PHYSICAL AND CHEMICAL TREATEMENTS OF ZEIN TO IMPROVE GLUTEN-FREE BREAD QUALITY

by

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To my mother, father, and brothers

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Tg	Glass transition temperature
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
DMA	Dynamic mechanical analysis
DSC	Differential scanning calorimetry
GF	Gluten-free
HMW	High molecular weight
LMW	Low molecular weight

ABSTRACT

Gluten is a complex mixture of proteins imparting unique viscoelastic properties to doughs. However, despite its outstanding role in producing high quality baked goods, gluten is also associated with health disorders such as celiac disease or gluten sensitivity. This generated an increased demand of gluten-free products as individuals suffering of gluten-related disorder need to follow a strict gluten-free diet. Zein is a gluten-free storage protein from corn possessing promising features for gluten replacement in breadmaking. Although numerous studies attempted to improve zein dough rheological properties to improve its breadmaking performance, zein bread making potential remain inferior if compared to gluten. When it is incorporated into a dough, zein can form fibrils which confers extensibility, however, it does not possess a strain hardening behavior, a fundamental feature to produce high quality bread.

To improve zein dough strain hardening we hypothesize that structures formed by zein are not large enough to generate an elastic response, however the application of physicochemical treatment can induce the formation of broader structures better suited to store energy and therefore generate elasticity. Thus, the objective of this dissertation is to determine if dough viscoelastic properties essential for bread production can be obtained through the application of treatment favoring the formation of larger zein structures.

Zein was electrospun using aqueous ethanol or acetic acid as solvent to form nanofibrils. Several additives were tested to enhance fibrils mechanical properties. Zein electrospun fibers were then incorporated into a dough and the extensional properties of the resulting dough were assessed with a texture analyzer. Results indicated that, the addition of electrospun fibrils into dough did not improve the generation of elasticity, suggesting that fibrillar morphology is not an essential feature for the generation of elasticity.

The effect of extrusion and thermal treatment on zein were tested. The formation of zein large assemblies has been assessed with the use of SDS-PAGE, showing the formation of larger aggregates for either treatment. FTIR and a texture profile analysis were performed to assess the secondary structure and elasticity of zein viscoelastic masses. Results indicated an increased amount of β-sheet structure together with increase of springiness and cohesiveness from 0.56±0.01 and 0.43±0.01 of untreated zein to 0.87±0.01 and 0.77±0.01 of samples treated at 190°C. Treated zein was then added to a dough which was tested for extensional properties with a texture analyzer.

Results indicated the generation of strain hardening behavior in dough, a fundamental property for dough gas retention, both in extruded zein and thermally treated samples at 160°C. Finally, extruded zein and thermally treated zein were incorporated into a dough and baked into a loaf of bread. Despite improvement in rheological properties, incorporation into bread did not show improvement in bread quality.

The findings of this dissertation showed that the use of zein extrusion and thermal treatment is an effective tool to improve the elasticity and strain hardening behavior of zein dough, potentially finding application as a substitute of gluten. Furthermore, this study showed that it is possible to form a gluten-free dough which is extensible and possess a strain hardening behavior.

CHAPTER 1. INTRODUCTION

1.1 Background

Zein is known as the prolamin fraction of corn proteins. For a long time, it has been considered an important industrial polymer in the American industry over the first half of 20th century. Starting from 1960 zein suffered a period of abandon due to the rise of synthetic polymers. However, in the last 30 years zein regained popularity among the scientific community due to its potential as a biodegradable polymer. Early studies of Lawton revealed noteworthy viscoelastic properties when heated above its glass transition temperature, making it an interesting alternative to gluten for breadmaking. The observation of zein doughs at a microscopic level showed the formation of fibrous strands comparable to structures found in wheat gluten (**Figure 1**). It is hypothesized that these structures play an important role in dough and bread textural properties. Successive studies suggested that viscoelastic properties of zein were linked to an increase in β-sheet conformation. β-sheet alignment in nature is frequently characteristic of fibrillar structure morphologies exhibiting superior mechanical properties. More recent studies conducted at Purdue university indicated that when zein is mixed with other polymers such has high molecular weight glutenin or casein, his rheological properties can be further enhanced. Yet rheological properties of zein dough are not matching gluten containing systems, especially in terms of elastic behavior, crucial for breadmaking. This thesis work will focus on the exploration of treatment favoring the improvement of elasticity of gluten-free dough such as electrospinning, extrusion, or thermal treatments.

1.2 Research objectives

Our first objective is to determine if dough viscoelastic properties essential for bread production can be obtained by physicochemical treatments of zein. The central hypothesis is that structures formed by zein are not large enough to generate an elastic behavior, however processing techniques such as, electrospinning, extrusion or thermal treatment can generate larger structures better suited to store energy and therefore generate elasticity. In this thesis, Chapter 2 is a literature review on the status of research and relevant techniques for gluten-free systems characterization. Chapter 3 focuses on the production of electro spun fibrils from zein that incorporates different plasticizers and co-protein. Chapter 4 explores extrusion technology as a tool to improve zein rheological properties and generate a more elastic dough. Chapter 5 studies the effect of thermal treatment on zein structure and dough rheological properties. Chapter 6 summarizes breadmaking studies incorporating functionalized zein from previous chapters.

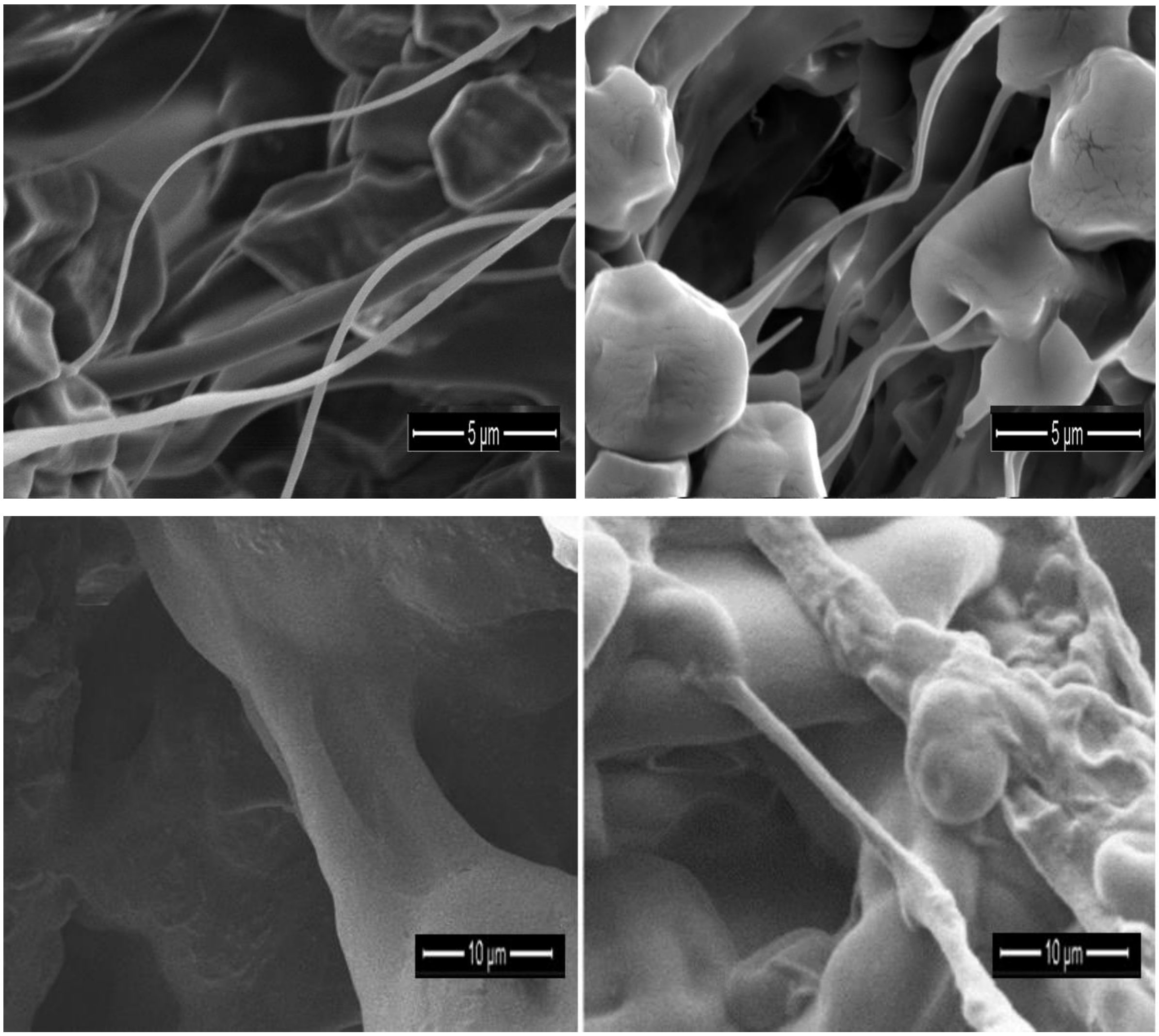


Figure 1 On the left micrograph of fibrils found in zein (top dough, bottom bread), on the right fibrils found in wheat flour bread (top dough, bottom bread)

CHAPTER 2. LITERATURE REVIEW

2.1 Gluten Structure and function

Gluten is a complex mixture of proteins, which can be isolated washing out the water-soluble components from a dough. Beside proteins, gluten also contains a less significant fraction of lipids and carbohydrates in variable quantities depending on the extraction method. Since it was first isolated, gluten quickly attracted the attention of scientist for its unique viscoelastic properties, which allowed wheat flour to produce high quality bread, contrary to other cereals. In order to better understand gluten variability in breadmaking performances, it was found that not only the quantity of gluten was important to determine good rheological properties, but also the quality of the proteins and the protein-protein interactions of the different protein fractions composing gluten (Lasztity, 1995).

In 1907, Osborne in his pioneering work extracted and classified wheat proteins based on their solubility, and for the first time, four classes of wheat proteins were proposed. The most abundant classes of proteins in wheat are glutenin and gliadin, which are the protein fractions soluble in acid or basic solutions (glutelin) and aqueous alcohol (gliadin) respectively. A minor fraction is constituted by the last two classes, albumin, and globulin, which are characterized for their solubility in distilled water (albumin) and in solution with low concentration of salt. Among these proteins, the ones forming gluten are prolamins and glutelins (Belitz, 2004) and each of them is composed by a complex mixture of molecules, which form the gluten matrix through a complex network of interactions such as disulphide bridges, hydrogen bonding and Van der Waals interactions (Lasztity, 1995). Gluten generally contain an equal proportion of gliadin and glutenin as it was reported by Osborne (Belitz, 2004). Numerous studies investigated the influence of gliadin and glutenin on dough rheological properties and there is general agreement that gliadin are responsible for the viscous behavior of dough while glutelin and in particular high molecular weight glutenin are responsible for the elasticity of wheat dough (Bonilla, Erturk, Schaber, & Kokini, 2020; Hoseney, 1986; H. Wieser, 2007).

2.1.1 Gliadins

Prolamin is a class of proteins which is characterized by the solubility in aqueous ethanol solutions, in wheat the prolamin fraction is better known as gliadin. Gliadins when hydrated form a continuous protein network in gluten favored by extensive non-covalent interactions such as hydrogen bonding between polar amino acids such as glutamine and hydrophobic interactions. However, an excess of interactions favored by extensive mixing is detrimental for dough quality (Bonilla et al., 2020). Covalent bonds such as disulfide bridges are also present and are considered to be mainly intra-molecular, and contributing in dough stability (Eliasson & Larsson, 1993; A. S. Tatham & Shewry, 1985). Gliadins are classified as α, β, γ and ω-gliadins based on approximate molecular weight determined through gel-electrophoresis (Lasztity, 1995; Herbert Wieser, 2007). Because of their dense structure highly stabilized by non-covalent intramolecular interactions, gliadins possess an outstanding stability to thermal treatment (Lasztity, 2017). Similarly to other prolamin from cereals, In aqueous ethanol α-, β- and γ-gliadins are characterized by a predominant α-helix conformation, formed by the repetitive sequences in the central domain of the proteins, and to a lower extent β-sheet which are favored by the high amount of glutamine (A. S. Tatham & Shewry, 1985). An alternative classification of gliadin categorizes them based on content of Sulphur: low in Cysteine (ω-gliadins) and rich in cysteine (α-, β- and γ- gliadins). Generally, the majority of the gliadins are rich in cysteine, allowing for the formation of extensive disulfide bonds (Koehler, 2003). Gliadins possess numerous peptide sequences responsible for the celiac disease (Koning, 2003).

2.1.2 Glutenins

Glutenins are knowns as the fraction of wheat gluten responsible for dough elasticity. Glutenins are very poorly soluble in alcohol, water, and basic buffers due to their high molecular weight. Glutenin contain numerous monomers with a very variable range of molecular weights (16,000 to more than 100,000 Da) which are linked together by an extensive number of disulfide bonds forming some of the largest proteins known, with molecular weight greater than 1,000,000 Dalton (Cornell & Cauvain, 2003; P. R. Shewry & Tatham, 1990). Contrary to gliadin, Glutenin are further classified in two groups based on their molecular weight, generally referred as high and low molecular weight glutenin (Eliasson & Larsson, 1993). Glutenin fraction approximate molecular weight have been observed using electrophoretic techniques, which showed that the smallest fraction (LMW) have a range of molecular weight of the monomers ranging from 15 to 45 kDa, while the larger fraction (HMW) monomers have been observed to have an apparent molecular weight ranging from 60 to 100 kDa (D'Ovidio & Masci, 2004). In general, the amino acid composition of glutenin resembles that of gliadin. Glutenin as well as gliadin possess some amino acid sequences responsible for the celiac disease (Koning, 2003).

2.1.3 Nature of viscous and elastic behavior in gluten

Gluten is a conglomerate of proteins which when hydrated has unique viscoelastic properties. Its unique rheological behavior plays a crucial role in the quality of baking goods. Therefore, a good understanding of the nature of gluten’s rheological behavior is important to decode structure/function relationships and identify analogies and differences with non-wheat cereal proteins. Due to its great importance for wheat-based products, gluten rheological behavior has been extensively studied in the past. As previously mentioned, gluten contain mainly two proteins, gliadin and glutenin. Gliadin are known to be responsible for the viscous behavior of a dough, on the other hand, glutenins are responsible for the elastic component of dough rheological behavior (Hoseney, 1986). The elastic behavior in gluten is particularly relevant, in fact, extensive literature exist on the crucial role of HMW glutenins and their effect on rheological behavior and ultimately bread quality (Branlard & Dardevet, 1985; Gupta, Singh, & Shepherd, 1989). Two models have been proposed to explain the viscoelasticity in gluten, Disulfide interchange model and train and loops.

Among the two proposed models, the Disulfide interchange model has been the most popular. This model stresses particularly the importance of disulfide bridges linking together the high molecular weight (HMW) fraction with the low molecular weight fraction (LMW). The generation of dough strength and elasticity is attributed to the disulfide bridges formation linking together HMW and LMW fraction forming the gluten macro polymer (Herbert Wieser, 2007). On the other hand the viscous behavior of the gliadin is attributed to non-covalent bonds (P. Shewry & Tatham, 1997). A major strength of this model is that it can explain well the early observation of bread volume improvement due to addition of ascorbic acid (Jørgensen, 1945). More recent studies using confocal microscopy confirm the interaction of HMW and LMW glutenin showing co-localization (Jose C Bonilla, James A Schaber, Arun K Bhunia, & Jozef L Kokini, 2019) followed by loss of co-localization at higher mixing times possibly due to breakage of disulfide bridges (Bonilla et al., 2020). On the other hand, the main pitfall of the disulfide interchange model is that it fails to account for elasticity at a molecular level. Dough elastic behavior is indeed attributed to LMW and HMW disulfide bridges, however, disulfide bridges by their nature cannot store energy. When disulfide bonds are broke by extension, there is no mechanism which will tend to restore the original dimension of the material (Belton, 1999). On the other hand other mechanism proposed of elasticity like for instance extensive β-sheet structure (A. S. Tatham, Shewry, & Miflin, 1984) have a molecular basis of energy storage, where hydrogen bonds and Van Der Waals interactions can break during stretching and reform quickly after the stress is released.

These considerations on the molecular basis of elasticity bring us to the second model: train and loops. This model was first introduced by Belton (Belton, 1999) which involved the formation of two distinct structures, trains formed by a sequence of β-sheet in the central domain of the protein and loops, formed by repetitive β-turns rich in glutamine (Erickson, Campanella, & Hamaker, 2012). Applied strain to gluten breaks hydrogen bonds in the loop and train region to a thermodynamically unstable state, which will return to original position once the stress is released. This model was also supported by spectroscopic evidence showing the β-sheet and eventual β-turn structures upon hydration of HMW subunits (Belton, 2005; Wellner et al., 2005). One of the main limitations of the train and loop model is that it only considers the high molecular weight glutenin, which, despite their importance in dough elastic behavior, constitute only a minor fraction of gluten. On the other end, the model is in agreement with numerous spectroscopic observation, which show an extensive formation of β-sheet structures (Erickson et al., 2012) which could form numerous sacrifice bonds and allow high molecular weight glutenin to generate elasticity working as entropic springs. Despite numerous studies on the nature of gluten viscoelasticity, great progress still needs to be made to fully understand the molecular mechanisms at the basis of the unique viscoelastic behavior that gluten possesses.

For a long time, gluten has been believed to be the only cereal protein able to form a viscoelastic dough. Nevertheless, this is not true, as zein, a storage protein from corn, showed to generate a remarkable viscoelastic behavior when incorporated into a dough.

2.2 Breadmaking

Bread in its many forms is a staple food consumed worldwide. Currently wheat and with poorer performance, rye and barley are the only cereals that after being milled and properly processed can produce good quality bread. It has been known for long time that the most important component of wheat for bread production is wheat gluten. Contrary to other cereal proteins, gluten has a unique ability of forming a viscoelastic mass with an optimal balance between extensibility, important to undergo the deformations imparted in the fermentation, and elasticity, which is fundamental for a gas retention. Furthermore, gluten unique rheological properties allow to form a dough which can be shaped and molded as desired (S. P. Cauvain & Young, 2007). Wheat is not the only cereal that contains gluten. Other cereals, as for instance rye and barley, can form a viscoelastic dough but it results inferior compared to gluten because the lower amount of proteins and their inferior quality than normally seen with wheat flours. Till now no other flour was able to achieve the unique characteristics that only the gluten contained in it can give to the dough. Still is possible to produce bread from other ingredient without using gluten but this always require the use additional ingredient such as protein, carbohydrate, lipids etc. Nowadays the main challenge of bread producers remains to increase the shelf life of the product. The most important problem is the bread staling, a phenomenon in which the starch component of the bread tend to crystallize conferring to the crumb a firm no more elastic hard texture (Gray & Bemiller, 2003).

As generally known, the basic recipe for producing a loaf of bread is extremely simple, including only flour, water, yeast, and salt. Anyway, bread makers know that for obtaining a good quality bread the use of only flour water yeast and salt is not enough, and it is necessary to add more ingredients. Most breads also add oil or fats. Soft fats, for instance lard in countries in cold climates or olive oil in the Mediterranean area, have been extensively used for traditional breads(Pyler & Gorton, 2008) In more recent time a more fundamental and wide knowledge about bread and bread making process led to the development of improvers. Improvers for breadmaking can be divided in the following categories.

- Oxidizing agents: for improve the gas holding capacity and so a better increase in volume and softness of the product.
- Reducing agents: they are added to make a dough weaker, helping to maintain the shape
- Emulsifiers: they help the gas retention and slow down the staling process

- Enzymes: amylases, hemicellulases, and less diffused proteases, with several distinct functions.

2.2.1 Breadmaking quality

Breadmaking evolution through human history took different paths in various region of the world leading to the diversification of a plethora of different typologies of bread. Quality of bread can also be a very subjective topic as different population have distinct perception of quality (S. P. Cauvain & Young, 2007). Nevertheless, food scientists have tried to develop methods to determine bread quality parameters to describe bread quality in a more objective way. One of the simplest parameters that is used to determine bread quality is its specific volume, as bread volume is considered by many the most important external characteristic of bread. Different methods are available to determine bread volume, with the official methods recognized by AACC rapeseed displacement (AACC Method 10-05.01) and laser topography (AACC Method 10-14.01). Specific volume, while simple to measure, is not always a good indicator of gas retention and texture, as it does not include information on the bubble size of the bread. Bubble size distribution is indeed another important parameter used to determine bread quality. Numerous methods have been developed in the last decades to measure bubble size distribution in bread. Generally, bubble size of high-quality bread must be small and homogeneous, however that depend on the typology of bread, with products requiring the presence of large pores to be considered of high quality. One of the simplest methods is using image analysis, however this technique has poor reproducibility. More sophisticated techniques has been used, as for instance microcomputed tomography (Bellido, Scanlon, Page, & Hallgrimsson, 2006) which could provide 3D reconstruction of the internal bubble distribution, giving a more comprehensive insight into the aeration phenomenon in breadmaking. Another method used is MRI (magnetic resonance imaging) which can be used to obtain advanced structural information on bread (Wagner, Quellec, Trystram, & Lucas, 2008).

For long time, dough quality has been directly associated with breadmaking performance. Starting from early 20th century, numerous methods have been developed to try to determine the rheological properties of dough and relate them with breadmaking performance (Bailey, 1940). Since then the understanding of dough rheological properties and bread quality made great steps forward, with numerous methods being developed to determine bread quality (B. J. Dobraszczyk & M. P. Morgenstern, 2003). In 1992, Van Vliet proposed to use strain hardening as an indicator of bread quality, investigating the different mechanism that affect gas retention and the importance of the generation of strain hardening to improve dough gas retention (T Van Vliet, Janssen, Bloksma, & Walstra, 1992). Numerous investigations showed the importance of HMW (high molecular weight) glutenin in relationship with wheat quality (Anjum et al., 2007; Gupta et al., 1989; Payne, Nightingale, Krattiger, & Holt, 1987). It was thus hypothesized that HMW weight glutenin are responsible of the elastic behavior of dough and therefore of its pronounced strain hardening behavior. A review of strain hardening behavior as an indicator of bread quality has been performed by Van Vliet after much evidence was collected since strain hardening was first proposed as an indicator of bread quality (T. van Vliet, 2008). Numerous evidences has been collected in support of the theory that strain hardening has a direct impact in the gas retention of dough with an important role in stabilizing the gas cells and to prevent the rupture of cell walls being responsible of coalescence. Furthermore, strain hardening is important in determining an homogeneous expansion of the CO_2 inside the cells (T. van Vliet, 2008), which is important to have a evenly distributed bubble size, preventing the occurrence of defects. Recent studies on microscopy confirm the importance of HMW glutenin on keeping dough strength and stability, showing a marked loss of strength when HMW aggregate during mixing in weak flours (Bonilla et al., 2020).

2.3 Need and State of the Art for Gluten-free products

2.3.1 Celiac disease and gluten sensitivity

Gluten is responsible of different pathologies in humans. A full list of the different gluten related disorders is shown in **Figure 2**. The principal ones are: Celiac disease, gluten sensitivity and allergy. Celiac disease is described as an inflammatory status of the small intestine triggered by the peptides present in gluten, causing the occurrence of an autoimmune reaction in individuals with genetically predisposition (da Silva Neves et al., 2010). Worldwide, celiac disease is an endemic pathology, and among life time disorder, is one of the most prevalent (Loftus & Loftus Jr, 2002) and can trigger the occurrence of other pathologies if patients don't adhere to a gluten-free diet. Up to date, there is no effective therapy for celiac disease patient, and the only solution to avoid major complications is the complete elimination from the diet of food containing gluten.

Another pathology associated to gluten is the gluten sensitivity, which recently raised the attention of the scientific community.

Non-celiac gluten sensitivity is a wheat associated affliction diagnosed through exclusion diagnosis (Tanveer & Ahmed, 2019). The disease state is defined as an onset of a spectrum of clinical manifestations (in the absence of celiac disease or wheat allergy) related to consumption of foods containing gluten (Ludvigsson et al., 2013). Its incidence is estimated to be between 0.6-6% of the world population with geographical difference. The symptoms of Non-celiac gluten sensitivity vary largely from intestinal such as diarrhea, flatulence and bloating, to other symptoms indirectly linked to the gut such as chronic fatigue, joint pain, muscular contraction, arriving till behavioral disorder such as lack of attention, depression, ataxia (Tanveer & Ahmed, 2019).

Food allergy is an adverse reaction to ingested proteins, including gluten. Differently than other gluten related disorders, wheat allergy has immediate effect after the ingestion of gluten. Wheat allergy associated with ingestion can produce respiratory symptoms, and skin symptoms, among others (Cabanillas, 2019). Similarly, to Celiac disease and non-celiac gluten sensitivity the only available therapy is the strict avoidance of wheat. This generated the need of gluten-free products, as the only available option for people suffering of gluten related disorders

2.3.2 Areas of improvement for gluten-free products

The large success of baked product can be associated with the unique properties of gluten, by far the most important component in many baked goods. Gluten significantly affect the quality of dough, having a fundamental role in developing fundamental dough rheological properties such as extensibility and elasticity (Hoseney, 1986). The rheological behavior of dough is a crucial determinant for technological operations involved in the production of bread (Bloksma, 1990). Moreover, gluten play a major role in quality attributes of bread and pasta (fresh and dry), cakes, cookies, and biscuits. The high content of gluten makes hard wheat suited for breadmaking, while the lower gluten content of soft wheat is ideal to produce cakes, cookies, and crackers. For instance, gluten has a major role in many of the irreversible process that occur during bread production; it’s fundamental for dough gas retention, for a correct expansion during cooking and finally to prevent staling when bread is formed, giving wheat bread its unique, and currently unmatched by other cereals, features (Delcour et al., 2012). Likewise, the among and the quality of gluten proteins, has been an important indicator for peculiar properties in the production of dry pasta, as for instance low cooking loss and texture (Delcour et al., 2012; Marti & Pagani, 2013).

Despite gluten outstanding performance for baked goods quality, there is a great necessity of developing gluten-free products for celiac and gluten-sensible individuals, since in all the cases the only current available therapy is the lifetime avoidance of gluten. In the last years, much effort has been done by researchers to replace gluten, yet further improvement is necessary to match the quality of gluten-containing products. Specifically, gluten-free products need improvement in organoleptic properties, accessibility, and nutritional properties.

Given its intrinsic role for the quality of baked good, it follows that gluten removal generate technical issues for bakers, even though considerable progress has been done in the last 20 years to reduce the gap with wheat bread. The perceived bread quality is a complex process that involves product appearance, odor, taste, flavor and oral texture (Heenan, Dufour, Hamid, Harvey, & Delahunty, 2008). Texture is especially important in gluten-free bread sensory perception with positive attributes including chewy, soft, moist and fluffy (Muggah, Duizer, & McSweeney, 2016). In terms of flavor consumer tend regards as positive attributes closer to wheat bread like bland, sweet and yeasty, while they regard as negative flavors attributes such as nutty, grainy and bitter generated by the presence of unfamiliar flours (Muggah et al., 2016). Despite recent progress, many gluten-free products currently commercialized are still considered inferior to their gluten counterparts, exhibiting mediocre organoleptic acceptance by consumers. To replace gluten, formulations frequently use either hydrocolloids such as HPMC, Xanthan gum, Guar gum and Psyllum or proteins rich ingredients such as soy, egg white, egg and pea (Roman, Belorio, & Gomez, 2019). Nevertheless, the lack of a single ingredient such as wheat flour create difficulties in product development, due to the complex formulations needed to substitute the gluten's functionality. Differences are more dramatic for all the aerated product such as bread because of their structural complexity. Currently, most of the gluten-free formulation after mixing results in the formation of a batter instead of a dough, leading to problems in industrial processing and in shaping numerous products such as pretzel or baguettes (T. J. Schober, S. R. Bean, D. L. Boyle, & S.-H. Park, 2008).

