Formation and Characterization of Legume Protein Amyloid Fibrils

by

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B.Sc., University of Alberta, 2019

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Food Science)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

December 2021

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

Formation and Characterization of Legume Protein Amyloid Fibrils

submitted by Sara Zamani in partial fulfillment of the requirements for

the degree of Master of Science

in Food Science

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Abstract

Amyloid fibrils are gaining attention as novel food ingredients and nanomaterials, due to their unique structural and chemical properties, and functionality as stabilizers and gelling agents. Amyloid fibrils are long, thin, unbranched protein aggregates that are important in medicine, biology and nanotechnology. Many proteins, including food proteins (e.g., from milk, eggs, and legumes), can be converted into amyloid fibrils under the proper conditions, most commonly by heating at low pH. Most of the research on nanofibrils has dealt with animal proteins, so a fundamental understanding of the self-assembly, structure, and functionality of nanofibrils derived from plant proteins is lacking. Plant protein is considered a sustainable source of protein. However, the functional properties of plant proteins, such as foaming, gelling, and emulsification, are generally inferior to that of animal proteins. Consequently, one method to improve the functionality of plant proteins is to produce nanofibrils, which are promising materials.

The goal of this research was to relate nanofibril structure and functionality by comparing nanofibrils made from various legume proteins. Nanofibrils were formed from peanut, lentil, pea, and mung bean during incubation at pH 2 and 85 °C. The results showed that protein extracts from peanut, mung bean, pea, and lentil formed nanofibrils, which were detected using thioflavin T and transmission electron microscopy (TEM). SDS-PAGE revealed that extensive protein hydrolysis occurred during the onset of fibril formation, indicating the significance of hydrolysis to fibrillation under these conditions. Using TEM, fibrils from different legumes showed morphological variability with differences in length, width, and flexibility. This research revealed that peanut, lentil, and mung bean fibrils were most soluble at pH 2 and least soluble at isoelectric point (pI) pH. Also, the fibrils showed smaller particle size at pH 2 to that of pH 7 which is consistent with the solubility result. The presence of the fibrils results in an increase in

viscosity compared to the unheated samples. The findings showed that a better understanding of legume fibrils is needed to increase their usage as functional materials in food systems, and that this would probably extend theoretical knowledge of the structure-function relationship between plant-based fibrils.

Lay Summary

Consumers are seeking alternatives to animal protein to improve their health and the sustainability of their diet. Legumes could be a good source of protein to meet this demand. Since the functionality of plant proteins is lower than that of animal protein, nanofibrils could improve plant functionality. The objective of this study was to induce proteins from different legumes (e.g., peanuts, pea, lentils, mung bean) to form amyloid fibrils, and their formation kinetics, structure and functionality were characterized. According to TEM images, lentil and peanut have a longer and straighter fibril, while mung bean and pea fibrils are curly and short. Peanut, lentil, and mungbean fibrils were most soluble at pH 2 and least soluble at IP pH. The fibrils showed a smaller particle size at pH 2, which is consistent with the solubility result. In comparison to unheated samples, the presence of fibrils causes an increase in viscosity.

Preface

These experiments were conceived and designed by myself and Dr. Derek Dee. I conducted all the experiments except for lentil protein sample preparation and ThT kinetics measurements for lentil fibrils (performed by Charlotte Shi) and was assisted by Fan Bu in doing the emulsion and viscosity measurements. I conducted all the TEM measurements for this study myself.

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List of Abbreviations

A.U.	Arbitrary Unit
AFM	Atomic Force Microscopy
PAGE	Polyacrylamide Gel Electrophoresis
PI	Isoelectric Point
RPM	Revolutions per Minute
SDS	Sodium Dodecyl Sulfate
TEM	Transmission Electron Microscopy
ThT	Thioflavin-T
UV-Vis	Ultraviolet-Visible

Acknowledgements

I would like to thank a number of people for their help and support during the production of this thesis. This accomplishment would not have been possible without them.

First of all, I would like to express my sincere gratitude to my supervisor, Dr. Derek Dee for giving me the opportunity to pursue my study and research at the University of British Columbia. Thank you for your patience, guidance, and support. I have benefited greatly from your wealth of knowledge, and it was an honor to work under your supervision. Thank you to my committee members, Dr. Anubhav Pratap-Singh and Dr. Simone Diego Castellarin. Your encouraging words and thoughtful, detailed feedback have been very important to me.

To my collaborators, Charlotte Shi and Fan Bu for lentil protein sample preparation, lentil ThT kinetics measurements, and assistance on the functionality tests. Andrew Sanders, Yuran Zhang, John Dupuis, and Lennie Cheung it was great knowing you, and thank you for sharing your experiences, providing support and help in my experiment and study.

Special thanks to Joana Pico, for your support and friendship. You were always there, wherever and whenever that was.

Finally, I am grateful for my family whose constant love and support kept me motivated and confident. My accomplishments and success are because they believed in me. I owe my deepest gratitude to my husband. I am forever thankful for his unconditional support throughout my years of education, both morally and financially.

Dedication

This thesis work is dedicated to my husband, who has been a constant source of support and encouragement during the challenges of graduate school and life. I am truly thankful for having you in my life. This work is also dedicated to my parents, and my brother who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

Chapter 1: Introduction and literature review

1.1 Interest in plant protein

The world population is expected to increase by around 15% to more than 9 billion people by 2050, so there are massive environmental and nutritional challenges to overcome this matter (Gerland et al., 2014). In addition, the global demand for meat is likely to increase by 73%, which means that an additional 160 million tonnes of meat per year is needed to encounter this demand. The world simply cannot keep up with the expected meat demand. For example, the production of beef requires large quantities of water (15,415 liters for a kilogram of beef), and large sections of lands (Heffernan, 2021). 30% of the earth's land is occupied by livestock production, so we will not have enough land space to grow enough livestock. (Heffernan, 2021). Changing to more sustainable protein source reduces water use, increase land availability for other uses and help to handle climate change (Heffernan, 2021).

Interest in plant protein has increased globally due to concerns around environmental sustainability and food security (Yang et al., 2021). Seed storage proteins from legumes could be a good substitute for animal protein (Munialo et al., 2014). Legumes such as lentils, beans, and peas are an important source of dietary protein in many cultures, particularly where animal proteins are expensive or rare. Legume proteins have an extensive variety of functional properties for use in food. However, the functionality of plant proteins is generally lower than animal proteins, such as gelling, emulsifying, and foaming properties. Therefore, one approach to improve the functionality of plant proteins is to induce their self-assembly into 'nanofibrils', which are promising materials (Munialo et al., 2014).

1.2 Amyloid fibrils

The word "amyloid" was used by Schleiden and later by Virchow in 1854 when they observed deposits in the liver upon staining with iodine during autopsy (Tjernberg et al., 2016). Amyloid-like nanofibrils are long, thin, unbranched fibrils with certain essential properties, including great aspect ratio, high stiffness, and a wide availability of functional groups on their surfaces (Eisenberg & Sawaya, 2017; Cao & Mezzenga, 2019). These fibrils are able to form a very ordered fibrillar structure that contains a cross- β-sheet structural core in which β-strands are associated perpendicularly to the fibril axis via hydrogen bonds (Díaz- Caballero et al., 2018). The β-sheet structures can form in parallel or antiparallel manner, while the parallel alignment is very common (Toyama & Weissman, 2011).

Amyloid fibrils are unbranched, 5-15 nm in diameter, and 100-1000 nm in length (Kumar et al., 2017). Due to their dual β-sheet structure, amyloid fibrils are thermodynamically stable (Nelson & Eisenberg, 2006). Congo red and thioflavin-T are florescent dyes that bind to amyloid fibrils and allow for identifying and analyzing the kinetics of amyloid fibril formation. Despite their wide use, the interaction of these dyes and amyloid is still not fully understood. It is believed that ThT binds parallel to the long axis of the fibrils between adjacent β-sheets or protofilaments. ThT fluorescence is enhanced after binding of the dye to amyloid fibrils (Malmos et al., 2017).

Two commonly accepted models for amyloid formation are the unfolding-fragmentation-fibrillation and the nucleation-elongation models (Figure 1). In the unfolding-fragmentation-fibrillation model, the protein unfolds and hydrolyzes into smaller fragments, followed by formation of protofilaments and oligomers, and finally mature fibrils are formed upon incubation. Hydrolysis usually occurs as the consequence of elevated temperature and low pH

during incubation. In the nucleation-elongation model, the formation of a nucleus from a partially unfolded monomeric form of the protein is essential, followed by the accelerated growth through the addition of monomeric structures to the nucleus leads to the formation of protofilaments and oligomers. Following prolonged incubation, the mature fibrils form, and the reaction plateaus (Adamcik & Mezzenga, 2011a).

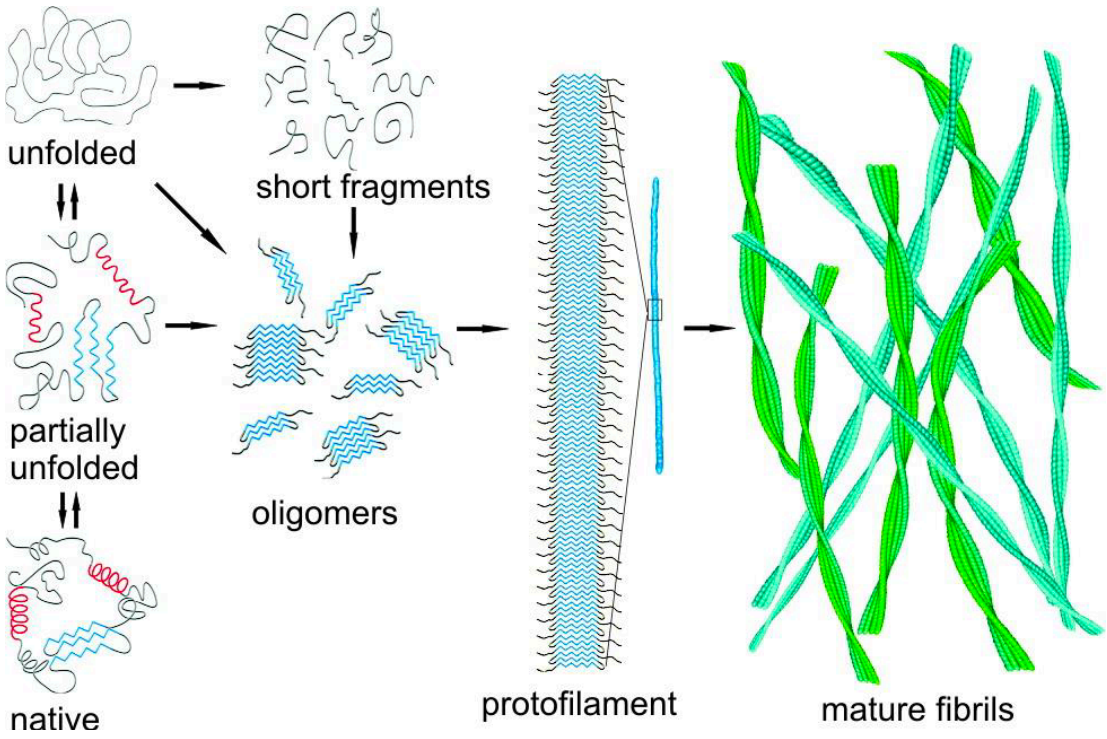


Figure 1. Mechanism of amyloid fibril formation from globular proteins. Figure is adapted with permission from (Adamcik & Mezzenga, 2012).

1.3 Functional amyloid fibrils

"Functional amyloids" are amyloid fibrils that occur naturally in many biological systems and comprise useful biological activities without acquiring toxicity (Chapman et al., 2002). Functional amyloids occur in a variety of organisms, such as bacteria, insects, fungi, and humans (Ow & Dunstan, 2014). One of the significant uses of amyloid fibrils in humans is Pmel17, which is involved in the production of the pigment melanin. This pigment is produced in specialized lysosomes that contain an amyloid fibril that acts to separate toxic intermediates involved in the production of melanin (Fowler et al., 2007). In addition, several hormones are stored as amyloid fibrils in humans, such as insulin, glucagon, and calcitonin (Berchowitz et al., 2015).

Various other organisms utilize functional amyloids for their functional characteristics, including biofilm formation (curli) in *Escherichia coli* and *Salmonella*, (Barnhart & Chapman, 2006), URE2p in the regulation of nitrogen catabolism in yeast (Ranson et al., 2006), adhesive formation in marine invertebrates (Mostaert et al., 2009), and in insect immune response (P102) (Falabella et al., 2012). Nanofibrils made from whey proteins, bovine insulin, β-lactoglobulin, and hen lysozyme were used in biosensors to detect different materials such as β-lactamase, metal ions, water, and glucose (Viguier et al., 2011).

1.4 Protein nanofibrils as food ingredients

Amyloid fibrils, or nanofibrils, are gaining attention as novel food ingredients and nanomaterials due to their unique structural and chemical properties, high aspect ratios, and rheological behavior in solutions as stabilizers and gelling agents (Loveday et al., 2011). Many proteins, including food proteins (e.g., from milk, eggs, and legumes), can be converted into amyloid fibrils under the proper conditions, most commonly by heating at low pH (Cao &

Mezzenga, 2019). Amyloid fibrils have also been formed with solvents (alcohol) and concentrated urea (Dave et al., 2013).

Fibrils were shown to be beneficial in functional materials and numerous food applications due to their improved functional characteristics (Cao & Mezzenga, 2019). Food protein nanofibrils can be utilized as foam stabilizer, emulsion stabilizer, gelling agents, texturizers in food production, and encapsulating agents (Mohammadian & Madadlou, 2018). Gelation is one of the most essential properties of proteins, and fibrillated food proteins are considered effective gelation agents because they can form gels at much lower concentrations compared to native proteins (Veerman, Sagis, et al., 2003). Moreover, food protein nanofibrils can increase viscosity at relatively low concentrations, so they improve the texture properties of the food (Mohammadian & Madadlou, 2018). Food protein fibrils can stabilize different types of emulsions, including water-in-water and oil-in-water. Since protein nanofibrils have excellent emulsification and film-forming abilities, it is a promising method to be used in the structure of different encapsulation systems like microgels, colloidosomes, and microcapsules (Mohammadian & Madadlou, 2018).

1.5 Safety of Food Nanofibrils

Certain amyloid fibrils are associated with diseases, where each disease is characterised by a specific protein or polypeptide that aggregates and forms insoluble amyloid fibrils (Makin et al., 2005), such as Alzheimer's (Ab peptides), Parkinson's disease (alpha-synuclein), transmissible spongiform encephalopathy (PrPsc), and type II diabetes (IAPP or amylin). Protein nanofibrils are being considered as new food ingredients because of their functional and biophysical properties. However, there are some concerns about their safety and toxicity for consumption given that they are amyloid-like (Lassé et al., 2016). There has been limited

research studying the safety of non-disease-related amyloid fibrils in their potential application in food. Amyloid fibrils derived from whey, soybean, kidney bean, and egg white (lysozyme, ovotransferrin) did not show cytotoxicity (Lasse et al., 2016), and could be used in food (Cao & Mezzenga, 2019). According to Lasse et al. (2016), there are 30 amyloid fibrils associated diseases that develop common amyloid aggregate structures and pathological pathways regardless of the source of protein. (Lassé et al., 2016). Oral feeding of mice with disease-related apolipoprotein A-II amyloid fibrils developed fibrils in the small intestine, tongue, stomach, heart, and liver (Xing et al., 2001). Also, oral administration of mice with murine amyloid-A or with amyloid-containing bovine liver resulted in amyloid deposition primarily in the spleen, liver, and kidneys (Cui et al., 2008). Studies performed in vivo, in vitro, and in silico suggest that small, soluble pre-fibrillar oligomers are the pathogenic form (Glabe & Kayed, 2006), while some studies in vivo and in vitro report that mature fibrils result in amyloid toxicity rather than oligomers (Stefani, 2010; Xue et al., 2009).

Amyloid fibrils are unique in their capacity to self-propagate and cause identical proteins to form fibrils (Halfmann et al., 2012). The seeding-nucleation polymerization mechanism describes how amyloid aggregates are created. Misfolded protein "seeds" are formed during the nucleation or lag phase, which is a process containing partially denatured proteins and small unstable oligomers of different sizes (Morales et al., 2013), while cross-seeding is when the oligomers containing one misfolded protein promote the polymerization of a different protein. (Morales et al., 2013). Studies have shown cross-seeding in prion proteins (Stepkowski & Bienias, 2012) and in mice, which showed the injection of serum amyloid-A resulted in systemic amyloidosis in transgenic mice (Solomon et al., 2007). In contrast, some studies suggest that for cross-seeding of nanofibrils (Krebs et al., 2004), and for coaggregation of different proteins

(Wright et al., 2005), a high degree of sequence identity is required. According to Raynes et al. (2014), low sequence identities (<40%) can protect against cross-seeding. Cross seeding is a concern when the proteins have a close sequence identity (*e.g.*, animal and human proteins) (Udomprasert et al., 2014). Therefore, plant proteins that have little similarity to human proteins are expected to have a lower likelihood of seeding.

According to Araghi and Dee (2020), hen egg white lysozyme (HEWL) can cross-seed human lysozyme (HLZ) in vitro. At the physiological condition, seeding and cross-seeding occurred between HEWL and HLZ at the 45°C condition, and only one out of 36 samples was aggregated, which shows the low efficiency of cross-seeding (Araghi & Dee, 2020a). A study by Morera et al. (2020) showed that amyloid fibrils form during the boiling of egg whites with 1 to 3% of proteins (ovalbumin) in egg white are converted into amyloid fibrils during cooking. The existence of amyloid fibrils in hard-boiled egg whites demonstrates the presence of nanofibrils in the human diet, which raises the question of whether amyloid fibrils are safe, or at least not all fibrils are toxic. Boiled eggs contain HEWL fibrils (Monge-Morera et al., 2020); HEWL fibrils can cross-seed HLZ in vitro (Araghi & Dee, 2020a); despite this, eggs are probably safe to eat and there is no known link between eggs and amyloidosis. It is critical to conduct additional research on the consumption of amyloid fibrils specifically for some at-risk individuals (Raynes et al., 2014).

1.6 Major Legume Proteins

The major legume proteins are classified as albumin and globulin fractions, where albumin represents 10–20% and globulins comprise about 70–80% of the total pulse protein. Also, there are other proteins that represent minor pulse proteins, such as prolamins and glutelin.

Legume and pulse proteins are primarily globulins that are classified as either 11S (legumins) or 7S (vicilins) proteins according to their sedimentation coefficients (Singhal et al., 2016).

Legumin is a hexameric protein with an overall molecular weight of 300–400 kDa. Legumin contains six subunits (Mw of ~60 kDa each) linked by non-covalent interactions. Each subunit pair consists of an acidic (Mw of 40 kDa) and basic (Mw of 20 kDa) chain joined by a disulfide bond, with the acidic sections at the molecule's surface and the hydrophobic sections buried at the interior, minimising their contact with water. Legumin is not coagulated by heat and is non-glycosylated (Shevkani et al., 2019). Vicilin is a trimeric protein, made up of several different subunits, with a total molecular weight of between 150 and 180 kDa, and held together by non-covalent hydrophobic interactions. Generally, vicilins lack disulfide bridges (Shevkani et al., 2019).

