microwave and conventional heat were around 15-16nm wide on average however the 15 hour microwave fibrils were smaller. These had an average width of about 7 nm. (Table 2)

Table 2 Fibril widths. Average widths of fibrils, calculated using the measure tool in ImageJ on TEM images of samples.

	Heat	Average Width	Standard Deviation		Heat	Average Width	Standard Deviation
5 Hour	MW	16 nm	4.1	5 Hour	CH	15 nm	4.4
15 Hour	MW	7 nm	1.8	15 Hour	CH	11 nm	3.7

Easyworm software was used to get the persistence length and contour length of the fibrils from the images taken on the JOEL instrument (Table 3). The persistence length of both the 5 hour and 15 hour microwave samples were similar, however the lengths of the 5 hour and 15 hour conventional heat samples had large variation. Both microwaved samples had persistence lengths around 1700 nm and contour lengths around 2000 nm. The 5 hour conventional heat sample's persistence length was calculated to be 897 nm with the contour length being 1880 nm whereas the 15 hour conventional heat sample's persistence length was around 2326 nm and a contour length of 916 nm. The easyworm tool allows for the user to isolate individual fibrils and then the software calculates multiple output functions.

Table 3 Fibril persistence and contour lengths.

Time	Heat	Persistence Length	R^2	Contour Length
5 Hr	MW	1728 nm +/- 476	0.995	2046 nm +/- 847
5 Hr	CH	897 nm +/- 245	0.984	1880 nm +/- 557
15 Hr	MW	1707 nm +/- 488	0.997	2337 nm +/- 950
15 Hr	CH	2326 nm +/- 513	0.999	916 nm +/- 648

3.3 Compositional Analysis

First, SDS-PAGE was performed to examine the peptide composition of the different fibril samples (Figure's 8-10). Untreated lysozyme was loaded, along with standards, and a large band was seen around 14 kDa. Microwaved fibril samples all contained a significant band at around 14 kDa as well, which was assumed to be the in-tact protein. It is not clear whether this protein was incorporated into the fibril aggregates or did not aggregate under these conditions. As the time increased with the microwave heat, the large band seen at 14 kDa did decrease in size but did not disappear. The samples also showed an increase in band intensity for peptides below 14 kDa but above 5 kDa. The 2 hour microwave sample contained the largest and most packed band at the 14 kDa and only faint bands corresponding to peptides. Over time the peptide bands became more intense. These results indicate that as fibril formation progressed, the amounts of intact protein decreased (yet remained substantial) and the peptides increased.

Comparing the microwaved samples to the conventionally heated samples showed large differences. The sample heated for 6 days conventionally showed just a slight band at the 14 kDa mark but a very large band at 4.6 kDa. This is in contrast to the microwaved fibril samples. SDS-

PAGE indicated that as the fibrils formed over six days using conventional heat, the intact protein was largely degraded.

The effects conventional and microwave heating were compared between 2 and 24 hours. The sample that was conventionally heated for 2 hours showed a large band of intact protein at 14 kDa with that band decreasing over the 24-hour period. The 2 hour conventionally heated sample had bands that looked very similar to the solution of untreated lysozyme. There was an increase in peptide bands as the heating time increased. The SDS-PAGE profile of the sample that was conventionally heated for 24 hours was similar to that of the samples that were microwaved for 2 or 5 hours.

SDS-PAGE was also performed using aliquots taken in the first two hours to determine the earliest stages of hydrolysis of the samples (Figure 9). In the first 120 minutes of conventional heating, very little degradation occurred as evidenced by the lack of bands below 14 kDa. The four aliquots showed large bands of intact protein at 14 kDa and few peptides. In contrast, during the first 120 minutes of microwave heating, several peptide fragments were formed. Peptides were apparent in the microwaved samples in as little as 30 minutes.