Complex formulations and difficulties in industrial processing cause the price of gluten free products to be higher compared to the same category of product containing gluten (Jnawali, Kumar, & Tanwar, 2016). Furthermore, complex formulations hardly match with the consumer demand of clean label products, nevertheless, most gluten-free product currently on the market are composed of complex mixtures of ingredients (Roman et al., 2019). As it is generally known for all categories of consumer, the main driver of purchase for any food item are price and taste, the same apply for consumer suffering of Gluten Intolerance. The issue of high cost of gluten-free products is especially true for Low-income families in countries in which is not provided a government help for celiac as for instance USA and Australia. In other geographical areas of the world like Africa and Asia, the demand for gluten-free products is not driven much by health issue, but rather by the lack of local wheat cultivation, paired with tumultuous population growth and migration from rural environment toward larger urban aggregates (Pingali, 2007). This will require the development of new solutions involving gluten-free cereals such as corn or buckwheat.

Although generally more expensive, the quality of gluten free-products is generally inferior also from a nutritional stand point, with several important macronutrients in lower quantity such as low amount of fibers, low proteins, scarce presence of micronutrients given the use of highly refined starches (Matos & Rosell, 2015). On the other hand, Gluten-free diets are more rich in macronutrients such as saturated fats, simple sugars, and NaCl, if compared with diet that are including products containing gluten (Makovicky et al., 2020). Gluten is also known to affect the digestibility of starch (Jenkins et al., 1987), decreasing its digestibility and improving its nutritional quality. Therefore, gluten removal can decrease the nutritional quality of food products potentially increasing starch digestibility. Multiple factors need to be taken into account regarding gluten-free products (Barbiroli et al., 2013; Wolter, Hager, Zannini, & Arendt, 2013).

Despite in the last two decades major progress in formulations and nutritional quality of gluten-free products, further progress needs to be done to guarantee equal dietary options to people suffering from gluten related disease. This dissertation will focus on improvement in dough rheological properties, texture, and structure, potentially leading to improvement in organoleptic quality of gluten-free bread. To replace gluten successfully it is important to first understand well what gluten is and what make it such an outstanding material for breadmaking purposes, thus in the next section gluten structure and function will be reviewed.

2.4 Gluten-free cereals storage proteins; zein and kafirins

2.4.1 Zein

The protein content of maize is variable, it ranges between 6-18% and is divided in 4 different classes: albumins, globulins, glutelin and prolamin. The most abundant protein is zein, a prolamin that constitutes between 44 to 79% of the endosperm protein (B. Hamaker, Mohamed, Habben, Huang, & Larkins, 1995). Zein is a mixture of different peptides varying for solubility, amino acid composition, and size, and (Shukla & Cheryan, 2001). According to the solubility in reducing or non-reducing conditions zein is classified in α, β, γ, and δ zein. The first description of zein is almost 200 years old, being described as a soft, ductile, tenacious and elastic material (Gorham, 1821). Similar to gliadin, zein was first categorized by the early work of Osborn in the class of prolamins due to its solubility and high content of proline (Osborne, 1924). During the first half of the 20st century, in the United States, zein has been a highly studied material due to its large use as an industrial polymer (John W Lawton, 2002). However, in the second part of the 20st century research on zein as industrial polymers was discontinued due to the rise of synthetic polymers boosted by their low price and superior technological properties. After that, Zein research suffered an extensive period of oblivion from the 1960 to 1990s. After decades of abandon, in the last 30 years there has been a rediscovered interest of this material by scientists, leading to a continuously increasing number of publications on zein. Numerous new applications have been found for zein. For instance to produce electrospun fibrils (Gordon W Selling, Biswas, et al., 2007; C. Yao, Li, & Song, 2007), Drug delivery (Jiang & Yang, 2011) microcapsules or nanoparticles for encapsulation purposes (Cheng, Ferruzzi, & Jones, 2019; Padua & Guardiola, 2015), pharmaceutical (Y. H. Wang, M. Zhao, S. A. Barker, P. S. Belton, & D. Q. M. Craig, 2019), food packaging (Rakotonirainy, Wang, & Padua, 2001) and also for breadmaking applications.

2.4.2 Zein extraction

The different zein extraction methods have been extensively reviewed by Shukla (Shukla & Cheryan, 2001). The variables between the zein extraction process available are 1) Processing of raw material 2) solvent for extraction 3) purification method 4) recovery method. There are mainly four methods of processing corn kernels: dry milling, alkaline processing, wet milling and dry grind process for ethanol production (Shukla & Cheryan, 2001). However, only wet milling and dry grind yield materials from which zein can be extracted. Wet milling is a process designed mainly to produce corn starch and oil, while the protein rich fraction generated are considered by-products consisting mainly of corn gluten meal and corn gluten feed (about 60% protein content). On the other hand, dry grinding main purpose is the production of ethanol, generating dried grains as by-product, which still contain a considerable amount of proteins (30%). Gluten meal and to a lesser extent dried grains are the starting raw materials for zein extraction.

After determining the starting material from which to extract zein, it is necessary to choose a solvent to use for its extraction. An exhaustive list of solvents is available to solubilize zein (Evans & Manley, 1941; Manley & Evans, 1943). However, among the plethora of solvent available for zein solubilization, the main solvent used for extraction are aqueous isopropyl alcohol and aqueous ethanol. Better solvent than aqueous alcohol systems are available, for instance diethylene glycol, triethylene glycol, propylene glycol, and dipropylene glycol can form zein solutions stable for years(Evans & Manley, 1941). Despite glycol solutions showed better performance, the principal reasons for the selection of aqueous alcohol solutions are the relatively low price and low toxicity. After the addition of aqueous alcohol mixtures to corn gluten meal to extract zein, the successive step consists in the purification process. At this point, beside a good solvent for zein, to further purify the protein fraction a nonpolar solvent for residual fat and pigment extraction need to be selected. Plus, other purification steps are added to the process such as filtrations and centrifugation to further purify the aqueous ethanol zein solution. Finally, after the purification steps, zein need to be recovered from the aqueous alcohol solution. There are two principal method used to recover zein: antisolvent precipitation and drying. Antisolvent precipitation consist in adding cold water to the zein solution inducing its precipitation, followed by filtering, drying, and grinding steps. Drying process simply consist in the evaporation of the solvent, which depending on the drying technology employed can affect zein structure.

2.4.3 α zein

α zein accounts for roughly 75-85% of the prolamins in corn, consequently, is the fraction generally present in commercial format (John W Lawton, 2002). For these reasons α-zein has been the most studied fraction. α-zein has two main peptides with molecular weights of 19 and 22kDa and their dimers and trimers (Y. Li, Xia, Shi, & Huang, 2011). Numerous studies investigated α-zein primary, secondary, and tertiary structure. The primary structure of the two dimers of α-zein has been known for long (Argos, Pedersen, Marks, & Larkins, 1982; Coleman & Larkins, 1999; Geraghty, Peifer, Rubenstein, & Messing, 1981). α zein contain a high proportion of Gln, Pro, Ala, and Leu but is poor in essential amino acid such as Lys and Trp (Coleman & Larkins, 1999), making it a protein with a poor nutritional quality.

Early studies using circular dichroism to study zein secondary structure indicated the prevalence of α-helix and β-turn and no-β-sheet in α-zein molecules (Argos et al., 1982). Based on the primary structure sequencing, Argos proposed a model where a central repeated sequence of zein α-helix is alternated with β-turns rich in glutamine. While this model accounted for various empirical observation such as fiber formation (Argos et al., 1982), its nature was merely speculative. Further progress in understanding zein structure was made through genetic studies, dividing zein in three fractions: An initial N-terminal domain, followed by a central domain characterized by sequences with high homology and finally a short C-terminal domain composed of only 10 amino acids. Building on Argon's previous work, Matsushima proposed a new model for zein (Matsushima, Danno, Takezawa, & Izumi, 1997). Using small angle X-ray scattering, the morphology of α-zein molecule in aqueous ethanol was determined as a prism with a cross section of 1.39nm by 4.00nm and a length of 13nm. Based on these results a new model of α-zein was proposed where the repeated central domain of α-helix and β-turn is aligned, disproving a previous model where helices had a much more compact structure (Garratt, Oliva, Caracelli, Leite, & Arruda, 1993). This model is in agreement with DLS and AFM experiments (Q. Wang, Yin, & Padua, 2008). Based on NMR, SAXS and FTIR analysis, another model by Forato has been proposed, proposing a hair pin structure composed of loops, sheets and turns (Lucimara A Forato et al., 2004). Finally, using molecular dynamic simulations a model with three super helical structures joined by glutamine rich β-turn has been proposed (Momany et al., 2006). No new model has been recently proposed on zein structure, however a recent study on zein conformation indicated the model of Forato more accurately describe zein transition to β-sheet conformation (Erickson et al., 2020). Nevertheless, Aggregative behavior was observed when zein is exposed to solvents such as aqueous ethanol (Uzun, Ilavsky, & Padua, 2017) suggesting that aqueous ethanol is not an ideal solvent for zein, potential impairing the validity of previous models assuming full solubility of zein. Zein aggregative behavior is believed to be important for the formation of nanostructure in zein, potentially linked with the conversion of α-helical to β-sheet structures (Y. Wang & Padua, 2012). Aggregative behavior induced by protic solvents such as water, have shown to induce orientation of peptides which lead to the formation of nanofibers through the formation of hydrogen bonding. (Ua-Arak, Jakob, & Vogel, 2016).

2.4.4 β, γ and δ zein

While the major prolamin fraction in corn is α-zein, constituting 75-80% of zeins proteins (Esen, 1986), the remainder 20-25% is constituted by three other fractions namely β, γ and δ zeins. β and δ zein are rich in sulfur-containing amino acids and in reducing conditions are constituted by single polypeptides, β-zein (Mr 14,000) and δ- zein (M, 10,000). On the other hand the two γ-zeins in terms of amino acid composition are poor in Cys but abundant in Pro (G. A. Thompson & Larkins, 1989). Simulation of δ zein secondary structure indicated a complex tangle of coils interconnected with α-helical regions spaced by β-sheet (Geourjon & Deleage, 1994) On the other hand, β-zein form less α-helix and is more prone to form stable β-sheet if compared with the more abundant α-zein, which is predominantly formed by α-helices (Lucimara A Forato, Bicudo, & Colnago, 2003). β-zeins are characterized by rich amount of sulfur containing amino acids and therefore can generate covalent cross-linking (D. Sessa & Woods, 2011) which is at the basis of their instability and high tendency to gel. While γ zein is mainly composed of α-helices (Bicudo et al., 2008).

2.4.5 Kafirins

The major protein class of sorghum is constituted by Kafirin, which constitute about 70-80% of total protein in sorghum (B. R. Hamaker & Bugusu, 2003). Kafirin are divided in three different classes, α-Kafirin, β-Kafirin and γ-Kafirin (Shull, Watterson, & Kirleis, 1991). α-Kafirin, are the most abundant fraction, constituting around 80% of total kafirins. α-Kafirin are characterized by an amino acid composition with large analogies with α-zein (DeRose et al., 1989). On the other hand, β and γ kafirin constitute only 5% and 15% of total kafirins (B. R. Hamaker & Bugusu, 2003) and are characterized by high amounts of cysteine contrary to α-kafirin. Kafirin are reported to be in protein bodies, which prevent them from participating in the development of food matrices. For this reason, it is necessary to extract kafirins from the proteins bodies to allow them to contribute to the mechanical properties of food structures. Cooking alone has been reported to not be sufficient to release kafirins from proteins bodies, and processing techniques with higher mechanical energy such as extrusion are necessary to release them from protein bodies (B. R. Hamaker & Bugusu, 2003).

Kafirins and zein present several analogies as well as some key differences. One of the main analogies between kafirins and zein is the similarity in amino acid composition, which is likely due to a common evolution path of corn and sorghum (DeRose et al., 1989). Both α-zein and α-kafirin, possess a similar molecular weight of 19 and 22kDa. Furthermore, both fractions are rich in glutamine and hydrophobic amino acids while they don't contain lysine (DeRose et al., 1989). Beside amino acid composition, the two molecules also present a similar structure, with a central domain characterized by repeated sequences with high homology (DeRose et al., 1989; Matsushima et al., 1997). Furthermore, α-kafirin are likely to possess an hairpin structure similarly to α-zein, including the various elements like β-sheet, β-turns, and α-helix (Belton, Delgadillo, Halford, & Shewry, 2006). These high amount of analogies lead to high similarities of zein and kafirin mechanical properties (Oom, Pettersson, Taylor, & Stading, 2008). The high homologies between kafirin and zein make them good for similar application as for instance the production of films (J. Taylor, Anyango, & Taylor, 2013) or to produce viscoelastic resins for breadmaking purposes (Oguntoyinbo, Taylor, & Taylor, 2018)

Despite major analogies, important differences between zein and kafirins have been reported. First, kafirins are retained to be the most hydrophobic prolamins, given their extremely limited solubility (Belton et al., 2006). Kafirins tend to contain a higher amount of cysteine compared to zein, which is believed to be responsible of the poorer rheological properties of kafirin compared to zein (Gillgren & Stading, 2008). Another important difference between the two classes of proteins is the difference in digestibility after cooking. Kafirin are known to reduce their digestibility upon cooking, contrary to most other proteins. Contrary, a similar phenomenon has not been observed in highly homologue α-zein molecules. One of the main hypotheses to explain this peculiar difference is a difference in protein conformation after cooking, where kafirin shows a higher proportion of β-sheet structures compared to zein (Bean, Ioerger, Smith, & Blackwell, 2011; B. R. Hamaker & Bugusu, 2003).

2.5 Treatments to improve zein functionality

Beside optimal consistency, a good dough for breadmaking needs to have two essential rheological properties, be extensible, and possess a strain-hardening behavior (Kokelaar, Van

Vliet, & Prins, 1996). These properties in wheat flour are provided by the presence of gluten. Gluten, when hydrated, naturally possess extensibility provided by the gliadin fraction, and elasticity, provided by the glutenin's. On the other hand, when we hydrate zein at room temperature, it will result not extensible nor elastic, forming doughs with poor breadmaking performances. Mixing zein above its glass transition temperature, will allow zein to become extensible (J. W. Lawton, 1992a) however, zein extensibility will be quickly lost when temperature will cool back below its glass transition. Stabilizing zein extensible behavior at room temperature will be highly desirable to perform more easily all the operations before proofing, after which dough will ferment at temperature above zein glass transition. While extensible, doughs containing zein exhibit strain-thinning behavior (Berta, Gmoser, Krona, & Stading, 2015) rather than the desired strain-hardening that is typical of wheat doughs. Strain-hardening behavior in doughs is highly desirable as it is a key feature for gas retention (T Van Vliet et al., 1992). As strain hardening contributes to stabilize film walls against coalescence of gas cells, we can assume that the same mechanism of stabilization will potentially apply to every dough, including gluten-free. In conclusion, while possessing interesting viscoelastic properties, zein usage for breadmaking remains limited as it still needs improvement to be an effective substitute of gluten (Taylor, J. Taylor, O. H. Campanella, & B. R. Hamaker, 2016) notably in terms of stability of extensibility and in terms of elasticity. In this section, several strategies used to improve zein functionality are reviewed. First, the use of plasticizers to reduce zein glass transition (Tg) will be discussed. Second, the use of antisolvent precipitation will be reviewed. Third, electrospinning of zein will be analyzed to form fibrils potentially imparting a more elastic behavior to zein. Finally, zein extrusion will be examined as a tool to create cross-linking generating large protein assemblies potentially enabling a enhanced elastic behavior of zein.

2.5.1 Plasticizers for zein

A plasticizer is defined as an additive that is incorporated into a material to impart flexibility, or extensibility (Godwin., 2011). For a plasticizer to work effectively, it needs to be incorporated into the polymer matrix and the polymer must be in its amorphous state. A strict requirement for a plasticizer to work effectively is the ability of freely move around the polymer and not being strictly bound to a certain location (Godwin., 2011). For biopolymers, plasticization is generally associated with decrease of glass transition. Numerous plasticizers are available for zein functionalization (Hansen, 1938; Huey-Min Lai & Padua, 1997; J. Lawton, 2004). Nevertheless, given the high complexity of protein it's impossible to find a plasticizer that is perfect for every application (John W Lawton, 2002).

The most effective plasticizer for zein is water. While zein, as a prolamin, does not dissolve in water, it greatly interacts with water showing extensive plasticization. For instance, water greatly affect zein glass transition, reducing it from 150-170ºC of dry powders to 28ºC when zein is fully hydrated (J. W. Lawton, 1992a; Madeka & Kokini, 1996). The plasticization effect of water on zein is particularly important to produce zein based doughs, as water enable zein to be worked above its Tg and showing a viscoelastic behavior which is key to the formation of good quality doughs. However, extensive interaction with water is considered a negative feature for numerous application such as films or coatings.

The most common plasticizers for zein films are glycerol and oleic acid, nevertheless, good solvents for zein discussed in section 2.3.1 will all work as good plasticizers for zein. Numerous studies used glycerol as an effective plasticizers showing decrease of Tg and improved elongation (Wongsasulak, Tongsin, Intasanta, & Yoovidhya, 2010b) and affecting film properties such as oxygen permeability (Liang et al., 2015) or film microstructure (Huey-Min Lai & Padua, 1997). Oleic acid plasticization of zein has also been extensively studied, showing a decrease of glass transition temperature (Xu, Chai, & Zhang, 2012), alteration of films water barrier properties (Y. Wang & Padua, 2006), and changes in tensile properties and water absorption (F. X. B. Santosa & Padua, 1999). Oleic acid acted as a plasticizer when incorporated in zein resins, decreasing its glass transition temperature (Erickson, S. Renzetti, A. Jurgens, O. H. Campanella, & B. R. Hamaker, 2014). The use of plasticizers can be coupled with electrospinning to change the physical properties of the resulting fibrils, which will be reviewed in chapter 2.5.3.

2.5.2 Antisolvent precipitation

Antisolvent precipitation is a popular technique to crystallize or precipitate a molecule in solution by the addition of another solvent to the solution, which act as a destabilizing agent on the mixture and induce the precipitation of solids in form of crystals or amorphous material, depending on the nature of the solution (Thorat & Dalvi, 2012). While largely employed in the pharmaceutical industry to crystallize drugs, the principle of antisolvent precipitation can be used to precipitate proteins as well. When solubilized, proteins are solvated and have limited interactions with other protein molecules in solution. The addition of an antisolvent destabilize the system, favoring protein-protein interactions inducing aggregation and subsequent precipitation of aggregates. Numerous applications are present in the food industry, in particular in the encapsulation of bioactive compounds (Zou, Xie, Zhu, & McClements, 2019). For instance several works used antisolvent precipitation for the production of micro and nano particles from zein (Hu & McClements, 2014; Yuan et al., 2019). Antisolvent precipitation has been largely used in the pharmaceutical industry to crystallize drugs and to improve their bioavailability (Thorat & Dalvi, 2012). Beside encapsulation of bioactive compounds, recent interest has raised in using antisolvent precipitation on zein as a technique to improve zein functionality (Mattice & Marangoni, 2020b)

Figure 3 shows a resin produced with antisolvent precipitation from a solution of 25% zein (w/w) in acetic acid. One of the simplest method consists in dissolving zein in warm (75ºC) aqueous ethanol solutions and subsequent addition of cold water in higher proportion to induce zein desolvation (Huey-Min Lai & Padua, 1997). After zein desolvation, excess solvent can be removed and a zein resin is obtained. It has been documented that the addition of water to zein solubilized in ethanol first results in coacervation, followed by zein precipitation (Yi Wang & Padua, 2010). Nevertheless, other solvents as for instance acetic acid can be used to form zein resins using antisolvent precipitation. Zein resins can be further functionalized by the addition of plasticizers and co-proteins (Erickson et al., 2014) using an adapted procedure previously developed by Lai & Padua (Huey-Min Lai & Padua, 1997). Furthermore, the resins obtained through antisolvent precipitation can be employed in the formation of gluten-free doughs, adding water as antisolvent to zein-acetic acid solutions (Oguntoyinbo et al., 2018). Although, we speculate that the presence of residual acetic acid is necessary to retain viscoelastic properties of the generated resins. A recent publication indicated potential interest of the use of zein antisolvent precipitation to produce meat analogues (Mattice & Marangoni, 2020a). One of the greatest advantages of antisolvent precipitation for zein fibrils formation is that it is relatively simple to use and has a moderate cost of the process. On the other hand, this technique makes it more challenging to control fibril dimensions and orientation compared to extrusion and electrospinning.

2.5.3 Electrospinning

Electrospinning is a technique use to force polymers to assume fibrillar morphology through the application of electric fields. Practical applications of electro spun fibers include structuring agents in films (Deng et al., 2018; Z.-C. Yao, Chang, Ahmad, & Li, 2016), textiles (Han, Huang, He, Liu, & Wu, 2006; Mirjalili & Zohoori, 2016), and controlled delivery vehicles for active molecules (Séon-Lutz, Couffin, Vignoud, Schlatter, & Hébraud, 2019; X. Zhang et al., 2020). Electrospinning can also be used to structure gluten-free cereal proteins, orienting them into fibrils, with potential applications in gluten substitution or in structuring meat analogues (Kyriakopoulou, Dekkers, & van der Goot, 2019). A schematic of electrospinning is illustrated in **Figure 4**. In the electrospinning process, a very thin jet of liquid is forced through an orifice; during this process the solvent evaporate and leave behind a very thin polymeric fiber, which is electrostatically attracted to a metallic plate (Reneker & Yarin, 2008). Numerous biopolymers relevant to the food industry can be electrospun, mainly carbohydrates and proteins (Mendes, Stephansen, & Chronakis, 2017). Among the food proteins that can be electrospun, zein is particularly interesting due to its facility of spinning in solvent regarded as food grade such as ethanol and acetic acid. Numerous studies have been conducted on zein electrospun fibrils to optimize its electrospinning conditions.

Some of the solution's critical parameter for electrospinning are solution viscosity, surface tension and conductivity. Zein solution viscosity depend mainly on the protein concentration and solvent, nevertheless, commercial zein lot variability can greatly influence viscosity as well (G. W. Selling et al., 2005). To favor the occurrence of fibrils formation instead of electrospraying, it is important that the polymer in solution are in a sufficient concentration to guarantee s sufficient level of entanglement (Neo, Ray, Easteal, Nikolaidis, & Quek, 2012). Surface tension importance is straightforward for electrospinning processing as electrical force need to be higher than the surface tension of a polymer to allow it to form a jet of liquid (Lu & Ding, 2008). Zein solution in aqueous ethanol and acetic acid generally present low surface tensions (Gordon W Selling, Biswas, et al., 2007) largely dictated by the solvent type rather than the biopolymer concentration (Haghi & Akbari, 2007). The solution conductivity play a crucial role in electrospinning as the higher the conductivity the more the polymer solution will be able to transport ionized molecules, which will drive the breaking of the cohesive forces on the surface of the liquid (Uyar & Besenbacher, 2008). While the effect of basic solution parameters and spinning conditions has been already extensively studied for zein electrospun fibrils, the research has now shifted more toward new applications such as composite electrospinning.

As mentioned earlier, zein is among the easiest spinnable food polymers. However, the use of zein alone has some limitations for food applications, for instance zein is greatly plasticized by water which limit its application in packaging, or its poor nutritional profile can limit its incorporation in food. Early attempts of spinning zein with other proteins such as casein and whey proteins resulted in a failure due to solvent incompatibility (Nieuwland et al., 2013) although the authors were able to successfully spin gelatin with other proteins. Recently, several studies were conducted with hybrid spinning of zein with gelatin (Deng et al., 2018). Zein/gelatin fibers cross-linked with glucose showed water resistance, plus they allowed to engineer the degree of hydrophilicity of the fibers and finally they are non-cytotoxic, which make them ideal for pharmaceutical applications (Deng, Li, Feng, & Zhang, 2019). Another study showed how zein/gelatin fibrils can be potentially used as packaging material for pork preservation, allowing encapsulation of antimicrobial compounds (L. Li et al., 2020). Recently, Casein was included into zein electrospun fibrils resulting in an enhanced β-sheet content and being associated with higher beading (Y. H. Wang et al., 2019). A study of incorporation of plasticizers and co-protein in zein electro spun fibrils will be extensively discussed in chapter 3 of this dissertation.

2.5.4 Extrusion

Extrusion is processing technique which primarily involve the compression of a plastic material through an orifice (Bouvier & Campanella, 2014). An extruder is generally composed by a motor drive which through a gear reducer system transfer the mechanical energy to the screw, located inside a barrel. Extruders are generally divided into two different types, single screw, or twin screw. Twin screw extruders can be divided based on the screw rotation direction and the position of the screw in relation to one another (fully intermeshing vs non-intermeshing). The screw barrel assembly is generally divided in different sections, classified on the function of the different zones, for instance a feeding section where the material is fed into the extruder or compression section. Finally, at the end of the screw section there is the possibility of the insertion of a die depending on the extrusion process. For instance in the extrusion of cellulose a die is not present (Bouvier & Campanella, 2014) contrary, in the production of meat analogues the presence of a cooling die is crucial for the development of the desired texture (Murillo, Osen, Hiermaier, & Ganzenmuller, 2019). Extrusion is a common technology in the food industry, and serval food components can be processed through this technology. Traditionally, extrusion processing was largely employed in the cereal and oilseed processing industry. More recently the need to find more sustainable alternatives to meat has raised attention on the use of high moisture extrusion of protein rich ingredient such as soy or pea protein, to produce meat analogs.

Similar to other plant proteins, zein fibrils can be formed through the application of extrusion technology (**Figure 5**). While texturized vegetable proteins form fibrillar structures due to phase separation of proteins in the cooling die (Murillo et al., 2019), zein fibrils formation is more likely due to molecular orientation due to the high temperature and shear stress inside the extruder. Early studies on zein extrusion showed that after release from protein bodies, the formation of what appeared to be fibrils was observed (Batterman-Azcona, Lawton, & Hamaker, 1999), which can impact the mechanical properties of products containing extruded zein. An insightful investigation of the effects of extrusion processing on zein has been conducted by Selling (Selling, 2010). Numerous modifications occur at increasing extrusion temperatures; Protein cross-linking begins at 120ºC, while a reduction in α-helix and β-sheet content was reported at increasing extrusion temperature, finally structure loss is further increased above 240 ºC (Selling, 2010). Multiple extrusion passages of zein can be used to regulate the amount of cross-linking formed during extrusion (Gordon W Selling & Utt, 2013). Zein extrusion can be used also to produce sheets or films (Ha & Padua, 2001; Y. Wang & Padua, 2003). A detail discussion on the incorporation of extruded zein in dough will be given in section 4 of this dissertation.