Peanuts, lentils, peas, and mung beans are economically important and nutritious legumes that contain a high content of protein. Canada is one of the largest producers of peas and lentils globally, with an annual production of 4.9 and 3.2 million tons, respectively (Canada: Outlook for principal field crops, 2020). Mung bean is an essential pulse grown in Asia, and it is also grown in the dry regions of southern Europe and the warmer parts of Canada and the United States (Yi-Shen et al., 2018). With a production of 2.8 million tons, the USA is the world’s third producer of peanuts after China, India, and Nigeria (International Production Assessment Devition, 2021). Peanuts are not typically produced in Canada due to the climate, although they can grow in southern parts of the province of Ontario (Peanuts in Canada, 2017). These legumes are rich in the major storage proteins, legumin and vicilin. The common names for each legume, total protein, and molecular weight are listed in Table 1. The available crystal structures for peanut vicilin, pea legumin, and mung bean vicilin are shown in Figure 2.

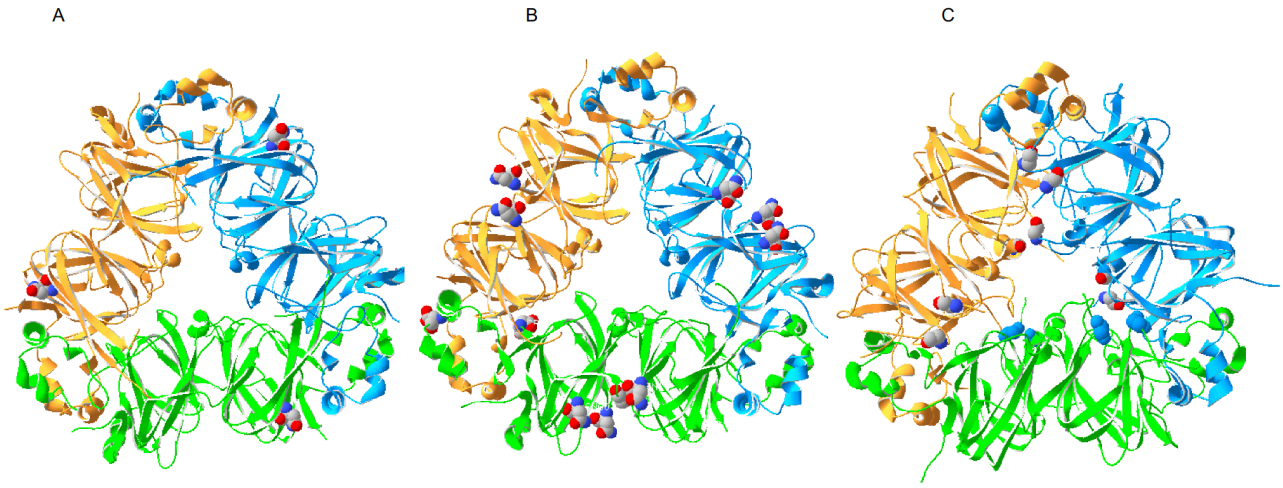


Figure 2. Legumin and vicilin structures. The crystal structures for (A) peanut conarachin (7S) PDB:3S7E, (B) mungbean vicilin (7S) PDB: 2CV6, and (C) pea canavalin (11S) PDB: 3KSC.

Peanuts contain 16–36% protein with their major storage proteins, arachin (legumin) and conarachin (vicilin), which account for 87% of the total protein of the peanut (Barać et al., 2015). Arachin and conarachin, which account for 63% and 33% of the total peanut protein respectively and are the main agents responsible for peanut protein properties. Surface properties cause a decline in interfacial tension, thus stabilizing the interfaces, and among peanut proteins. Conarachin comprises about three times the amount of sulphur (1.09% as compared to 0.4% in arachin) and is more soluble than arachin, which assists in separating these two components (Monteiro & Prakash, 1994). All of the twenty amino acids present in peanuts include the essential amino acids, which are necessary for body growth and metabolism (Ivanova et al., 2016).

Lentil contains 20.6–31.4% protein, with 19.6% albumins and 53.9% globulins (Neves & Lourenço, 1995). According to Garcia-Mora et al. (2014), the major SDS-PAGE bands found in

the lentil protein correspond to vicilin 50–65 KDa while lower molecular weight bands belong to legumin (40, 20, <15) and a mixture of γ-vicilin and albumin polypeptides. Also, the isoelectric points of lentil protein vary from pH 3.0–5.9 (Jarpa-Parra, 2018). Peas contain about 22–23% protein on a dry basis, with globulins and albumins accounting for 55–65% and 18–25%, respectively. The isoelectric point, or minimum solubility, of pea protein is around pH 4.5. Pea globulins are classified into 7S (vicilin and convicilin) and 11S (legumin) fractions which differ in their structure, composition, and thermal stability (Barać et al., 2015).

The thermal transition of pea legumin is 90 °C. Pea legumin is a heat-stable protein due to its compact structure. The quaternary structure of legumin is more sensitive to pH and salt concentration. At pH 7.0 and high ionic strength (0.1 M), pea legumin exists as a hexamer, and it dissociates at pH 3.5 and 10 into a mixture of trimers, dimers, and monomers depending on the ionic strength. Moreover, there are more sulphur-containing amino acids present in legumin per unit of protein than in vicilin per unit of protein, and it is a more available segment from a nutrition point of view (Barać et al., 2015). On the other hand, pea vicilin heterogeneity is more complex than that of legumin. Two factors lead to vicilin heterogeneity, including different proteolytic processing, different glycosylation, and the production of vicilin polypeptides from several small gene families encoding diverse primary sequences. The thermal denaturation temperature of vicilin is 71.7 °C at low ionic strength conditions ($\mu = 0.08$), and 82.7 °C at high ionic strength conditions ($\mu = 0.5$) (Bara et al., 2015). Mung bean has a 17–26% protein content (dry basis), with 60% globulin and 25% albumin content. The major mung bean storage protein is vicilin (8S), which comprises 89% of the total globulin. Legumin (11S) is a minor storage protein with a content of 7.6% (Tang & Sun, 2010).

The mung bean vicilin is comprised of four polypeptides with molecular weights of about 60, 48, 32, and 26 kDa, and mung bean legumin has acidic and basic subunits with molecular weights of 40 and 24 kDa, respectively (Tang & Sun, 2010a). According to a study done by Tang & Sun (2010a), mung bean legumin and vicilin differ in their thermal properties, with higher thermal stability for legumin (11s). Also, emulsifying ability of these globulins depend on their protein solubility (or net charge), polypeptide heterogeneity, and surface hydrophobicity (Tang & Sun, 2010a). Analysis of DNA revealed mung bean vicilin (8S) exists in three isoforms of cDNA, including 8Sα, 8Sα′, 8Sβ. The three different isoforms are homologous with each other (Bernardo et al., 2004).

Even though legumin and vicilin have very similar native folded structures, they differ in their primary structures. Soy glycinin (11S) and soy β-conglycinin (7S) both contributed to fibril formation. Both proteins share a high degree of structural and sequential identity, but their quaternary structure differs (Josefsson et al., 2019). Since they are different in structure and sequence, they will result in fibrils with different structure and function properties.

Table 1. Protein composition for peanut, mung bean, pea, and lentil

Name	*Class*	*Common name*	*% of total protein*	*MW (kDa)*	*References*
Peanut	Legumin	Arachin	63	54.57	Wang et al, 2014
	Vicilin	Conarachin	33	48.09	
Mung bean	Legumin	Legumin	7.6	24,40	Tang& Sun, (2010)
	Vicilin (8s)	Vicilin	89	26, 60, 48, 32	
pea	legumin	Canavalin	18-25	40,20	Munialo et al, 2014
	Vicilin	Vicilin	35	47,50,34,30	Barac, et al, 2015 Pedrosa et al., 1997
Lentil	Legumin	Legumin	50	40, 32, 18, 20	Jarpa- Para, (2015)
	Vicilin	Vicilin	____	50-60	

1.7 Functional properties of protein nanofibrils

Globular food proteins can self-assemble and convert into semiflexible amyloid fibrils once heated at low pH, including lysozyme (Mishra et al., 2007), ovalbumin and bovine serum albumin (Pearce et al., 2007), and soy glycinin (Tang & Wang, 2010). In addition, proteins from pea (Munialo et al., 2014), cottonseed (Zhou et al., 2014), rice bran (Hui et al., 2014), and kidney bean (Liu& Tang, 2012) formed amyloid fibrils. It appears that both legumin and vicilin contribute to legume protein fibril formation, although the details are unknown. During fibrillation, not all protein monomers are converted to fibrils (e.g., less than 40% for whey protein), and aggregated spherulites, in addition to non-aggregated peptides, exist in fibril solutions (Bolder et al., 2006).

1.7.1 Solubility properties as a function of pH

One of the most important functionalities of proteins is their solubility. Other functionalities of protein, such as foaming, gelling, and emulsion, are closely related to the solubility properties (Kristinsson & Rasco, 2000). The solubility of a given protein strongly depends on the pH, temperature, ionic strength, properties of the solvent, and the relative content of hydrophobic and hydrophilic amino acids. The globular protein's solubility increases with the increase in temperature up to 40 °C and decreases quickly, which leads to protein denaturation (Sikorski, 2001). Globulins are more soluble than other classes of plant proteins. Their solubility is low in a range of pH values where their net charge density (α) is low, normally between pH 4 and pH 6 (Nicolai & Chassenieux, 2019).

One of the foremost challenges in making plant protein fibrils is their limited solubility in the aqueous phase. Fibrils form as a result of assembling hydrophobic peptides that have the ability to form β-sheets (Kroes et al., 2012). According to Mohammadian and Madadlou (2016), the solubility degree of whey protein isolate (WPI) and whey protein hydrolysate (WPH) is decreased by fibrillation. Increasing the surface hydrophobicity of whey proteins by fibrillation decreases the solubility of fibrillated whey protein (Mohammadian & Madadlou, 2016a).

1.7.2 Emulsifying properties

An emulsion is a dispersion of two or more immiscible liquids such that one liquid is dispersed in the other as small droplets (0.1–100 μm). The emulsion droplet size is usually in the 0.1–100 μm range, and nano-emulsions contain dispersed droplet sizes in the ⩽100 nm range. Emulsions are formed by applying mechanical shear to the solution using a homogenizer, a valve (high pressure) homogenizer, or by sparging to create small droplets of one liquid dispersed in the other (Lam & Nickerson, 2013). In the food industry, the two most common emulsions are

oil-in-water (O/W) and water-in-oil (W/O), such as milk and butter, respectively (Lam & Nickerson, 2013).

Food protein fibrils can stabilize oil in water (O/W), water-in-water (W/W), and particle-stabilized emulsions (Mohammadian& Madadlou, 2018). Protein fibrils could adsorb onto the oil-water interface and reduce the interfacial tension, so it stabilizes an oil-in-water emulsion. Moreover, protein fibrillation could be a new technique to form stable plant protein-based emulsions without the use of synthetic surfactants (Wynnychuk et al., 2021). A recent study investigated lentil protein fibrils as a stabilizer to form oil-in-water emulsions (Wynnychuk et al., 2021). This research reported that the lentil fibrils could form a stable emulsion due to their elongated structure. Fibrils could adsorb and assemble at the oil/water interface, forming a solid coating to limit coalescence and creaming (Wynnychuk et al., 2021).

Compared to their native form, transforming proteins into nanofibrils may improve their emulsifying properties in a Pickering type of droplet stabilization (Serfert et al., 2014). At a fixed oil content, WPI provided a superior emulsifying activity at a fixed oil content and extremely higher elasticity at the O/W interface compared to native WPI (Serfert et al., 2014). Protein fibrils could adsorb onto the oil-water interface and reduce the interfacial tension, so it stabilizes an oil-in-water emulsion.

1.7.3 Viscosity properties

Fibril formation can affect the viscosity and flow behavior of food proteins (Mohammadian & Madadlou, 2018). Food protein nanofibrils can increase a solution's viscosity at a very low concentration, which could be used as a texture modifier and thickening agent. The apparent viscosity of whey gel solutions as a function of shear rate has been investigated, and the viscosity of whey protein isolate solution increased upon fibrillation (Mohammadian &

Madadlou, 2016b). In whey proteins, the increase in viscosity is due to the increase in the hydrodynamic radius of whey proteins during fibrillation and the formation of entangled networks (Liu & Zhong, 2013).

Fibril entanglement forms a highly packed assembly which could resist the flow in the direction of the shear field (Zhang & Huang, 2014). Soy fibrils, which formed after 15 hours of heating at 80 °C and pH 2, increased the viscosity and decreased the flow behavior index (n) of 1% soy globulin dispersions (Tang & Wang, 2010). Zhang & Huang (2014) stated that the viscosity of rice bran protein solution was increased by the addition of pre-formed rice bran protein fibrils. The viscosity and shear thinning of all the fibrillated samples were higher than non-fibrillated. Moreover, the study stated that the viscosity of the samples was higher at pH 2 than at pH 7. As the pH increases, the structure of the fibril's changes, which leads to weaker molecular interactions and a decrease in viscosity (Zhang & Huang, 2014).

The morphology and the protein concentration are important factors that influence the viscosity of the fibrillated protein solutions. Another study investigated whey protein nanofibrils and found that curly fibrils provided a higher viscosity than longer, straighter fibrils. Moreover, the concentration of the fibrils has a direct correlation with viscosity and shear-thinning behavior (Loveday et al., 2012). In whey protein, stirring during the fibrillation and a longer heating time can increase the fibrillation yield and result in a higher apparent viscosity (Bolder et al., 2007).

1.7.4 Gelling properties

One of the most important functional properties of food proteins is gelation. Fibrillated food proteins can form gels at very low concentrations compared to native proteins, so they are effective gelation agents (Mohammadian & Madadlou, 2018). Their high gelation ability is due to their high aspect ratio (length vs. diameter) and their capability to create entangled networks

(Akkermans et al., 2008). One of the techniques used to prepare gels using fibrillated proteins is the cold-set method. This method involves two steps: heating proteins at a pH far from the isoelectric point and at low ionic strength, followed by the addition of salt to induce gelation. Ionic strength is the main factor affecting gel properties (Kuhn et al., 2010). The most common salts for cold-set gelation of whey proteins are $CaCl_2$ and NaCl (Mohammadian & Madadlou, 2016b).

A process for calcium-induced cold gelation of lactoglobulin at very low concentrations was developed by Veerman et al. (2003). In this new technique, β-lactoglobulin fibrils are formed at pH 2.0 and cross-linked by adding $CaCl_2$. On the other hand, in the conventional method of cold gelation, the free salt β -lactoglobulin is heated at neutral pH, cooled down, and then cross-linked by adding salt. The study reported that the critical concentration necessary to form a gel in the new technique is much lower than the conventional cold gelation methods (Veerman et al., 2003). Monovalent salts are not suitable for the gel formation of β-lactoglobulin nanofibrils. Salt bridge formation is an essential step in the gel formation of B-lactoglobulin nanofibrils. A study conducted by Mohammadian & Madadlou reported that the divalent cations, including $CaCl_2$, $MnCl_2$, and $ZnCl_2$, could affect the textural and functional properties of whey protein nanofibrils hydrogels. The hydrogel formed by zinc was firmer than manganese and calcium hydrogels (Mohammadian & Madadlou, 2016b).

Munialo et al. (2014) characterized pea protein fibrils and studied the rheological behaviour of pea fibril gels. In this study, gelation of pea fibrils was done by acid-induced cold gelation using glucono-δ-lactone (GDL). The results showed that gels prepared from pea fibrils were weaker than gels made from whey fibrils (Munialo et al., 2014). Another method for forming gels is thermal gelation, in which the gel forms by heating. Thermal gelation

involves protein unfolding, aggregation, and association of aggregates. The thermal gelation method was used to form gels from lentil fibrils at pH 2. Compared to native lentil protein, lentil nanofibrils formed translucent, homogeneous gels (Jo et al., 2020). The formation and properties of gels made from plant fibril proteins have been studied much less than whey proteins.

Chapter 2: Rationale, hypothesis and objectives

2.1 Rationale

Previous research on food protein fibrils mainly focused on the animal proteins from milk whey and egg white, while a few studies investigated fibril formation from legume proteins such as soybean, pea, kidney bean, and mung bean. Tang and Wang (2010) investigated the formation of amyloid fibrils from soy β-conglycinin and glycinin. The β-conglycinin and glycinin were separated from soybean flour, and the solutions were adjusted to pH 2, and then heated for 12 hours at 80°C. According to the ThT fluorescence analysis, β-conglycinin (7S) had a much higher potential to form heat-induced amyloid fibrils than glycinin (11S). This result could be due to the differences in their conformational changes and the degree of polypeptide hydrolysis by heating (Tang & Wang, 2010). All of the soy globulins were formed into twisted screw-structured fibrils. However, the structural characteristics of the fibrils, including fibril heights, coil periodicity, width at half-height, and average counter lengths, were significantly different.

Tang and Wang (2010) discovered that -conglycinin had higher coil periodicity and lower width at half-heights than glycinin. Another study on soybean protein showed that long and semiflexible fibrils from soy glycinin and soy protein isolate (SPI) when heated at 85 °C for 2 or 20 h at pH 2 (Akkermans et al., 2007). According to their findings, fibrils of SPI and soy glycinin fibrils are more branched than fibrils of β-lactoglobulin and WPI fibrils when they are prepared at similar conditions (pH 2). Also, SPI has formed more branched and curved fibrils than soy glycinin, which could be due to the higher concentration of β-conglycinin that could impact the morphology of SPI (Akkermans et al., 2007).

Wang & Gau, 2019, investigated the thermal aggregation behaviors of the constituent peptides of SPI fibril (CPSF) at different pH values (2–10) and investigated the

structural changes in SPI fibril with increasing pH (2–11). The fibrils were made from soy protein isolate by heating it at 85 °C for 20 hours with constant stirring at pH 2. At pH less than 6, the aggregates comprised multiple β-sheet structures, and these β-sheets were stacked together to form fibrils only at pH 2 (Wan & Guo, 2019). The SPI fibrils were curved and branched at pH 2. At pH 3, these fibrils were connected and were wrapped around each other, and they became thicker at pH 4. As the pH approached the isoelectric point (~4.5), the association between fibrils increased, and this is because of a loss of positive electrostatic repulsion between fibril molecules. The SPI fibrils convert into some shorter fibril segments at pH 7, and the ThT fluorescence intensity begins to decline extremely at pH higher than 6. The fibrils with the original structure did not exist at pH 9. At pH 11, only small amorphous protein debris scattering was observed.

Electrostatic neutralization (pH ≤ 6) results in morphology changes, while by an extensive loss in β-sheets (pH > 6), the fibril structure disintegrates (Wan & Guo, 2019). Lasse et al. (2016) investigated the amyloid fibril formation from kidney bean protein isolate (KPI). The pH of the KPI solutions was adjusted to pH 1.6 (HCl) and incubated at 80°C for 22 hours. The result showed that the fibrils formed at pH 1.6 were stable at this pH for several weeks, while the fibrils at pH 7.5 were stable at this pH for at least 48 hours (Lassé et al., 2016). ThT fluorescence analysis showed a gradual increase in fluorescence intensity over 24 hours and stable fluorescence levels were reached after a 7-days which mature fibril formation was completed. Fibrils from KPI were curly and formed an entanglement network (Lassé et al., 2016).