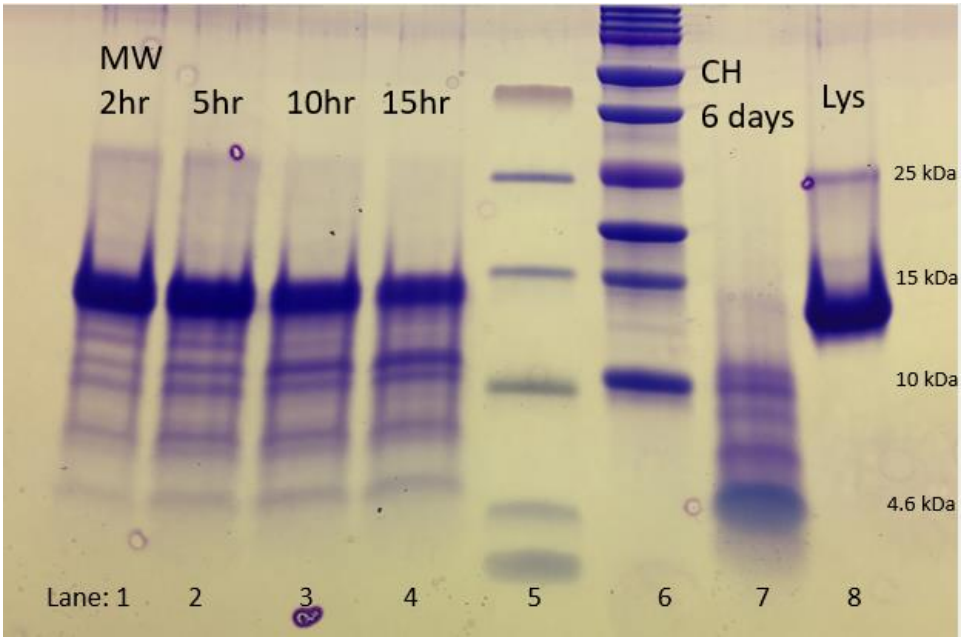

Figure 8. SDS-PAGE- Microwaved and Conductively Heated Samples. Sample lanes are labeled. Lane 8 is untreated Lysozyme (14.313 kDa).

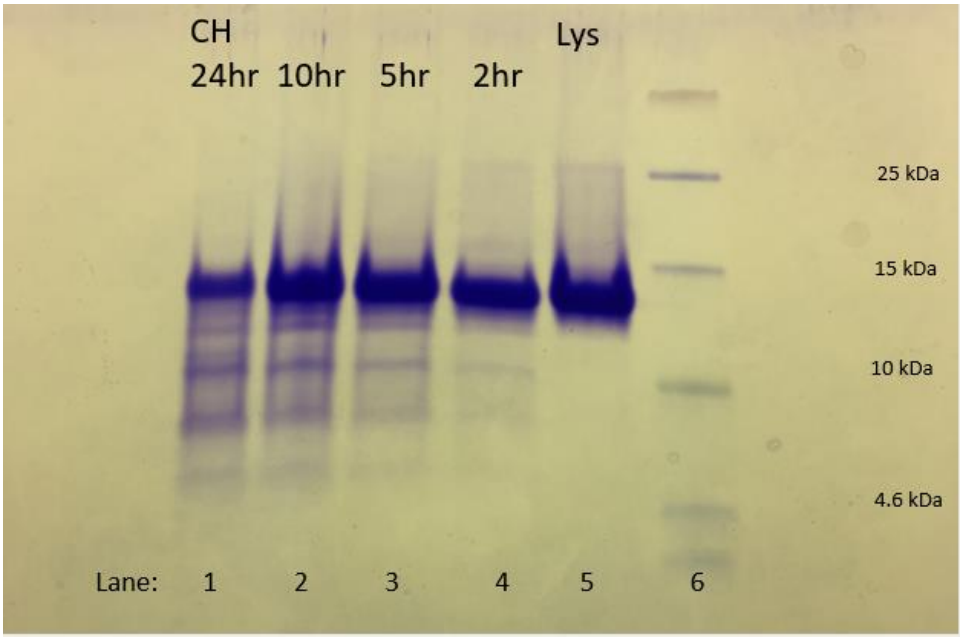

Figure 9. SDS-PAGE- Conductively Heated Samples. Time lengths of heating are labeled, lane 5 is untreated Lysozyme (14.313 kDa).