2.6 Spectroscopic techniques for zein characterization

Numerous spectroscopic techniques are available for the characterization of zein, among the most popular techniques are FTIR (Fourier-transform infrared spectroscopy), CD (Circular dichroism), NMR (Nuclear magnetic resonance) and XRD (X-ray diffraction). Fourier transform infrared spectroscopy (FTIR) is a spectroscopic technique that is based on the absorption of infrared radiation by organic molecules. A molecule will absorb IR radiation if the wavelength will alter the electrons orbital distribution generating a vibration in the covalent bond (Nielsen, 2010). The frequency at which each molecule absorbs IR radiation is specific for each functional group and therefore confer structural information about a molecule. Protein, due to their structural complexity yield complex spectra when they are analyzed with FTIR spectroscopy. A spectrum of zein is reported in **Figure 6**. Particularly important for protein structure is the signal generated by the amide group, which can be divided in 4 different peaks, amide A, amide I at 1640cm^{-1}, amide

II at 1540cm^{-1}, and amide III around 1230-1330 cm^{-1} (Nielsen, 2010). Beside amide bands, amino acids forming protein possess numerous other functional groups. Alcohols groups in serine, tyrosine and threonine absorb around 3200-3600 cm^{-1} and 1300-1500 cm^{-1} due to OH stretch and bend respectively, and around 1000-1220 for C-O stretching. Aromatic rings of Phenylalanine, Tyrosine, and Tryptophane show a typical absorption around 3000-3100 cm^{-1} due to CH stretch and at 1600 cm^{-1} for C=C bonds (Nielsen, 2010). A complete list of absorption of amino acids side chains has been reviewed in detail by Barth (Barth, 2000).

The amide signals can be used to obtain information on proteins secondary structure. However, particular care must be taken in the attribution and especially in case of quantification of protein secondary structure using FTIR (Jackson & Mantsch, 1995). Generally for the determination of the secondary structure of a protein, the preferred peak is the amide I peak as it arise only from one functional group (Jackson & Mantsch, 1995). In order to determine the secondary structure of a protein it is necessary to identify the different structures composing the amide I peak. To do so, Fourier self-deconvolution (FSD) and second derivative are applied to determine the peaks forming the amide I peak. Then, based on existing literature on the structure of the protein the peaks can be attributed to specific secondary structures. In the case of zein, generally 5 different peaks are identified, which are attributed to α-helix, β-sheet and β-turns (Erickson et al., 2014). Zein is known to have a prevalence of α-helical structure and in lower amounts β-turns constituted by the high amounts of glutamine and low levels of, β-sheets (Argos et al., 1982; Matsushima et al., 1997). After the different peaks forming the amide I peaks are properly identified, it is possible to proceed with the deconvolution of the peaks and the determination of the secondary structure. FTIR is a powerful analytical technique to be used for the quantification of the secondary structure of proteins, presenting several advantages such as the possibility to obtain the spectra of solid materials such as powders or viscoelastic masses. Nevertheless, it’s important to be cautious with the secondary structure attribution using FTIR as many factors can interfere in the generation of the amide I peak. Therefore, it is a good practice to strengthen the results obtained with FTIR with more robust techniques.

Another common technique for the determination of the secondary structure in zein is Circular Dichroism (CD). CD is a spectroscopy technique that use polarized UV radiation to obtain information on proteins secondary and tertiary structure based on different absorption of polarized radiation caused by protein structural asymmetry. To be analyzed with CD, proteins must be fully solubilized. Generally, zein is dissolved in either aqueous ethanol or aqueous methanol for CD analysis. Starting from spectra of proteins with known structure it is possible to determine the secondary structure of another protein (Argos et al., 1982). For raw zein most of the reports indicate a prevalence of α-helix structure and lower amounts of β-turns and β-sheets (Cabra et al., 2005; A. Tatham et al., 1993). CD spectroscopy has been used to study several factors that can potentially influence zein structure. The most studied factor affecting zein structure is the effect of the solvent. Bugs et al studied the effects of different water ethanol concentration on zein secondary structure finding that α-helix content of zein increased slightly at increasing water content of the solution(Bugs et al., 2004). Contrary, Erickson observed that increasing amount of water decreased the amount of α-helix and favored more β-sheet conformation (Erickson et al., 2020). The latter results were further confirmed by thioflavin T essay, a chemical component that binds to β-sheet structures. Another factor studied using CD on zein structure is pH. At neutral pH the structure of zein was reported to be mainly α-helical (V. Cabra, R. Arreguin, R. Vazquez-Duhalt, & A. Farres, 2006), suggesting that hydrophobic interaction favor the formation of α-helix. Likely, a neutral pH is promoting interactions between polar amino acid with hydrophobic amino acids such as alanine, leucine, and isoleucine. On the other hand, a low pH favored the reduction of α-helix (V. Cabra et al., 2006). The effect of temperature, solvent and pH was extensively studied by Selling, concluding that zein has a resistant structure as it is necessary to use harsh treatments to generate any structural changes on it (Gordon W Selling, Hamaker, & Sessa, 2007).

NMR is a popular analytical technique able to provide important structural information. For instance, NMR can provide information that can be used to identify functional groups of a molecule. Moreover, it’s the only spectroscopy that, without the aid of any other analytical too can fully resolve the complex structural arrangement of biomolecules (Nielsen, 2010). The principle of NMR is based on the ability of specific atoms to absorb radio frequency when they are exposed to a strong magnetic field. The most common atoms analyzed in NMR spectroscopy due to their abundance in biological systems are proton (H), ^{13}C, ^{19}F, and ^{31}P. Only nuclei with an angular momentum and a characteristic spin number can be analyzed using NMR (Nielsen, 2010). NMR found numerous applications related to food systems, for instance, studies of staling bread or gluten hydration and plasticization (Bertocchi & Paci, 2008). Beside the more informative high resolution NMR, a low resolution mode has been also developed for several applications in food and agriculture (Barker & Stronks, 1990).

NMR has proven a useful technique to gather important structural information on zein. Forato, used ^{13}C and ^{1}H NMR on protein bodies and extracted α zein to reveal the presence of fatty acids inside zein structure (L. A. Forato, Colnago, Garratt, & Lopes, 2000). Subsequently, using a combination of FTIR, XRD and NMR proposed a structural model of α-zein. Notably, the fast N-H to N-D exchange observed through NMR indicated an unfolded structure of zein molecules (Lucimara A Forato et al., 2004). Another application of NMR on zein is the determination of secondary structure using Cross Polarization Magic Angle Spinning. For instance a passage from predominantly α-helix to a secondary structure more dominated by β-sheet has been observed after wet cooking of maize or sorghum (Duodu et al., 2001). Chemical changes induced by zein treatments can also be observed using NMR. For instance, zein extrusion was performed by Selling and NMR analysis possible formation of esters bonds (Selling, 2010). Other application of NMR for study of zein has been interactions with different solvent (Yamada, Noguchi, & Takahashi, 1996) or to study the interactions between zein and other molecules (Rao, Wang, & Zhang, 2019)

Together with NMR, XRD is a technique that can provide key information on protein structure. The principle of XRD is based on X-ray radiation being diffracted when they encounter some molecular structures. However, a constructive interference, necessary to generate a clear signal, only occurs at specific wavelengths depending on the spacing between atoms. Thus, different crystalline structure generates different diffraction patterns, from which is possible to determine the interspacing distance of atoms using Bragg’s law (Warren, 1941). X-ray diffraction is performed only on crystalline materials as amorphous materials random arrangements cause beams to randomly interfere and no distinctive patterns is generated (Drenth, 2007). X-ray diffraction can be performed in two different modes, wide angle, and small angle. The main difference consists in the distance from sample to detector; in wide angle the distance from sample to detector is shorter, thus diffraction maxima are observed at wider angles.

Several publications used X-ray technique to study zein structure. Matsushima used Small-angle X-ray scattering (SAXS) to study the structure of zein in 70% ethanol solutions (Matsushima et al., 1997). SAXS experiments allowed to determine the dimension of zein molecules in solution, with a cross-section of 4 and 1.39nm and a length of 13nm, respectively. These information were used to develop a model of zein molecules in solution. Studies involving the use X-ray diffraction on dry zein and films plasticized with oleic acid (H-M Lai, Geil, &

Padua, 1999) indicated the presence of non-crystalline structures. Furthermore, spacing of 4.6 and 9.5Å confirmed the presence of alpha helices as observed in previous studies. More recently, X-ray scattering has been used to better understand zein self-assembling behavior (Uzun et al., 2017). Different structure was identified such as, molecular zein, two dimensional sheets and three-dimensional spherical aggregates. In conclusion, XRD is a powerful tool able to provide important information on zein structure.

2.7 Thermal analysis

Numerous food components have properties that changes with modification in temperature. For instance, biopolymers present in food undergoes phase transitions such as crystallization or glass transition (Noel, 1990). Typically, food proteins due to their complexity and unordered structures undergoes glass transitions rather than enthalpic peaks of crystallization or melting. In order to determine a material glass transition, two different techniques are widely adopted: DSC and DMA. *Tg* in DSC can be measured using the midpoint approach (Thomas, 2005b), while three different parameters can be used to determine it in DMA (G', G'', and Tan δ) (Instruments, 1999). An example of DSC and DMA glass transition determination is shown in **Figure 7**. DSC's main advantage in determining the glass transition of a material is its versatility; it can analyze materials in different physical states, from powders to liquids. Furthermore, the sealed conditions of the pans prevent any evaporation of volatile plasticizers such as water. On the other hand, glass transition signals using DSC are generally weak, and an optimization step is frequently necessary to enhance the signal (Thomas, 2005a; Xivillé, Lorente, & Kordikowski, 2012). On the other hand, DMA yield stronger signal which generally do not need preliminary work for signal enhancement. However, DMA need samples to be in specific physical states i.e. a film or a mat, and signal glass transition can change depending on the method with which it is measured (Instruments, 1999).

Numerous studies have been conducted on cereal proteins to determine their glass transition. Phase diagram have been developed for zein (Madeka & Kokini, 1996) and for gluten (Kokini, Cocero, Madeka, & De Graaf, 1994) showing extensively the plasticization role of water in cereal proteins. While gluten proteins are well known to be in the rubbery state when hydrated and mixed into a dough. Glass transition of zein has generally been reported to be around 160°C for dry zein, rapidly dropping to 60°C at 10% moisture content and slowly decrease to close to room temperature (28ºC) for moisture content around 25-30% (J. W. Lawton, 1992a). This imply that zein will be in its glassy state if it is mixed with starch into a dough at room temperature. Indeed, zein doughs are brittle and unable to stretch and expand when mixed below its glass transition temperature, lacking fundamental characteristics of a good dough. Further, being above glass transition temperature dramatically increase molecular mobility, allowing zein to reorganize its molecular structure, which is thought to be fundamental for the generation of its unique viscoelastic properties (Mejia, Mauer, & Hamaker, 2007). Beside water, numerous other plasticizers are known for zein, such as glycols like glycerol or fatty acids such as oleic acid, (J. Lawton, 2004).

2.8 Dough microscopy

Imaging techniques were widely used in many studies on dough and bread to evaluate their structure. A deeper comprehension of the microscopic structure of these systems is fundamental to better understand the macroscopic behavior as for instance the rheological properties of dough systems. Many microscopy studies were done to investigate the structure of gluten after rheological studies proved its importance in the bread making. Different microscopy techniques were adopted in the last decades to investigate the microscopic structure of dough and bread, the most used were Confocal, SEM, Cryo-SEM.

An important technique used to study dough microstructure is confocal microscopy. Advances on the use of monoclonal antibodies allowed for a more in-depth studies of dough formation (Bonilla, Bozkurt, Ansari, Sozer, & Kokini, 2016). In a recent study, Bonilla investigated the molecular interactions between the main protein fractions of dough, gliadin, LMW glutenin and HMW glutenin (Jose C. Bonilla, James A. Schaber, Arun K. Bhunia, & Jozef L. Kokini, 2019). Results indicated that initially development is associated to the assembly of the three different fractions of dough. Afterwards, the continuous mixing induces the aggregation of LMW glutenin was observed, leading to decrease of dough strength. Finally, 10 minutes after departure time, aggregation of HMW glutenin has been observed colocalized with LMW glutenin aggregates, resulting in further decrease of dough strength. Further studies confirmed the important role of the formation of a cohesive network between the three main components of gluten, and that the breakdown of the network is the main reason of loss of dough strength in weak dough (Bonilla et al., 2020).

Another widely used technique to study dough microstructure is electron microscopy. The first report of electron microscopy on flour and dough was reported in 1968 (Aranyi & Hawrylewicz, 1968). Soon after these early observation, the question on the exact morphology of gluten rose among scientist, with the more accredited hypothesis being either fibrillar or sheet like (Bernardin, 1973). The fibrous nature of gluten is associated with the high molecular weight (HMW)-glutenin (P. Wang, Jin, & Xu, 2015). While constitute only a minor fraction of wheat flour, it was hypothesized that fibrillar structure formed by high molecular weight glutenin could have a major role in the development of gluten elasticity (J.-s. Wang, Zhao, & Zhao, 2007). The importance of elongated glutenin structure was further reinforced by a study showing a significant reduction in elasticity and fibrous microstructure among hydrated gluten samples that were previously hydrolyzed and/or re-cross-linked by enzymes (X. Y. Wang, Guo, & Zhu, 2016). Other observation suggested the formation of fibrillar structures from glutenins, favored by the formation of extensive hydrogen bonding in the presence of water (McIntire et al., 2005). Further Zounis et. Al. using cryo SEM was able to show gluten strand in a frozen dough system (Zounis, Quail, Wootton, & Dickson, 2002).

Other studies indicated that at the mesoscopic scale, gluten form sheets and not fibers, meaning that the gluten network exist in lower length scale (Amend & Belitz, 1990). These observations suggest that it is possible that gluten formation of fibrils is only an artifact that is generated by the sample preparation or observation conditions, inducing aggregation and formation of fibrils like structure. More recent studies proved that for the formation of fibrils occur only under very specific conditions (Athamneh & Barone, 2009; Bache & Donald, 1998; Reddy & Yang, 2007).

Zein microstructure has also been extensively studied with different microscopy techniques. Contrary to gluten, there is more agreement on the fibrillar nature of zein when inserted into a dough. Fibrillar structures were observed from early studies from Lawton, showing that when mixed above the *Tg* and in the presence of dibutyl tartrate (J. W. Lawton, 1992a). Successive studies using confocal microscopy confirmed the ability of zein to form fibrillar structure (Sly, Taylor, & Taylor, 2014). While progress has been made recently in understanding the chemical changes inducing the formation of fibrils from zein (Erickson et al., 2020) a model accurately explaining how zein molecules dispose to form fibrils is not available. Nevertheless, a hypothesis has been proposed suggesting analogies with a model proposed to explain the formation of zein nanoparticles (Taylor, J. Taylor, et al., 2016; Y. Wang & Padua, 2012).

2.9 Dough rheological properties

Bakers and food scientist knew for a long time about the importance of gluten rheological properties, which are known to deeply affect every stage of breadmaking processing, from the formation of dough during early mixing operation to the quality of the bread (Bloksma, 1990). For these reasons, a deep understand of the rheological properties of dough is fundamental to produce good quality bread. Usually the rheological techniques are divided in two main categories, empirical and fundamental (Weipert, 1990) A list of empirical and fundamental tests to assess dough rheology is shown in Table 1. The empirical rheological testing furnish data which are not very well defined, however they are vastly used by the industry for the evaluation of flour performance and in general for quality control purposes (B. J. Dobraszczyk & M. P. Morgenstern, 2003; Menjivar, 1990; Weipert, 1990). On the other hand, fundamental analysis gives well-defined parameters, thus the data generated are expressed in well-defined unit of measure. Thus, fundamental tests have the advantage of being more reproducible compared to the empirical test which can be harder to reproduce because of their intrinsic nature. However, they require more expensive instrument and trained users. Numerous rheological techniques has been used in many studies on dough, including various apparatus for uniaxial and biaxial extension measurements, creep recovery, various oscillation tests, stress relaxation, lubricated squeezing, to investigate gluten and wheat doughs rheological properties (B. J. Dobraszczyk & M. P. Morgenstern, 2003). Further, the increasing demand for gluten free product increased the necessity of good rheological technique for the characterization of the dough rheological property of gluten free system. In this chapter we will focus on the review of the rheological test that were performed in this dissertation.

Dynamic oscillatory measurements have the advantage to not be destructive on the sample analyzed. They generally yield parameters such as the storage modulus (G’) or the viscous modulus (G’’) and can investigate the effect of variable such as frequency, stress, temperature or time dependent phenomenon as well. These test are generally considered to be in the low deformation spectrum as it’s important to test the samples at stresses or strain that lie on the linear viscoelastic region of the material (Steffe, 1996). The equipment that is commonly employed is a rheometer with attached a parallel plate geometry. There are two types of rheometer available, instrument with constant stress which measure the applied strain or with constant strain measuring the stress. In dough, like for other more “solid like” materials such as strong gels, the storage modulus is higher than the viscous modulus. On the other hand, High G’ are generally correlated with less extensible dough. Tan δ of dough is in general low indicating a prevalence of elastic modulus over viscous modulus. It is important to remember that the dough need to be tested in the right conditions to obtain information relevant to breadmaking processing. Unfortunately, A great deal of literature produced on dough rheological fail to take into account this important prerequisite in order to perform rheological test that can truly simulate the conditions under which dough undergoes during baking phases (B. J. Dobraszczyk & M. P. Morgenstern, 2003).

A creep recovery test consists in applying a constant stress to the sample ($\sigma = \sigma_0$) for a certain time after which the stress is removed. When the stress is removed the sample can recover over time. wheat doughs studies using creep-recovery showed a viscoelastic behavior which is a combination of viscous and elastic components (Janssen, Van Vliet, & Vereijken, 1996). Flour doughs were reported to exhibit a strain hardening behavior under biaxial extension, contrary, when tested under shear, dough exhibited a shear thinning behavior (Rouillé, Della Valle, Lefebvre, & Sliwinski, 2005). Shear thinning behavior was also reported at high shear rates. Creep recovery test can be conducted either under small (Rouillé et al., 2005) or large deformation conditions. (Edwards, Dexter, Scanlon, & Cenkowski, 1999).

The literature counts a large amount of extensional flow measurements for doughs, which can generally be divided into two categories uniaxial extension and biaxial extension. Uniaxial tests are one of the oldest tests for dough rheology and are still very popular for their relative simplicity. Numerous instrumentations have been employed to perform uniaxial extension test as for instance kefir rig using a texture analyzer, extensograph, or other typologies of extensometers. Although, none of these equipment gives fundamental rheological units, because the sample geometry is not well defined (B. J. Dobraszczyk & M. P. Morgenstern, 2003). Numerous studies have been conducted using uniaxial extension to predict baking quality of wheat dough(Bagley, Christianson, & Martindale, 1988; Yue et al., 2020). Scarce information is available on gluten-free dough, with a few studies showing low extensibility of dough formed using gluten-free flours resulting in bread of poor quality in terms of specific volume (Burešová, Kráčmar, Dvořáková, & Středa, 2014; Buresova & Kubinek, 2016). The explanation for the lack of use of these test is the poor rheological properties of gluten-free doughs; Many of the gluten-free dough formulation employed for the production of gluten-free bread formed batters rather than doughs (Salehi, 2019), making it impossible to test the doughs with uniaxial extension tests.

While there is only one way to conduct a uniaxial extension test of a dough, consisting in pulling the dough from the extremities, different methods imparting a biaxial extension have been developed such as inflation, lubricated compression, or extension test. Inflation was one of the first method used to assess dough rheological properties (Chopin, 1921). Numerous studies have been conducted using an alveograph to asses dough quality (Bettge, Rubenthaler, & Pomeranz, 1989; Codină, Mironeasa, Bordei, & Leahu, 2010). For the same reason articulated for uniaxial extension, there is a lack of studies using inflation methods to characterize gluten-free dough rheological properties. Nevertheless, Taylor was able to analyze with an alveograph a gluten-free dough composed of a mixture of zein and either starch or flour (Khuzwayo, Taylor, & Taylor, 2020; Sly et al., 2014), highlighting zein doughs outstanding rheological properties compared to other gluten-free doughs. Lubricated compression is another popular method using a biaxial compression to determine key rheological properties. A popular method is lubricated squeezing flow, with lubrication to minimize contribution of friction to the extension (Campanella & Peleg, 2002). Numerous studies have been conducted in wheat doughs (Kouassi-Koffi, Launay, Davidou, Kouamé, & Michon, 2010; Launay & Michon, 2008) while only one report on gluten-free dough has been reported (Fevzioglu, Hamaker, & Campanella, 2012). Finally, another method to measure extensional properties of doughs sheets consist in the extension through an aperture of a sheet of dough hold between two parallel plates (Morgenstern, Newberry, & Holst, 1996).

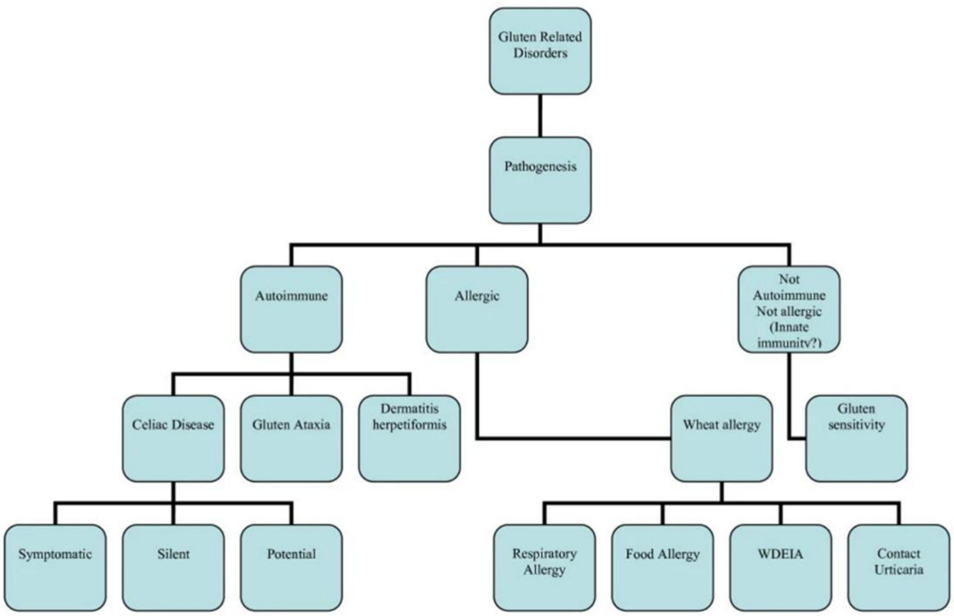


Figure 2 Classification of gluten related disorders (Sapone et al., 2012)

Figure 3 Zein resin obtained with antisolvent precipitation from a 20% (w/w) zein-acetic acid solution.

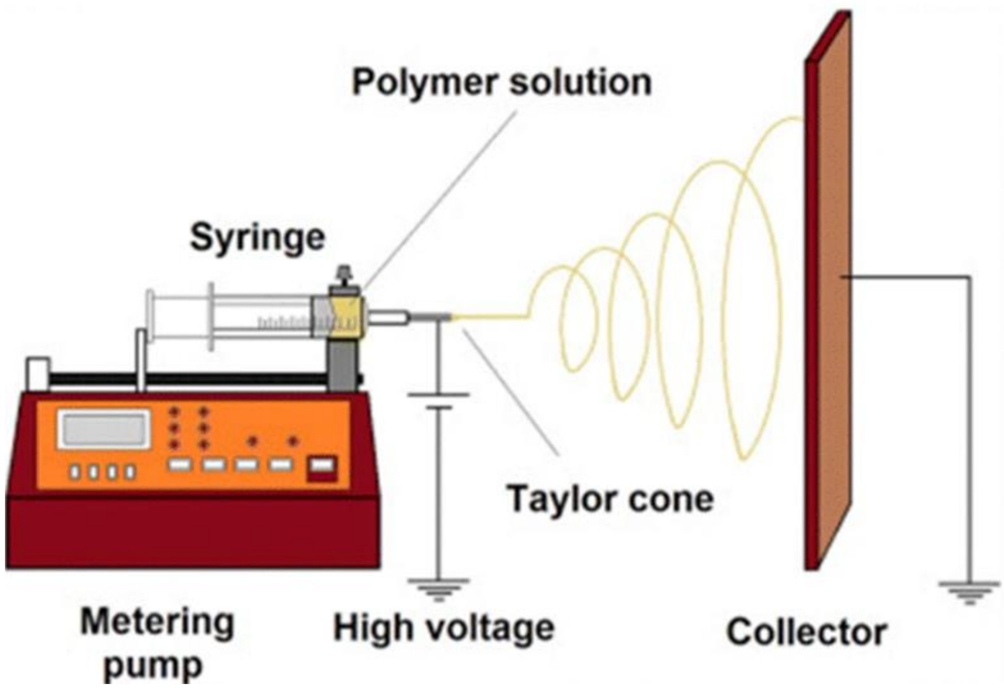


Figure 4 Schematic diagram of set up of electrospinning apparatus (Islam, Ang, Andriyana, & Afifi, 2019)

Figure 5 Zein fibrils generated with extrusion processing.