Another study investigated the reaction kinetics and changes in fibril morphology of vicilins from kidney beans, mung beans, and red beans (Liu & Tang, 2013). The fibrils were made by adjusting the pH to 2 and heating them at 85 °C. According to the result, kidney bean

fibrils displayed a higher ability to form highly ordered fibrils than mung bean or red bean fibrils. The ThT fluorescence intensity showed that the maximum intensity increased extremely during the initial incubation period (less than 0.5 hours), and then the rate of the increase slowly reduced. Mung bean fibrils presented the highest maximum fluorescence intensity at incubation periods of more than 0.5 h, followed by kidney bean and red bean fibrils, respectively. Kidney beans formed much longer fibrils in length than mung bean fibrils, which were curly (Liu & Tang, 2013).

Fibrils were produced from pea protein isolate by heating at 85 °C for 20 hours at pH 2 (Munialo et al., 2014). All of the pea proteins were hydrolyzed into peptides, and only 50 % of these peptides were constructed into fibrils. The maximum fluorescence intensity was observed within 2 hours of incubation. After 2 hours of heating, the fluorescence intensity slowly reduced, which could be due to partial disruption of the highly ordered structure by further polypeptide hydrolysis. A gradual increase in fluorescence intensity was detected after heating of the samples for more than 6 hours. Pea protein formed into curly and worm-like fibrils, which are similar to soy protein fibrils (Munialo et al., 2014).

According to Robertson, 2019, fibrils were formed from peanut protein crude extract at 80 °C stirring for 24 hours at pH 2. Peanut protein appears to be able to form long and straight fibrils, which look very different from the data published on soy, kidney bean, and pea proteins, with short and curly fibrils. (Figure 3).

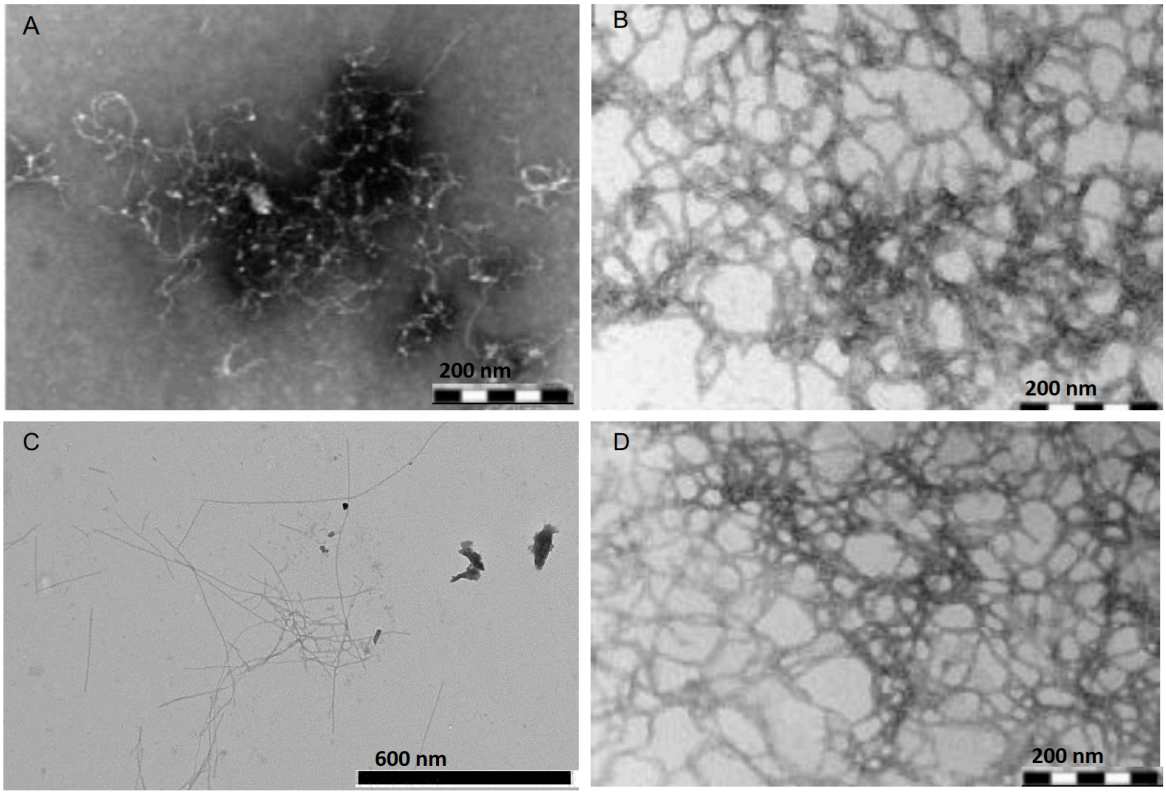


Figure 3. Comparison of fibril morphology from various legume proteins. TEM images of nanofibrils made from A) Pea, B) Soybean, C) Peanut, and D) Kidney bean. (Panels A was adapted from Munialo et al, 2014; Panel B and D were adapted from Lasse et al, 2016; Panel C was adapted from Robertson, 2019. Images reprinted with permission).

These findings indicate most of the legume crude extracts produce worm-like, curly fibrils that look very different from the whey protein, lysozyme, or any other animal protein fibril. In this project, we want to do a side-by-side comparison to see if peanuts, lentils, peas, and mung beans behave the same way under the same conditions or not (in terms of structure and function).

2.2 Research hypothesis

The hypothesis of this study was that proteins from different legumes can be induced into nanofibrils with different structures and functional properties.

2.3 Research objectives

The objective of this research was to compare several legume proteins, in terms of fibril formation kinetics (ThT), structure (TEM, SDS-PAGE), and functionality. To test the central hypothesis, three specific objectives were examined:

Objective 1: *Determine and optimize conditions for protein extraction and fibrillation.*

Many proteins can form fibrils under the presence of organic solvents, low pH, heating, stirring, salts, microwave, and sonication (Cao & Mezzenga, 2019). In this project, we optimized conditions for extraction and fibril formation from peanut, lentil, pea, and mung bean proteins.

Objective 2: *Examine fibrillation kinetics and structure of the fibrils from different legume proteins.*

To get a comprehensive understanding of food protein nanofibrils, their kinetics, and morphology were investigated.

Objective 3: *Compare the functionality of the legume protein amyloid fibrils.*

The morphology and structure of fibrils likely affects their functionality, such as their foaming, emulsifying, and gelling properties (Jansens et al., 2019). Therefore, we investigated how the functionality of fibrils from various legume proteins differ. The ability of nanofibrils made from peanut, mung bean and lentil proteins to form emulsions, gelling, apparent viscosity, and the solubility of fibrils was investigated.

Chapter 3: Materials and methods

3.1 Materials

Whole green lentil, pea and mung bean were purchased from a local supermarket (Vancouver, BC). Raw peanuts of the runner variety from Hampton Farms, Inc. (Severen, NC, USA), were purchased from a grocery store (Bell's Food Store, Athens, GA, USA). Tris base (molecular biology grade) was purchased from Thermo Fisher Scientific (Waltham, MA). Sodium chloride, thioflavin T, sodium hydroxide, hydrochloric acid, zinc chloride, manganese chloride, calcium chloride were purchased from MilliporeSigma (St. Louis, MI). All buffers and solutions were prepared using ultrapure water.

For SDS-PAGE, a precast 16% Mini-PROTEAN tricine gel purchased from Bio-Rad (USA) was used. The tricine sample buffer (catalog #161-0739), β-mercaptoethanol (catalog #161-0710), and tricine running buffer (catalog #161-0744), and dual Xtra protein standard were also purchased from Bio-Rad. The Coomassie (Bradford) Protein Assay Kit, including bovine serum albumin (BSA) protein standard and Coomassie protein assay reagent, was purchased from Thermo Fisher Scientific.

3.2 Methods

3.2.1 Protein extraction

Proteins were extracted from the raw peanut, mung bean, and pea using a method adapted from Koppelman et al. (2001) in order to isolate the protein to form fibrils. Briefly, de-shelled, de-hulled peanut, mung bean, and pea were ground using a coffee grinder (BODUM Bistro) to create a powder. Three grams of the ground peanut, mung bean, and pea powder were mixed with 30 mL of 20 mM tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) buffer at pH 8.2 and stirred at room temperature (23 ℃) for 2 hours. The resulting solutions were centrifuged

at 3000 x g for 10 minutes at 21°C (Avanti J-E, High-Speed Centrifuge, Beckman Coulter) to remove any insoluble particles followed by subsequent centrifugation of the supernatant at 12000 x g for 20 minutes to further remove fat and any traces of insoluble particles. The protein extracts were stored at 4 °C.

Lentil protein was extracted under acidic conditions according to the procedures described in Liu & Tang (2013) with some modifications. Briefly, de-hulled lentils were ground using a coffee grinder (BODUM Bistro) to create a powder. 50 g of lentil flour was mixed with 500 mL 0.5 M NaCl. The pH was adjusted to 3.5 using 1 M HCl and stirred for 2 hours at room temperature (23 °C). The insoluble solids were removed by centrifugation at 10,000 x g for 20 minutes at 4 °C (Avanti J-E, High- Speed Centrifuge- Beckman Coulter). The aqueous fraction was diluted with 5-fold volumes of 4 °C MilliQ water. The precipitates obtained after dilution were collected by centrifugation at 12,000 x g for 20 minutes at 4 °C and redissolved in a 5-fold volume of MilliQ water, adjusted to pH 7 using 0.5 M NaOH and stored at 4 °C until use.

3.2.2 Protein concentration

Protein concentration was estimated using the Bradford assay. BSA standards were prepared in the range of 100–1500 µg/mL in triplicate using serial dilution according to the Standard Test Tube Protocol provided in the user guide (Thermo Fisher Scientific Inc., 2013). Dilutions of the different protein samples were prepared in triplicate. 30 µL of sample or standard was mixed with 1.5 mL of 1x Coomassie G-250/R-250 stain and were incubated at room temperature (23 ℃) for 10 minutes. The blank was prepared using water and 1x Coomassie G-250/R-250 stain.

The absorbance of each BSA standard was measured at 595 nm using a Nanodrop 1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples of peanut,

mungbean, pea, and lentil protein extract with unknown concentration were mixed with 1x Coomassie G-250/R-250 stain and the absorbance was measured three times for each legume. A standard curve was created by plotting the absorbance at 595 nm values of the BSA standards versus the concentrations of the BSA standards and used to determine the sample protein concentrations.

3.2.3 Fibril formation

Peanut, pea, mung bean and lentil protein extracts were adjusted to pH 2 using HCl followed by centrifugation at 15000 x g for 30 min at 21 ℃ to remove any insoluble particles. The protein solutions were transferred in 4 mL aliquots to a screw-capped glass vial containing a magnetic stir bar. Fibrillation was then induced by incubating the vials at 80 ℃, with constant stirring at 400 rpm in a hotplate/stirrer (Profession Round Top, VWR® Radnor, PA, USA). The incubation of each of the protein extracts was done in triplicate. Aliquots of 200 µL were collected at different time points (0 h, 1 h, 2 h, 5 h, 8 h, 24 h, 30 h) and were stored at 4 °C for subsequent analyses.

3.2.4 Thioflavin-T (ThT) assay

Amyloid formation was detected by the increase in thioflavin T (ThT) fluorescence intensity. ThT was added to the samples to a final concentration of 20 µM. The measurements of ThT binding kinetics were performed using an Agilent Technologies Cary Eclipse Fluorescence Spectrophotometer (Santa Clara, CA). The temperature was set to 80 ℃ and the fluorescence was measured every 5 minutes, with excitation at 440 nm and emission at 480 nm, and with 5 nm slit widths. Nanofibrils are detected based on their interaction with ThT and the resulting increase in ThT fluorescence (Xue et al, 2017). ThT binds (relatively) specifically with amyloid fibrils, and once bound, the maximum emission wavelength of ThT shifts from ~430 nm to ~480

nm. This method is widely utilized to analyze amyloid fibril formation (Biancalana & Koide, 2010).

The incubation was stopped after the growth curve reached the plateau phase. The real-time ThT fluorescence measurements were repeated three times. A graph of the intensity over time was created for each sample to show the kinetics of fibril formation.

ThT fluorescence time progress curves were fitted with OriginLab 2019 software according to the following equation:

$$y=y_0+A/(1+\exp(-k(t-t_{0.5}))) \quad \text{eq (1).}$$

Where y_0 is the y-intercept, A is the transition amplitude, k is the apparent rate constant for fibril growth, and $t_{0.5}$ is the transition half-time. The lag time was determined as the intersection of linear fits to the pre-transition and growth phases for each trace.

3.2.5 TEM imaging

Fibril morphology was evaluated using transmission electron microscopy (TEM) at the Bioimaging facility at the University of British Columbia. A sequential two-droplet negative-staining method was used for TEM sample preparation. Samples were diluted in MilliQ water (200 μg/mL protein concentration) and 5 μL was placed on the 200-mesh copper Formvar/carbon-coated grids (TED PELLA, Inc.). Five μL of 2% (w/v) uranyl acetate was placed on the grid and after 30 seconds the excess uranyl acetate was removed by touching the edge of the grid with filter paper. After drying the grids at room temperature, grids were stored in a grid box, ready for TEM analysis with a Hitachi H7600 at 80 kV with a high contrast 2k x 2k AMT mid-mount digital camera.

3.2.6 SDS-PAGE

Samples were diluted to 4 mg/mL and 5 μL was mixed with 5 μL of 2× tricine sample buffer that contained 2% β-mercaptoethanol, heated at 95 °C for 5 min, and centrifuged at 10,000 g for 3 min.10 μL of each sample was loaded and run on 16.5% tris-tricine Mini-Protean

precast gels (Bio-Rad) at 100 V for about 150 min. The gel was submerged in a fixative solution (40% methanol, 10% acetic acid) for 30 min, stained (0.025% w/v Coomassie blue G-250, 10% acetic acid) for 1 h, and destained in 10% acetic acid solution (replaced destaining solution every 15 min) and remained in 10% acetic acid solution overnight. The gel image was captured with a Bio-Rad ChemiDoc MP Imaging System (Hercules, CA).

3.3 Methods for functional studies

3.3.1 Fibril Solubility

Solubility analysis was performed according to the method adapted from Wang et al. (2020) with slight modifications. After 24 h of the fibril reaction, the pH of fibril samples (20 mg/ml) was either maintained at pH 2 or adjusted to a pH of 3, 4, 5, 6.2, 7, or 8, with 40 mM acetate, phosphate, or Tris buffers. The samples were then stirred continuously for 30 minutes and centrifuged at 4500 x g for 25 minutes to remove the insoluble protein. The protein concentration in the supernatant was determined using the Bradford assay (section 3.2.2). The ThT fluorescence of the solutions before and after the centrifugation was measured.

3.3.2 Emulsion measurement

Emulsion properties were tested according to the method adapted from Wynnychuk et al. (2021) with slight modifications. Briefly, the emulsions were prepared using canola oil as the dispersed phase and peanut, mungbean, and lentil protein fibril solutions as the continuous phase. Fibril samples of 20 mg/ml were mixed with 50% canola oil at pH 2 and pH 7. The oil and fibril mixtures were homogenized (Ultra Turrax, T25 digital, IKA)) at the speed of 14000 rpm for 5 minutes with the probe positioned at the oil-water interface. Protein solutions without heat treatment (i.e., no fibrillation) were prepared for comparison. Immediately following the homogenization, the emulsion droplet size distribution was measured using a particle analyzer

(Litesizer 500, Anton Paar, USA). The refractive index of the dispersed phase (canola oil) was set to be 1.5800. The refractive index of the continuous phase (demineralized water) was set to be 1.3303. The emulsions were tested in triplicate and were diluted (40x) for the analysis.

3.3.3 Gelation measurement

The ability of gel formation of peanut, mungbean, and lentil nanofibrils was investigated according to the method adapted from Mohammadian & Madadlou (2016) with slight modifications. Cold-set gelation was performed by mixing fibril samples (20 mg/ml) with an appropriate volume of $CaCl_2$, NaCl, $MnCl_2$ and $ZnCl_2$ stock solutions at final concentration of 100 mM,150 mM, 200 mM, and 300 mM, of cations (Ca^{2+}, Na, Mn^{2+} and Zn^{2+}), respectively. Then, the samples were stored for 24 hours at 4 °C for stabilizing the gel network. The samples did not form a self-supporting gel, so no textural analysis was completed for this part.

3.3.4 Viscosity measurement

The rheological properties of fibril solutions and protein solutions with no heat treatments were investigated, according to the methods adapted from Mohammadian & Madadlou (2016) and Tang & Wang (2010), with slight modifications. The peanut, mungbean, and lentil fibril samples (20 mg/ml) were mixed with an appropriate volume of NaCl and $ZnCl_2$ stock solutions to a final concentration of 200 mM. The mixtures were stored for 24 hours at 4 °C to stabilize the solution, and the apparent viscosity of the samples was measured using a rheometer (MCR 302, Anton-Paar, Graz, Austria) at 23 °C with a parallel plate geometry (diameter of 50 mm and a gap of 1 mm). A shear experiment was conducted with shear rates between 0.1 to 100 s^{-1} within 30 s intervals. Each sample was tested in triplicate and data points were obtained when a steady- state was reached after 30 s.

3.3.5 Data analysis

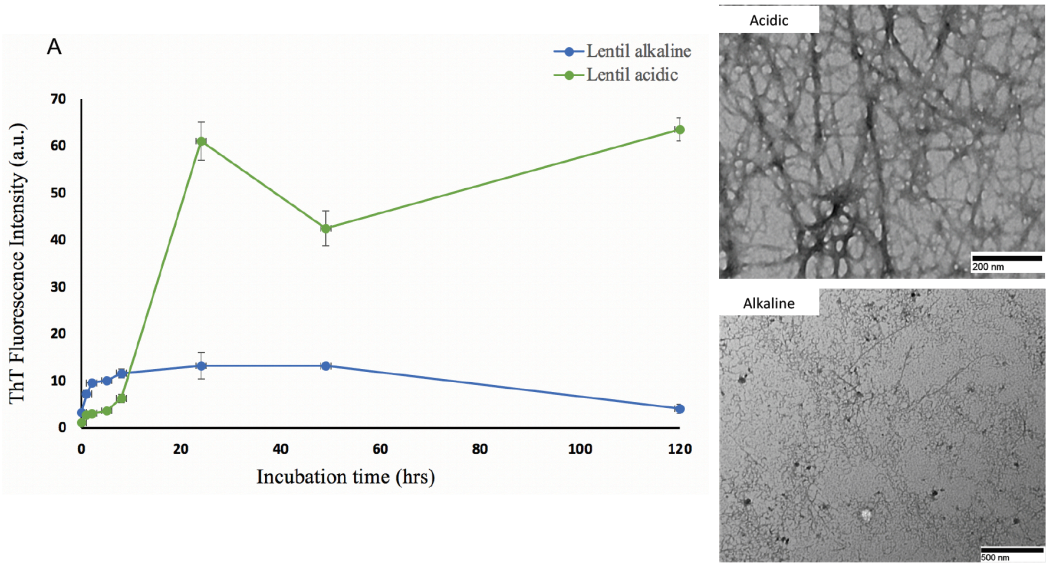
All experiments were done in triplicate. Data analysis was performed using Excel and Origin software (data plotting and fitting). Solubility data was analyzed using One-way analysis of variance (ANOVA) to test the significant difference between samples. Emulsion data was analyzed using Two-way analysis of variance (ANOVA) to test the significant difference between samples. Viscosity data was analyzed using Two-way analysis of variance (ANOVA) and T-test to test the significant difference between samples. Significant differences among the samples were considered at $p<0.05$.