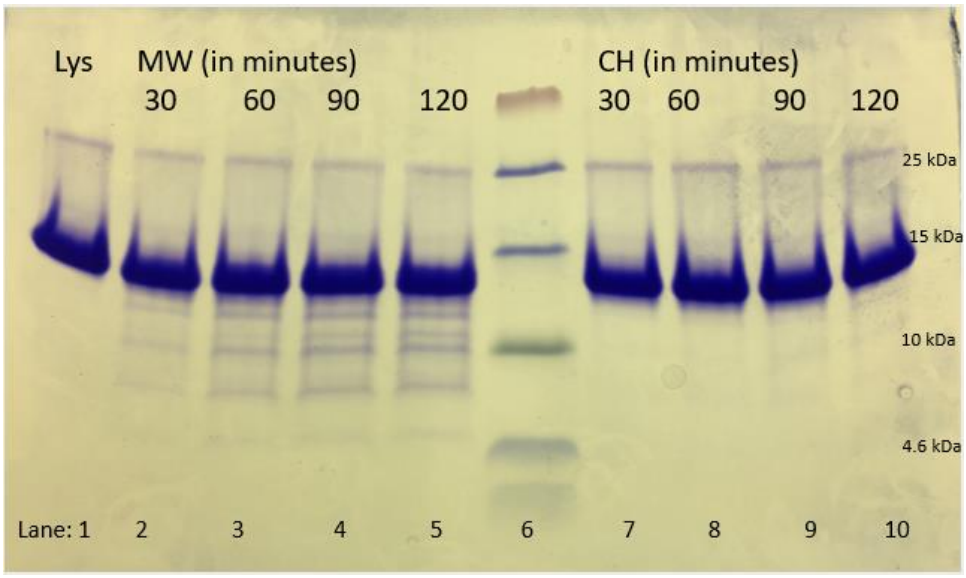

Figure 10. SDS-PAGE- Microwave and Conductively Heated Hydrolysis Samples. Aliquots taken within the first 120 minutes. Sample lanes are labeled. Lane 1 is untreated Lysozyme (14.313 kDa).

Fibril samples were sent to University of Georgia Proteomics and Mass Spectrometry Core Facility and analyzed by MALDI-TOF mass spectrometry. A peak around 14 kDa was found in both samples. This peak was correlated to the intact hen egg white lysozyme left in the sample after fibril formation. The area under these curves were analyzed to compare heating methods and support results seen with SDS-PAGE. The conductively heated sample had an area under this curve of around 770,000 whereas the microwave heated sample had one of 1,790,523. In an effort to analyze fully formed fibrils, 6 days of conductive heat and 15 hours microwave heat were specifically used with MALDI. These time points are the longest heated samples for each method.