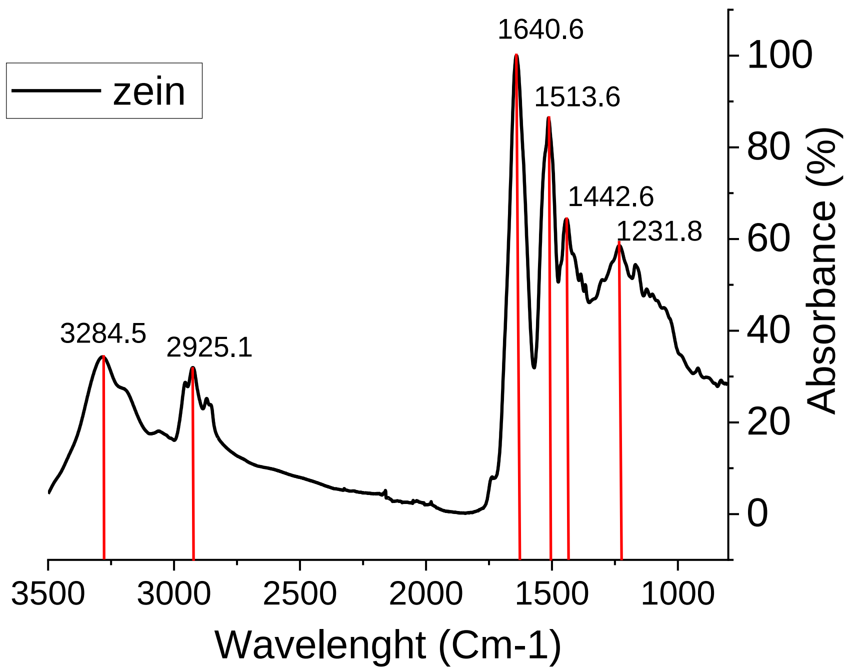


Figure 6 FTIR spectra of dry zein powder

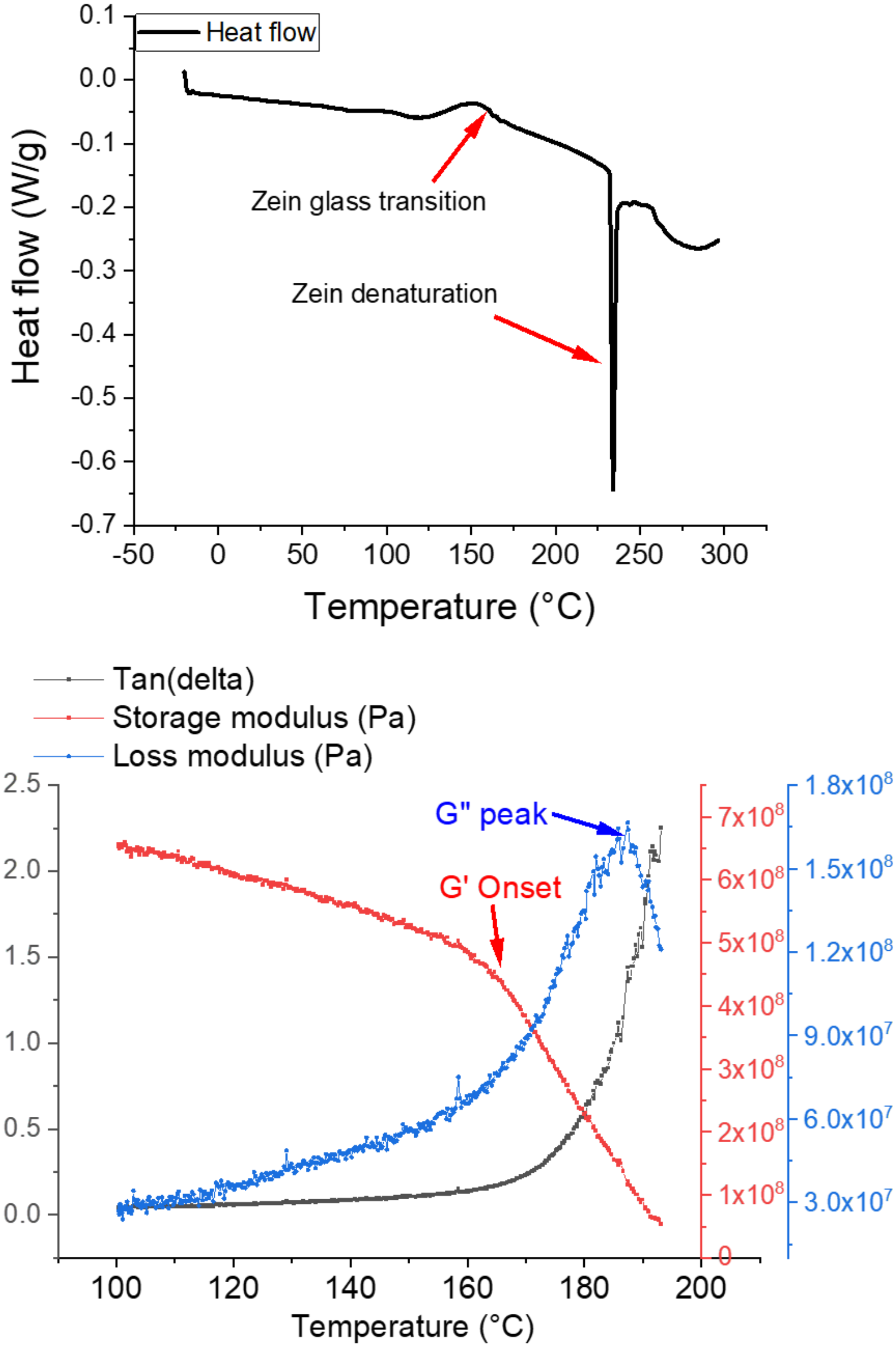


Figure 7 Determination of zein glass transition using DSC (upper figure) and DMA (lower figure)

Table 1 Empirical and fundamental rheological methods for cereal products (B. J. Dobraszczyk & M. P. Morgenstern, 2003)

Method	Products	Property measured
Empirical methods		
Mixers: farinograph, mixograph, reomixer	Dough	Mixing time/torque apparent viscosity
Extensigraph	Dough	Extensibility
Taxt2/Kieffer Rig	Dough, gluten	Extensibility
Alveograph	Dough, gluten	Biaxial extensibility
Amylograph, RVA	Pastes, suspensions	Apparent viscosity, gelatinisation temp
Consistometer	Sauces, fillings	Apparent viscosity
Flow cup	Fluids, sauces, batters	Apparent viscosity
Falling ball	Fluids	Apparent viscosity
Flow viscometers	Fluids, pastes	Apparent viscosity
Fermentometers	Dough	Height, Volume
Penetrometers	Semi-solid foods, gels	Firmness, hardness
Texturometer, TPA	Solid foods	Texture, firmness
Fundamental methods		
Dynamic oscillation, concentric cylinders, parallel plates	Fluids, pastes, batters, doughs	Dynamic shear moduli, dynamic viscosity
Tube viscometers: capillary, pressure, extrusion, pipe flow	Fluids, sauces, pastes, dough	Viscosity, viscosity, in-line viscosity
Transient flow: concentric cylinders, parallel plates	Semi-solid (visco-elastic) materials	Creep, relaxation, moduli and time
Extension: uniaxial, biaxial, dough inflation system, lubricated compression	Solid foods, doughs	Extensional viscosity, strain hardening

CHAPTER 3. INCORPORATION OF PLASTICIZERS AND CO-PROTEINS IN ZEIN ELECTROSPUN FIBERS

The following content has been reproduced from an article published on the Journal of Agricultural and Food Chemistry.

Federici, E., Selling, G. W., Campanella, O. H., & Jones, O. G. (2020). Incorporation of Plasticizers and Co-proteins in Zein Electrospun Fibers. Journal of Agricultural and Food Chemistry, 68(49), 14610-14619.

3.1 Abstract

As a means to alter the physical properties of electrospun zein fibers, plasticizers (glycerol, lactic acid and oleic acid) or co-proteins (casein, whey proteins, rice proteins) were mixed with zein using the solvents acetic acid or aqueous ethanol with or without sodium hydroxide. Incorporating plasticizers or co-proteins had a negligible impact on solution viscosity, solution surface tension, and fiber formation, although electron microscopy of fiber mats showed an increase in bead formation with added co-proteins. Gel electrophoresis identified casein and whey protein in spun mats, yet it could not be verified if they were located in the beads rather than the fibers. Infrared spectra demonstrated inclusion of plasticizers in fiber mats. Glycerol, lactic acid, and oleic acid reduced glass transition temperature of bulk fibers, while nano-indentation showed reduced Young's moduli of individual fibers. Thus, zein fibers can be physically modified by incorporating food-grade plasticizers, yet protein incorporation remains a challenge.

3.2 Introduction

Electrospinning is a technique to create fibrous structures by extruding small jets of a polymer solution by aid of an electric field. Specifically, a polymer solution or suspension is extruded from an electrically-charged needle towards a charged metallic target(Reneker & Yarin, 2008). The generated electric field pulls and elongates the extruded liquid into a thin jet so that the solvent is evaporated and leaves behind a very thin-diameter fiber, which is drawn and deposited against the metallic target (Reneker & Yarin, 2008). Potential utilization of electrospun fibers include structuring agents in films (Deng et al., 2018; Z.-C. Yao et al., 2016), textiles (Han et al., 2006; Mirjalili & Zohoori, 2016), and controlled delivery vehicles for active molecules

(Séon-Lutz et al., 2019; X. Zhang et al., 2020). Electrospinning of food-grade proteins is an excellent technique to increase surface area to volume ratio, porosity, unique interfacial or film functions, and mechanical behaviors (Lu & Ding, 2008).

Zein, the most abundant protein in maize, is one of the few proteins that can be electrospun utilizing solvents regarded as safe for the food industry (Nieuwland et al., 2014). Zein is classified as a prolamin and is present in the corn gluten meal or distillers dried grains (useful as animal feed products) from the endosperm fraction of maize which is isolated during commercial milling. If a suitable end-use is found, it could be obtained in large quantities as a byproduct of the bioethanol and starch industries(Shi, Kokini, & Huang, 2009). Studies have found that acetic acid is a slightly better solvent than ethanol for zein and is still food-grade (Gordon W Selling & Woods, 2008).

Important parameters defining fiber formation and dimensions during electrospinning include viscosity, surface tension, and solvent volatility. Spinning of fibers rather than particle-spraying occurs at semi-dilute polymer concentrations because there is an increased chance of entanglement; this occurs for zein solutions with solution viscosity greater than 50 mPa s (Neo et al., 2012). Liquid surface tension of the spun solution further defines fiber morphology for a given electric field strength (Doshi & Reneker, 1993). The type of solvent has a much greater impact on the surface tension than the concentration of dissolved biopolymer (Haghi & Akbari, 2007). Earlier studies demonstrated the capacity to electrospun zein fibers from solutions with acetic acid or aqueous alcohol, which are preferred for their low cost and low toxicity (Gordon W Selling, Biswas, et al., 2007; C. Yao et al., 2007). Electrospinning of zein with aqueous ethanol can occasionally form round fibers (C. Yao et al., 2007), but the relatively fast evaporation of ethanol more commonly forms ribbon morphologies (Gordon W Selling, Biswas, et al., 2007) and also increases clogging near the syringe needle (Kanjanapongkul, Wongsasulak, & Yoovidhya, 2010). Using acetic acid as the solvent generally yields round fibers (Gordon W Selling, Biswas, et al., 2007).

Incorporating plasticizers and additional proteins (co-proteins) alters the viscoelastic properties in zein-based films and resins (D. P. Erickson, S. Renzetti, A. Jurgens, O. H. Campanella, & B. R. Hamaker, 2014). We hypothesize the same could be achieved if zein is electrospun with plasticizers or co-proteins. Examples of food-grade plasticizers successfully incorporated in zein films and resins include glycerol, lactic acid, and oleic acid (Emmambux & Stading, 2007; Huey-Min Lai & Padua, 1997). While lactic or oleic acids have never been incorporated in spun zein fibers, glycerol was previously found to increase the tensile strength of electrospun zein fiber mats with a 8% solids content while increasing extensibility when the concentration increased to 10% solids content (Wongsasulak, Tongsin, Intasanta, & Yoovidhya, 2010a). A recent work on this hybrid electrospinning process showed successful incorporation of gelatin in zein fibers, providing an improved resistance to dissolution of fibers when the mats were soaked in water or ethanol (Deng et al., 2018). Although dairy proteins have shown promise in improving the extensibility of zein resins, a prior attempt at spinning zein with casein or whey proteins using aqueous ethanol as a solvent was unsuccessful (Nieuwland et al., 2014). This was attributed to the poor mutual solubility of the proteins, as zein is insoluble in water and casein is insoluble in aqueous mixtures with high ethanol content. Recently, casein was spun with zein at up to 16% solids content by using 70% acetic acid in water as the solvent, and fibers were nearly identical to zein-only fibers apart from a noted increase in bead formation (Y. H. Wang et al., 2019). Whey proteins and rice proteins have never been incorporated within zein electrospun fibers.

In this study we sought techniques to prepare zein fibers by electrospinning with incorporated plasticizers (glycerol, lactic acid, oleic acid) and co-proteins (casein, whey protein, rice proteins). Electrospinning was performed with two commonly utilized solvents (90% aqueous ethanol and acetic acid). Further attempts were made to improve mutual solubility of co-proteins with zein by also using a mixture of ethanol and 0.1M sodium hydroxide (NaOH) solution. Suitability for electrospinning was verified by characterizing the solution viscosity and its surface tension, while morphology of spun fibers was determined microscopically. The presence of the plasticizers and co-proteins had to be confirmed by subsequent testing to ensure their co-extrusion with zein during the spinning process. It was hypothesized that incorporation of these plasticizers and co-proteins in spun fibers could be accomplished in acetic acid or aqueous ethanol solvents with only slight modifications, or by incorporating plasticizers able to confer changes to their thermomechanical properties. To this end, nano-indentation of fibers using Atomic Force Microscopy (AFM) was used for the first time to identify changes in the Young's modulus of plasticized zein fibers.

3.3 Materials and methods

3.3.1 Materials

Zein was obtained from Flo Chemical Corporation (Ashburnham, MA, Lot #F40000081C, 14.26% Nitrogen, 4.8% moisture). Ethanol, acetic acid, sodium hydroxide (NaOH), glycerol, lactic acid, and oleic acid were obtained from Sigma-Aldrich (St. Louis, MO). Sodium caseinate 180 and whey protein isolate 894 were kindly donated by Fonterra (Chicago, IL). Rice proteins were kindly donated by Axiom Foods (Los Angeles, CA). Ultrapure water was obtained from a filtration system operating at resistivity $\geq$ 18 mΩ-cm (Barnstead E-pure, Thermo Scientific, Waltham, MA).

3.3.2 Sample preparation

Samples containing 27% (w/w) zein and 3% (w/w) plasticizers (glycerol, lactic acid, oleic acid) were prepared using 90% aqueous ethanol or glacial acetic acid as solvents, while samples containing 25% (w/w) zein and 2% (w/w) co-proteins (casein, whey protein, rice protein) were prepared using a 70:30 ethanol:0.1M NaOH aqueous solution and glacial acetic acid as solvents. Samples were classified by the solvent and additional components included in each solution; abbreviations for the samples are reported in **Table 2**. In each sample, the plasticizers and co-proteins were dispersed in the solvent prior to addition of zein, which was slowly added to each mixture and stirred for 30 min. The mixtures were centrifuged which provided a clear solution. Final protein content for all samples was fixed at 27% (w/w), comprising either 27% (w/w) zein or 25% (w/w) zein with 2% (w/w) co-protein. Plasticizer content was fixed at 2.5% (w/w) of the total mixture.

Fibers were prepared according to the method used by Selling et al. (Gordon W Selling, Biswas, et al., 2007). A syringe pump (100 Series, KD Scientific, Holliston, MA) was utilized to deliver 10 mL of the solutions to the target plate at a rate of 12 mL/hr. The spinning (1000 fpm) roll was covered with a Teflon-coated aluminum foil and was separated from the tip of the needle by a distance of 10 cm. A voltage of 25 kV was supplied to the needle from an external power source (Model ES50P-10W, Gamma High Voltage Research, Inc., Ormond Beach, FL). As a portion of the solution dries on the tip of the needle during fiber production which could potentially clog the needle opening, the hardened polymer solution was periodically removed using a paper towel. After spinning, the foil target was removed from the spinning wheel and allowed to fully dry overnight.

3.3.3 Solution characterization

The rheology of zein suspensions with or without plasticizers or co-proteins was determined using a rheometer (ARG-2 Model, from TA Instruments, Newcastle, DE, USA) with a cone and plate geometry (60 mm diameter plate) and temperature controlled with a Peltier-system. The solutions were transferred on the bottom plate and the upper cone was lowered to achieve a gap-size of 50μm. A solvent trap was used to reduce solvent loss during testing. Measurement temperature was kept constant at 25°C. Viscosities were measured in a shear rate range 1 - 100 s^{-1}. The values of shear viscosities were obtained using Trios software.

Surface tension of the electrospun solutions was tested by measuring the surface-air contact angle of pendent drops using a droplet shape analyzer (KRÜSS DSA30; Hamburg, Germany). Solutions were dispensed using a syringe with a 0.182 mm diameter steel needle. Interfacial tension was determined from droplet shape using DSA4 software (Kruss GMbH; Hamburg, Germany). All measurements were performed at room temperature and determined from a minimum of 5 droplets/suspension.

3.3.4 Fiber characterization

Images of the surface of fiber samples were obtained with a FEI NOVA nanoSEM Field Emission Scanning Electron Microscope (Hillsborough, Oregon, USA) attached to an Everhart Thornley detector. Fibers mats were placed on adhesive carbon paper and sputter-coated with platinum for 120 seconds prior to imaging. Sample was then inserted into the microscope chamber and vacuum was applied. An operating distance of 5 mm was used.

Infrared spectra of fibers were measured with a nitrogen-cooled attenuated total reflectance Fourier-transform infrared spectrometer (Thermo Scientific Nicolet Nexus; Waltham, MA). Background and sample spectra were recorded in a wavelength range between 1000 cm^{-1} and 3500 cm^{-1} with a resolution of 1 cm^{-1}. Each measurement was an average of 64 scans. All the spectra were baseline-corrected after acquisition. Absorbance is reported as a percentage of the highest value in the spectra. Analyses were performed at room temperature.

Polyacrylamide gel electrophoresis was performed to determine the approximate molecular weight of the constituent proteins. Samples were prepared by adding sodium dodecyl sulfate reducing buffer with tris(hydroxymethyl) aminomethane, stained with bromophenol blue tracking dye, and boiled for 5 min at 100°C to ensure full dispersion of the solid matter. Sample aliquots (10 μL) were then loaded onto a polyacrylamide gel with 1mm thickness composed of a 4% stacking gel and a 12% resolving gel. Runs were conducted at 200V for 60 minutes. After separation, gels were stained with Coomassie Blue R-250. Molecular weights of protein bands were determined using known molecular weight standards (Precision Plus Protein All Blue Standards, Bio-Rad Laboratories; Hercules, CA). Gels were imaged with a GelDoc XR system using transwhite illumination (BioRad; Hercules, CA).

Dynamic mechanical analysis (DMA850 New Castle, DE, USA) was used to determine the glass transition temperature (T_g) of electrospun fibers containing zein and co-proteins. Dry samples were cut into mats of 3cm of length and 1 cm of diameter and thickness was measured using calipers. The mats were then fixed on a film clamp. First, a strain sweep was performed to determine the region of linear response of the sample. Then a temperature ramp ranging from 100-200ºC was performed at a heating rate of 5ºC/min. A frequency of 1Hz and amplitude of 1μm (found to be within the linear viscoelastic regime) were used for the temperature ramp. T_g was determined by evaluating the onset decrease of the elastic modulus.

Mats made using ethanol as the solvent and containing plasticizer were too brittle to be analyzed by dynamic mechanical analysis, so T_g of these samples was measured using modulated differential scanning calorimetry in a TA Q2000 calorimeter (TA Instruments; New Castle, DE, USA). Dry samples were weighed (5 ± 0.5 mg) into hermetically sealed pans. An empty pan was used as reference. The calorimeter cell was heated at a rate of 2°C/min from 100°C to 200°C with a temperature modulation of 1ºC over 60s periods. T_g was determined using Trios Software using the midpoint approach.

Surface topographical imaging and nano-indentation of fiber surfaces were performed using an atomic force microscope (MFP-3D Model from Asylum Research, Santa Barbara, CA). Fibers were spun directly onto copper slides with dimensions of 2.5 x 7.5 cm. Aluminum-coated silicon probes were used with a nominal spring constant of 5 N/m and a resonance frequency of 150 kHz (Ted Pella, Redding, CA). Specific spring constants were measured using the thermal method. Surface topography measurements were performed in intermittent-contact mode at a scanning rate of 1 Hz. Nano-indentation measurements were performed in contact mode at loading/unloading rates of 500nm/s with no dwell time. Data collection was limited to indentations with a determined penetration depth of 20-50 nm. Surface indentation was verified by evaluating the surface topography of fiber surfaces in intermittent-contact mode (**Figure 8**). Young modulus of each indentation was calculated according to the Oliver-Pharr model (Pharr & Oliver, 1992). A total of 75 indentations per sample were performed.

3.3.5 Dough characterization

Gluten-free dough formulations were prepared using pre-made flours containing 15% zein/electrospun fibrils and 85% rice starch. All procedures and subsequent analyses were performed in a room with the temperature controlled at 25°C. Water was added in the proportion of 90% of flour weight. The samples were mixed for 5 min using a KitchenAid Ultra Power KSM95WH – 300 W with a paddle-mixer attachment. Extensional properties of dough samples were determined on a Texture Analyzer HD Plus (Newcastle, DE, USA) with a 50-kg load cell following the method of Morgenstern and others (Morgenstern et al., 1996). Dough was sheeted in squares of 100mm at a thickness of 3mm using a pasta sheeter. It has been shown that sheeting of wheat dough can promote its development (M. Morgenstern, H. Zheng, M. Ross, & O. Campanella, 1999), so only 3 passes were used to prepare the sheeted dough. Unless otherwise stated, the dough was allowed to rest for 15min on aluminum foil and then placed on the test support possessing an aperture diameter of 30mm. The dough was fixed between two plastic plates with 4 metallic pins. The extension of the dough was performed with a cylindrical probe having a diameter of 19 mm. Every test lasted less than two minutes, therefore the effects of moisture evaporation from the surface were considered negligible.

Dough samples were mounted, flash-frozen in liquid nitrogen slush, and cryo-transferred into the preparation chamber of a Gatan Alto 2500 system set at -185°C (Pleasanton, CA, USA). Cryotomed sections of the frozen dough samples were then transferred to the cryo stage and moisture was sublimated at -90°C. Samples were imaged until a visible structure was observed and then returned to the Gatan cryo preparation chamber for 120 seconds of sputter coating using a platinum target. Images of coated samples were taken at -140°C.

3.3.6 Statistical methods

At least three replicates were performed for every analysis. Values are reported as average ± standard deviation. Significant differences ($p \leq 0.05$) among samples were calculated by one-way-analysis of variance (ANOVA) with a Tukey-high significant difference test.

3.4 Results and discussion

3.4.1 Solution properties

The first challenge to address in preparing electrospun fibers of mixed materials was the identification of appropriate solvents. As a prolamin, there are numerous solvents and solvent mixtures with moderate dielectric constant that are suitable for complete solvation of zein (Evans & Manley, 1941; Manley & Evans, 1943). However, few of these solvents are food-grade materials, and the most common solvents utilized for zein isolation and solubilization are acetic acid or aqueous mixtures of ethanol. All plasticizers and proteins chosen for this study were soluble in glacial acetic acid. Plasticizers were fully soluble in 90% aqueous ethanol, although separation of oleic acid as a thin upper layer was observed during storage. Further mixtures with plasticizers were prepared with 27% zein (w/w) and 2.5% plasticizer. Casein, whey protein, and rice protein were insoluble in 70-90% aqueous ethanol but were soluble at a 2% concentration in a 7:3 mixture of ethanol and 0.1M NaOH aqueous solution. In order to maintain consistent protein content all mixtures containing co-proteins were prepared with 25% zein and 2% casein, whey, or rice proteins .

Solution viscosity in the range of 0.1-2 Pa·s is ideal for production of fibers using electrospinning (H. Zhang et al., 2018), with lower viscosities promoting bead formation (Fong, Chun, & Reneker, 1999). Shear viscosity of zein solutions and mixtures with plasticizers or co-proteins were all found to be within the acceptable limits for fiber production by electrospinning (**Table 3**). The shear viscosity of zein solutions in acetic acid and 90% ethanol were slightly lower than previously reported values of 1 Pa·s and 0.4 Pa·s, respectively (Gordon W Selling, Biswas, et al., 2007), which could be attributed to batch-specific variations (G. W. Selling et al., 2005). No effects of incorporated plasticizers on viscosity were observed in 90% ethanol solutions, while viscosities of acetic acid solutions slightly decreased by addition of glycerol and oleic acid. Incorporation of casein, whey protein, or rice protein increased shear viscosity in both solvents, indicating co-protein solubilization and contribution to solution viscosity. Preliminary attempts to incorporate amounts greater than 2% of whey protein or casein in the solvents led to gel formation (data not shown) and were not studied further.

Another important solution property defining fiber formation in electrospinning is surface tension (S. Q. Wang, He, & Xu, 2008). Solutions with high surface tension favors bead formation, whereas reduced surface tensions favors fiber formation (Fong et al., 1999). Zein solutions showed slight variations in surface tension based on the solvent used, although all values were within the range of 28-31 mN/m. Similar surface tension values were observed in zein solutions that were successfully spun into fibers (Gordon W Selling, Biswas, et al., 2007), so all of the prepared samples were considered acceptable. At 25ºC, reported surface tension of acetic acid is 27 mN/m (Álvarez, Vázquez, Sánchez-Vilas, Sanjurjo, & Navaza, 1997), while surface tension of 70% and 90% aqueous ethanol (v/v) approximately 24.4 mN/m and 22.5 mN/m, respectively, based upon prior reports (Vazquez, Alvarez, & Navaza, 1995). The addition of plasticizers and co-proteins decreased the surface tension of the aqueous ethanol solutions but increased the surface tension of the acetic acid suspensions. The decrease of surface tension for the proteins and oleic acid in ethanol-water mixtures could be attributed to their surface activity(Dee, Puleo, & Bizios, 2002). Decreased surface tension of ethanol-water interfaces was unexpected for glycerol, which has a relatively high surface tension as a pure component and has been shown to increase the surface tension in ethanol mixtures (Alkindi, Al-Wahaibi, & Muggeridge, 2008). As expected, surface tension values of zein-acetic acid mixtures increased most in the presence of the polar plasticizers, glycerol, and lactic acid.

These measurements showed that all the solutions analyzed had acceptable range of shear viscosity and surface tension for successful electrospinning. It should be noted that centrifugation of mixtures revealed that a portion of samples with co-proteins were insoluble during storage, and this may have limited the impact of co-proteins on viscosity and surface tension. Precipitate content after centrifugation represented approximately 75% of added rice protein or casein in acetic acid mixtures, ~40% of added whey protein in acetic acid mixtures, and ~60% of added rice protein in aqueous ethanol mixtures (**Table 2**). Casein and whey protein remained fully soluble in aqueous ethanol mixtures with zein during storage. Regardless, sedimentation of co-proteins during high-speed centrifugation did not translate into any difficulties in the electrospinning process, and fibers were successfully formed for all samples.

3.4.2 Morphology of Electrospun fibers

Scanning electron microscopy was used to determine the morphology of fibers prepared by electrospinning zein dissolved in 90% and 70% aqueous ethanol and acetic acid solvents (**Figure 9**). In all samples prepared with 90% ethanol, there was a prevalence of ribbon structures rather than fibers. Ribbon formation has been observed in prior studies of zein spun from aqueous ethanol solutions (Gordon W Selling, Biswas, et al., 2007; Wen, Yang, Yu, Li, & Zhang, 2016). Ribbon formation during electrospinning has been attributed to fast evaporation of solvent, which causes formation of a skin in formed jets and subsequent collapse at atmospheric pressure (Koombhongse, Liu, & Reneker, 2001). Zein spun with acetic acid formed round fibers that was unaffected by incorporation of lactic acid, whereas those containing oleic acid formed a mix of round fibers and broad ribbons. Diameter of fibers containing glycerol or oleic acid using acetic acid as solvent were approximately three times larger than those containing lactic acid or with only zein (**Figure 9 and 10**). Continuing the logic of ribbon formation being ascribed to the collapse of jets formed during spinning, oleic acid incorporation could have promoted skin formation of the zein jets. These results are in general agreement with prior observations of fibrous morphologies for zein spun from acetic acid (Yixiang Wang & Chen, 2012)

Zein spun using 70% ethanol-0.1M NaOH as a solvent only formed round fibers (**Figure 9B**) in the presence of casein, whey protein, and rice protein. Change in morphology to fibers rather than ribbons was likely due to the use of a 0.1 M NaOH aqueous solution rather than pure water in the ethanol mixtures. Greater alkalinity of the solution would increase the relative negative charge of zein due to deprotonation of the amine residues. Charge density is an important parameter to define fiber diameter during spinning (C. Thompson, Chase, Yarin, & Reneker, 2007) and could have decreased tendency to form a skin on the jet and form ribbons during the spinning process. Mixtures of 70% ethanol with 0.1 M NaOH have been shown to lead to partial hydrolysis and deamidation of zein after extended exposure (Cabra, Arreguin, Vazquez-Duhalt, & Farres, 2007), potentially contributing to greater solubility and more negative charge. However, the alkaline conditions used in this study were relatively weak, and the contribution of hydrolysis and deamidation were likely to be minor. Small beads could also be seen in these spun mats, and an image was chosen to include such a bead even though fibers were predominant.