Chapter 4: Results

4.1 Protein extraction and fibrillation

Seed storage proteins are generally most soluble below and above their pI of ~4-5, and protein extraction of various legumes is often carried out at either an acidic (Liu & Tang, 2013) or an alkaline pH (Munialo et al., 2014; Klupait & Juodeikien, 2015). Here, a comparison was made between alkaline and acidic extraction to prepare peanut, lentil, pea, and mung bean proteins and their fibrillation assessed by ThT and TEM analysis. The results suggested that, under alkaline extraction, peanut, pea, and mung bean worked well in terms of fibril formation and morphology. However, as shown in Figure 4, lentil protein did not show a satisfactory result in terms of fibril formation and morphology. Lentil fibrils displayed short and rigid morphology under alkaline conditions.

The results from ThT and TEM suggested that there is no benefit to using acidic extraction for mung bean and pea since they were not significantly different from alkaline extraction samples (Figure 4). There was not enough peanut protein obtained to run the experiments under acidic extraction. Lentil protein functioned well in terms of fibril formation and morphology under acidic extraction, and the results will be shown later in this project.


A
Lentil alkaline
Lentil acidic
ThT Fluorescence Intensity (a.u.)
Incubation time (hrs)
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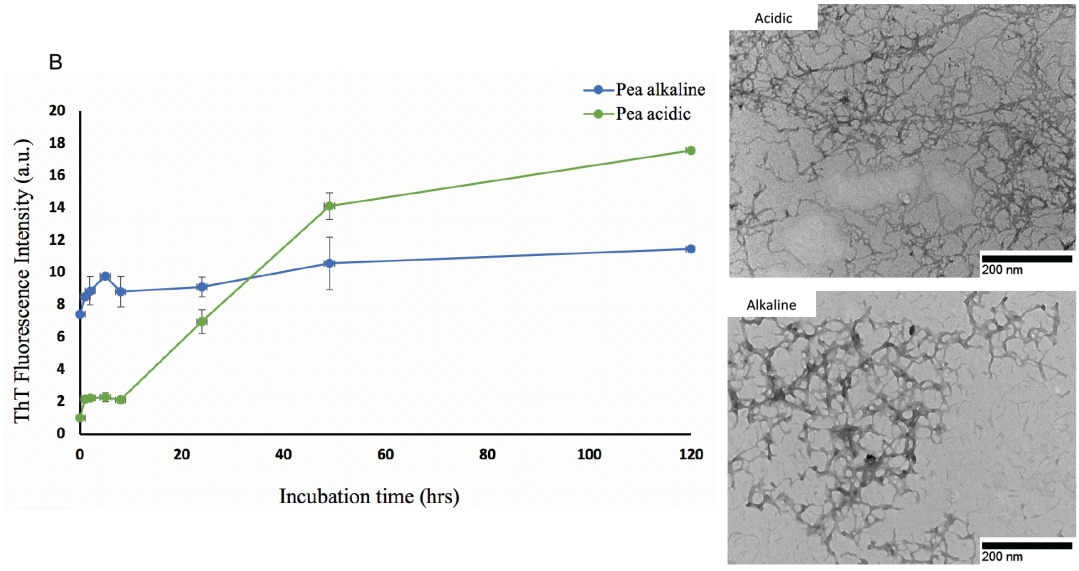
B
Pea alkaline
Pea acidic
ThT Fluorescence Intensity (a.u.)
Incubation time (hrs)
0
2
4
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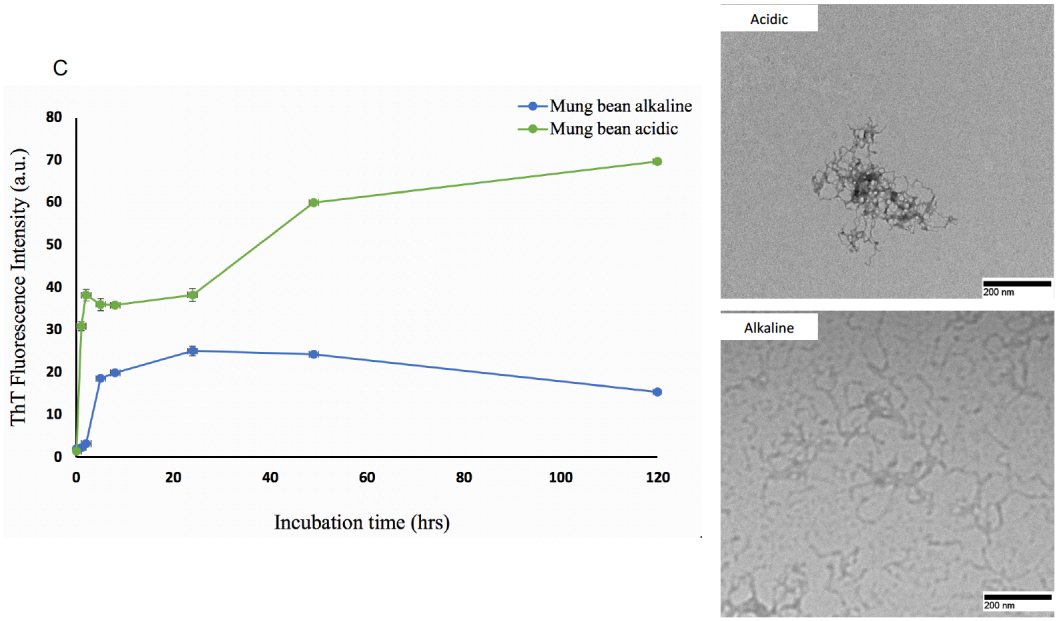


Figure 4. Comparison of fibril morphology and ThT from alkaline and acidic extraction. kinetics and TEM image of A) Lentil, B) Pea, and C) Mung bean fibrils from acidc and alkaline extraction. All the proteins (20 mg/ml) were incubated at 80 °C, pH 2, with 400 rpm stirring. Experiments were performed in triplicate, and each point represents the mean value and standard deviation (n=3).

The final extracts had average protein concentrations of 48.8 ± 6.9 mg/ml for peanut, 50.2 ± 12.3 mg/ml for mung bean, 32.4 ± 3.3 mg/ml for pea, and 31.0 ± 4.3 mg/ml for lentil. The protein extracts were diluted to 20 mg/ml for fibrillation reactions to maintain a consistent concentration across samples. SDS-PAGE patterns of alkaline extracts of peanut, pea, and mung bean proteins, and an acidic extract of lentil protein from total crude extract are shown in Figure 5. The profiles show a wide variety of polypeptide subunits of molecular weight (Mw) between 25 and 75 kDa. Most bands are the polypeptides of legumin and vicilin. Based on the literature, the subunits of 70 kDa correspond to convicilin, and the subunits around 50 kDa are ascribed to vicilin. The polypeptide subunits around 37 and 20 kDa are acidic and basic subunits, of legumin

(Ettoumi et al., 2016; Wang et al., 2014; Mendoza et al., 2001). As expected, the results suggested that both vicilin and legumin proteins were extracted from the legumes.

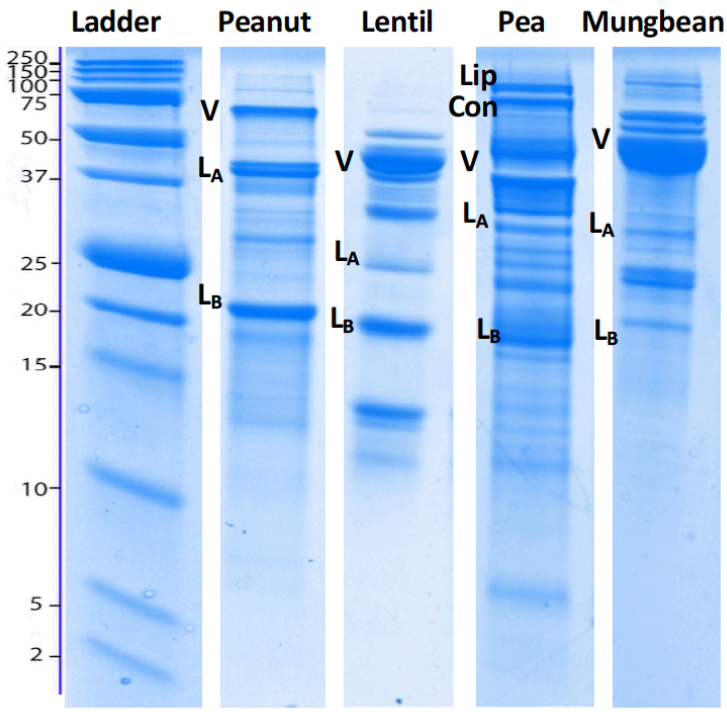


Figure 5. Legume protein crude extracts. SDS-PAGE (reducing) of alkaline extracts of peanut, pea and mung bean proteins, and acidic extract of lentil protein. Vertical: Lip: lipoxygenase; Con: convicilin; V: vicilin; L_A: legumin acidic subunit; L_B: legumin basic subunit.

4.2 ThT kinetics measurements

Fibril formation of peanut, mung bean, pea, and lentil proteins was monitored by ThT kinetics. ThT is a fluorescence dye that binds specifically to the β-sheet structure present in the protein fibrils. An increase in the ThT fluorescence intensity confirmed the amyloid formation of peanut, mungbean, pea, and lentil. Fluorescence was normalized to the value of the plateau phase in each reaction. As shown in Figure 6, there is a significant intensity increase for lentils. Also, lentil and peanut showed a lag phase where for mungbean and pea the formation

showed no apparent lag phase. The lag-times and apparent fibril growth rate constants were determined by fitting the ThT fluorescence intensity vs. time results using equation 1.

The results suggested a difference in the duration of the lag phase and the slope of the growth phase. Lentil showed the lowest growth rate of 0.0041 min^{-1}, and mung bean showed the highest growth rate of 0.0158 min^{-1} (Table 2). Peanut and pea growth rates were 0.0065 min^{-1} and 0.0032 min^{-1}, respectively. Moreover, mung bean and pea did not show any lag time, while lentil had a long lag phase of 342 min, followed by peanut for 231min.

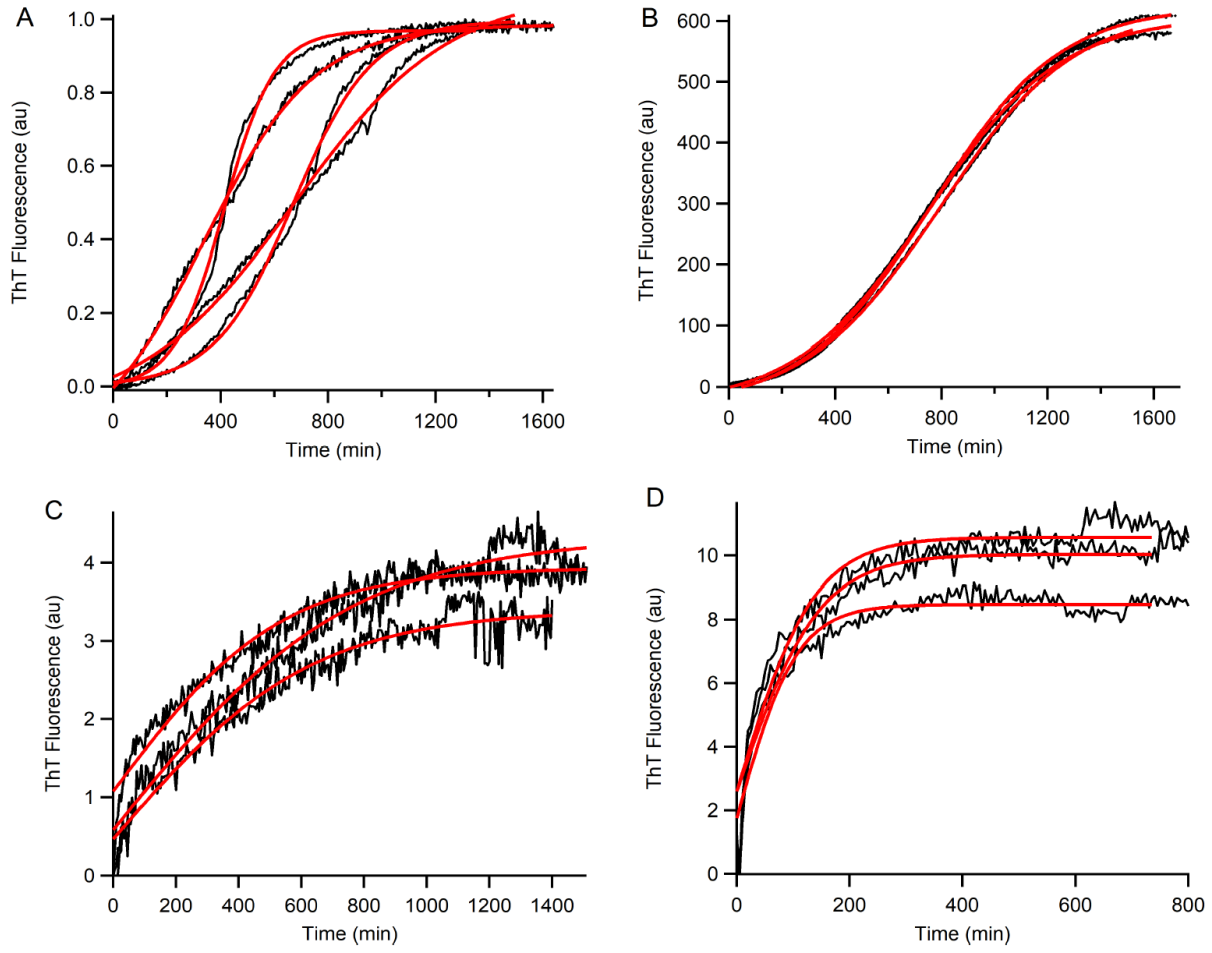


Figure 6. Kinetics of amyloid formation. ThT fluorescence traces for A) peanut, B) lentil, C) pea, and D) mung bean. Data points show three representative traces, and lines indicate fits to equation 1. Proteins (20 mg/ml) were heated at 80 °C, pH 2, with stirring.

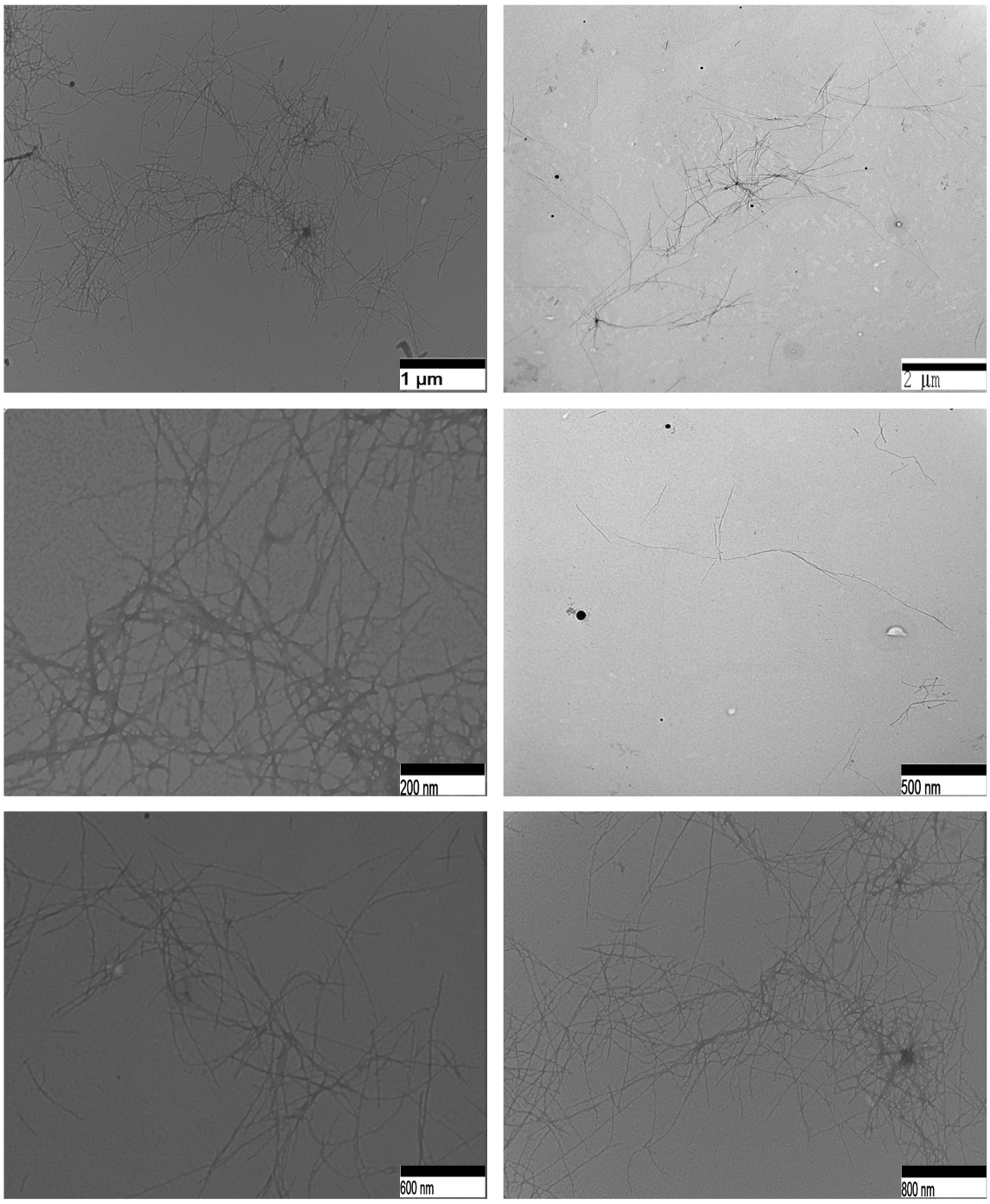
Table 2. Fitting results from ThT real-time kinetics.

Sample	Rate constant (min^{-1})	Lag time (min)
Peanut	0.0065 ± 0.0032	231 ± 157.2
Lentil	0.0041 ± 0.0001	342 ± 0.4
Pea	0.0032 ± 0.0006	-
Mung bean	0.0158 ± 0.0028	-

4.3 TEM imaging

Transmission electron microscopy (TEM) was used to visualize the fibrils that have formed. As shown in Figure 7, the TEM images confirm that fibrils were formed from peanut, mung bean, pea, and lentil protein. From the TEM results, the morphological variability of peanut, mung bean, pea, and lentil fibrils are evident. Fibrils from lentils and peanut are long and unbranched, while mung bean and pea fibrils are curly and branched (Figure 8).