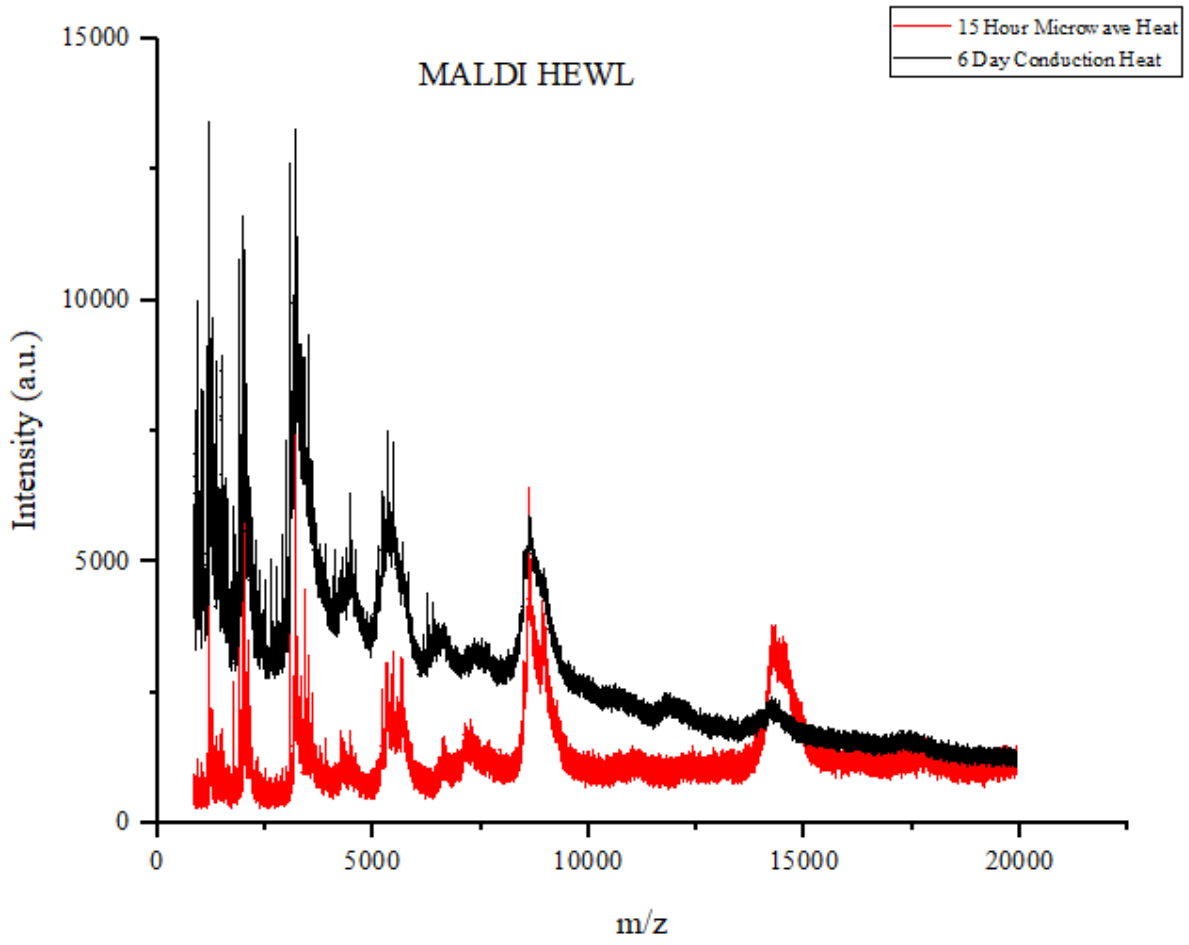

Figure 11. MALDI-TOF mass spectra of microwave and conductively heated HEWL fibrils. Samples treated with 15 hours of microwave heat (red line) and 6 days of conventional heat (black line) were compared.

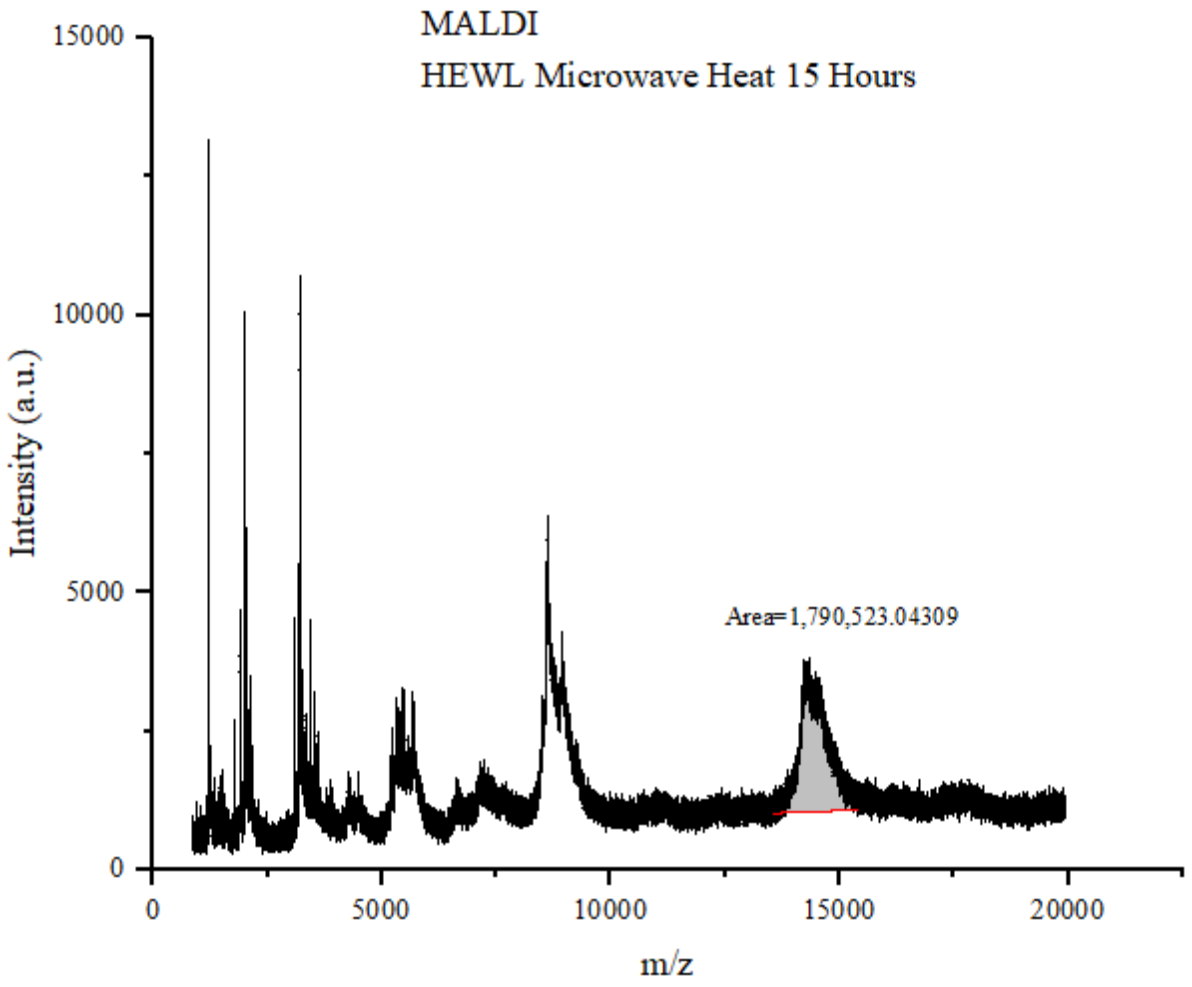

Figure 12. MALD-TOF mass spectra of microwave heated fibrils. The area of the peak at 14290.6 m/z was analyzed, which corresponds to intact HEWL.