No significant change in fiber diameter or shape was observed with addition of co-proteins among samples prepared from acetic acid or 70% ethanol-0.1M NaOH solutions (**Figures 9B**).

However, beads were observed that were similar in shape but much larger than those in the pure zein fibers prepared with 70% ethanol-0.1M NaOH. These beads were more frequently observed in samples electrospun with co-proteins, while no beads were observed in samples containing plasticizers. Increased bead formation with co-proteins agreed with a recent study involving electrospinning of zein with casein (Y. H. Wang et al., 2019) and suggested that some proteins were not fully incorporated in the fibers formed during the electrospinning process. As it was not feasible to physically separate beads from fibers or to analyze composition *in situ*, the identity of the beads was not verified.

3.4.3 Plasticizer and co-protein incorporation

Infrared spectroscopy was used to verify the presence of zein and plasticizers in electrospun fibers (**Figure 11**). All samples presented typical spectral signatures of zein (F. Li et al., 2017). Specifically, the peak at 1642 cm^{-1} with highest intensity which was attributed to amide I (C=O stretch), while the peak observed at 1514 cm^{-1} was attributed to amide II (N-H and C-N stretch) (Ying Li, Lim, & Kakuda, 2009). A third major peak at 3281 cm^{-1} can be attributed to hydroxyl groups (Wongsasulak et al., 2010a). Samples with incorporated glycerol displayed a new peak at 1045 cm^{-1} that could be attributed to C-O bending, a signature that has previously been used to identify glycerol incorporation within zein (Wongsasulak et al., 2010a). Samples with incorporated lactic acid also showed a small peak at 1044 cm^{-1} and a stronger peak at 1128 cm^{-1}, attributable to C-O of lactic acid (Păucean et al., 2017). Stretching vibrations at 2927 and 2854 cm^{-1} are attributable to $-CH_2$ and $-CH_3$ functional groups found in all materials of this study (**Figure 11A, 11D**). However, relative intensity of these peaks increased with incorporation of oleic acid due to the high density of methylene groups in the fatty acid chain (Wu et al., 2016). The shoulder reported between 1720-1760 cm^{-1} can be attributed to C=O stretching of carboxylic acid groups, which increased in samples containing lactic acid and oleic acid (**Figure 11B, 11E**). Therefore, the infrared spectra demonstrated the presence of glycerol, lactic acid and oleic acid within the electrospun fiber mats.

Gel electrophoresis was performed to identify which proteins were present in the fiber mats (**Figure 12**). As expected, Etoh-B and Acoh-A exhibited 2 major bands corresponding to zein (22 and 24 kDa), which has been reported in other studies (Sly et al., 2014). Samples prepared from mixtures with casein exhibited one or two new bands of greater molecular weight than zein when prepared from acetic acid or 70% ethanol-0.1M NaOH, respectively; these bands were attributed to α- and β-caseins based upon similar gel migration patterns observed in prior studies (Chen, Chen, & Hsieh, 2015). Similarly, when whey proteins were incorporated, two bands appeared above and below the 15kDa marker that could be attributed to β-lactoglobulin (18.2 kDa) and α-lactalbumin (14.2 kDa) (Basch, Douglas, Procino, Holsinger, & Farrell, 1985). There was no evidence of new bands observed in samples containing rice protein, although rice protein should have displayed various bands with molecular weights of ~15 kDa, 20-25 kDa, and 38-52 kDa (Agboola, Ng, & Mills, 2005). We can then conclude that casein and whey proteins were successfully electrospun and present within the mats, while no evidence was found for successful incorporation of rice proteins. This finding verifies a recent study showing incorporation of casein in electrospun zein fibers (Y. H. Wang et al., 2019). This is the first report of successfully electrospinning whey proteins with zein from the same solution.

3.4.4 Thermo-mechanical properties of fibers

Glycerol, lactic acid and oleic acid are proven plasticizers of zein films and resins (J. Lawton, 2004; Wei & Baianu, 1999). In order to determine the impact of their presence, the T_g of the electrospun fibers was determined (**Table 3**). The T_g of fibers prepared with acetic acid was comparable to other reports (186-190ºC) (Di Gioia, Cuq, & Guilbert, 1999). Fibers produced from a 90% ethanol solution were too brittle to be measured by dynamic mechanical analysis and were instead analyzed by differential scanning calorimetry. The T_g of fibers produced from 90% ethanol was 12ºC less than fibers produced with acetic acid, which was consistent for similar fibers analyzed by calorimetry (Di Gioia et al., 1999). The incorporation of glycerol, lactic and oleic acids all decreased T_g of fibers prepared with acetic acid, and the T_g depression using glycerol matched prior reports for glycerol-plasticized zein electrospun fibers (Wongsasulak et al., 2010a). Depression of T_g was also observed among fibers prepared in 90% ethanol after incorporation of glycerol, lactic acid, and oleic acid. These results confirm the incorporation of plasticizers into the electrospun fibers by demonstrating their ability to plasticize the zein material.

To test the impact of plasticizers on individual fibers produced with acetic acid, Young's moduli of each fiber was determined by nanoindentation in an AFM instrument. All electrospun fibers showed hysteresis between indentation and retraction typical of a plastic material (**Figure 13**), as well as deformation of the surface after nanoindentation measurements (**Figure 8**).

Accordingly, Young's moduli were determined from indentation curves with the Oliver-Pharr model, and determined moduli are presented in **Figure 14**. Distribution of measured Young's moduli of zein fibers without added plasticizers was monomodal with a Gaussian average of 597±154 MPa. This value is greater than that reported in a prior study with electrospun zein fibers (30-200MPa) (C. Yao et al., 2007) but within the range of values commonly reported for zein-based films (10^0-10^3 MPa) (J. Lawton, 2004). Incorporation of lactic acid or oleic acid decreased the mode of Young's moduli below 500 MPa, but distributions were relatively skewed with a tail to the right. A large number of observations among fibers with lactic acid or oleic acid still possessed Young's moduli of ~600 MPa, comparable to the Gaussian average of non-plasticized zein fibers. A greater number of observations at ~300 MPa and ~600 MPa suggested two distinct populations, with the former representing more populous regions of plasticized zein fibers and the latter representing regions of fibers with relatively minimal plasticizer content. Such bimodality in the distribution was more apparent for fibers containing oleic acid (**Figure 14c**). Inhomogeneous distribution of oleic acid in fibers could be partially attributed to the observed incompatibility, which has been observed in prior studies of oleic acid in composite zein films (F. B. Santosa & Padua, 2000).

Thermomechanical properties of zein fibers with added co-proteins were not evaluated because the increased number of beads indicated that co-protein was not well-distributed within the fibers (**Figure 9**). A prior study indicated that added casein has minimal impact on the glass transition temperature of zein, although it did increase Young's moduli of wetted resins (D. P. Erickson et al., 2014). Further studies are needed to determine whether these relatively hydrophilic proteins could have greater impact on zein fibers in higher moisture conditions, such as during wetting of fiber-based materials.

In summary, this study demonstrated successful electrospinning of zein fibers with non-prolamin proteins and plasticizers by careful selection of solvent conditions. Glycerol, lactic acid, and oleic acid could all be incorporated within electrospun zein fibers using 90% aqueous ethanol or acetic acid as solvents. Incorporation of the plasticizers within the zein fibers was supported by recognized signatures in infrared spectroscopy measurements, depressed T_g values, and reduced moduli of individual fibers (containing oleic and lactic acid). Mixtures of zein with casein, whey protein, and rice protein could also be electrospun using acetic acid or 70% aqueous ethanol with sodium hydroxide, although gel electrophoresis could not verify rice protein in electrospun mats.

Because of the large spheroidal structures observed in samples containing co-proteins, distribution of casein or whey protein in electrospun zein fibers and their impact on physical properties needs further exploration, particularly in moist conditions where the proteins could enhance absorption. The demonstrated capacity to electro-spin zein fibers in the presence of plasticizers and co-proteins offers new approaches to alter physical behaviors of zein fibers.

3.4.5 Incorporation of zein electrospun fibrils into a dough

Extension test of zein dough with 5% (w/w) plasticizer addition are reported in **figure 16A**. When zein dough was mixed at room temperature, during extension it showed an early breakage at strain close to 2, symptom of poor extensibility. These results are expected as it was previously observed that zein viscoelasticity is generated only when a dough is formed above the *Tg* of zein (J. W. Lawton, 1992a). Similarly, to the zein control, the addition of glycerol and oleic acid did not show any marked improvement in dough extensibility (**figure 16A**), but only a decrease in peak force. On the other hand, the addition of lactic acid (**figure 16A**) induced a sharp increase in dough extensibility, concomitantly with the observation of the generation of oriented fibrils during the dough extension. These results are in agreement with what was previously observed in other studies on addition of organic acid to zein based doughs (Sly et al., 2014).

In **figure 16B** the extension test of doughs where zein was replaced with electrospun fibrils produced with different plasticizers are reported. Addition of electrospun fibrils did not show any improvement on the rheological properties of the dough, conversely a similar behavior like untreated zein was observed. Similarly, the addition of fibrils plasticized with glycerol did not yield any substantial improvement. On the other hand, fibrils incorporating lactic acid and oleic acid showed a marked increase in extensibility, however the dough presented a marked strain thinning behavior rather than strain hardening. We can speculate here that the presence of organic acids, and in particular the presence of a carboxylic acid group (**figure 16C**), favor plasticizer-zein interactions, altering zein molecular structure and allowing the formation of fibrillar architectures even at room temperature. Surprisingly, oleic acid showed improvement only when incorporated into electrospun fibrils, contrary to the addition of oleic acid to zein. The reason can be ascribed to the addition of oleic acid to zein in solution with acetic acid; zein structure in acetic acid is more open, facilitating the interaction between zein and oleic acid. This behavior of oleic acid showed analogies to what was observed by Padua for incorporation of oleic acid in zein films, where heating of zein-ethanol solutions was necessary to allow oleic acid to bind to zein (Huey-Min Lai & Padua, 1997).

Electron micrograph of dough with the addition of zein and electrospun fibrils is reported in **figure 17**. All dough presented a recurrent structure, where granules of starch were surrounded by a continuous protein matrix. Although, we can observe that both zein dough and dough prepared from the addition of electrospun fibrils did not show the presence of fibrillar structure. These results suggest that during hydration and mixing, the fibrillar morphology imparted by electrospinning was largely lost. Due to the ephemeral stability of electrospun fibrils against hydration and mixing it was not possible to unequivocally establish if fibrillar morphologies could have yielded an improved viscoelastic behavior. An hypothesis to explain fibrils instability can be found in the lack of stabilization of zein at a molecular level, when it is kept at a temperature below its glass transition (Mejia et al., 2007). In conclusion addition of electrospun fibrils to dough did not result in significant improvement of rheological properties. On the other hand, the addition of plasticizers, in particular plasticizers with a carboxylic acid groups were able to stabilize zein extensibility even at room temperature.

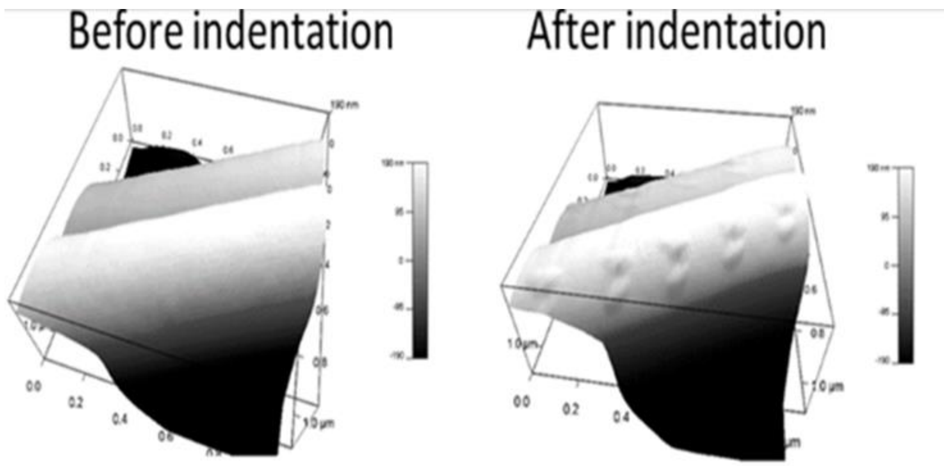


Figure 8 Surface topography of zein fibers electrospun from acetic acid solution before and after a series of indentation measurements. Material displacement on the fiber surface after indentation demonstrates the plasticity of the sample and proves significant penetration of the material during indentation measurements.

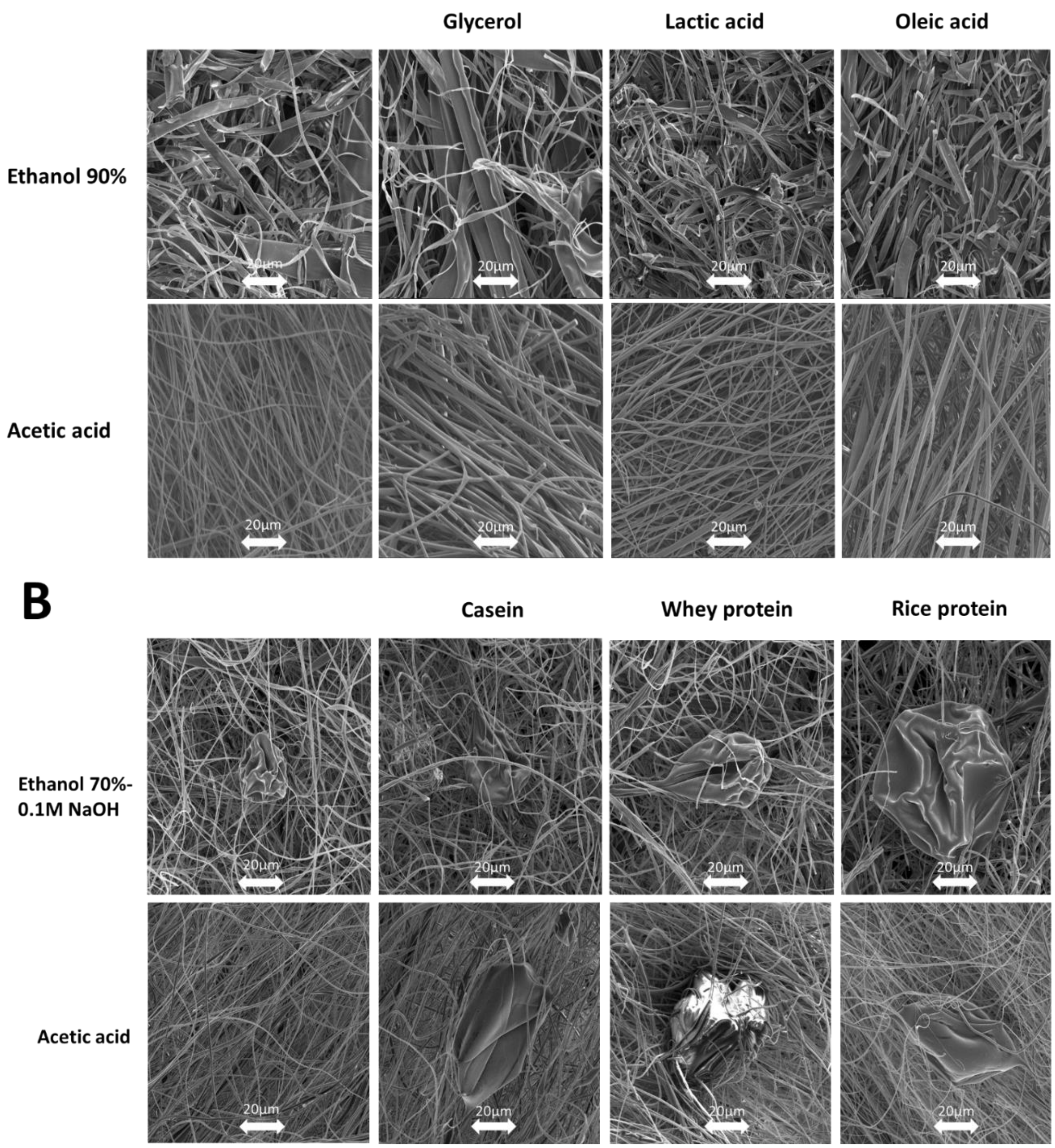
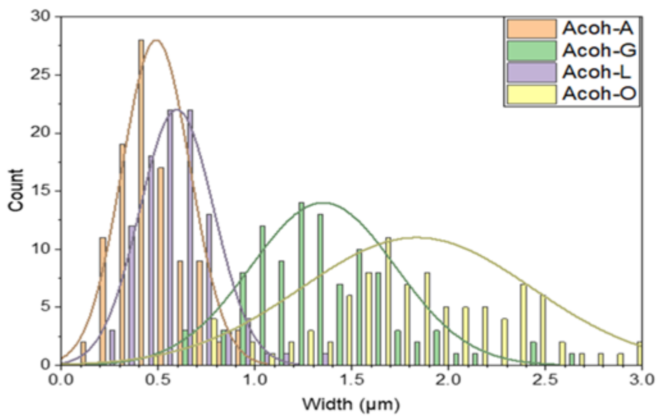
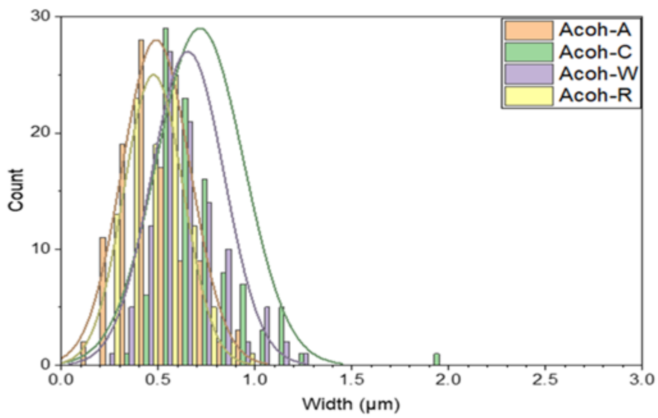


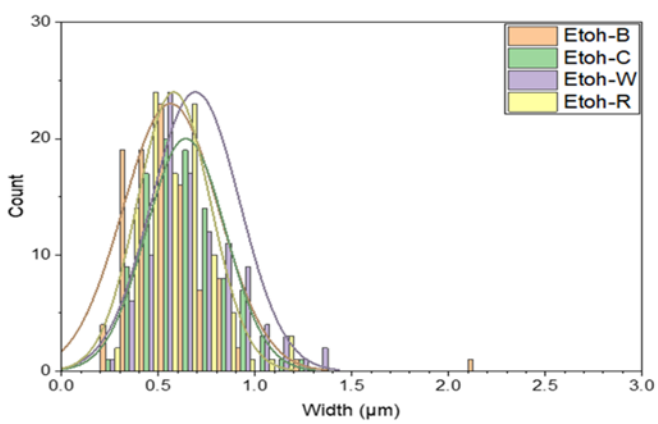
Figure 9 A) Scanning electron micrographs of zein electrospun fibers with specified plasticizers using 90% aqueous ethanol or acetic acid as solvent B) Scanning electron micrographs of zein electrospun fibers with specified co-protein using a 7:3 ethanol: 0.1M



Sample	Fibers diameter (nm)
Acoh-A	$0.488^{cd}\pm0.181$
Acoh-G	$1.351^{c}\pm0.374$
Acoh-L	$0.595^{cd}\pm0.192$
Acoh-O	$1.805^{a}\pm0.595$



Sample	Fibers diameter (nm)
Acoh-A	$0.488^{cd}\pm0.181$
Acoh-C	$0.716^{d}\pm0.225$
Acoh-W	$0.652^{bcd}\pm0.167$
Acoh-R	$0.475^{d}\pm0.153$



Sample	Fibers diameter (nm)
Etoh-B	$0.562^{bcd}\pm0.249$
Etoh-C	$0.641^{bcd}\pm0.203$
Etoh-W	$0.691^{bc}\pm0.228$
Etoh-R	$0.579^{bcd}\pm0.187$

Figure 10 Figure S2. Measured width of zein fibers spun from acetic acid or aqueous ethanol from SEM images. Abbreviations for samples can be found in Table 1 or Table S1. Lines shown present fittings to a Gaussian distribution.

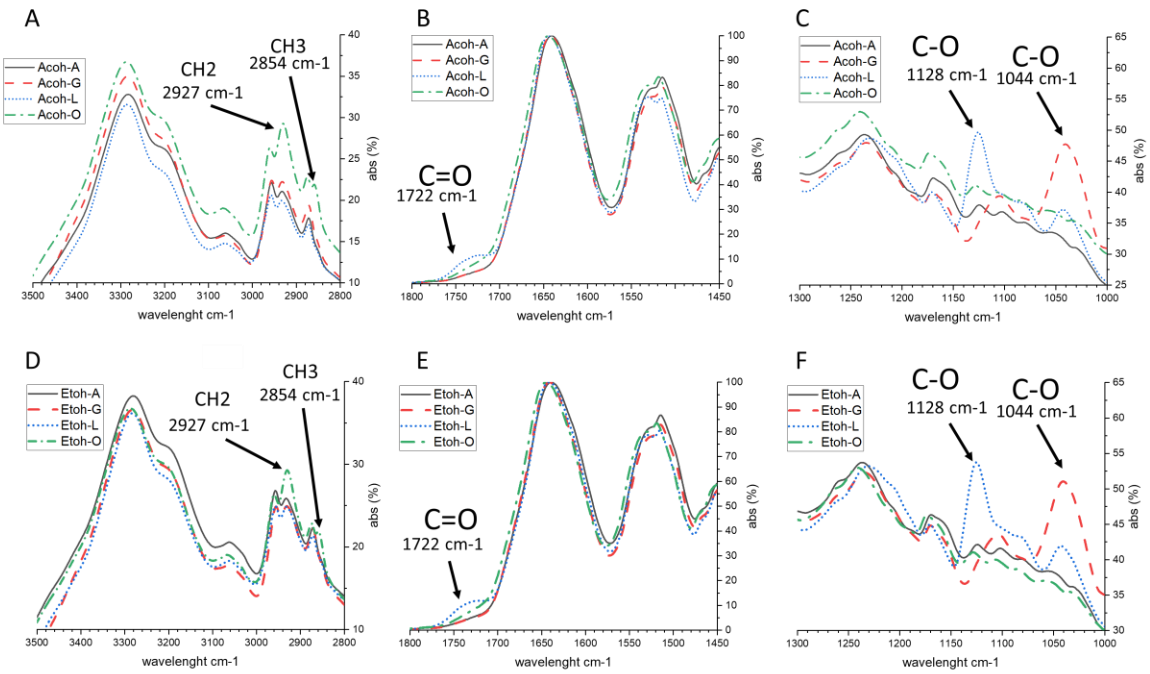


Figure 11 Infrared spectra of zein mats electrospun with or without plasticizers using (a-c) acetic acid (Acoh) or (d-f) 90% aqueous ethanol (Etoh) solvents and highlighting wavenumbers: (a,d) 2800-3500 cm-1, (b,e) 1450-1800 cm-1, and (c,f) 1000-1300 cm-1. Drawn arrows specify peaks attributed to characteristic vibrations that are further described in-text. Use superscripts in all cm-1

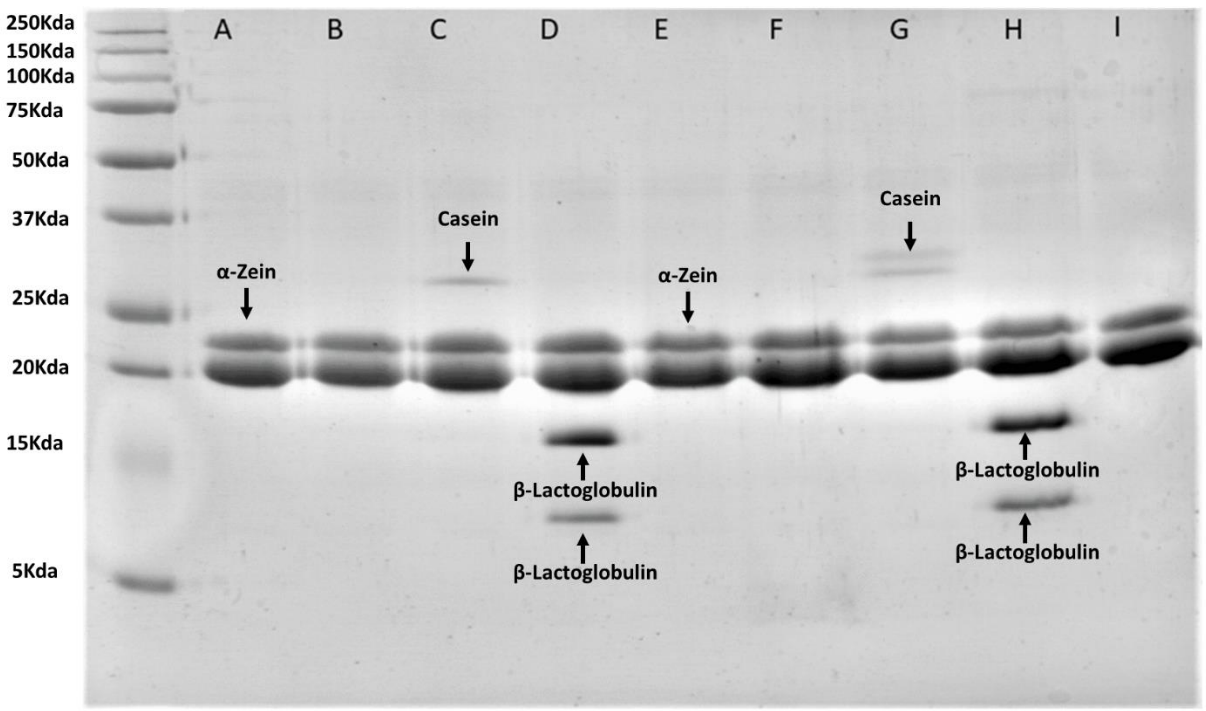


Figure 12 SDS-PAGE gel of zein, zein fibers, and zein fibers spun with co-proteins. Lane descriptions: A) zein, B) Acoh-A, C) Acoh-C, D) Acoh-W, E) Acoh-R, F) Etoh-B, G) Etoh-C, H) Etoh-W, I) Etoh-R. Drawn arrows specify protein identities further discussed in-te

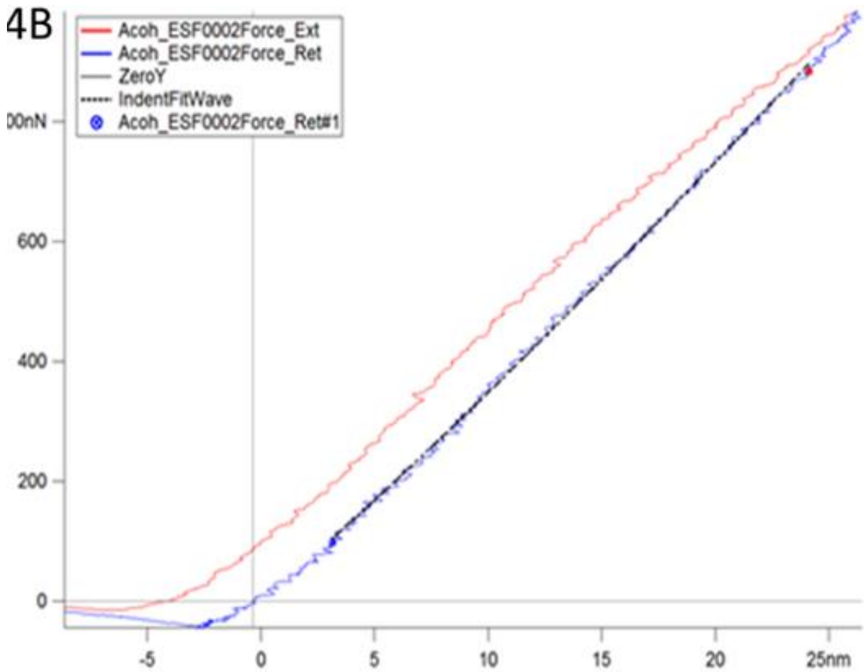


Figure 13 Example nano-indentation plot of zein fiber spun from acetic acid solution displaying hysteresis typical of plastic materials.