A



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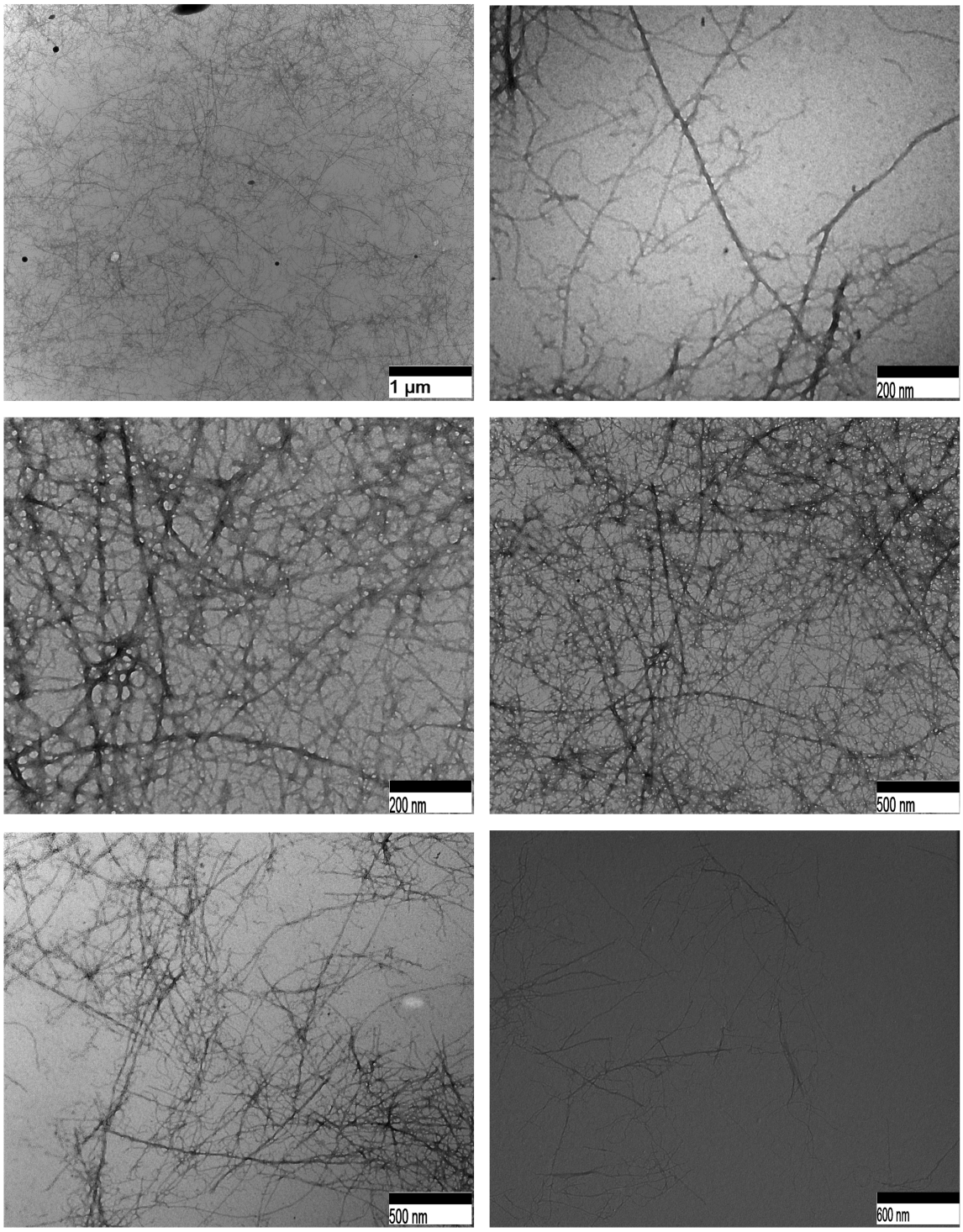

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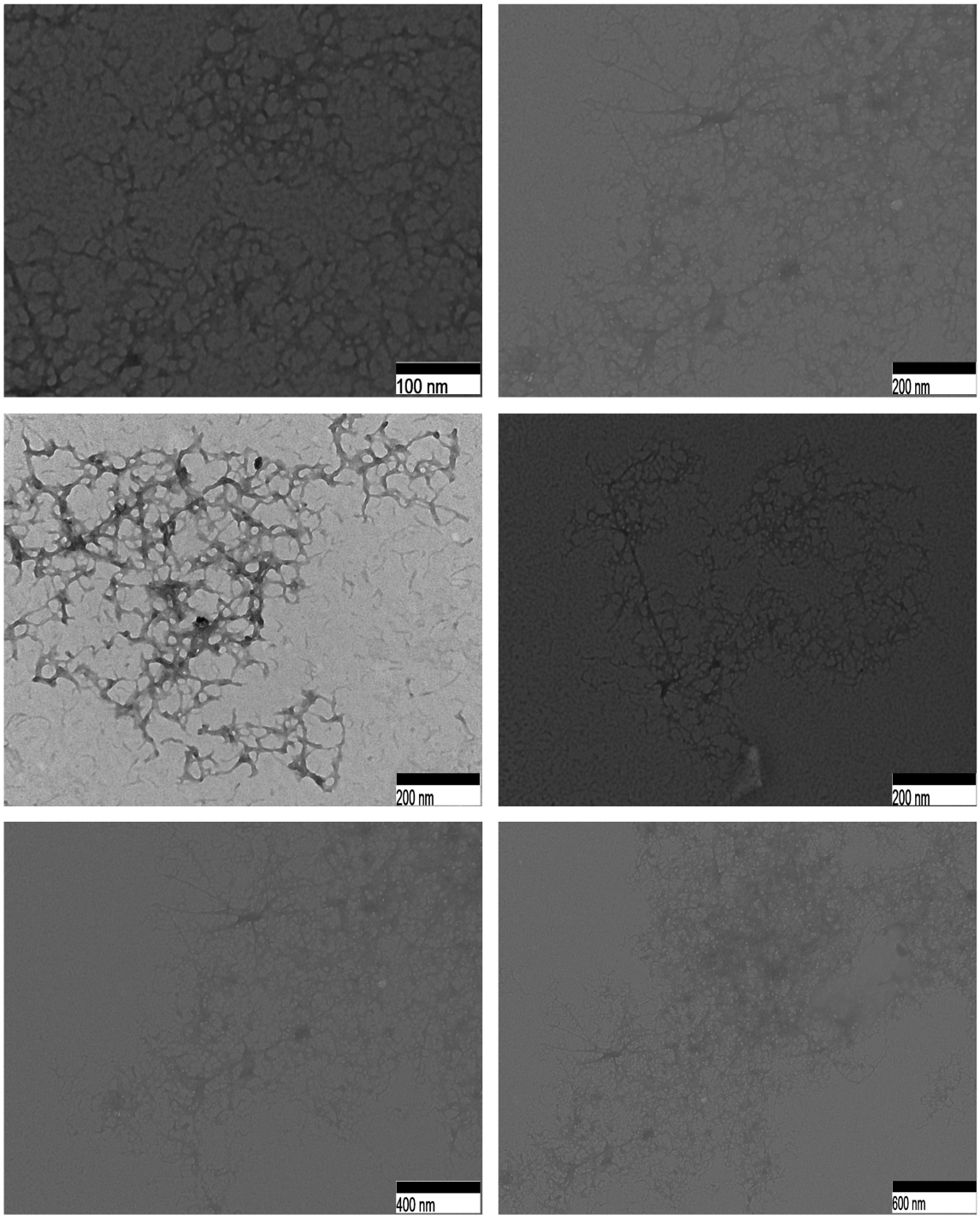

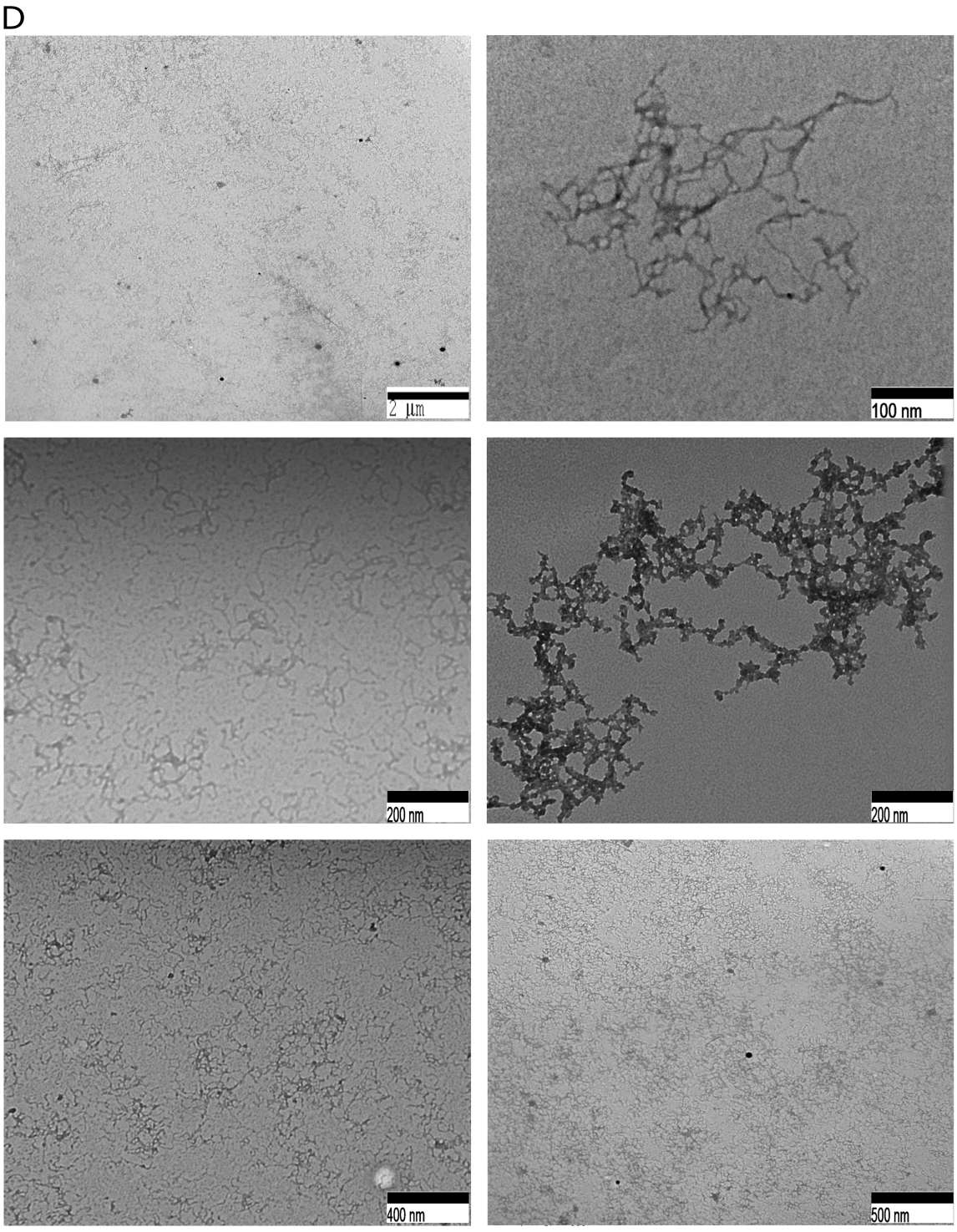

Figure 7. Fibril morphology by TEM. A) peanut, B) lentil, C) pea, and D) mung bean. Fibril samples were collected after 24 hours of incubation at 80 °C, pH 2 with stirring.

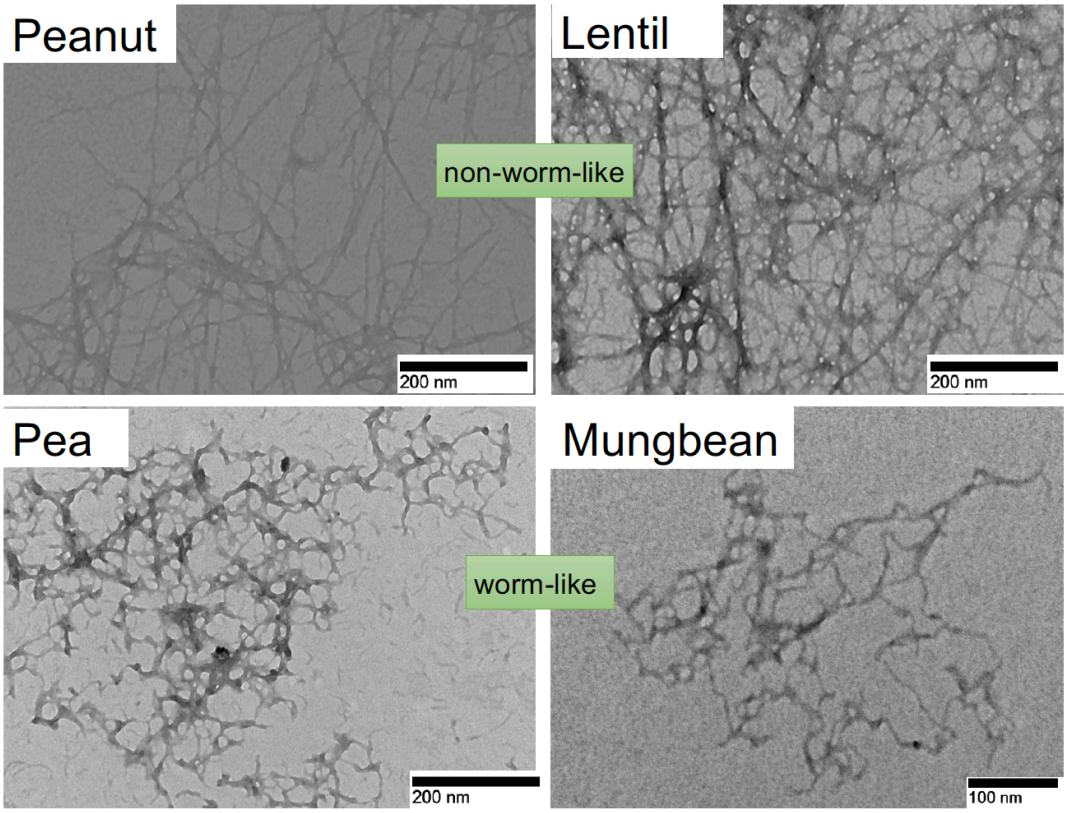

Figure 8. Peanut and lentil fibrils (top) generally showed longer, straighter fibrils than those from pea and mung bean (bottom). Fibrils were collected after 24 hours at 80 °C, pH 2.

4.4 SDS- PAGE

Hydrolysis of proteins usually occurs as the result of high temperature and low pH during incubation (Wang et al., 2020a). SDS-PAGE was carried out on peanut, mung bean, pea, and lentil fibrils solutions to study the molecular weight degradation. Fibril samples were collected at different time intervals (0, 1, 2, 5, 8, 24, and 30 h) and protein extracts at pH 8 were loaded onto gels. As shown in **Error! Reference source not found.**, both vicillin/convicilin and legumin fractions were still present in the samples at pH 2 at 0 hour. The dominant bands at ~70, 50 are vicilin/convicilin fraction subunits, whereas the less dominant bands at 20 kDa represent the legumin fraction subunit.

The hydrolysis of protein started at pH 2 and then heat-induced hydrolysis. The bands at 75 kDa and 37-50 kDa corresponds to vicilin bands that disappear faster than the 20 kDa, which is the

legumin band. After heating for 24 h, various legume proteins were hydrolyzed into peptides with Mw below 15 kDa. Also, the high Mw bands are not visible at time 30 hours for all of the samples. By comparison, the bands of vicilin were more easily degraded than legumin during heating at pH 2. In legumin, the acidic subunits were more easily hydrolyzed than alkaline subunits.

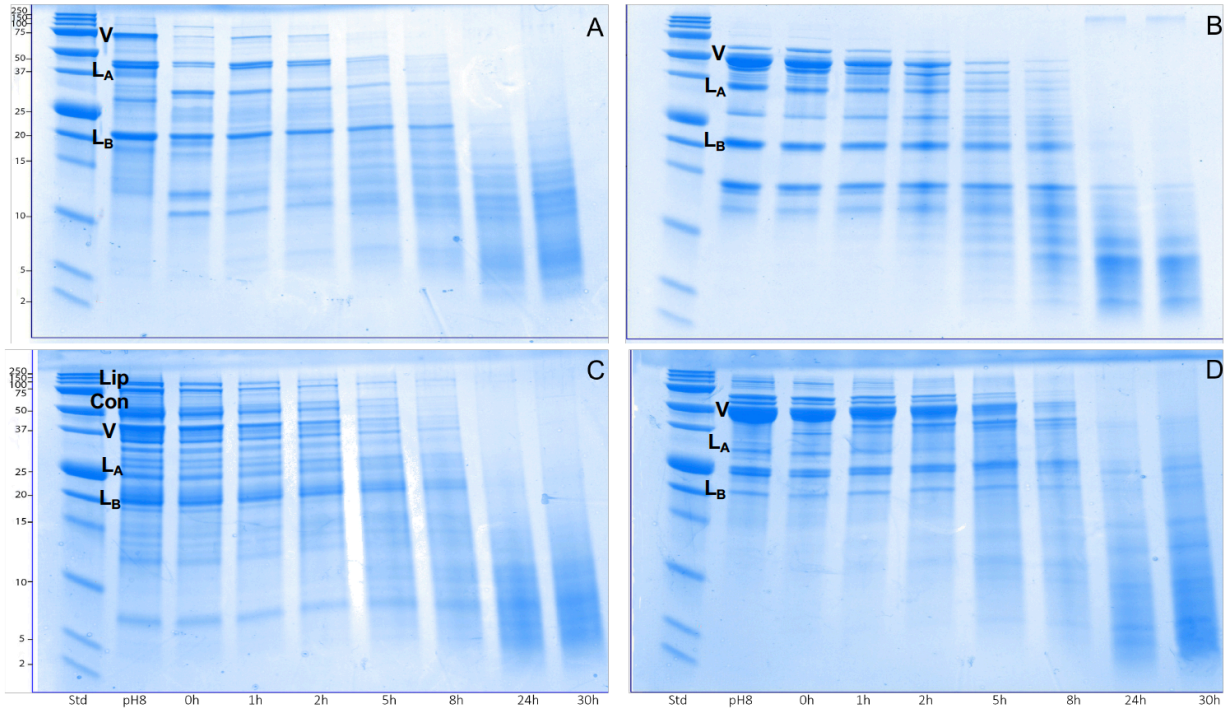

Figure 9. Protein hydrolysis during fibrillation. SDS PAGE profiles of unheated (pH 8) and heated (pH 2) samples of A) peanut, B) lentil, C) pea, and D) mung bean. The heating was carried out with the protein concentration of 20 mg/ml, at 80 °C for different time intervals of 0-30 hours. The standard bands (std) from bottom to top correspond to 2, 5, 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa. Vertical: Lip: lipoxygenase; Con: convicilin; V: vicilin; L_A: legumin acidic subunit; L_B: legumin basic subunit.

4.5 Solubility

The fibril solubility was tested at different pH values (2, 3, 4, 5, 6.2, 7, and 8). The solubility of peanut, mung bean, and lentil fibrils was calculated. The results suggested that the

solubility of all three legumes near the isoelectric point (approximately pH 4.8) decreased (Figure 10A).

Among the pH values tested in this study, the maximum protein solubility was observed at pH 2 for all three lentils (1.61 mg/ml), mung bean (1.5 mg/ml), and peanut (0.95 mg/ml). The lowest solubility for lentil (0.44 mg/ml), mung bean (0.22 mg/ml), and peanut (0.40 mg/ml) were found at pH values of 5, 6, and 5, respectively. For lentil, mung bean, and peanut the solubility is significantly difference at pH 2 versus pH 3.