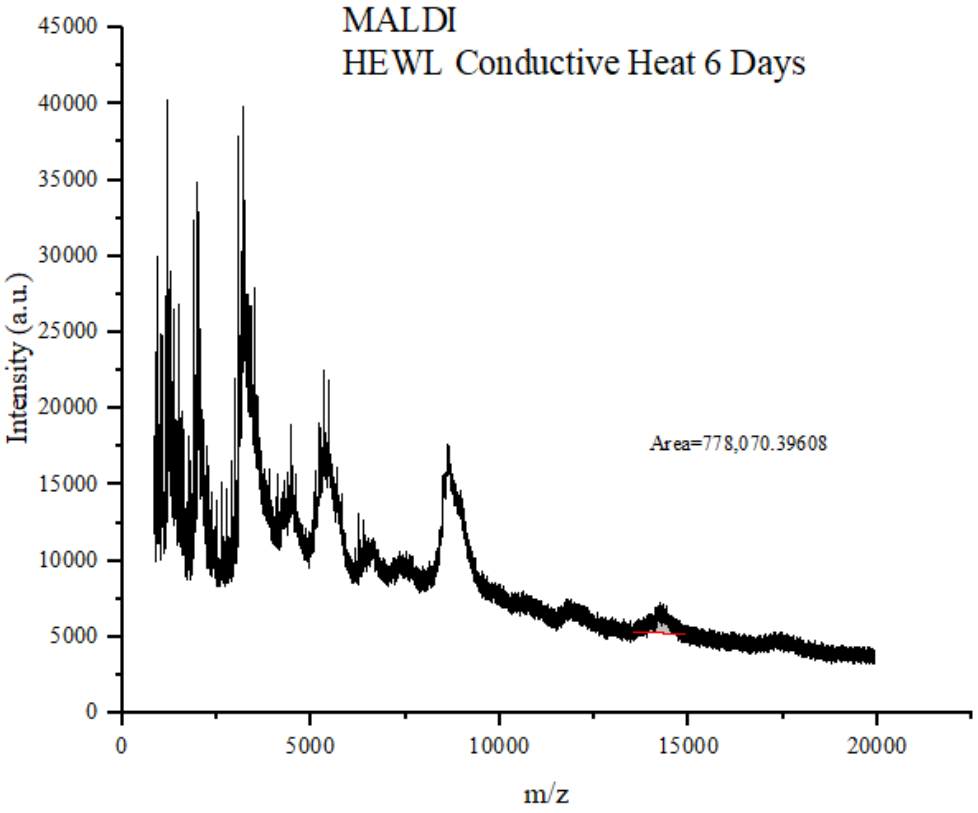

Figure 13. MALDI-TOF mass spectra of conductively heated fibrils. The area of the peak at 14236.7 m/z was analyzed, which corresponds to intact HEWL.

CHAPTER 4

DISCUSSION

Previous research on microwaving food proteins to form fibrils has not analyzed hen egg white lysozyme. Microwaved beta lactoglobulin fibrils are typically used to study the kinetics, morphology and composition. The specific behaviors of beta lactoglobulin verses hen egg white lysozyme (HEWL) when heated conventionally and under microwave are believed to contribute to the differences in the results compared to what was expected. The expected results were to see a peak in fluorescence intensity around two hours, with the intensity then decreasing (30). This type of peak is not seen when heating beta lactoglobulin under conventional heat. When gathering preliminary data with Beta Lactoglobulin this trend was seen, but with the experimental changes of using HEWL different behaviors emerged. HEWL under microwave heat seems to behave very similar to that of HEWL under conventional heat just on a quicker time scale. The kinetic curve of both heating methods has a similar shape with a lag phase, growth phase and plateau.

Measuring the kinetics of fibril formation under microwave heating was the first objective of this research. The overall fluorescence intensity throughout the experiment was analyzed as a kinetic profile. With HEWL the microwave heat produces a kinetic curve similar to conventional heat but on a reduced time scale. Under microwave heat the lag phase ends around 7 hours and the growth phase is ending around 10 hours as opposed to conventional heat where the lag phase ends around 10 hours and the growth phase doesn’t end until around 80 hours.