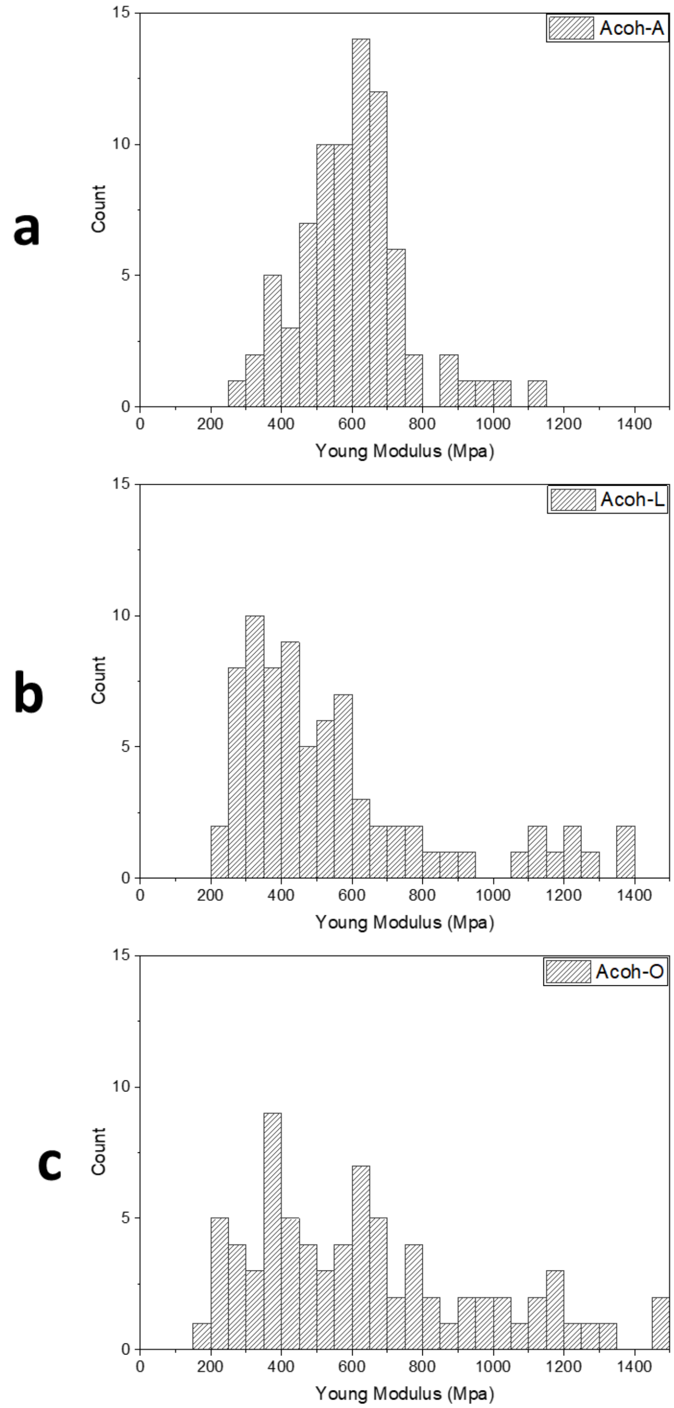


Figure 14. Young’s moduli from nanoindentation measurements of zein fibers electrospun from acetic acid solutions (a) alone, (b) with lactic acid, or (c) with oleic acid

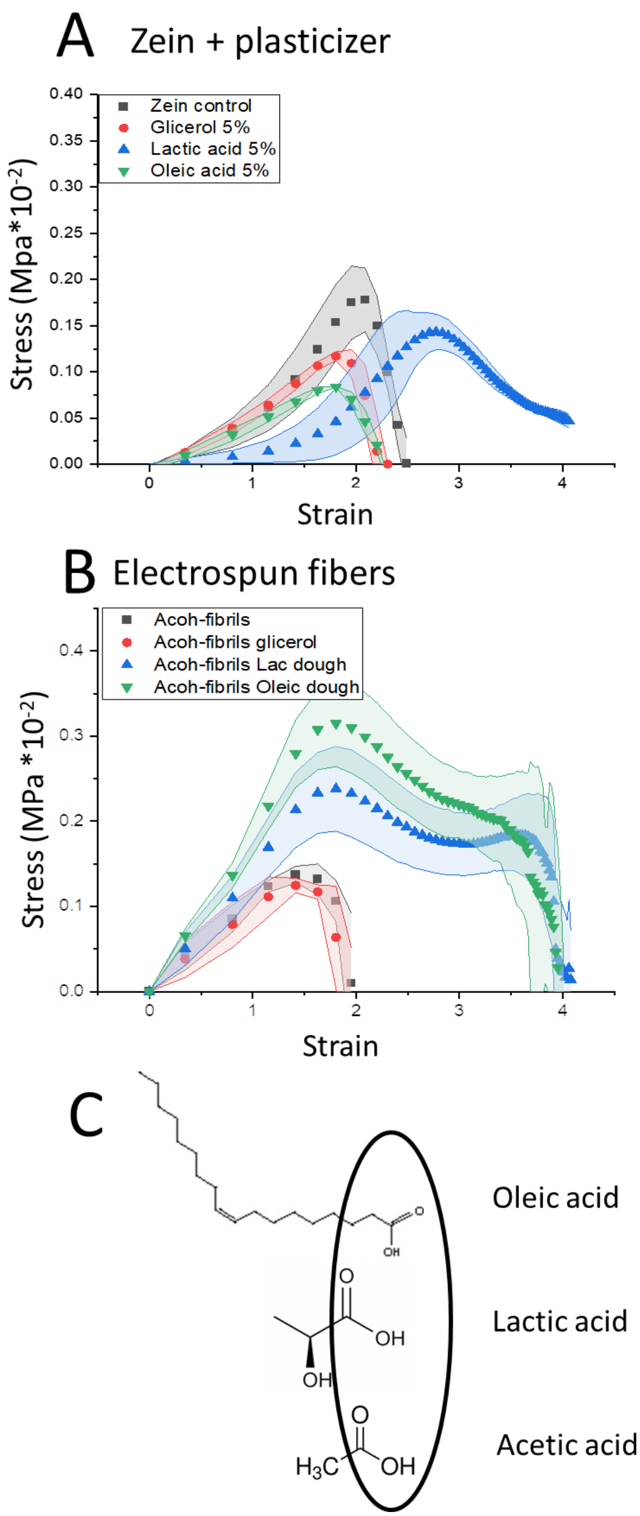


Figure 15 A) Extension test of zein dough with 5% plasticizer addition w/w, Extension test of dough incorporating zein electrospun fibrils, C) molecular structure of plasticizer stabilizing viscoelasticity of zein at room temperature.

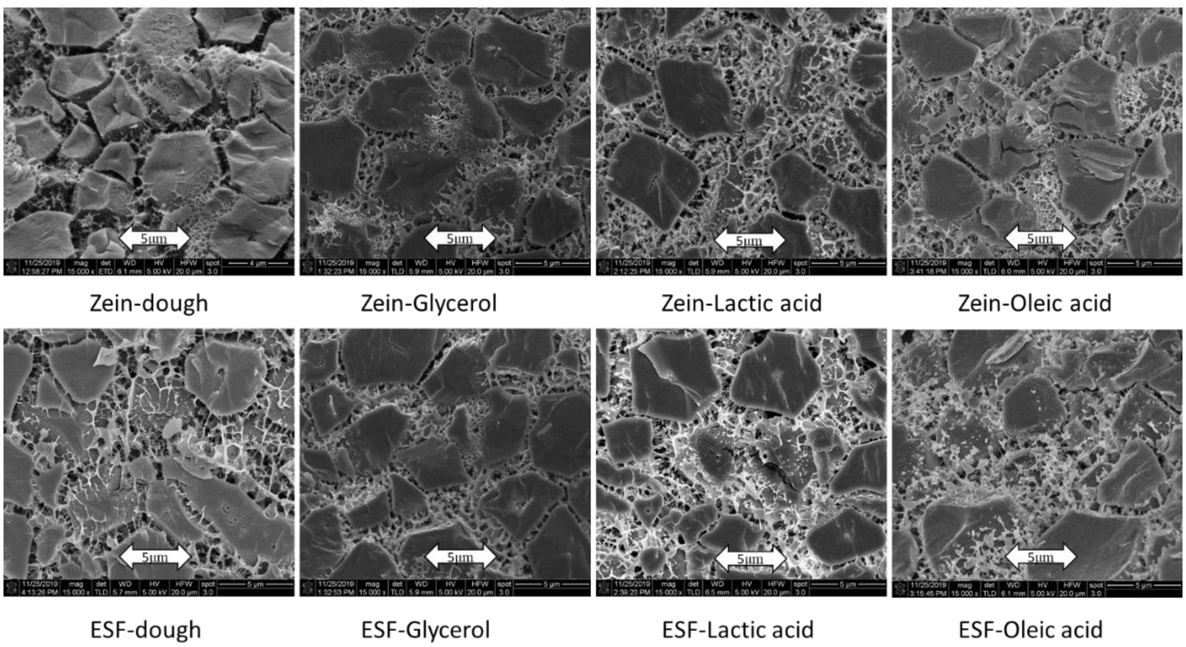


Figure 16 Micrograph of zein dough with addition of plasticizer (top) Dough incorporating electrospun fibrils with plasticizer incorporated before spinning (bottom)

Abbreviations and Nomenclature

Tg: Glass transition temperature

Table 2 Identity of solvents and additives used to prepare samples for electrospinning, including abbreviated names for each sample.

Abbreviation	Solvent	Additive*
Acoh-A	Acetic acid	None
Acoh-G	Acetic acid	Glycerol
Acoh-L	Acetic acid	Lactic acid
Acoh-O	Acetic acid	Oleic acid
Acoh-C	Acetic acid	Casein
Acoh-W	Acetic acid	Whey protein
Acoh-R	Acetic acid	Rice protein
Etoh-A	Aqueous Ethanol, 90%	None
Etoh-G	Aqueous Ethanol, 90%	Glycerol
Etoh-L	Aqueous Ethanol, 90%	Lactic acid
Etoh-O	Aqueous Ethanol, 90%	Oleic acid
Etoh-B	Ethanol / NaOH soln**	None
Etoh-C	Ethanol / NaOH soln**	Casein
Etoh-W	Ethanol / NaOH soln**	Whey protein
Etoh-R	Ethanol / NaOH soln**	Rice protein

** Additives include plasticizers (glycerol, lactic acid, oleic acid) and proteins (casein, whey protein, rice protein) both dispersed at initial contents of 10% (w/w) and 7.4% (w/w), respectively, in relation to zein.*

*** Solvent comprises a 7:3 volumetric mixture of ethanol and 0.1M NaOH aqueous solution*

Table 3 Shear viscosity and surface tension of zein sample solutions prepared with or without additives.

Sample	**Viscosity (Pa.s)***
Acoh-A	$0.688^{d} \pm 0.005$
Acoh-G	$0.643^{fe} \pm 0.022$
Acoh-L	$0.672^{de} \pm 0.016$
Acoh-O	$0.626^{fe} \pm 0.007$
Acoh-C	$0.867^{a} \pm 0.021$
Acoh-W	$0.788^{b} \pm 0.014$
Acoh-R	$0.749^{c} \pm 0.001$
Etoh-A	$0.197^{jhi} \pm 0.06$
Etoh-G	$0.238^{h} \pm 0.023$
Etoh-L	$0.229^{hi} \pm 0.027$
Etoh-O	$0.213^{jhi} \pm 0.023$
Etoh-B	$0.156^{k} \pm 0.004$
Etoh-C	$0.196^{ji} \pm 0.005$
Etoh-W	$0.179^{jk} \pm 0.006$
Etoh-R	$0.208^{jhi} \pm 0.005$

** Subscripts with different letters next to the data represent values that were significantly different within each column*

Table 4 Surface tension of zein sample solutions prepared with or without additives.

Sample	**Surface tension (mN/m)***
Acoh-A	28 ± 1
Acoh-G	31 ± 1
Acoh-L	30 ± 1
Acoh-O	30 ± 1
Acoh-C	27 ± 1
Acoh-W	28 ± 1
Acoh-R	27 ± 1
Etoh-A	29 ± 1
Etoh-G	29 ± 1
Etoh-L	28 ± 1
Etoh-O	28 ± 1
Etoh-B	30 ± 1
Etoh-C	29 ± 1
Etoh-W	28 ± 1
Etoh-R	28 ± 1

Table 5 Glass transition temperatures of electrospun fibers

Sample	**Method of determination**	**Glass transition (*Tg*)**
Etoh-A	DSC	$174.4^{b} \pm 0.6$
Etoh-G	DSC	$166.7^{cd} \pm 2.9$
Etoh-L	DSC	$163.3^{de} \pm 0.2$
Etoh-O	DSC	$159.9^{e} \pm 1.4$
Acoh-A	DMA	$186.1^{a} \pm 1.4$
Acoh-G	DMA	$162.2^{de} \pm 1.2$
Acoh-L	DMA	$173.9^{bc} \pm 3.1$
Acoh-O	DMA	$167.9^{cd} \pm 3.4$

** Subscripts with different letters next to the data represent values that were significantly different*

CHAPTER 4. EFFECT OF ZEIN EXTRUSION AND STARCH TYPE ON THE RHEOLOGICAL BEHAVIOR OF GLUTEN-FREE DOUGH

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Federici, E., Jones, O. G., Selling, G. W., Tagliasco, M., & Campanella, O. H. (2020). Effect of zein extrusion and starch type on the rheological behavior of gluten-free dough. Journal of Cereal Science, 91, 102866.

4.1 Abstract

Previous works showed that zein, above its glass transition temperature may adopt molecular structures able to form doughs with viscoelastic properties comparable to those of wheat gluten. It is hypothesized that extrusion can promote molecular changes in zein and favor interactions with starches that enhance dough viscoelasticity. Thus, the effects of extruding zein at 90-160°C on the rheological properties of doughs prepared with potato, rice, and maize starches were determined.

Formulations were optimized to provide similar mixing profiles to that of a standard wheat dough. For all zein-types, creep-recovery tests demonstrated that doughs prepared with maize and potato starches were less elastic, when compared to doughs with rice starch, which were comparable to wheat-dough. Extensional tests showed that zein extruded at 160°C provided a larger increase in strain-hardening behavior, which is important for bread production. These samples also exhibited larger extensional stresses. Gel electrophoresis of zein extruded at 160°C revealed an increase in protein aggregates and smaller peptides when compared to samples subjected at lower extrusion temperatures. Scanning electron micrographs of doughs containing zein showed starch granules embedded within an amorphous material and fibrous structures attributable to elongated zein.

4.2 Introduction

Celiac disease is an inflammatory condition of the small intestine caused by an autoimmune reaction after ingestion of gluten and similar proteins from barley and rye in genetically susceptible individuals (Miñarro, Normahomed, Guamis, & Capellas, 2010). Currently the only solution for celiac patients is the avoidance of gluten. An increase in the disease incidence has been reported in many countries (Murray et al., 2003). Thus, there is a pressing need to provide gluten free proteins from cereal-based products because the prolamin protein fractions of wheat, barley and rye are the trigger of Celiac disease. Furthermore, there is also a need to develop non-wheat and low-wheat bread formulations in developing countries of Asia and Africa where wheat is not a locally produced crop and its use depends on imports.

The production of gluten free products has been a major problem for bakers. A frequent issue is given by the dough consistency, indeed most of gluten-free bread is produced from batters rather than doughs, generating issues in dough handling and shaping products. This is particularly true for leavened products, such as bread, mainly due to their structural complexity that affect texture, and the impact of the dough viscoelastic properties on the bread final quality. The reason is because gluten proteins play a key role in determining the unique baking quality of wheat flour by conferring water absorption capacity, gas retention, cohesivity, viscosity and viscoelasticity on the dough used to prepare the bread. For a long time, among other cereal proteins, only wheat gluten has been considered suitable to form viscoelastic doughs with acceptable properties for bread production. It is hypothesized that the ability of gluten proteins to form fibrillar/amyloid structures provide doughs with the capacity of retain gases in leavened products (J. R. Taylor, J. Taylor, O. H. Campanella, & B. R. Hamaker, 2016).

Previous studies have demonstrated the ability of the maize protein, zein, to behave comparably to gluten in dough formulations because it can form a viscoelastic dough when mixed at a temperature above its glass transition (J. W. Lawton, 1992a). However, the appropriate level of strain hardening is generally lacking in zein based doughs. This is hypothesized to be an important factor for bread quality, in fact strain hardening behavior is considered a fundamental rheological attribute associated with dough gas retention during proofing (T Van Vliet et al., 1992). In fact, when zein alone is used, poor quality breads are produced and other additives, such as hydroxypropyl methylcellulose (HPMC) which can interact with zein by coating it, are required to produce acceptable breads (Tilman J Schober et al., 2008).

Alternatively, some studies have shown that extrusion can modify the molecular structure of zein(Gordon W Selling & Utt, 2013). For instance, extrusion of zein above 160°C has been found to increase the molecular weight significantly (Selling, 2010). Concerning the present work, it is hypothesized that the applied shear in the extrusion process at temperatures under which the protein is in a molten state may favor the formation of fibrils and aligned structures. Zein fibril may have a positive impact on the dough rheological properties and consequently in the development of strain hardening behavior. At extrusion temperatures above 180°C and long residence times, zein may degrade and change color so that it may no longer be suitable for food applications.

While the protein fraction plays a fundamental role in the dough viscoelastic properties, starch may also have an important role in bread making. For instance, it can interact with the proteins present in the formulation and affect viscosity development of the bread formulation upon gelatinization (Abdel-Aal, 2009). Starch, the main component in cereal flours, significantly affects the properties of dough such as water absorption and viscoelasticity. Starch also has a fundamental role in the formation of the bread crumb. Multiple sources of starches have been employed in order to produce gluten-free products, such as maize, wheat, tapioca, rice and potato. It has been found that the origin and type of starch impacts microstructure, rheology of the dough, water retention and final structure and quality of the final products (D. Zhang, Mu, & Sun, 2017; Ziobro, Juszczak, Witczak, & Korus, 2016). Nevertheless, starch alone is not able to impart viscoelasticity to dough and needs to be paired with other ingredients, notably proteins, to improve its viscoelastic properties. Other ingredients such as gums (e.g. xanthan gum) have been used as single ingredients to produce gluten free products but their inclusion results in sticky doughs and breads with poor texture. In fact, gluten free breads using gums as ingredients tend to have a low volume, pale crust, crumbly texture, bland flavor and a high rate of staling (O'Shea, Arendt, & Gallagher, 2014)

The objective of this study was to investigate the effects of different types of starches and extruded commercial zein extract on the rheological properties of dough. This study is novel since extruded commercial zein has never been incorporated into dough systems. Consequently, the effects of the extrusion process on zein and the resulting dough rheological properties have not been studied. While the effects of different botanical sources of starch has been investigated in model gluten free dough systems (D. Zhang et al., 2017) their effects have not been studied when they are incorporated in doughs containing extruded zein. An experimental factorial design of 15 total samples, including three different starches (rice, maize and potato) and five different types of zein (non-extruded or extruded with barrel temperatures of 90°C, 120°C, 140°C and 160°C), were employed to determine the effects of zein physical treatment and starch type on the dough rheological properties.

4.3 Materials and methods

Commercial all-purpose wheat flour (Great Value, Walmart) was purchased from a local market. Maize starch was kindly donated by ADM (Decatur, Illinois, USA) , while potato starch (Penpure 10) and rice starch (Novation) were kindly donated by Ingredion incorporated (Bridgewater, NJ, USA). Commmercialextract, composed mainly of α zein with a moisture content of 4.1% and a protein content of 85.2±0.5 was purchased from Flo Chemical (Ashburnham, MA). Ultrapure water (resistivity ≥ 18 mΩ-cm) was obtained from a Barnstead E-pure filtration system (Thermo Scientific, Waltham, MA).

4.3.1 Zein extrusion

200 g of zein powder was added to a Hobart mixer (Troy, OH), and 19.1g of water was slowly incorporated to increase the water content of the zein to 12.5%. After mixing for ~10 min at the low speed setting, the wetted zein mix was extruded in an 18 mm twin-screw extruder (Micro 18, Leistritz, Somerville, NJ) without a die. Four samples were produced where the final section of the barrel was set at temperatures 90, 120, 140 and 160 °C. A screw speed of 150 rpm was used for the 90, 120 and 140 °C treatments. Whereas, for the zein extruded at 160 °C the screw speed was increased to 405 rpm to reduce motor torque (this is indicative of the increased melt viscosity, likely to be produced by an increase in the molecular weight of zein extruded at this temperature – shown in section c) and reduce the product residence in the extruder and polymer degradation. Screw configuration, throughput, torque and temperature profile used can be found in the (table. 6 and 7). After extrusion, the extrudates were collected, ground using a Wiley Mill (Thomas Scientific, Swedesboro, NJ) using a 2 mm screen. The sample was further ground using the Freezer/Mill 6870 (SPEX Sample Prep, Metuchen, NJ) where the samples was placed in a large polycarbonate grinding vial in 40 g aliquots with the metal rod. After precooling (10 min), the sample was shaken at 10 cycles/sec for 2 min and then followed by a 2 min cool down time. After 10 of these cycles, the sample was equilibrated to room temperature, collected and stored in plastic bags. Images of extruded zein are shown in **Figure 17**. Final moisture of zein after extrusion and grinding were: 6.1%±0.09 for extrusion at 90°C; 5.0%±0.13 for extrusion at 120°C; 3.5%±0.14 for extrusion at 140°C and 1.4%±0.15for extrusion at 160°C. These moisture contents were used to obtain doughs with the set moisture contents.

4.3.2 Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed to analyze the size of zein and extruded zein samples. Samples were prepared by adding SDS reducing buffer with tris(hydroxymethyl)aminomethane, stained with bromophenol blue tracking dye, and boiled for 5 min at 100°C. After visual inspection, no lumps or sedimentation were observed. Sample aliquots (10 μL) were then loaded onto a polyacrylamide gel with 1mm thickness composed of a 4% stacking gel and a 12% resolving gel. Runs were conducted at 200V for 60 minutes. After separation, gels were stained with Coomassie Blue R-250. Molecular weights of protein bands were determined using known molecular weight standards (Precision Plus Protein All Blue Standards, Bio-Rad Laboratories, Hercules, CA). Gels were imaged with a GelDoc XR system using trans white illumination (BioRad).

4.3.3 Dough preparation

Gluten-free dough formulations were prepared using pre-made flours containing 15% commercial zein and 85% starch. The different sources of starch and zein, dough final moisture content and sample names are reported in Table 8. The optimal amount of water for each gluten-free formulation was calculated using a mixograph (Swanson-Working from National Mfg. Co., Lincoln, NE, USA), according to the method of Fevzioglu (Fevzioglu et al., 2012) with the exception of potato starch dough which has been calculated using the viscoelastic properties of doughs measured by a creep test and the parameter α that indicates the elasticity of the dough sample (described in section f).The amount of water at 50°C added was 87.5% for the formulation prepared with rice starch and all zein forms, 70% for the mixture prepared with maize starch and all zein forms and 72.5% for the formulation prepared with potato starch and all zein forms. For the wheat flour standard formulation, water added was 63%. All water content are based on flour weight. The optimal amount of water for wheat flour dough was also estimated using a Farinograph (Brabender Farinograph AT) yielding the same result as the mixograph 63%. Mixing of samples was conducted in a 10.0 g mixer from National Mfg. Co. (Lincoln, NE) for 5 minutes in a room conditionated at 35°C. All the ingredients with the exception of water were stored in a room at 35°C for 24h.

4.3.4 Dough properties

The effect of starch on the mixing properties of starch-protein mixtures was determined using a 35-g Swanson-Working mixograph (National Mfg. Co., Lincoln, NE, USA). The spring tension was set at 7 during the operation. Starch (85%, w/w), commercial zein (15%, w/w), and the appropriate amount of water were mixed at 35 °C for 10 min into a dough system. Mixograph properties were evaluated in terms of water absorption, and mixing tolerance as reported by (Fevzioglu et al., 2012). In order to determine the optimum amount of water to add to the mixtures, several trials were made. Lack of water prevented the proper blending of the ingredients and development of the dough elasticity, while an excess of water resulted in a dough with a batter consistency. Mixing tolerance is an indicator of the dough stability during mixing(H. Zheng, M. P. Morgenstern, O. H. Campanella, & N. G. Larsen, 2000). For instance in wheat dough, the strength of the dough tends to decrease with increase in mixing time after an optimum is achieved, an effect attributed to the depolymerization of the protein. Mixing tolerance is traditionally and qualitatively evaluated by the decrease of the mixing torque (dough strength) after peak time, A good wheat flour produces dough with a strength that is maintained and approximately constant after the appearance of the peak (Zheng et al., 2000).