The ThT intensity of the peanut, mung bean, and lentil solutions before and after the centrifugation were tested. As shown in Figure 10B, C, and D, overall, the ThT fluorescence intensity for the solutions was higher before the centrifugation step compared to the ThT fluorescence intensity after centrifugation at all of the pH values. The ThT intensity for mung bean before and after centrifugation did not change considerably at pH 2 and pH 3. The ThT intensity after the centrifugation dropped at pH values of 4,5, 6, 7, and 8. For peanut, the ThT intensity decreased after the centrifugation at pH values of 4,5, 6, 7, and 8. In terms of lentils, the ThT intensity of lentil fibrils decreased after centrifugation but for 2, 3, 4, and 6 pH, the ThT intensity is still present.

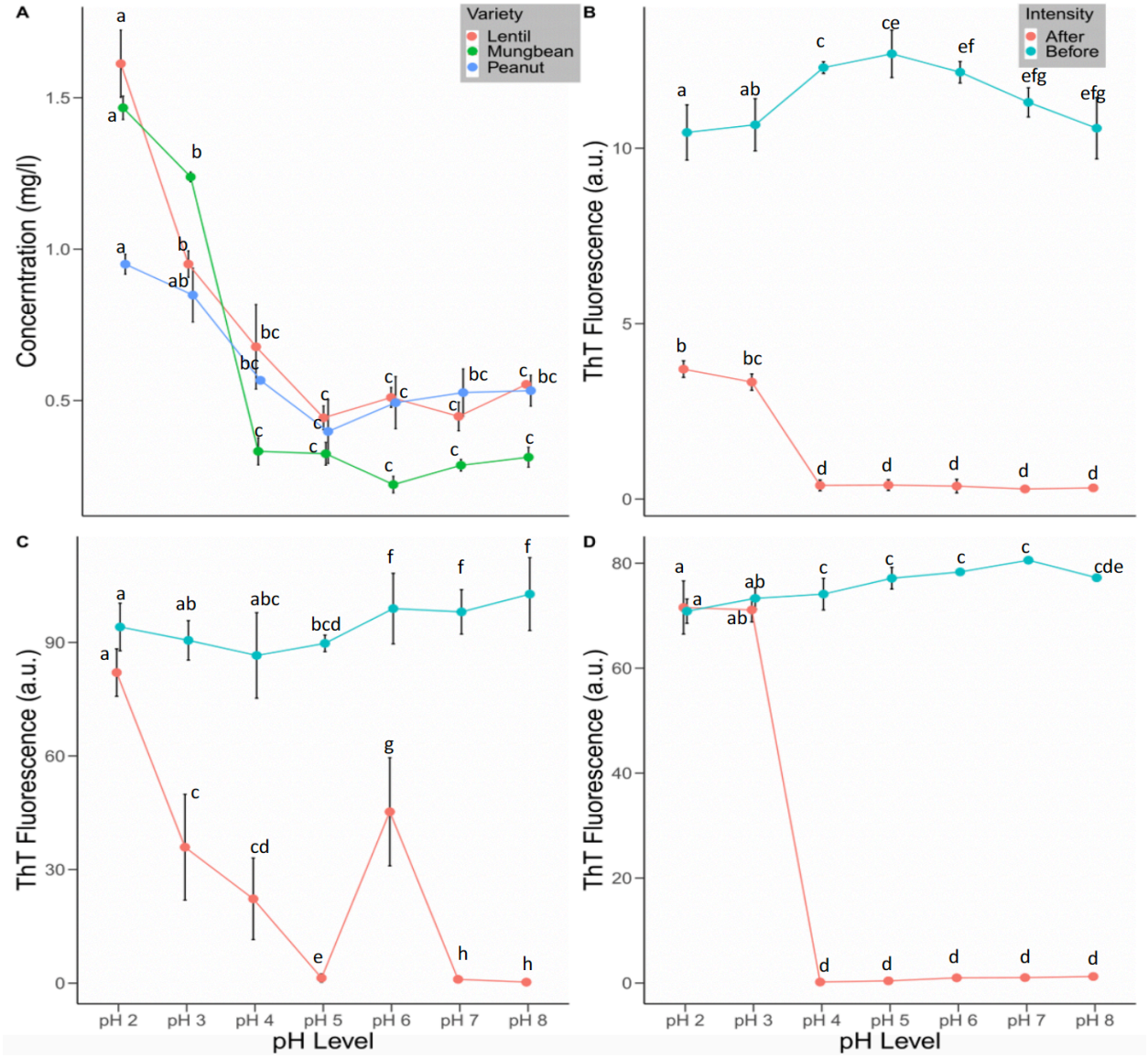

Figure 10. Fibril solubility as a function of pH. Solubility of peanut, mung bean and lentil fibrils at pH values of 2, 4, 5, 6, 7, 8 (A). ThT fluorescence intensity of A) peanut, B) lentil, and C) mung bean fibril solutions before and after the centrifugation. Experiments were performed in triplicate, and each point represents the mean value and standard deviation (n=3). Statistical analysis determined by one- way ANOVA to test significant difference between samples Superscript letters denote significant differences ($p<0.05$) between treatments.

Emulsifying ability is an important functional property of proteins that could affect the application of proteins in food formulations. The emulsifying properties of the peanut, mung bean, and lentil fibrils were evaluated at pH 2 and 7 at the same protein concentration (2 % w/v) and an oil fraction of 50% using droplet size analysis. The average droplet size of the peanut, mung bean, lentil proteins, and fibril emulsions were determined at pH 2, and pH 7 (Figure 11). All legume emulsions except for mung bean have a significantly smaller droplet size at pH 2 compared to pH 7. Slightly larger droplets were observed for non-heated mung bean protein emulsion at pH 2 (1.15 μm) compared to pH 7 (1.05 μm), which no significant difference in that case.

At pH 2, lentil protein has a smaller droplet than the lentil fibrils, while this result is reversed at pH 7. Mung bean protein has a larger droplet size than the mung bean fibrils at pH 2, whereas at pH 7, mung bean fibrils have a droplet size far bigger than the control. The droplet size of peanut fibrils at pH 2 and pH 7 is considerably smaller than that of the control. The average of three replicates of the emulsion oil droplet size distribution is shown in Table 3.

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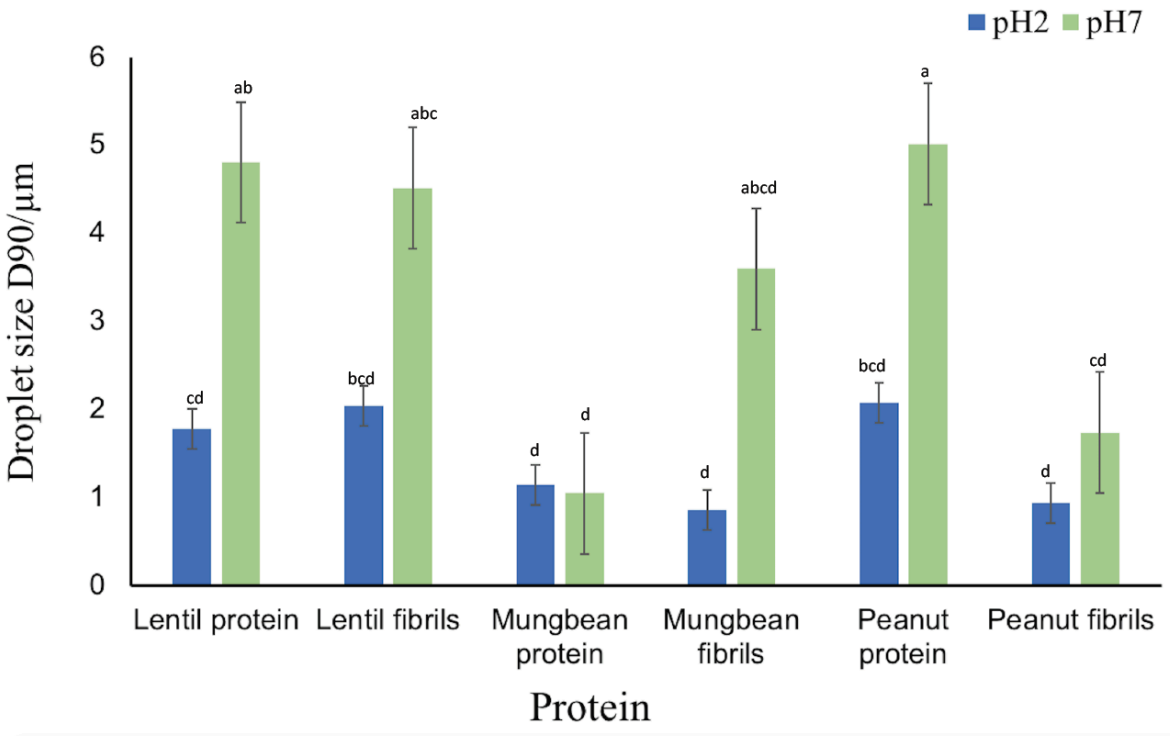

Figure 11. Emulsion oil droplet size (d90) of peanut, mung bean, lentil proteins and fibrils at pH 2 and pH 7. All emulsions were prepared at 50% oil fraction and 2% protein concentration. Experiments were performed in triplicate, and each point represents the mean value and standard deviation (n=3). Statistical analysis determined by two- way ANOVA to test significant difference between samples. Superscript letters denote significant differences ($p<0.05$) between treatments.

4.6 Gelling

The ability of gel formation of peanut, mung bean, and lentil nanofibrils was investigated. Cold-set gelation was performed by mixing fibril samples (20 mg/ml) with an appropriate volume of $CaCl_2$, NaCl, $MnCl_2$, and $ZnCl_2$ stock solutions to a final concentration of 10 mM, 150 mM, 200 mM, and 300 mM of cations (Ca^{2+}, Na^{+}, Mn^{2+}, and Zn^{2+}). None of the samples formed a self-supporting gel at the protein concentration investigated, so no textural analysis was completed for this property.

4.7 Viscosity

The rheological properties of fibril solutions and protein solutions with no heat treatments were investigated to compare the viscosity of peanut, mung bean, and lentil protein fibrils and their controls. Monovalent and divalent salts display electrostatic interactions between charged protein molecules, resulting in a decrease in electrostatic repulsions and protein aggregation (Kuhn et al., 2010). Monovalent cations are not suitable for production of gels from β-lactoglobulin nanofibrils because the formation of salt bridges is a prerequisite for gel formation (Veerman et al., 2003). NaCl (monovalent) is one of the most common salts for cold gelation of whey proteins, and $ZnCl_2$ is a divalent salt which has recently been used as a thickening agent (Mohammadian & Madadlou, 2016b).

In this study, we compared the effects of both NaCl and $ZnCl_2$ on non-heated and fibrillated samples' viscosity. The non-heated (control) and fibril samples (20 mg/ml) were mixed with an appropriate volume of NaCl and $ZnCl_2$ stock solutions to a final concentration of 200 mM. The flow curve of viscosity versus shear rate for fibril solutions and protein solutions are shown in Figure 12. All of the dispersions exhibited shear-thinning behaviors upon increasing shear rate; however, the changing pattern of η upon shear rate varied with the type of

fibrils and salts. Both salts, $ZnCl_2$ and NaCl, increased the viscosity of the protein solutions. Fibrillation improved the apparent viscosity of all the legume proteins significantly (Figure 12). In terms of NaCl, the highest apparent viscosity was observed for mung bean fibrils, 48695 mPa·s at 1 s^{-1} followed by peanut fibril, 2812 mPa·s, and lentil fibril, 1839 mPa·s (Figure 12). For detailed data and statistical analysis please refer to Table 4 in appendices. In terms of $ZnCl_2$, the highest apparent viscosity was observed for lentil and mung bean fibrils, 3252 mPa·s at 0.1 s^{-1}, and followed by peanut, 282 mPa·s at 0.1 s^{-1}. For detailed data and statistical analysis please refer to Table 4 in the appendix.

Lentil fibrils showed higher apparent viscosity of 21.764 mPa·s at 50 s^{-1} for $ZnCl_2$ than NaCl at 18.607 mPa·s at 50 s^{-1} (Table 3). Peanut demonstrated a higher apparent viscosity of 23.431 mPa·s at 50 s^{-1} for NaCl than $ZnCl_2$ at 4.520 mPa·s at 50 s^{-1} (Table 3). For mung bean, fibrillated protein improved the apparent viscosity of the solution by 65.039 mPa·s at 50 s^{-1} for NaCl significantly while it demonstrated lower apparent viscosity, 18.708 mPa·s, for $ZnCl_2$ (Table 3).

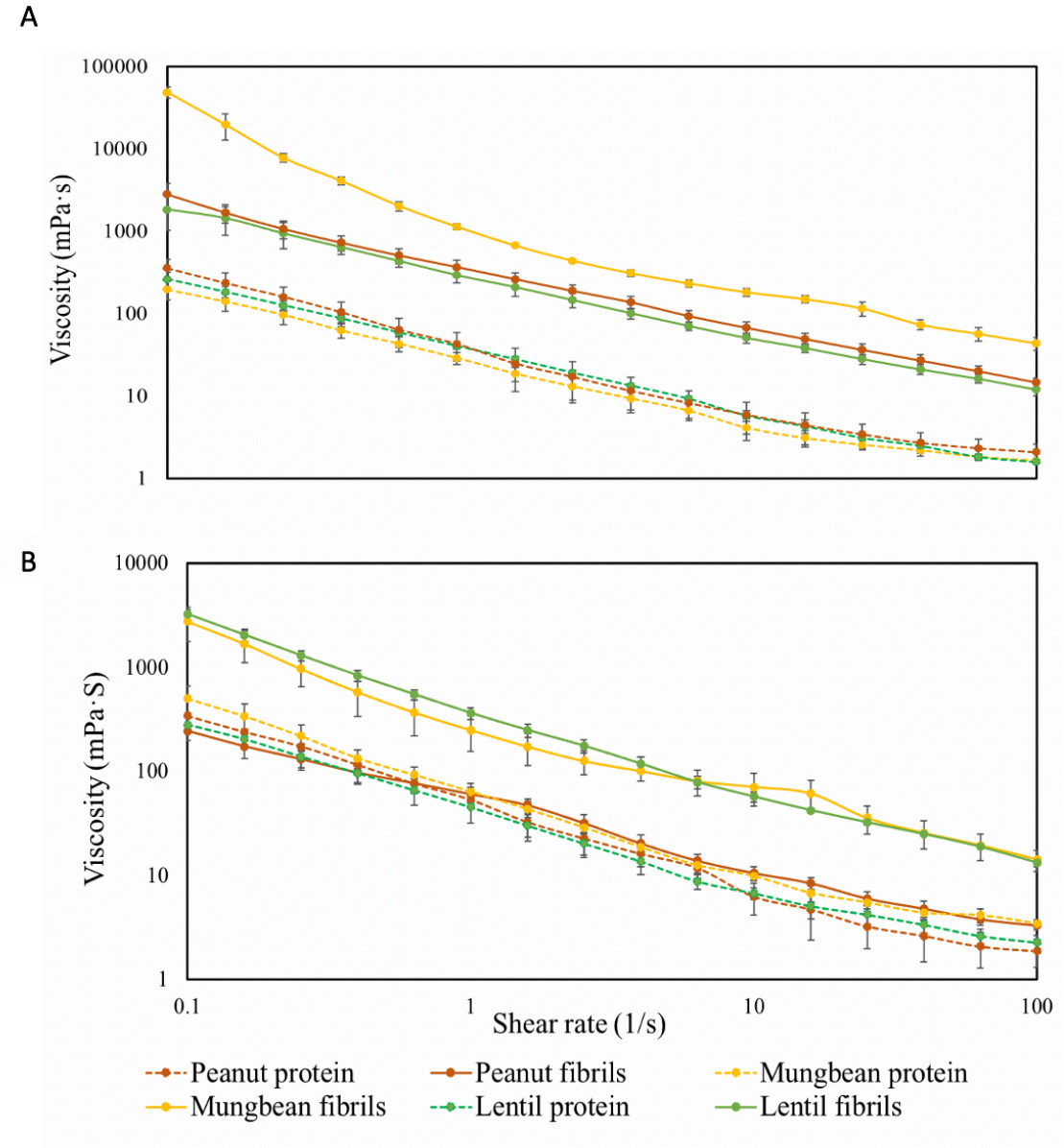

Figure 12. Viscosity of legume protein nanofibrils. Viscosity as a function of shear rate for untreated and fibrillated peanut, mung bean and lentil protein solutions with 200 mM A) NaCl or B) $ZnCl_2$. Results are reported as the mean ± standard deviation (n = 3).

Table 3. Apparent viscosity of untreated and fibrillated peanut, mung bean and lentil protein solutions with different salts at dt=50 1/s constant shear rate.

Samples	Apparent viscosity (mPa·S)		P value (T-test)
	$ZnCl_2$ (200 mM)	NaCl (200 mM)	
Peanut protein	2.166±0.733[ns]	2.519 ±0.765[ns]	0.5972
Peanut fibrils	4.520±0.482**	23.431±3.853**	0.0125
Mung bean protein	4.251±0.675**	2.160±0.256**	0.0220
Mung bean fibrils	18.708±7.953**	65.039±10.386**	0.0044
Lentil protein	2.973±0.485[ns]	2.142±0.132[ns]	0.0882
Lentil fibrils	21.764±0.656**	18.607±2.354**	0.0375

[1] results are reported as mean ± standard deviation (n=3)
[2] superscript letters denote significant differences ($p<0.05$) between treatments, as determined by T- test to test significant difference between samples.
[3] [ns] not significant
[4] [**] significant

Chapter 5: Discussion

5.1 Formation of nanofibrils by peanut, mung bean, pea, and lentil and their kinetics

A general mechanism for amyloid formation begins with protein unfolding, followed by the formation of protofilaments and oligomers, and finally mature fibrils are formed upon incubation (Adamcik et al., 2010). Fibril formation kinetics generally follows a sigmoidal shape, with an initial lag phase followed by exponential growth until a plateau is reached upon saturation of the fibrils (Arosio et al., 2015).

Here, ThT kinetic traces were analyzed to characterize the lag phase, half time, and growth rate of fibril formation. The differences in the real-time kinetics show the variances in the duration of the lag phase, the slope of the growth phase, and the plateau of different protein fibrils that could result in variations in the morphologies of the amyloid fibrils. Increasing ThT intensity confirms the formation of nanofibrils from peanut, mungbean, pea, and lentil. An essential step for fibrilization for globular proteins is partial unfolding because the fibrillization-prone segments are normally buried in protein interior structures (Linden & Venema, 2007). This suggests that for all proteins studied, partial unfolding occurred before fibril assembly was possible. The sigmoidal shape of the fibrilization curves was observed only for peanut and lentil fibrils, as neither mung bean nor pea fibrillization had a lag phase.