Previous research has shown that several factors apart from chemical changes can affect the formation of fibrils in solution, such as the rate of stirring (7). Similarly, here it was found that HEWL fibrillation can be highly sensitive to the environmental conditions. When heating conventionally, the container and stir bar seemed to make a large effect on the rate of fibril formation. When the solution wasn't stirred it created an even greater change.

Storm *et al* states that "a filament is considered to be flexible when $l_p << L$ and rigid when the opposite holds" (36) with l_p being the persistence length and L being the contour length. The data calculated from a representative TEM image for each sample was used to determine flexibility of the fibrils (Table 3). First looking at the 5 hour samples, both microwave and conductive heat, calculating from the chosen TEM images gives a ratio of persistence lengths to contour lengths that may be considered flexible. The contour length to persistence length ratio is somewhat larger in the 5 hour microwave sample versus the 5 hour conductive heat sample; however, they both show flexibility at this stage. Increase in microwave time does not show a significant effect. The contour length and the persistence length remain unchanged from 5 hour to 15 hour microwave but with the margin of error increasing with time. Lastly, the 15 hour conductive heat sample does not show flexibility. The contour length is shorter than the persistence length of this sample.

Fibrils formed at 5 hours of heating are somewhat flexible. This is true regardless of whether the heat is conductive or microwave, and they are in fact similar to each other in many aspects. This is supported by persistence and contour lengths as well as the widths, and tentatively by SDS-PAGE results.

Transmission electron microscopy highlights the differences in the samples heated for 15 hours, both conventionally and microwave. Visually, images of the two samples look quite

different. The conductively heated sample has smaller pieces as well as long twisted strands. The microwaved sample contains all very long, twisting strands with no presence of smaller aggregates. The conductively heated sample's fibrils are more rigid and wider on average. As time increased, the microwaved fibrils continued to remain flexible while decreasing in width, whereas the conductively heated fibrils did not. The conductively heated fibrils persistence lengths more than doubled from 5 to 15 hours and the contour lengths shortened significantly. The conductive heat created fibrils that thickened, shortened and became more rigid with time.

Both conventional heat and microwave heat samples start with the same stock solution. When the native lysozyme in solution is subjected to heat and acid it is thought that the protein partially unfolds and then specific regions begin the fibril formation. This is the usual thought for conventional conductive heat but it is speculative as to whether this same mechanism is employed by the protein in solution when microwave heat is used. The peptide content of the conventionally heated samples increases with time. It is also seen that the amount of intact protein in solution decreases as time increases. This is consistent with a mechanism where the protein first takes time to unfold and then the fibril forming regions have to begin to aggregate and grow.

The microwave heated samples don't show the same trend. As time increases, the amount of peptide in solution does also increase however the intact protein does not seem to decrease to the point of being negligible after 15 hours. Both the SDS-PAGE and the MALDI results show a large portion of intact protein along with peptides at the end of the 15 hour time period. Assuming that fibrils are fully formed at this point (see TEM images and ThT intensity), it would be logical to assume that the microwave heat forces the protein to use a different mechanism to aggregate.

The fact that there is excess intact protein could be the result of many different mechanistic aspects. One possible way the process of fibril formation is changed by microwave heat is the rate of unfolding. The microwaves not only heat the solution more directly and quicker, but there are a variety of nonthermal effects that are not entirely known. Microwaves may not only change the process of unfolding but also refolding. It has been seen that microwaves can change the secondary structure of HEWL, it is highly possible that it can change the specific structures that will be used in the fibril aggregation as well. Another possible process that is happening in this instance is that the microwaves are somehow inducing local folding along the protein. Local protein folding can lead to fibril formation, if microwaves are inducing this process than the mechanism would follow a different pattern than that of conventional heat.

The protein hydrolysis patterns were measured using SDS-PAGE in order to examine the differences between the mechanisms of fibril formation induced by conductive and microwave heating. Microwave heating produced bands corresponding to peptides within the first 30 minutes, but peptides were barely present in the conventionally heated samples after two hours. Microwave heat is hydrolyzing the protein at a quicker rate, or in a different manner, than conventional heat and either allowing fibrils to form significantly quicker than conventional heat or the peptides seen are hydrolyzed pieces of protein before aggregation.