4.3.5 Creep recovery

Creep and recovery measurements were carried out in a rheometer (ARG-2 Model, TA Instruments, Newcastle, DE, USA) using a parallel plate geometry (40 mm diameter plate) at temperatures above the glass transition of the doughs which was determined to be about 35°C for these zein doughs at the set moisture contents, (Chanvrier, Della Valle, & Lourdin, 2006) and 25°C for wheat dough (Kalichevsky, Jaroszkiewicz, & Blanshard, 1992). After mixing, the dough was compressed to a gap of 2mm and excess was trimmed. A solvent trap was used to prevent moisture evaporation. Dough was allowed to rest for 15 min before the analysis started. Strain (%) of the samples was recorded after an applied constant shear stress of 100 Pa for 150 s followed by a recovery period in where no stress was applied for 150 s. Compliance of the sample (J) was determined by the instrument software and described by the rheological model expressed by Eq. 1. The model has three parameters which describe the viscoelastic properties of the dough such as its degree of elasticity (α), the inverse of dough elastic modulus (λ_1), and the inverse of the dough elastic modulus during the creep recovery (λ_2) as described by Spotti (Spotti, Tarhan, Schaffter, Corvalan, & Campanella, 2017). It is important to note that the test rheological parameters using this test can be determined at conditions of large deformations, or stresses, which reflect conditions expected during the handling of the dough. The parameters λ_1 and λ_2 have been associated to the structure of the sample and the difference λ_1- λ_2 provide an indication of the capacity of the sample to retain viscoelastic properties after deformation (Spotti et al. 2017). Application of this analysis to study the viscoelastic behavior of dough samples is straightforward and relevant given the characteristic rheological properties of the doughs tested. The fact that the test can be applied using large deformations and/or stresses is important because the rheological behavior of the dough can be determined at conditions that resembles those at which the dough is processed and handled.

$$J(t)=\frac{1}{\Gamma\left(\alpha+1\right)}\left[\lambda_1 t^{\alpha} H(t)-\lambda_2 (t-t_m)^{\alpha} H(t-t_m\right] \qquad [1]$$

4.3.6 Extension test

Extension tests were performed to determine the extensional properties of dough samples following the method of Morgenstern (Morgenstern et al., 1996). For the tests, a Texture Analyzer HD Plus (Newcastle, DE, USA) with a 50-kg load cell was used. Dough was sheeted in squares of 100mm at a thickness of 3mm using a pasta sheeter. It has been shown that sheeting of wheat dough can promote its development as a dough (M. P. Morgenstern, H. Zheng, M. Ross, & O. H. Campanella, 1999) so to avoid different dough development in the samples, care was taken to use 3 passes to prepare the sheeted dough. The dough was then allowed to rest for 15min in a closed box with air saturated with water and then moved to the test support, which has an aperture of 59mm of diameter. The dough was fixed between two plastic plates with 4 metallic pins. The extension of the dough was performed with a cylindrical probe having a 40mm diameter. Tests were performed in a room conditioned at 35°C.

4.3.7 Scanning electric microscopy

Images of the surface of extruded zein dry powders and different flour composites were obtained with a FEI NOVA nanoSEM Field Emission Scanning Electron Microscope (Hillsborough, Oregon, USA) with an Everhart Thornley detector. Dry powder samples were dispersed on adhesive paper and sputter-coated with platinum for 120 seconds prior to imaging. Dough samples were mounted, flash-frozen in liquid nitrogen slush, and cryo-transferred into the preparation chamber of a Gatan Alto 2500 system set at -185°C (Pleasanton, CA, USA). Cryotomed sections of the frozen dough samples were then transferred to the cryo stage and moisture was sublimated at -90°C. Samples were imaged until a visible structure was observed and then returned to the Gatan cryo preparation chamber for 120 seconds of sputter coating using a platinum target. Images of coated samples were taken at -140°C.

4.3.8 Statistical methods

At least three replicates were performed for every analysis. Significant differences ($p \leq 0.05$) among samples were calculated by one-way-analysis of variance (ANOVA) with a Tukey-high significant difference test.

4.4 Results and discussion

4.4.1 SDS-PAGE

Approximate molecular weights of zein fractions after extrusion were characterized in order to identify the impact of extrusion on the zein molecular weight distribution and SDS page results are illustrated in **Figure 18**. As expected, there was observed major bands of α-zein (~22 and 24 kDa) and minor bands of β-zein (~14 kDa) or dimers (~36 kDa) in the gel. These have been reported in previous studies (Paulis, 1981). Extrusion from 90 to 140 °C slightly increased the formation of small peptides. Zein extruded at 160°C exhibited the presence of high molecular weight proteins (bands > 50 kDa) with a reduction in the band densities of native zein. These changes were attributed to the formation of di-sulfide bonds and ester formation (Selling, 2010). With these changes in molecular weight, the solubility and rheology of the protein changes significantly (Gordon W Selling & Utt, 2013).

4.4.2 Mixograph properties and dough moisture optimization

Torque responses of wheat flour dough and composite zein-starch dough samples during mixing evaluated by the mixograph are shown in **Figure 19**. The amounts of water determined from these tests were added to the rice starch-zein and the maize starch-zein mixtures to achieve dough with optimum moisture contents of 52.4% and 47.5%, respectively. The optimum moisture content for the potato starch-zein dough could not be determined from the mixograph test because no dough development was observed at any water addition level but transitioned cleanly from weak particulate masses to batter-like consistency with increasing water content. The failure of potato starch may have been due to the mixing action of the mixograph. Mixograph is not a suitable method to determine optimal moisture content of mixture of zein and potato starch.

Optimum amount of water for preparation of potato-starch dough was instead based on the αvalues determined from creep tests (**Figure 19**). At the optimum water content, the α value was comparable to that of the Maize starch-zein dough. A better mixing was achieved using a 10g mixer (National Mfg. Co., Lincoln, NE, USA) and formation of a dough with a moisture content of 48.3% was confirmed and used for potato starch-zein composites. Although the approach used to calculate the moisture content in potato starch-zein composite differs from the one used with the other samples, the large deformation conditions used in the test resemble realistic dough handling conditions and it is expected that these results provide similar information as from the mixograph tests. The formulation containing potato starch did not yield a proper dough during the mixograph test because not enough elasticity could be achieved in the dough to recover and spring back to the center of the bowl to be further deformed by the mixer impeller. Furthermore, the industrial use of potato starch would require mixer modifications to scrape the bowl surface. It is possible that the large granule size of potato starch prevents the correct formation of a continuous zein network thus, reducing the elasticity of the dough. The optimum amount of water for doughs prepared with extruded zein were similar and are not reported.

Wheat dough is known to reduce its strength during prolonged mixing. In fact, after a certain time ("mixing tolerance") its strength decreases due to gluten depolymerization (Skerritt, Hac, & Bekes, 1999). Doughs containing zein, however, did not display a decrease in strength during the measured time, suggesting that the protein matrix formed by zein during mixing was stable even after mixing for long times. Previous studies associated the development of viscoelasticity in zein based dough with an increase in β-sheet structures (Mejia et al., 2007). It's possible that the higher stability to mixing of zein dough compared to wheat dough is due to the higher resistance of β-sheet structure to overmixing compared to the formation of disulfide bridges, which tend to break after long mixing times (Skerritt et al., 1999).

4.4.3 Creep and recovery

All the doughs analyzed in this study presented typical creep-recovery curves of viscoelastic materials. From the creep and creep recovery curves α and λ_1-λ_2 were determined. Values of α for all the tested samples are illustrated in **Figure 20** and show differences due to the starch type and the type of zein used. Values of α range from 0 to 1, where 0 indicate a pure elastic behavior and a value of 1 indicates a pure viscous behavior (Spotti et al., 2017). Comparing doughs prepared with different starches, the lowest value of α, which is comparable to that of the wheat standard, was found for the rice-starch dough. Higher values of α were obtained from doughs prepared with maize and potato starches, describing doughs with lower elasticity. In addition, doughs prepared with maize and potato starches did not have good recovery after the applied stress was removed. Thus, these results suggest that for the same amount and type of protein, the type of starch used to prepare zein-based, gluten-free doughs have a significant effect on the dough viscoelastic properties. These results agree with previously published research (Petrofsky & Hoseney, 1995). Rice starch, which positively contributed to the viscoelasticity of the dough, has a small granule size distribution in the range 3-8μm whereas Maize and potato starches have larger granule sizes of 1-20 μm and 15-110μm, respectively (Eliasson, 2004). It is possible that the small size distribution of the rice starch granules may favor the formation of a more continuous protein matrix, leading to higher elasticity. Additional studies at the molecular level are necessary to better understand why rice starch can form doughs with viscoelastic properties comparable to those of wheat flour dough.

Creep recovery tests also showed that extrusion of zein significantly influenced starch-zein dough elasticity as determined by the parameter α (**Figure 20**). Values of α increased with extrusion temperature when maize and potato starches were used in the preparation of the doughs (**Figure 20**). The decrease in elasticity with increase of extrusion temperature could be linked to structural changes of the zein molecules. However, zein extruded at 160°C displayed a significantly decrease of α values (higher elasticity of the doughs) when prepared with flour containing rice starch and maize starch (compared to zein extruded at 120°C and 140°C). This apparent increase in elastic behavior of the dough could be attributed to the increased molecular weight of zein upon extrusion, which have been reported to occur at temperatures higher than 150°C (Selling, 2010). These results agree with electrophoresis results showing that zein extruded at 160 underwent a significant increase in molecular weight (**Figure 18**).

4.4.4 Extension test

It has been recognized that small strain oscillatory shear tests do not necessarily measure rheological properties of dough systems that could be relevant to bread making (B. Dobraszczyk & M. Morgenstern, 2003). For instance, during leavening dough undergoes biaxial extension rather than shear deformation. During mixing operations, depending on the type of mixer, the dough is subjected to extension and shear deformations. In addition, shear tests are not sensitive to molecular structures like the ones formed by gluten networks, which affect baking quality and performance. Furthermore, viscoelastic properties of networks formed by cereal proteins such as storage modulus, exhibit little variation with changes in the protein molecular weight when doughs are characterized by shear tests (B. Dobraszczyk & M. Morgenstern, 2003). Thus, the extension test provides a more suitable examination of the dough taking into consideration the effects of the protein structure and with a view on dough handling.

Stress-strain curves obtained in the extension test are given in **Figure 21**. All doughs exhibited extension curves having different characteristics when compared to the extension behavior of wheat dough. Wheat dough displayed strain-hardening behavior before achieving rapid failure, matching previously reports observed behavior (Kokelaar et al., 1996). Conversely, zein dough composites except the ones incorporating zein extruded at 160ºC, didn’t show a continuous strain hardening behavior before dough rupture and did not follow the typical power law trend that wheat dough normally shows. The lack of strain hardening in zein dough result in poor bread quality, since it is a fundamental factor for obtaining good bread quality (T Van Vliet et al., 1992). All zein-starch composites except for zein extruded at 160ºC exhibited peak stresses lower or comparable than that of wheat dough, showing a similar resistance to extension at the optimal moisture contents (**Figure 21**). Zein-starch dough samples exhibited weaker strain-hardening followed by a rapid decline in stress with increased strain prior to the dough rupture. The gradual decline in stress-strain slope prior to rupture is characteristic of soft plastic materials in which the internal structure gradually weakens as the sample approaches to rupture conditions.

The rheological behavior of samples prepared with zein and zein extruded at low temperatures shows a decrease in the slope of the stress-strain curve clearly indicating a weakening of the structure that affect the dough elasticity.

Doughs made with starch and zein extruded at 160ºC showed a much higher ability to extend and greater strain hardening during extension (**Figure 21**). This increased strain was observed for all sources of flour. Gluten-free doughs generally lack this type of behavior, having a batter-like consistency that creates problems for large scale bread production. For instance, doughs made with good quality flours have higher resistance to biaxial deformation, as well as a more pronounced strain hardening behavior; strain hardening has also been related to the amount of gas that the dough retains during baking (Kokelaar et al., 1996). Therefore, the addition of zein extruded at 160ºC can potentially help to improve the quality of bread prepared with these gluten-free composite flours. Changes in molecular weight of zein extruded at high temperature, as observed in **Figure 18**, can be associated with the observed changes in the extension properties of doughs. It is possible that large molecules of zein formed during extrusion enhanced the strength of the protein matrix within the doughs, generating higher resistance during extension. The formation of a continuous protein phase is critical for an improved storage life of gluten-free bread (Moore, Schober, Dockery, & Arendt, 2004)

As illustrated in **Figure 21** the type of starch employed in the dough formulation also played an important role in the stress-strain response of the doughs subjected to extensional test. Maximum strain before complete breakage had lower values for dough samples containing rice starch (average strain 4.2±0.21) followed by maize starch (average strain 4.4±0.23) and finally by potato starch (average strain 4.6±0.12), indicating a statistically significant effect of the type starch on sample breakdown. The starch granule size might explain these different behaviors, with the hypothesis that depending on their size starch granules may either promote or inhibit interconnectedness of the zein protein network within the dough and therefore affect its ability to stretch either with or without fracture. The type of starch also influenced the magnitude of the peak stress and stresses before reaching the sample fracture. Differences can be observed in dough formulated with rice starch showing higher peak stresses (0.56MPa ±0.11), followed by maize (0.39 MPa ±0.08) and potato starches (0.24 MPa ±0.09). These differences could be attributed to the different interconnectedness of zein, in its different forms, with the starch granules to form a network. These peak extensional stresses values are also in accordance with previously reported results on standard wheat doughs prepared with wheat flours of different protein content and prepared with different levels of mixing (H. Zheng, M. Morgenstern, O. Campanella, & N. Larsen, 2000). From these results appear that a more fragile or easily breakable network matrix is formed when small size granules are used in the formulation, notably rice starch. While networks having lower strengths but more extensible are created when starches have large granule sizes, notably maize and potato starches. Similar findings have been reported by other researchers which are in a general agreement with these results (Singh, Singh, Kaur, Sodhi, & Gill, 2003). Results from the extensional tests are also in agreement with those obtained in creep-recovery tests and the associated parameters, specifically α values, indicated that formulations including rice starch result in doughs with higher elasticity when compared with formulations containing maize and potato starches. Thus, the rheological behavior of doughs subjected to shear and extensional tests appears to be related to the interaction of the different forms of zein proteins with the different types of starch used in the formulation.

4.4.5 Scanning electron microscopy

Electron microscopy was used to characterize the morphology and potential changes of the extruded zein before and after its incorporation into the dough. Dry zein powders (unextruded and extruded) showed a particulate structure with a wide range of sizes as shown in **Figure 22A**. Although the majority of extruded zein also displayed a particulate, amorphous structure, a small but consistent fraction of fibrous structures was detected in all extruded zein samples **Figure 22D**. Fibrous structures could be observed visually following extrusion (**Figure 17**), yet they appear to be mostly broken because the extrudate material was milled (**Figure 22D**). Fibrous aggregates observed in extruded zein samples are also present in small fractions of the samples, likely escaped from the grinding process or created during sample preparation.

The microstructure of the dough samples containing starch and zein showed dominance of approximately spherical starch granules and with some interstitial material that could be attributed to zein during dough formation (**Figure 22B, C, E, F)**. Fibrous structures, comparable to those observed in freshly prepared extruded zein samples, were found partially adhered to granules in samples containing extruded zein (**Figure 22E, F**). Those fibrous structures could have been created during the extrusion process and then survived both grinding and dough preparation. Alternatively, fibrous structures may have formed during mixing of the dough at temperatures above zein's glass transition (35°C). Regardless, fibrous structures in the doughs are contributing to increase elasticity and extensibility of zein-doughs.

4.5 Conclusion

This study shows that both the starch source and the temperatures at which zein is extruded had a significant effect on the viscoelastic properties of doughs prepared with these components. Mixograph and rheometry using different types of tests showed that by using extruded zein and different source of starches, it is possible to form doughs with viscoelastic properties like that of doughs prepared with wheat flour. Creep tests showed that the use of rice starch promoted the formation of a dough with a high elasticity (lowest α) and results were comparable to the one observed in wheat standard doughs. Conversely, potato and maize exhibited similar lower levels of elasticity. The zein extrusion conditions, also influenced dough elasticity, with the greatest effect shown when zein was extruded at 160°C. Gas-retention is highly important during bread production and is a complex property that involves many factors and relationships to the properties of the dough used in the production of the bread. Strain hardening is one of those important factors, and this work has shown that doughs formulated with zein extruded at 160°C has a good strain hardening performance. Microscopy showed that doughs with extruded zein exhibited a small fraction of fibrous structures, yet it is not clear if extrusion or the mixing process at 35°C had a role in their formation. Electrophoresis of samples showed production of both small peptides and larger molecular structures with increasing extrusion temperatures, which is a molecular characteristic associated with formation of amyloid-like fibrils (Ridgley, Ebanks, & Barone, 2011). Furthermore, the presence of high molecular weight protein (HMW) fractions such as HMW glutenins have been positively associated to the quality of wheat-based breads (Fevzioglu et al., 2012). Future studies will need to assess the effects of extruded zein on bread production and evaluate whether fibrous structures have a role in the quality of the bread.

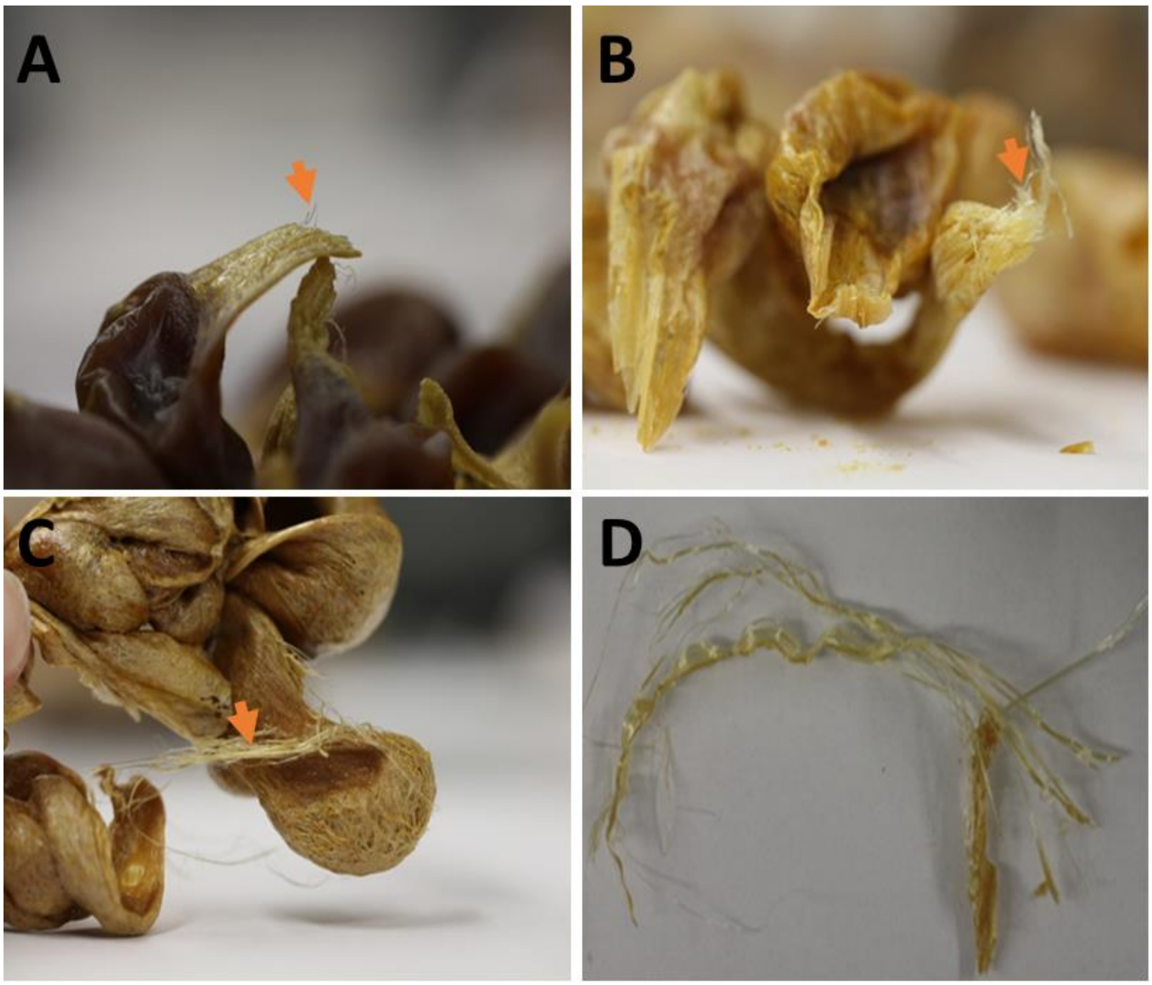

Figure 17 appearance of zein extrudate, arrow shows fibrils: A) zein extruded at 90, B) zein extruded at 120 C) zein extruded at 160 D) fibrils formed during extrusion

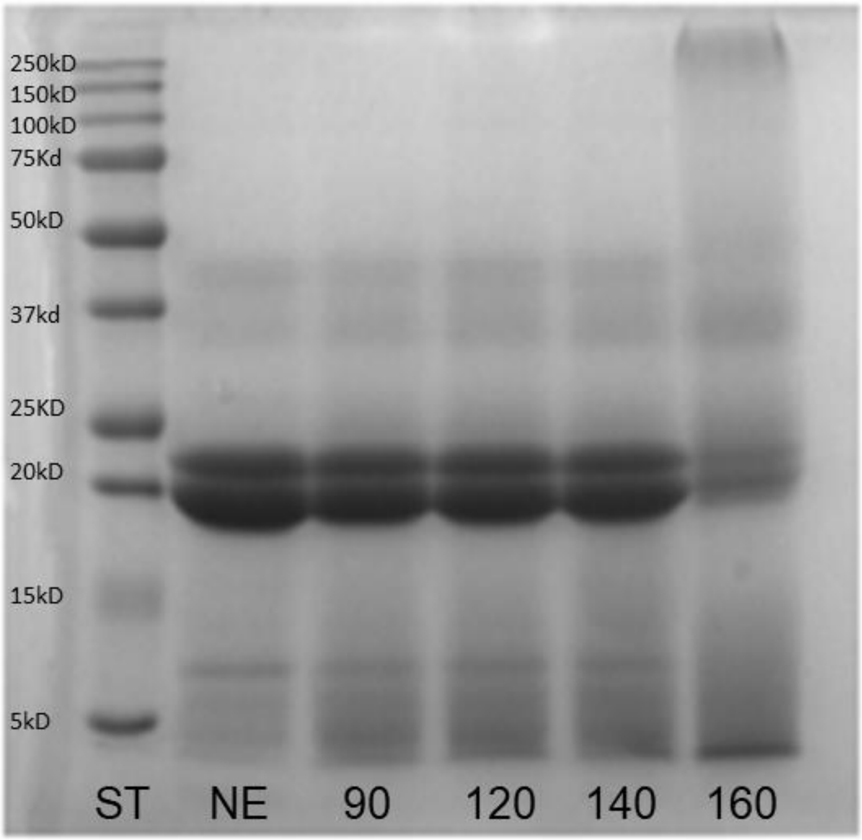

Figure 18 SDS Page of zein and Extruded zein. ST= protein standard NE=non-extruded zein, 90=zein extruded at 90℃, 120= Zein extruded at 120℃, 140= zein extruded at 140℃, 160=zein extruded at 160℃

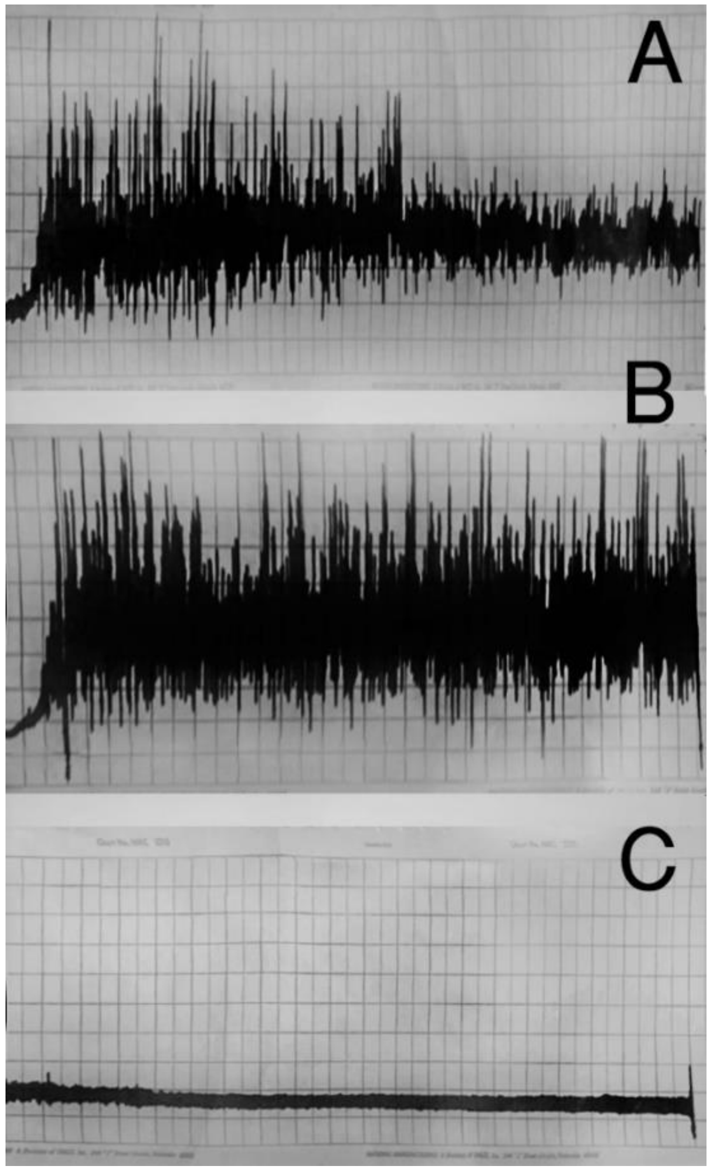

Figure 19 A) Mixograph of wheat dough control at 35°C B) Mixograph of zein-rice starch dough at 35°C C) Mixograph of zein-potato starch dough at 35°C