The lag phase is a time during which nuclei form but amyloid growth is minimal. Peanut and lentils demonstrate a lag phase and a steep increase in the growth phase, while mung bean and pea fibrils demonstrate no lag phase and a less steep rise in the growth phase. Lentil shows the significantly highest maximum fluorescence intensity, followed by peanut, mung bean, and pea (Figure 6). Lentil kinetics showed a distinct lag time, and the intensity increased gradually and reached the plateau phase at the heating time of 24 hours. However, another study reported

lentil fibrillation with no lag phase and a sharp increase in ThT fluorescence intensity (Li et al., 2021). Different fibril formation conditions including protein concentration (10 mg/ml), temperature (90°C), and no stirring, could result in differences in ThT fluorescence intensity.

Pea kinetics show no lag phase, which is consistent with the results of Munialo et al. (2014), however, the maximum ThT fluorescence intensity is much higher in their results. This could be due to the higher pea protein content (40 mg/ml) that was used in the fibril formation. The peanut fibrillation kinetics here are similar to that from chickpeas (Li et al., 2021), with comparable ThT fluorescence intensities and lag times, although the peanut growth rate constant is slower. Also, the conditions used in the study were different (*e.g.,* temperature, protein concentration, and no shaking), which could result in the differences in the growth rate constant. Therefore, these findings demonstrate that different conditions such as temperature, shaking, stirring, and protein concentration might result in variations of fibrils and ThT fluorescence intensity.

The growth rate constant is much higher for the mung bean fibrils than for the other proteins, while lentils were the slowest. The ThT fluorescence intensity of mung bean protein increased remarkably during the short period (0.5 h) and then increased slowly. After heating for 6 hours, it reached the plateau phase. The ThT fluorescence intensity and the lag time are consistent with other research (Liu & Tang, 2013), in which the ThT intensity of mung bean increased during a heating time of 0.5 hours and did not show a lag phase. However, the maximum fluorescence intensity of the mungbean fibrils is not consistent with their results. This outcome could be due to the higher protein content (78.9 % w/w) and purified vicilin that was used in their research.

According to Akkermans et al. (2007), during hydrolysis, a peptide is released from cleavage of the peptide bonds before or after aspartic acid residues of β-lactoglobulin, which is the source of building blocks for the fibrils at pH 2.0. Vicilin from legumes is rich in aspartic acid, asparagines, glutamic acid, and glutamine, which is suggested to be a good starting point material to form amyloid fibrils (Tang & Sun, 2011).

Several extrinsic factors influence the different microscopic reactions and consequently the length of the lag phase, including peptides and proteins, salts, pH, small molecules, and mechanical agitation (*e.g.,* shaking or stirring) (Arosio et al., 2015). Agitation could decrease the lag time for the fibrillating system. Agitation resulted in considerably increased reproducibility of the a-synuclein protein fibrilization process with regards to lag time, endpoint ThT emission level, and overall time profile shape (Giehm & Otzen, 2010).

Also, the length of the lag phase is dramatically decreased if the fragmentation rate increases, which may happen if samples are shaken, or if the mechanical stability of the fibrils is reduced due to, for example, impeded inter-peptide interactions within the fibril (Arosio et al., 2015). Any of these factors could control the shortening or lengthening of the lag phase. Li et al. (2021) reported that vicilin fractions were more favourable to lentil fibril formation in the ordered arrangement. Moreover, the growth rate of fibril assembly is influenced by the subunit composition of proteins. According to Wang et al. (2011), the growth kinetics of the fibrils form β-conglycinin and its subunits greatly varied among various subunits. Because the hydrolysis behaviour of the three subunits of -conglycinin differs, including hydrolysis rate and cleavage locations, this indicates a close link between polypeptide hydrolysis and amyloid fibril production kinetics (Wang et al., 2011). This result suggests the differing roles which are involved in amyloid formation and the growth rate.

5.2 Morphology of fibrils (TEM)

The morphology of the fibrils was observed by TEM (Figure 3). The TEM technique has been successfully used for the nanoimaging of proteins and fibrils and the qualitative comparison of features, such as the twists in ribbon-like fibrils, the curvature of fibrils, and the smoothness of their surfaces (Gras et al., 2011). As TEM images show, peanut, lentil, mung bean, and pea fibrils are different in morphology.

Peanut and lentil fibrils are long, non-worm-like, and straight, while pea and mung bean fibrils are short, worm-like, and curly. Peanut and lentil fibrils are similar to milk and egg white protein fibrils (*e.g.,* b-lactoglobulin and lysozyme) that are straight and long (Loveday et al., 2011; Rahimi Araghi & Dee, 2020). Moreover, a similar morphology was also discovered in other research, which was done on fibrils derived from partially purified soy proteins (Akkermans et al., 2007). The morphology of lentil fibrils observed here is not consistent with other studies on lentil fibril formation (Wynnychuk et al., 2021; Li et al., 2021). In the other studies, lentil fibrils are short, curved, and semiflexible, while the fibrils obtained in this research are long and straight. The difference in the morphology of lentil fibrils could be due to the different conditions used in protein extraction (*e.g.,* pH 3.5 vs pH 8, solvent, and defatting) and fibril formation. (*e.g.,* temperature and heating duration).

The morphology of our pea fibrils is consistent with the Munialo et al. (2014), study on pea fibrils, which shows curly and worm-like fibrils (Munialo et al., 2014). According to Amagliani and Schmitt (2017), pea protein fibrils had comparable structural characteristics to soy protein fibrils, however, the legume fibrils were more branched and flexible than whey protein fibrils. The TEM images for mung bean are consistent with the AFM images of a previous study (Liu & Tang, 2013) that was done on fibril morphology of vicilin from mung

bean protein. The differences in the characterization of fibrils depend on several conditions such as ionic strength, protein concentration, pH, and whether the samples were stirred during heating or not (Munialo et al., 2014).

5.3 Role of hydrolysis in fibrillation

During the fibrillation process peanut, lentil, mung bean, and pea proteins are hydrolyzed into smaller protein fragments over time, and the high MW bands are not visible by 30 hours (Figure 9). After heating for 24 h, the various legume proteins are hydrolyzed into peptides with a MW below 15 kDa. For peanut, lentil, and pea, the hydrolysis timescale correlates with the ThT kinetics that also reach the plateau phase after 30 hours. In these cases, most of the hydrolysis has occurred by the time of the fibril growth phase, indicating that hydrolysis may assist fibril formation.

For mung bean, however, the fibril formation (ThT kinetics) is faster than hydrolysis, with the fibrillation reaching the growth phase before extensive hydrolysis occurs. This result suggests that complete hydrolysis is not essential for mung bean fibril formation. Since the kinetics of mung bean is consistent with the other study done by Li & Tang (2013), which showed no lag time, it is reasonable to conclude that complete hydrolysis is not required for the mung bean fibril formation.

Even though hydrolysis of polypeptide bands normally leads to fibril formation under heating and acidic conditions, it is not a requirement for fibril formation (Lambrecht et al., 2019). In addition, Jung & Mezzenga (2010) reported that monomers were present in fibril by heating β-lactoglobulin at a low pH for a very short period. Moreover, monomers and large peptides were still present in β- lactoglobulin fibrils after microwave heating at 80 °C and pH 2.0 (Hettiarachchi et al., 2012). In contrast, most studies claim that hydrolysis of polypeptides and

the release of building blocks is required for protein fibril formation at pH 2 (Akkermans et al., 2008).

The result suggested that the β- lactoglobulin fibrils were not composed of intact β-lactoglobulin proteins but rather peptide fragments released from acid hydrolysis during the heat treatment at pH 2 (Akkermans et al., 2008). According to work done by Munialo et al. (2014), all of the proteins were hydrolyzed into peptides upon heat treatment of pea protein, with 50% of these peptides being constructed into the fibrils. This result confirms that hydrolysis of all proteins occurs following the heating of pea proteins at 85 °C for 20 hours at pH 2.0. In addition, Kroes et al. (2011) investigated the acid hydrolysis of β- lactoglobulin and found that the fibril formation occurs through two steps of the protein hydrolysis into peptide fragments and the assembly of some of these released peptide fragments into fibrils. Another study discovered that high MW proteins from kidney bean 7S globulin (50 kDa) were hydrolyzed into low MW proteins of less than 10 kDa with increasing heating time (Tang et al., 2010).

The ability of different proteins to form highly ordered fibrils is closely associated with the differences in the sensitivity of the polypeptides to acid hydrolysis (Liu & Tang, 2013). Regardless of whether hydrolysis is a prerequisite for fibrillation or not, the end result is that the proteins are hydrolyzed. This will affect the structure and surface properties of the fibrils.

5.4 Solubility of fibrils

The protein concentration as a function of pH was calculated for peanut, mung bean, and lentil fibrils. The result suggests that the solubility of all three legumes near the isoelectric point (approximately pH 4.8) decreased (Figure 5). This result is consistent with the other studies where fibrillation reduced the solubility of whey protein enzymatic hydrolysate and whey protein isolate, particularly at pH values near the pI (Mohammadian & Madadlou, 2016a).

According to several studies (Pelegrine & Gasparetto, 2005; Kakalis & Regenstein, 1986), a protein is least soluble at the pI due to increasing protein-protein interactions, which are caused by the minimum electrostatic forces and less protein-water interaction. Therefore, protein molecules contact each other, aggregate, and precipitate. Proteins are generally more soluble at pH values above or below their pI due to having a greater net negative or positive charge, resulting in greater electrostatic repulsion and water interactions. Charge repulsion and net charges result in greater solubility of the protein (Pelegrine & Gasparetto, 2005).

Overall, heat treatment leads to protein denaturation and aggregation of unfolded molecules, which leads to loss of solubility (Carbonaro et al., 1997). Therefore, even at pH 2, the solubility decreases from what it was at time 0, due to hydrolysis, unfolding, and amyloid formation. The ThT fluorescence intensity for the fibril solution for all of the pH values is higher before the spinning compared to the intensity after spinning. This suggests that fibrils are mostly present in the pellet or insoluble. For mung bean, the ThT fluorescence intensity before and after the spinning at pH 2 and pH 3 did not change greatly. This result is comparable with the solubility of the mung bean, which is most soluble at pH 2 and pH 3. Also, at pH values of 4, 5, 6, 7, and 8, the ThT intensity decreased after the centrifugation, which suggests that mung bean is completely insoluble at these pH values.

The solubility of mung bean protein isolate was higher at pH 2, 10, and 12 than at the other pH values due to electrostatic repulsion and hydration (Brishti et al., 2017). The minimum solubility of mung bean protein was observed at its pI (pH 4.6) (Du et al., 2018). In the work done by Tang and Sun (2010), mung bean native protein was more than 90% soluble at pH greater than 7, or less than 3, which shows the electrostatic repulsion interactions between the proteins were essential for the protein solubility of this globulin.

Lentil ThT fluorescence intensity after spinning is high at pH 2, followed by pH 6, 3 and 4, demonstrating that fibrils are present in the solution and soluble at these pH values. Lentil fibrils are least soluble or insoluble at pH values of 5, 7, and 8. This result is consistent with the other study on lentil legumin protein (Jarpa-Parra et al., 2015), where lentil legumin protein was highly soluble at pH 2 and above pH 8 and showed minimum solubility at pH 4.7. This demonstrates that lentil protein develops high surface hydrophobicity and solubility at pH far from pI, so a balanced level of hydrophilic and hydrophobic amino acid residues is exposed at the protein surface (Jarpa-Parra et al., 2015).

The ThT fluorescence intensity of peanut fibrils is still present at pH 2 and 3 after spinning. This result demonstrates that some of the peanut fibrils remained soluble, and this is comparable with the solubility, which has the highest solubility at pH 2 and 3 compared to other pH values. Also, ThT fluorescence intensity decreased at pH values of 4, 5, 6, 7, and 8 after spinning. This suggests that the peanut fibrils are completely insoluble at these pH values. According to work done by Uddin et al. (2018), the minimum solubility of peanut protein was observed at pH 3.5–4.5 and maximum solubility at pH 10 or higher. Furthermore, the results revealed that peanut is the least soluble at its pI near pH 4. However, heating the peanut

decreased the protein solubility, which could be due to the increase in surface hydrophobicity of protein through the unfolding of molecules upon heat (Uddin et al., 2018).

The solubility patterns of peanut, lentil, and mung bean protein fibrils are similar where they are most soluble at pH 2, and least soluble at pH 4-5. Fibrils have better solubility when the pH is far away from the isoelectric point (e.g., pH 2 and pH 3), which indicates a sensitivity of polypeptides to pH. These findings are comparable with soy protein fibrils, which had better solubility at pH lower than 3 or higher than pH 7, which was far away from the pI (Y. Wang et al., 2020b). Also, the difference in protein solubility could be the result of a difference in the degree of protein denaturation (Y. Liu et al., 2011).

5.5 Emulsion properties and particle size

The formation and particle size measurements of oil-in-water emulsions using non-heated protein and fibril samples were studied to investigate the emulsifying properties of peanut, lentil, and mung bean fibrils. In general, the smaller the droplet sizes of protein-stabilized emulsions, the better the emulsifying ability of the protein (Ettoumi et al., 2016). The result of particle size (d_{90}) analysis shows that almost all of the protein control and fibril samples except mung bean control have smaller droplet sizes at pH 2 than at pH 7. This result is consistent with the solubility of these protein fibrils (Figure 5), indicating that the solubility of proteins contributes to their emulsifying properties, as these fibrils are most soluble at pH 2. Protein solubility is an important factor in the quality of the emulsions (Mcwatters & Cherry (1977)). Our result is comparable with another study on the emulsifying properties of pea, chickpea, and lentil proteins, where all of the samples showed a similar dependency of emulsifying ability on their

solubility. These proteins exhibited minimum emulsion quality at their pI and increasing emulsifying properties at pH values far from their pI (ex., pH 3, 8) (Ettoumi et al., 2016).

Tang and Sun (2010) show that globulin properties are highly dependent on solubility, surface hydrophobicity, and polypeptide heterogeneity (Tang & Sun, 2010b). In work done by Tang and Sun (2010), the emulsifying ability index with pH was consistent with the mung bean protein solubility, which demonstrated a close correlation between the emulsifying ability and the protein solubility for mung bean globulins. Mung bean fibrils could form emulsions with smaller droplet sizes at pH 2, whereas lentil fibril emulsion is more efficient at pH 7. Also, emulsions prepared with peanut fibrils had a smaller droplet size at both pH 2 and 7, compared to their respective non-heated controls.

These variances in droplet sizes of different emulsions made from fibrils could be due to the ratio of legumin to vicilin. The emulsifying ability of pulse protein could be affected by the ratio of globulins to albumins and legumin to vicilin. For example, pure vicilin pea protein had a better emulsifying index compared to pure legumin (Scaviner et al., 1987). The mung bean globulins with a higher ratio of legumin (11S) to vicilin (7S) demonstrate better solubility and emulsifying abilities than mung beans with a lower 11S: 7S ratio (Tang & Sun, 2010b). Out of these three fibrils, only peanut fibrils showed smaller droplet sizes at both pH 2 and 7. These results indicate that the performance of proteins or fibrils is highly dependent on the pH of the samples, of which some display the greatest function at their natural pH (pH 7), and others perform much better by adjusting the pH to a level to increase the solubility of the proteins (Mcwatters & Cherry, 1977).

Even though the droplet size of lentil fibrils is larger than the control at pH 2, overall, the lentil emulsions prepared in this research show a much smaller droplet size (Table 3). Emulsions

with smaller average droplet sizes will have a slower creaming rate, so develop emulsions with long-term stability (Wynnychuk et al., 2021). In contrast, another study reported a much larger droplet size (d90), 25.82–37.83 μm, for lentil fibril emulsions prepared with a fibril concentration of 1.5, 3, and 50% and an oil fraction of 50% at pH 2 (Wynnychuk et al., 2021). Karaka et al. (2011) found that an emulsion could be stabilised by lentil protein with a small droplet size ($d_{3,2}$) (ranging between 1.2 and 21,7 μm). The stability of an emulsion is greatly dependent on its droplet size, and an emulsion with small droplets generally has a longer shelf life than one with larger droplets (Karaca et al., 2011).

Moreover, Peng et al. (2016) investigated the behaviour of oil-in-water emulsion in the presence of whey protein fibrils. The study discovered that longer fibrils (2 μm) are more efficient at stabilizing the emulsions than short fibrils (0.3 μm), and this stabilization does not depend on temperature and oil volume fraction. The shorter fibrils were less effective in depletion flocculation, and the longer fibrils had a greater excluded volume in the emulsion system (Peng et al., 2016).

5.6 Viscosity properties

The apparent viscosity of fibril samples and unheated protein solutions mixed with NaCl and $ZnCl_2$ was investigated to compare the viscosity of these samples. Protein concentration is essential to the properties of the viscous solutions or gels formed by adding salt. At low protein concentrations, heat-denatured protein will form a viscous solution instead of a gel (Bryant & McClements, 1998).

The apparent viscosity of different protein solutions (non-heated and fibrils) as a function of shear rate is shown in Figure 12. The presence of the fibrils results in an increase in viscosity compared to the unheated samples. This result is consistent with other studies which reported

protein fibrillation could greatly improve the viscosity of protein solutions and their flow behaviour (Zhang & Huang, 2014; Tang & Wang, 2010). The hydrodynamic diameter of protein is increased, and fibrils can form entangled networks that can result in increased viscosity (Mohammadian & Madadlou, 2018). Based on the result of apparent viscosity, all the dispersions exhibited shear thinning behaviours upon increasing shear rate (Figure 8). Fibrils can resist the flow in the direction of the shear field due to their highly packed structure (Zhang & Huang, 2014). However, as the shear rate increases, nanofibrils gradually disentangle and result in shear-thinning behaviour (Loveday et al., 2012). Variations in fibril entanglement result in variability in viscosity measurements (Zhang & Huang, 2014).

Polypeptide volume and molecular interactions are the important factors that affect the viscosity of the solutions (Zhang & Huang, 2014). The viscosity of native protein solutions is increased by fibrillation. In addition, in terms of peanut and mung bean fibrils, the viscosity rise of fibril solutions are greater in the presence of NaCl than in the presence of $ZnCl_2$. In the presence of $ZnCl_2$, lentil fibrils had a greater viscosity than with NaCl. The rheological effects of monovalent and divalent salts differ, which might be attributed to differences in network-forming behaviour and/or mechanical characteristics of fibrils, such as bending and extensional modulus (Loveday et al., 2012a). Mung bean fibrils demonstrated highest viscosity in the presence of NaCl than the other two legumes.

Monovalent (Na^+) and divalent (Zn^{2+}) cations modulate electrostatic interactions between charged protein molecules, which decreases the electrostatic repulsion and results in protein aggregation. Zn^{2+} directly stabilizes the interaction between negatively charged carboxyl groups on the protein chains and forms an intermolecular ion bridge. Na^+ decreases the electrostatic

repulsion between carboxylic groups, and then the interaction between hydrophobic groups occurs (Bryant & McClements, 2000).

In this study, lentil fibrils demonstrated a higher apparent viscosity of 57 and 51 mPa·s at 10 s^{-1} shear rates for $ZnCl_2$ and NaCl, respectively, while another study (Wynnychuk et al., 2021) reported a much lower viscosity of 4.7, 8.4, and 10.4 mPa·s at concentrations of 1.5%, 3.0%, and 5.0%, respectively. The differences in the apparent viscosity of lentil fibrils could be due to the addition of salts to prepare the viscous solution. The addition of salt could induce the thickening and gelling of protein better than the conventional (protein) method.