4.1 Impact and Future Research

Amyloid fibrils can form within the human body from a multitude of proteins and are prone to cause disease. Diseases caused by amyloidosis are well known, such as Alzheimer's, Huntington's, and spongiform encephalopathy, and include insoluble aggregates near the infection site that can be classified as amyloid like (37). With the similarity between human

lysozyme and hen egg white lysozyme, any research studying how hen egg white lysozyme produces fibrils can impact the medical field (6; 38). Although this study is also relevant to producing protein nanofibrils for food applications, any knowledge contributing to the field of amyloidosis is helpful in studying disease and prevention.

This research can be the first step toward a multitude of other projects. It would be valuable to know whether food protein fibrils formed from hen egg white lysozyme have the ability to cross-seed human proteins. Because of the similarity between HEWL and human lysozyme, studies should be done testing HEWL fibril's abilities to seed human lysozyme. This is also a food safety issue. If microwaved HEWL fibrils do carry the ability to help human lysozyme to form fibrils within the body, there would need to be control measures taken to limit the ingestion of these fibrils.

The kinetic differences microwave makes are valuable, and the compositional and morphological differences provide a potential opportunity to alter nanofibrils structure/functionality based on the heating source. Isolating the fibrils from solution and using them in a food matrix would be a novel project due to the unique physical appearance of microwaved HEWL fibrils. For example, gelling and foaming in regards to protein aggregates have been explored, but utilizing the isolated microwaved HEWL fibrils may show a difference in their capabilities. At present, there appear to be no, or few, published studies that compare the functionality of microwaved and conventionally heated protein nanofibrils, so this could be a useful area of research to pursue next.

CHPATER 5

CONCLUSIONS

Hen egg white lysozyme is a highly studied protein that can form fibrils under a multitude of conditions. While many of the conditions under which HEWL forms fibrils have been studied, using microwaves as a heat source is rare. Microwave heat effects the kinetics, morphology, and peptide composition of such fibrils. First, an increased rate of formation is seen with microwave when compared to conventional conductive heat. Second, longer, thinner, more flexible fibrils are formed under microwaves. These unique fibrils have the potential to be used in applications yet to be researched. Third, the peptide composition of the microwave fibrils is altered. There is increased initial hydrolysis of the microwaved solution and an increased presence of in-tact protein after fibril formation. Microwave does affect the formation of nanofibrils when compared to a conventional heating method.