Moreover, Li et al. (2021) investigated the viscosity properties of fibrils made from lentils, chickpeas, and cowpeas. They found that cowpea fibrils displayed better viscosity than chickpea and lentil fibrils due to the formation of flexible aggregates from vicilin fractions. Our results showed a lower apparent viscosity than their study for lentil fibrils (2 Pa·s) (Li et al., 2021), which could be due to the crude extract of proteins that were used in this research.

5.7 Relationship between fibril morphology and functional properties

The hypothesis of this study was that legume proteins are different in structure and morphology, so they can be induced into nanofibrils with different structures and functional properties. As the TEM images show (Figure 7), these fibrils are different in morphology. Lentil and peanut show longer and straighter fibrils, while mung bean and pea fibrils are curly and short. The morphology of lentil fibrils observed here is not consistent with other studies on lentil fibril formation (Wynnychuk et al., 2021; Li et al., 2021), while pea and mung bean have similar morphology to previous studies (Munialo et al., 2014; Liu & Tang, 2013). Several conditions could influence the formation and structure of fibrils, including protein concentration, different extraction methods, temperature, pH, shaking, and stirring (Munialo et al., 2014). Also, fibril

formation and the morphology of the fibrils are influenced by differences in amino acid composition and peptide sequences (Wang et al., 2011b).

Despite differences in morphology, the peanut, lentil, and mung bean fibrils were all most soluble at pH 2 and least soluble at pH 4 to 5, which is consistent with other studies (Brishti et al., 2017; Tang & Sun, 2010a). In terms of emulsifying properties and particle size, the smallest particle size at pH 2 was observed in mung bean fibrils with a curly structure. This result is not consistent with another study which stated that the longer whey fibrils, not the shorter ones, stabilized the emulsion (Peng et al., 2016). Also, only peanut fibrils show small particle size and less than the non-heated peanut protein at both pH 2 and 7. In another study (Wynnychuk et al., 2021), short and curved lentil fibrils formed emulsions with larger particle sizes compared to our results, in which long and straight lentil fibrils formed smaller particle sizes than peanut and mung bean fibrils. Previous studies suggested that fibrils formed stronger gels than non-fibrillated proteins (Mohammadian & Madadlou, 2018). However, in this research, none of the fibrils (straight or curly) exhibited gels under the conditions used.

In terms of viscosity, the presence of the fibrils resulted in an increase in viscosity compared to the unheated samples. Mung bean fibrils with curly and worm-like structures demonstrated the highest apparent viscosity compared to lentil and peanut with straight and non-worm-like structure. According to findings in the literature, curly whey protein nanofibrils have a greater viscosity than long straight fibrils. The hydrodynamic diameter and creation of entanglement networks of whey protein fibrils cause the rise in solution viscosity (Loveday et al., 2012). Mung bean's high apparent viscosity may be attributed to the curly structure of the fibrils. In this study, lentil fibrils with straight and non-branched structures demonstrated the high apparent viscosity. In contrast, Wynnychuk et al. (2021) reported lentil fibrils with curly and

curved structures that showed much lower viscosity. So, the differences in their viscosity properties could be related to different structures of fibrils (straight vs. curly) or could be due to different conditions that were used in fibril formation and the preparation of samples for viscosity measurement.

Based on our findings, not only the structure of the fibrils but a variety of conditions influence the functional properties, including pH, protein concentration, solubility, and polypeptide composition. Tang & Sun (2010) found that the emulsifying properties of the globulins are highly dependent on their solubility, surface hydrophobicity, and polypeptide heterogeneity (Tang & Sun, 2010b). Also, polypeptide volume and molecular interactions are the important factors that affect the viscosity of the solutions (Zhang & Huang, 2014). The ratio of vicilin to legumin concentration is positively correlated with the functionality of fibrils. Tang & Sun (2010) found that mung bean globulins with a higher ratio of legumin (11S) to vicilin (7S) demonstrate better solubility and emulsifying abilities than mung beans with a lower 11S: 7S ratio. According to SDS-PAGE gels (Figure 5), for mung bean, the major band appears to be vicilin with little legumin. Therefore, a lower 11S:7S ratio could explain the low emulsifying properties of mung bean fibrils.

Except for lentil fibrils, divalent salt ($ZnCl_2$) was much less effective at promoting viscosity enhancement than monovalent salt (NaCl) (Table 3). Monovalent and divalent salts have differing rheological effects, which could be due to changes in network-forming behaviour and/or mechanical properties of fibrils, including bending and extensional modulus (Loveday et al., 2012). The viscosity of fibril dispersions is determined by the volume fraction of fibrils in solution as well as their degree of entanglement or alignment. Entanglement or alignment

becomes very significant in viscous or gelled fibril dispersions, in both long semiflexible fibrils and curly fibrils (Loveday et al., 2012).

Moreover, the performance of fibrils is highly dependent on the pH of the samples, of which some display the greatest function at pH 7, and others perform much better by adjusting the pH to a level to increase the solubility of the proteins (Mcwatters & Cherry, 1977). According to Tang and Wang (2010), even though soy β-conglycinin (7S) and soy glycinin (11S) could form nanofibrils, β-conglycinin exhibited a higher capacity to form amyloid fibrils than glycinin. Both soy protein isolate and glycinin (11S) converted into fibrils at pH 2.0 and 85°C, but only glycinin displayed excellent functional properties (*e.g.,* flow properties) (Akkermans et al., 2007). As a result, changes in the amino acid composition and peptide sequence appear to be connected to the fibrillation kinetics and morphologies of different legume proteins (Wang et al., 2011).

Consequently, since we used crude extract of protein for fibril formation in this study, both vicilin (7S) and legumin (11S) could contribute to the fibril formation. From the crude extract of protein, we do not exactly know which of the protein subunits (7S or 11S) affected the functionality tests. Also, according to ThT data before and after spin, the estimated fibril content was about less than 20% (Figure 10 A, B, and C), therefore any non-fibrillar material is likely impacting the functioning outcome. Taken together, our research suggests that in addition to the structural and functional relationship, other conditions, including protein concentration, polypeptide concentration, pH, and temperatures, should be explored.

The functional qualities of legume fibrils and the impact of structural alterations on their functional properties are still unknown. Thus, in order to improve the use of legume fibrils as functional materials in food systems, a greater understanding of legume fibrils is required, and

this would definitely expand the theoretical knowledge of the structure-function link between plant-based fibrils.

Chapter 6: Conclusion and future research

6.1 Conclusion

Protein fibrillation is a promising strategy to expand protein functionalities due to the high stiffness, extreme aspect ratios and collective ordering features of amyloid fibrils. This study examined formation of fibrils from peanut, lentil, pea, and mung bean. Formation of fibrils was confirmed by ThT fluorescence and morphology characteristics of the fibrils were evaluated with TEM. At 20-30 mg/ml of protein concentration, the fibrils formed within five (mung bean) to 24 hours (lentil). As, TEM images showed, these fibrils are different in morphology. Lentil and peanut have shown a longer and straighter fibril and mung bean and pea fibrils are curly and short. It is possible to use peanut, lentil, and mung bean fibrils in the food industry as emulsifiers, and thickeners. However, due to the low protein content (20 mg/ml) and crude protein extract used in this investigation, no gel formation was detected.

In general, the formation and functional properties of legume protein nanofibrils are influenced by diverse conditions including, protein concentration, protein type (7S vs 11S), temperature, and morphology of the fibrils (straight vs curly).

6.2 Future Research

In this project, for fibril formation, characterization, and functionality tests, crude extracts from legume proteins are used. The next step in this study is to purify the major proteins from legume extract and examine their roles in nanofibril formation, morphology, and functionality in isolation and when combined. This research focused on the formation and characterization of the legume fibrils. However, more work is needed to optimise the functionality tests. Also, future studies could focus on using legume fibrils as an emulsifier, thickener, and foaming agent in a

real food application (*e.g.,* sausages, and ice-cream). Future research might help determine which proteins are most suited for particular types of foods.

All of the TEM images of fibrils in this study were obtained at pH 2, but it's important to examine these fibrils at different pH levels to see how changing pH affects their structure, morphology, and functioning. Other aspects to investigate include the safety of food made from legume protein nanofibrils, such as fibril cross-seeding between plant and human proteins.

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Appendix: Supplementary table for Chapter 2

Table 4. Viscosity of legume protein nanofibrils. Viscosity as a function of shear rate for untreated and fibrillated peanut, mung bean and lentil protein solutions with 200 mM NaCl or $ZnCl_2$.

Sample ID	Shear Rates	NaCl (200 mM)	$ZnCl_2$ (200 mM)
Peanut protein	0.1	355.94 ± 97.86^{defgh}	342.64 ± 22.40^{cd}
Peanut protein	0.159	234.98 ± 78.16^{efgh}	240.06 ± 11.69^{cde}
Peanut protein	0.252	160.50 ± 49.18^{fgh}	174.36 ± 12.18^{efgh}
Peanut protein	0.399	104.09 ± 35.17^{fgh}	116.09 ± 2.83^{efgh}
Peanut protein	0.632	63.72 ± 24.50^{gh}	76.51 ± 0.54^{efgh}
Peanut protein	1	42.98 ± 16.28^{h}	53.60 ± 2.64^{gh}
Peanut protein	1.59	24.85 ± 13.60^{h}	32.59 ± 9.22^{gh}
Peanut protein	2.51	17.11 ± 8.92^{h}	22.74 ± 7.04^{gh}
Peanut protein	3.99	11.55 ± 5.23^{h}	16.15 ± 3.92^{h}
Peanut protein	6.32	8.24 ± 3.24^{h}	11.80 ± 2.43^{h}
Peanut protein	10	5.92 ± 2.47^{h}	6.27 ± 2.13^{h}
Peanut protein	15.9	4.44 ± 1.86^{h}	4.72 ± 2.33^{h}
Peanut protein	25.1	3.43 ± 1.12^{h}	3.24 ± 1.27^{h}
Peanut protein	39.8	2.71 ± 0.86^{h}	2.62 ± 1.15^{h}
Peanut protein	63.1	2.333 ± 0.68^{h}	2.07 ± 0.78^{h}
Peanut protein	100	2.09 ± 0.53^{h}	1.87 ± 0.57^{h}
Peanut fibril	0.1	2812.27 ± 1001.96^{a}	$244.75 \pm 47.71^{cdefgh}$
Peanut fibril	0.159	1682.83 ± 428.26^{b}	174.31 ± 40.45^{efgh}
Peanut fibril	0.252	$1068.57 \pm 2\ 63.45^{cd}$	131.23 ± 28.31^{efgh}
Peanut fibril	0.399	$728.2500 \pm 154.99^{def}$	97.25 ± 19.43^{efgh}
Peanut fibril	0.632	$510.4967 \pm 105.72^{efgh}$	76.92 ± 15.35^{fgh}
Peanut fibril	1	369.5900 ± 74.68^{fgh}	60.48 ± 10.41^{gh}
Peanut fibril	1.59	263.3900 ± 50.08^{fgh}	47.27 ± 7.54^{gh}
Peanut fibril	2.51	189.9233 ± 32.84^{fgh}	31.90 ± 6.39^{gh}
Peanut fibril	3.99	138.1333 ± 24.74^{gh}	20.32 ± 4.18^{gh}

Peanut fibril	6.32	93.9353 ± 15.67^{gh}	13.88 ± 2.18 ^{h}
Peanut fibril	10	67.4860 ± 11.70^{h}	10.56 ± 1.59 ^{h}
Peanut fibril	15.9	49.5470 ± 8.66 ^{h}	8.45 ± 1.14 ^{h}
Peanut fibril	25.1	36.5817 ± 6.18 ^{h}	6.03 ± 0.90 ^{h}
Peanut fibril	39.8	27.1260 ± 4.49 ^{h}	4.82 ± 0.83 ^{h}
Peanut fibril	63.1	20.1177 ± 3.29 ^{h}	3.80 ± 0.50 ^{h}
Peanut fibril	100	14.6760 ± 1.94 ^{h}	3.33 ± 0.32 ^{h}
Mung bean protein	0.1	196.51 ± 48.29^{fgh}	500.94 ±161.04 ^{efgh}
Mung bean protein	0.159	140.48 ± 32.99^{fgh}	340.04 ± 104.17 ^{efgh}
Mung bean protein	0.252	97.08 ± 23.01^{fgh}	218.55 ± 63.02 ^{efgh}
Mung bean protein	0.399	62.61± 12.74 ^{gh}	134.33 ± 28.33 ^{gh}
Mung bean protein	0.632	43.24 ± 8.84 ^{h}	92.86 ± 18.28 ^{gh}
Mung bean protein	1	29.08 ± 5.01 ^{h}	64.14 ± 12.62 ^{gh}
Mung bean protein	1.59	18.73 ± 3.68 ^{h}	43.51 ± 7.81 ^{gh}
Mung bean protein	2.51	13.15 ± 4.15 ^{h}	28.99 ± 4.01^{h}
Mung bean protein	3.99	9.36 ± 2.62 ^{h}	18.75 ± 2.22 ^{h}
Mung bean protein	6.32	6.65 ± 1.34 ^{h}	12.55 ± 1.95 ^{h}
Mung bean protein	10	4.13 ± 1.23 ^{h}	9.86 ± 0.94 ^{h}
Mung bean protein	15.9	3.09 ± 0.70 ^{h}	6.79 ± 1.19 ^{h}
Mung bean protein	25.1	2.56 ± 0.33 ^{h}	5.51 ± 0.69 ^{h}
Mung bean protein	39.8	2.20 ± 0.18 ^{h}	4.36 ± 0.70 ^{h}
Mung bean protein	63.1	1.83 ± 0.13 ^{h}	4.13 ± 0.65 ^{h}
Mung bean protein	100	1.64 ± 0.12 ^{h}	3.46 ± 0.32^{h}
Mung bean fibril	0.1	48695.00 ± 7723.46^{gh}	2780.05 ± 1008.97^{b}
Mung bean fibril	0.159	19663.67 ± 6818.26^{gh}	1691.10 ± 566.82^{c}
Mung bean fibril	0.252	7800.067 ± 912.54 ^{gh}	969.94 ± 321.96^{cdefg}
Mung bean fibril	0.399	4094.27 ± 449.56 ^{gh}	580.44 ± 241.88^{efg}
Mung bean fibril	0.632	2031.37 ± 262.21 ^{gh}	369.78 ± 151.00 ^{efg}
Mung bean fibril	1	1136.73 ± 96.10 ^{gh}	250.16 ± 94.31 ^{efg}

Mung bean fibril	1.59	672.18 ± 18.25 ^{h}	173.23 ± 58.41^{gh}
Mung bean fibril	2.51	440.23 ± 16.37 ^{h}	125.94 ± 33.49 ^{gh}
Mung bean fibril	3.99	310.45 ± 25.58 ^{h}	100.74 ± 19.68 ^{gh}
Mung bean fibril	6.32	233.83 ± 23.00 ^{h}	80.37 ± 21.94 ^{gh}
Mung bean fibril	10	182.38 ± 20.80 ^{h}	71.03 ± 24.44 ^{gh}
Mung bean fibril	15.9	150.36 ± 14.22 ^{h}	61.40 ± 20.54 ^{gh}
Mung bean fibril	25.1	116.05 ± 22.83 ^{h}	35.70 ± 10.76 ^{h}
Mung bean fibril	39.8	73.76 ± 10.10 ^{h}	25.67 ± 7.67 ^{h}
Mung bean fibril	63.1	57.19 ± 10.95 ^{h}	19.50 ± 5.57 ^{h}
Mung bean fibril	100	43.68 ± 7.61 ^{h}	14.26 ± 3.29 ^{h}
Lentil protein	0.1	261.31 ± 55.61^{fgh}	282.91 ± 49.30 ^{efgh}
Lentil protein	0.159	182.61 ± 33.11 ^{fgh}	204.66 ± 37.15 ^{efgh}
Lentil protein	0.252	126.01 ± 18.20 ^{gh}	139.41 ± 30.99 ^{gh}
Lentil protein	0.399	87.40 ± 10.18 ^{gh}	96.43 ± 21.12 ^{gh}
Lentil protein	0.632	58.77 ± 5.87 ^{h}	66.06 ± 19.03 ^{gh}
Lentil protein	1	40.49 ± 2.95 ^{h}	45.45 ± 13.62 ^{gh}
Lentil protein	1.59	28.02 ± 2.16 ^{h}	30.10 ± 8.58 ^{h}
Lentil protein	2.51	19.20 ± 1.31 ^{h}	20.31 ± 5.48 ^{h}
Lentil protein	3.99	13.38 ± 0.42 ^{h}	13.67 ± 3.52 ^{h}
Lentil protein	6.32	9.31 ± 0.29 ^{h}	8.76 ± 1.40 ^{h}
Lentil protein	10	5.79 ± 0.84 ^{h}	6.74 ± 0.84 ^{h}
Lentil protein	15.9	4.32 ± 0.79 ^{h}	5.08 ± 1.2 ^{h}
Lentil protein	25.1	3.11 ± 0.09 ^{h}	4.20 ± 1.04 ^{h}
Lentil protein	39.8	2.50 ± 0.19 ^{h}	3.36 ± 0.56 ^{h}
Lentil protein	63.1	1.83 ± 0.11 ^{h}	2.62 ± 0.43 ^{h}
Lentil protein	100	1.58 ± 0.07 ^{h}	2.27 ± 0.38 ^{h}
Lentil fibril	0.1	1839.40 ± 809.52 ^{b}	3252.33 ± 376.75 ^{a}
Lentil fibril	0.159	1443.36 ± 548.10 ^{bc}	2056.57 ± 272.43^{b}
Lentil fibril	0.252	940.49 ± 326.84 ^{cde}	1303.67 ± 151.16^{c}

Lentil fibril	0.399	642.42 ± 123.33 ^{defg}	837.83 ± 103.58^{cdef}
Lentil fibril	0.632	436.42 ± 68.77 ^{efgh}	551.82 ± 62.25^{defgh}
Lentil fibril	1	293.55 ± 58.18^{fgh}	364.73 ± 48.51^{efgh}
Lentil fibril	1.59	210.5 ± 47.85 ^{fgh}	249.47 ± 36.70 ^{efgh}
Lentil fibril	2.51	146.57 ± 27.54 ^{gh}	176.37 ± 25.20 ^{efgh}
Lentil fibril	3.99	101.59 ± 15.09 ^{gh}	118.74 ± 20.61 ^{gh}
Lentil fibril	6.32	71.02 ± 8.73 ^{h}	78.86 ± 11.15 ^{gh}
Lentil fibril	10	50.92 ± 7.31 ^{h}	57.32 ± 6.08 ^{gh}
Lentil fibril	15.9	38.43 ± 4.92 ^{h}	42.37 ± 1.65 ^{gh}
Lentil fibril	25.1	28.26 ± 3.89 ^{h}	32.42 ± 1.74 ^{h}
Lentil fibril	39.8	21.14 ± 2.77 ^{h}	24.10 ± 0.98 ^{h}
Lentil fibril	63.1	16.30 ± 1.98 ^{h}	18.85 ± 0.50 ^{h}
Lentil fibril	100	12.03 ± 1.90 ^{h}	13.27 ± 1.07 ^{h}

[1] Results are reported as mean ± standard deviation (n=3)

[2] Superscript letters denote significant differences ($p<0.05$) between treatments in each experiment set, as determined using a two-way analysis of variance (ANOVA) to test the significant difference.