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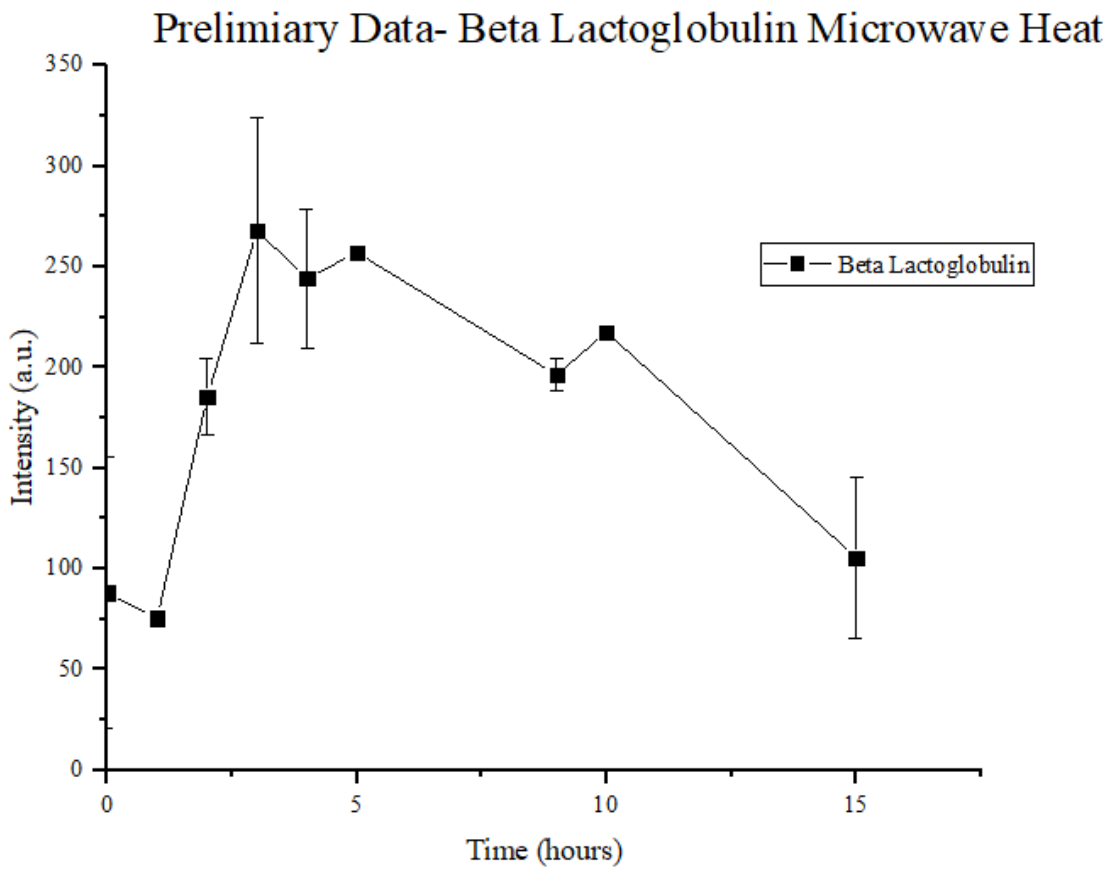
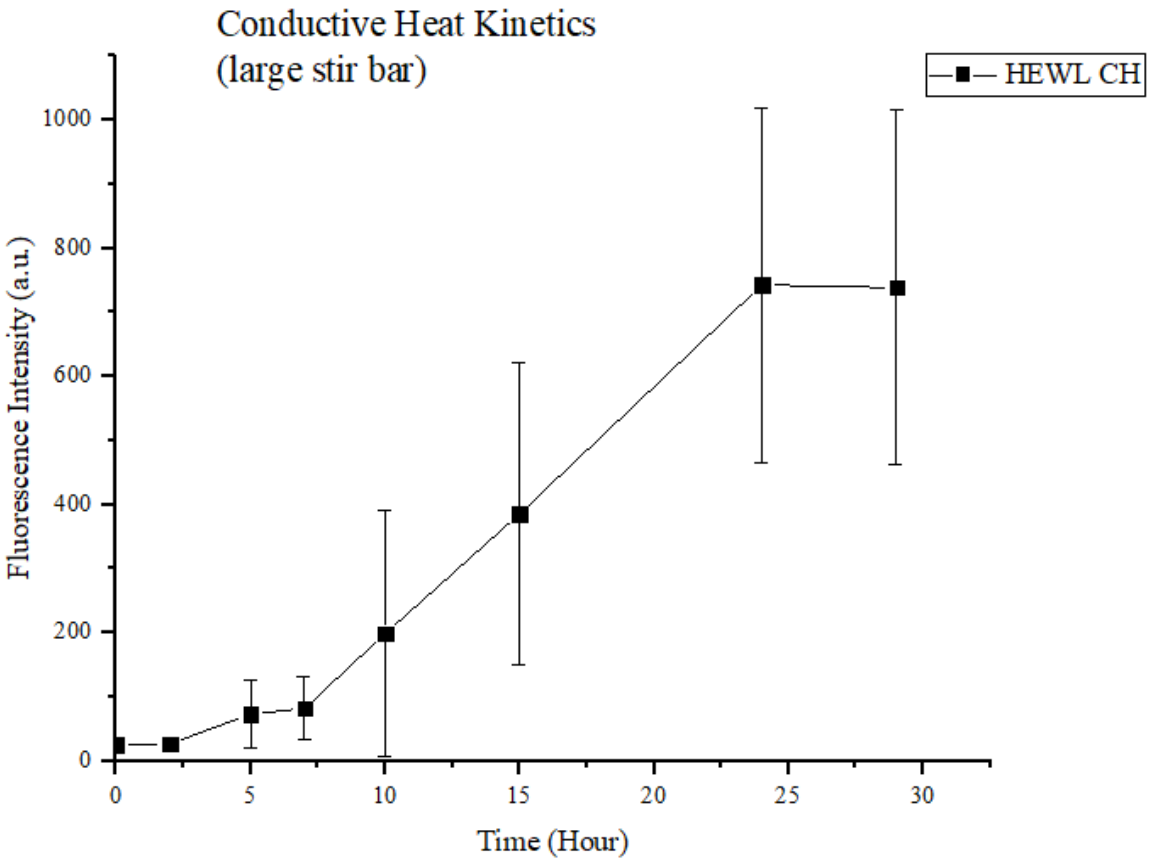
APPENDIX A



Preliminary data of beta-lactoglobulin fibril formation via microwave heat. Samples were heated at 80°C, pH 2, stirred at 440 rpm. Data points correspond to the average ± standard deviation. A peak seen around 2 hours with intensity decreasing over the 15-hour time period.

APPENDIX B



HEWL fibril formation via conductive heat. Data points correspond to the average ± standard deviation. Samples were heated in a stirring dry bath at 65°C, pH 2, stirred at 440 rpm with large stir bars.