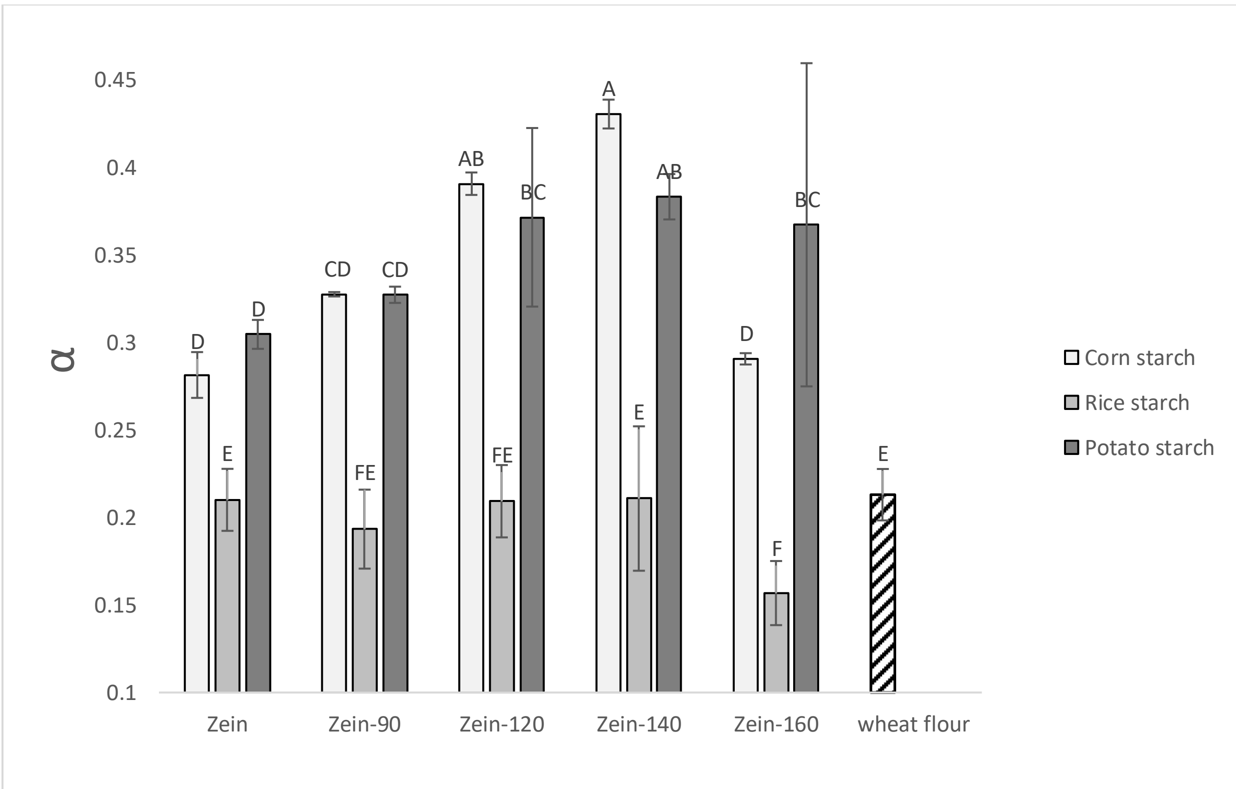

Figure 20 alpha value of doughs in creep and recovery at 35°C

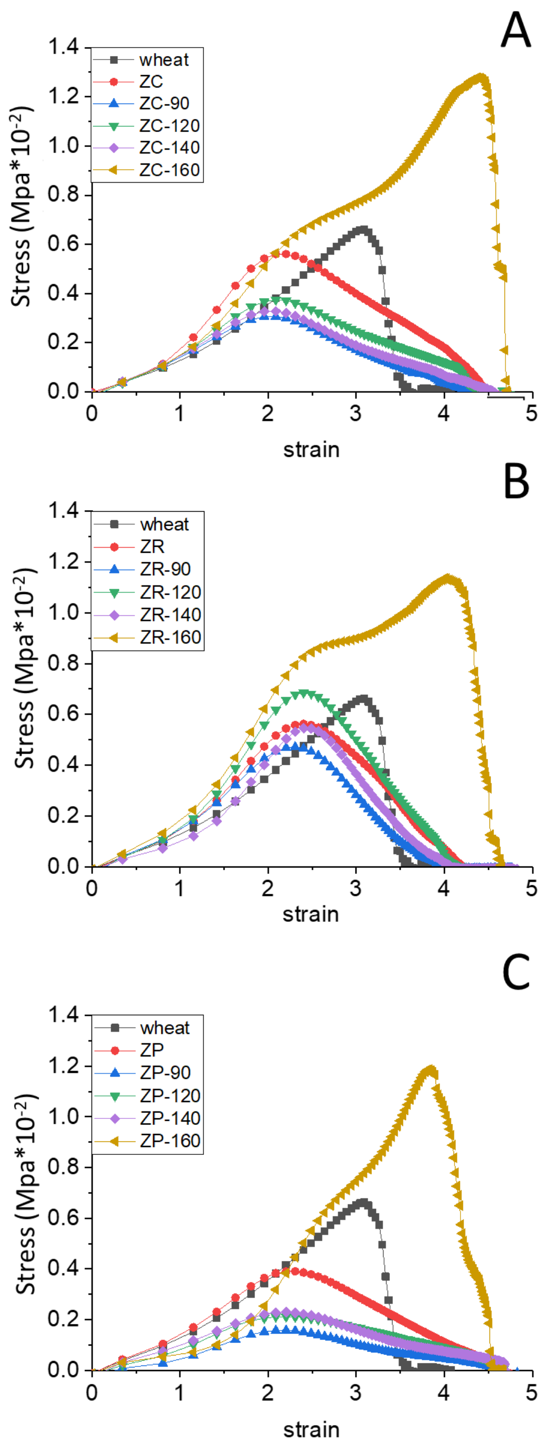

Figure 21 Stress-strain curves of doughs prepared with the different form of zein and different starches measured at 35oC. (A) Corn starch, (B) rice starch and (C) potato starch

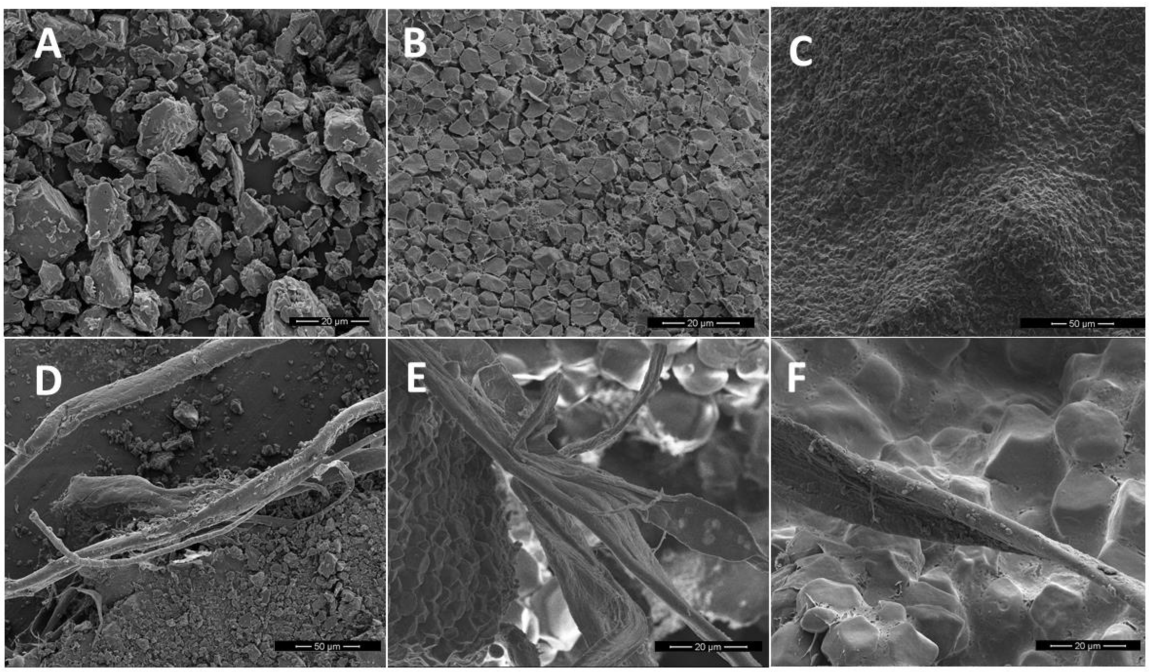

Figure 22 Scanning electron micrographs of A) non-extruded solid zein, mag 3000X B) dough of rice starch and zein (ZR), mag 3000X C) dough of corn starch and zein (ZC), mag 1000X D) zein extruded at 140°C mag 300X E) magnified image of fibrous structures observed

Table 6 Screw configuration

Leistritz Twin Screw

Shaft Length = 540 mm

Screw Elements

1	Shaft	12	20X30 Convey
2	30X90 Convey	13	30 deg KB4
3	30X30 Convey	14	60 deg KB4
4	30X30 Convey	15	45 deg KB4
5	30X30 Convey	16	15X30 Convey
6	20X30 Convey	17	60 deg KB4
7	20X30 Convey	18	60 deg KB4
8	30 deg KB4	19	45 deg KB4
9	20X30 Convey	20	15X30 Convey
10	60 deg KB4	21	End Cap
11	60 deg KB4		

Table 7 Temperature profile

rpm	T3	T4	T5	T6	T7	T8	Average Throughput	Ave Torque
150	60	80	90	90	90	90	21	46
150	65	90	110	120	120	120	24	22
150	80	100	120	140	140	140	32	23
405	80	101	145	159	160	160	30	55

Table 8 Composition and sample name of the zein-starch dough samples

Sample	Starch source	Zein extrusion temperature (°C)	% dough moisture (wet basis)
Wheat	Wheat flour	Not extruded	46
ZR	Rice	Not extruded	52.4
ZR-90	Rice	90	52.4
ZR-120	Rice	120	52.4
ZR-140	Rice	140	52.4
ZR-160	Rice	160	52.4
ZC	Corn	Not extruded	47.5
ZC-90	Corn	90	47.5
ZC-120	Corn	120	47.5
ZC-140	Corn	140	47.5
ZC-160	Corn	160	47.5
ZP	Potato	Not extruded	48.3
ZP-90	Potato	90	48.3
ZP-120	Potato	120	48.3
ZP-140	Potato	140	48.3
ZP-160	Potato	160	48.3

CHAPTER 5. THERMAL TREATMENT OF DRY ZEIN TO IMPROVE RHEOLOGICAL PROPERTIES IN GLUTEN-FREE DOUGH

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Federici, E., Selling, G. W., Campanella, O. H., & Jones, O. G. (2021). Thermal treatment of dry zein to improve rheological properties in gluten-free dough. *Food Hydrocolloids*, 106629.

5.1 Abstract

Zein is a gluten-free storage protein from corn that provides extensibility to starch-based doughs. However, zein does not confer a viscoelastic behavior, a fundamental feature to produce high quality bread. Previous research showed that extrusion above 160°C can improve zein dough viscoelasticity through the generation of a high molecular weight fraction. To determine whether thermal treatments alone could similarly improve zein function in doughs, zein was heated in a vacuum oven at temperatures of 160-200°C. Gel electrophoresis of treated samples showed that thermal treatment promoted development of higher molecular weight zein fractions, which were resistant to dissolution in reducing conditions, presence of surfactant, and urea. Infrared spectroscopy also revealed that thermal treatments increased β-sheet content and decreased subsequent rearrangement following hydration. Zein hydrated with equal parts of water displayed increased viscoelasticity when the zein was thermally treated at 160-190°C. Doughs prepared with rice starch and zein treated at 160°C exhibited improved strain-hardening behavior at higher extensional strain when compared to doughs prepared with unheated zein; no improvements were observed for higher temperature treatments. Electron microscopy revealed formation of discrete protein bodies among doughs prepared with zein treated at high temperatures, unlike fibrous strands observed for lower temperature treatment. Controlling heat-induced cross-linking and structural rearrangement of zein enhances its function in gluten-free doughs.

5.2 Introduction

The prevalence of gluten-related disorders is rising, and increasing numbers of individuals are trying a gluten-free diet for a variety of symptoms (Leonard, Sapone, Catassi, & Fasano, 2017). Despite many efforts, gluten-substitution in baked products remains a major challenge for food scientists, as gluten-free bread is generally of lower quality when compared to wheat bread (Conte, Fadda, Drabinska, & Krupa-Kozak, 2019). In the last decade, a great number of studies on gluten-free breadmaking identified improvements in bread quality attributes through the incorporation of hydrocolloids and gums (Naqash, Gani, Gani, & Masoodi, 2017). While great progress has been made, there is still room for improvement in formulations. Storage protein from gluten-free cereals can also be used to provide structure and stability to breads, although the breadmaking properties of gluten-free cereal flours or protein isolates are poor without some modification or treatment (Taylor, J. Taylor, et al., 2016).

A good dough for breadmaking needs to have optimal consistency, be extensible, and possess a strain-hardening behavior (Kokelaar et al., 1996). Notably, Strain-hardening behavior in doughs is highly desirable as it is a key feature for gas retention (T Van Vliet et al., 1992). These properties can be assessed by the resistance of the dough to extensional strain (Federici, Jones, Selling, Tagliasco, & Campanella, 2020). While optimal consistency is mainly dependent on hydration and formulation, obtaining desired dough extensibility and elasticity is more challenging. Wheat dough extensibility and elasticity are provided by gliadin and glutenin proteins (H. Wieser, 2007), implying that gluten-free cereal proteins must be modified or improved in some way to obtain ideal dough rheological properties.

Zein is the storage protein of corn that has demonstrated a small degree of the desired extensibility and viscoelasticity when incorporated into doughs within certain conditions. Early studies were able to prepare viscoelastic dough with a mixture of zein and starch if mixed above zein's glass transition temperature (J. W. Lawton, 1992a). At these conditions (~35^{o}C), zein possessed increased hydrogen bonding and β-sheet content when shared, which has led to speculation that generation of viscoelasticity is driven by self-assembly of zein into supramolecular structures (D. P. Erickson et al., 2020; Mejia et al., 2007). The addition of organic acids allows zein-containing doughs to achieve desirable dough extensibility at standard room temperatures and also improves stability of fibrous strands after dough stretching (Sly et al., 2014). Similar improvements in dough extensibility were achieved after adding peroxides, which was proposed to increase transient hydrogen-bonds among zein (Taylor, Johnson, Taylor, Njila, & Jackaman, 2016). Fractions enriched in α-zein, typically obtained from commercial isolation procedures, are more susceptible to disulfide bonding and increased extensibility in plasticized resins or doughs when compared to other fractions of zein (King, Taylor, & Taylor, 2016).

While these studies have improved zein's extensibility, further improvements are needed to enhance zein's contributions to viscoelasticity and the strain-hardening behavior necessary in dough systems if they are going to replace gluten in the breadmaking process. Doughs containing zein exhibited strain-thinning behavior (Berta et al., 2015) rather than the desired strain-hardening that is typical of wheat doughs. Addition of small quantities of high molecular-weight wheat glutenin fractions or bovine casein to zein was found to increase the strength and viscoelasticity of zein doughs and resins (Erickson et al., 2012; Erickson et al., 2014; Fevzioglu et al., 2012), suggesting the importance of larger protein assemblies in the generation of viscoelasticity. Furthermore, extrusion of zein using barrel temperatures above 150°C was recently shown to generate large protein assemblies and also contributed to increased viscoelasticity and strain-hardening attributes when used in gluten-free formulated doughs (Federici et al., 2020). It follows that zein could provide greater viscoelasticity and strain-hardening properties to gluten-free doughs by using prior thermal treatments to promote supramolecular assembly of the zein.

The objective of this study was to determine the effect of thermal treatments on zein properties and functions in dough systems using either a vacuum oven or an oven at atmospheric pressure. SDS-PAGE and ATR-FTIR were used to determine changes to the molecular size and chemical signatures within thermally treated zein. Viscoelastic masses containing zein were formed to investigate changes in mechanical properties due to thermal treatments. Finally, dough was produced with the treated zein and starch, and its rheological properties and microstructure were investigated.

5.3 Materials and methods

5.3.1 Materials

Zein, with a moisture content of 4.1% and a protein content of 85.2 ± 0.5 was purchased from Flo Chemical (Ashburnham, MA). Gluten was obtained from Sigma (St. Louis, MO). Rice starch (Novation) was kindly donated by Ingredion incorporated (Bridgewater, NJ, USA). Deionized water ($\sigma \geqslant 18$ mΩ cm) was obtained using a filtration system (Barnstead E-pure, Thermo Scientific, Waltham, MA).

5.3.2 Sample Preparation

Approximately 50 g of zein was distributed as a thin layer on a piece of aluminum foil. Samples were then heated for 60 min at temperatures of 160, 170, 180, 190, or 200˚C using a natural-convection oven at standard pressure or a vacuum oven (Isotemp model 281, Fisher Scientific, Waltham, MA) at a pressure of 30KPa. The full list of the treatments and their abbreviated designations are shown in Table 9. After thermal treatment, samples were cooled to room temperature, milled with a FOSS Cyclotec 1093 cyclone mill (Hilleroed, Denmark), and sieved to obtain a particle size < 125μm. The samples were then stored in sealed plastic bags at room temperature.

Select samples were prepared with lactic acid as an additional plasticizing ingredient, where specified. Specifically, 1g of zein was mixed with 1ml of water containing 5% lactic acid. Resulting viscoelastic masses were kept sealed in plastic film at room temperature.

5.3.3 Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed to analyze the approximate molecular weight of constituent proteins. Three different buffers were employed for protein dissolution: 1) Laemmli buffer, 2) Laemmli buffer with 5% (v/v) 2-mercaptoethanol, 3) 6M aqueous urea with 5% (v/v) 2-mercaptoethanol. 500 μL of buffer were added to 3μg of sample into a 2ml centrifuge tube and boiled at 100℃ until the solid was fully dispersed, unless otherwise noted in the text. Sample aliquots (10 μL) were then loaded onto a polyacrylamide gel with 1mm thickness composed of a 4% stacking gel and a 12% resolving gel. Runs were conducted at 200V for 60 minutes. After separation, gels were stained with Coomassie Blue R-250. Molecular weights of protein bands were determined using known molecular weight standards (Precision Plus Protein All Blue Standards, Bio-Rad Laboratories, Hercules, CA). Gels were imaged with a GelDoc XR system using Trans White illumination (Bio-Rad Laboratories, Hercules, CA).

5.3.4 Characterization of hydrated zein masses

Infrared spectra of zein was measured at room temperature with a nitrogen-cooled attenuated total reflectance Fourier-transform infrared spectrometer (Nicolet Nexus, Thermo

Scientific, Waltham, MA). Zein was measured as a dry powder or after hydration of 1g dry powder with 1 mL of 5% lactic acid aqueous solution. Background and sample spectra were recorded at wave numbers between 1000 cm^{-1} and 3500 cm^{-1} with a resolution of 1 cm^{-1}. Each measurement comprised an average of 64 scans. All the spectra were baseline-corrected after acquisition. Absorbance values were normalized to the highest recorded absorbance within each sample spectrum and are reported as a percentage. Amide I peak was used to determine changes in secondary structure of zein. Fourier self-deconvolution was used to evaluate spectra within 1580-1720 cm^{-1} using the software Omnic (Thermo Fisher Scientific, Waltham, MA).

In order to obtain a viscoelastic mass, zein powder was equilibrated for 8h in a room conditioned at 35℃ before textural analysis. Water was warmed to 50℃ before the analysis. Zein and water with a ratio of 1:1 were manually mixed in a bowl. The resulting zein viscoelastic mass was positioned between two metal slabs of 5mm height and compressed to 5mm thickness putting a third slab with 4kg weight on it. Cylinders of 20mm diameter were carved out of the compressed viscoelastic mass and allowed to rest for 15 min before analysis. A double compression test was conducted using a Texture Analyzer HD Plus (Newcastle, DE, USA), a 50-kg load cell, and a 20mm cylindrical probe. Tests were operated with a maximum strain of 50% and a speed of 1mm/sec. Area of the first compression, springiness and cohesiveness were determined. Cohesiveness was defined as the ratio of the positive force areas under the first and second compressions (A2/A1). Springiness was defined as the vertical recovery of the sample during the time that elapsed between the end of the first compression and the start of the second compression (Bourne, 2002). Young's modulus was determined from the slope of stress as a function of strain during the first compression.

Images of the surface of hydrated zein samples were obtained with a FEI NOVA nano SEM Field Emission Scanning Electron Microscope (Hillsborough, Oregon, USA) attached to an Everhart Thornley detector. In order to obtain a viscoelastic mass, zein was preconditioned inside a room at 35ºC for 8 hours while water was warmed just before the analysis. After preconditioning zein was mixed with water at 50℃ using a spatula (zein:water 1:1) to form a viscoelastic mass. The obtained zein mass was then manually stretched and attached on adhesive carbon paper. After drying the sample with an air drier, it was sputter-coated with platinum for 60 seconds prior to imaging. An operating distance of 5mm was used during imaging.

5.3.5 Dough Characterization

Gluten-free dough formulations were prepared using pre-made flours containing 15% zein and 85% rice starch. All procedures and subsequent analyses were performed in a room with the temperature controlled at 35°C. Water was added in the proportion of 90% of flour weight. The samples were mixed for 5 min using a KitchenAid Ultra Power KSM95WH – 300 W with a paddle-mixer attachment. Extensional properties of dough samples were determined on a Texture Analyzer HD Plus (Newcastle, DE, USA) with a 50-kg load cell following the method of Morgenstern and others (Morgenstern et al., 1996). Dough was sheeted in squares of 100mm at a thickness of 3mm using a pasta sheeter. It has been shown that sheeting of wheat dough can promote its development (M. Morgenstern et al., 1999), so only 3 passes were used to prepare the sheeted dough. Unless otherwise stated, the dough was allowed to rest for 15min on aluminum foil and then placed on the test support possessing an aperture diameter of 30mm. The dough was fixed between two plastic plates with 4 metallic pins. The extension of the dough was performed with a cylindrical probe having a diameter of 19 mm. Every test lasted less than two minutes, therefore the effects of moisture evaporation from the surface were considered negligible.

A piece of dough was collected at the end of the rheological analysis to observe the microscopic structure of the dough during extension using an FEI NOVA nano SEM Field Emission Scanning Electron Microscope (Hillsborough, Oregon, USA) attached to an Everhart Thornley detector. Gluten dough was also characterized as the control sample, which was prepared from a dry mixture of 15 wt% vital wheat gluten and 85 wt% rice starch that was moistened and mixed as describe above for zein-based doughs. Dough samples were deposited on carbon paper, quickly dried, and sputter-coated with platinum for 120 seconds prior to imaging.

5.3.6 Statistical methods

At least three replicates were performed for every analysis. Values are reported as average ± standard deviation. Significant differences ($p \leq 0.05$) among samples were calculated by one-way-analysis of variance (ANOVA) with a Tukey-high significant difference test.

5.4 Results and discussion

5.4.1 Gel electrophoresis

Gel electrophoresis was utilized to assess the impact of thermal treatments with or without vacuum on zein's apparent molecular weight distribution (**Figure 23**). Untreated zein and zein treated with lower temperature treatments showed bands typical of α zein with approximate molecular weights of 19 and 22 kDa, as well as two faint bands at approximately 35-45 kDa that were attributable to zein dimers and trimers (Sly et al., 2014). Zein bands at 19 and 22 kDa faded in the vacuum-oven-treated samples with increasing temperature and almost completely disappeared when treatments were above 190ºC (**Figure 23A**). Samples exhibiting those fading bands also increased the residue remaining at the top of the lanes and a fraction of insoluble matter remaining in sample buffers (not shown), indicating that a portion of zein became less soluble in the Laemmli buffer with an increased treatment temperature under vacuum. Insoluble matter in these high-temperature treated samples could not be dissolved even with extended boiling duration. Thermal treatments were previously reported to induce aggregation of zein (Federici et al., 2020; Nunes, Correia, Barros, & Delgadillo, 2005). Other authors have reported difficulties in solubilizing and dispersing aggregates of zein despite boiling for long periods with detergents, chaotropes, and reducing agents (Vanessa Cabra, Roberto Arreguin, Rafael Vazquez-Duhalt, & Amelia Farres, 2006).

Extraction of thermally treated zein with reducing Laemmli buffer slightly improved the solubility of zein due to breakage of disulfide bridges formed within zein and its aggregates (**Figure 23B**). However, insoluble residue was still observed at the bottom of the samples. Extraction using 6M urea and 5% 2-mercaptoethanol completely dissolved all zein samples, although material of large apparent molecular weight could still be observed at the top of the gels (**Figure 23C**). This demonstrated a relation between vacuum-oven temperature during thermal treatments and apparent molecular weight of zein samples. As each of the various buffer ingredients were unable to fully disrupt aggregates found in thermally treated zein, increased size of zein with thermal treatment was therefore attributable to not only disulfide formation or hydrogen bonding but to additional types of covalent linkages. Previous works on zein extrusion reported the formation of ester bonds at comparable temperatures (Selling, 2010), and similar covalent bonds may have formed during zein thermal treatments.

Zein treated in an oven at atmospheric pressure showed very similar results to those treated in vacuum-ovens at lower temperature treatments, whereas zein became completely insoluble and unresolved on the gels when treated above 170°C (**Figure 23D**). Zein solubility and the appearance of bands on gels were unchanged by use of reducing buffers (**Figure 23E**), indicating that disulfide bonds within the zein were either unimportant to solubility or inaccessible to the reducing agents. Reduced solubility at higher temperature treatments could be attributed to oxidation-related reactions among and between zein molecules when exposed to heating under standard atmosphere. Given the lower solubility of samples treated at atmospheric pressure, only samples produced with a vacuum oven were further investigated.

5.4.2 Infrared spectroscopy of dry and hydrated zein

Infrared spectra were acquired to determine changes in secondary structure due to thermal treatments. Fourier self-deconvoluted and normalized spectra of dry and hydrated zein samples are reported in **Figure 24**. Due to signal amplification from the application of Fourier Self Deconvolution, quantification of secondary structure content was not possible and only general trends are be discussed.

Spectra of dry zein showed five peaks associated with different components of zein secondary structure (**Figure 24A**). The peak centered at 1613 cm^{-1} has been associated with intramolecular β-sheet (Georget, Barker, & Belton, 2008). Peaks centered at 1638 cm^{-1} and 1649 cm^{-1} are associated with random coils and α-helices, respectively (Y. Wang, M. Zhao, S. A. Barker, P. Belton, & D. Craig, 2019). Finally, the two peaks centered at 1678 cm^{-1} and 1688 cm^{-1} are attributed to β-turn structures due to glutamine turns (Barth, 2000).

Previous studies showed a change in zein secondary structure after it had been hydrated and either warmed above its glass transition temperature or supplemented with additional plasticizers such as organic acids (Mejia et al., 2007; Sly et al., 2014). To determine how zein thermal treatment would affect such structural changes, spectra were obtained of zein samples after hydration with an aqueous solution of 5% lactic acid (**Figure 24B**). Hydration of zein caused the peak at 1638 cm^{-1} to shift to 1630 cm^{-1} and indicated an increase of β-sheet content. This agrees with a recent study that demonstrated an increase in random coil and β-sheet structures at the expense of α-helix following zein hydration (D. P. Erickson et al., 2020). With increasing treatment temperature, intensity of peaks increased at 1613 cm^{-1} and 1630 cm^{-1} and implied an increase in β-sheet structure (**Figure 24B,C**). These results agree with previous work on zein films, in which the authors reported an increase of β-sheet structure after zein was thermally treated (Magoshi, Nakamura, & Murakami, 1992). Development of β-sheet secondary structures in zein has been related to fibrous protein structure development in doughs with similarities to wheat gluten systems (Mejia et al., 2007), yet the β-sheet content diminished if cooled below the glass transition temperature (Erickson et al., 2012). Interestingly, **Figure 24B,C** demonstrated maintenance of β-sheet structures in zein hydrated at room temperature, suggesting that structural changes induced by thermal treatment in a vacuum oven can promote stable β-sheet structures in zein.

5.4.3 Microstructure and mechanical properties of hydrated zein

Zein forms a viscoelastic mass after it is hydrated and mixed above its glass transition temperature (Schober, Moreau, Bean, & Boyle, 2010). All the zein samples tested were able to form such viscoelastic masses after mixing with an equal part of water at 35°C. Photographs and electron micrographs of stretched zein viscoelastic masses are shown in **Figure 25A**. Masses were easily stretched and showed prevalently viscous behavior that was comparable to prior observations (J. Taylor, Anyango, Muhiwa, Oguntoyinbo, & Taylor, 2018). Electron micrographs also revealed significant orientation of stretched fibrous structures in the direction of the applied stress, as previously observed by other authors (Mattice & Marangoni, 2020a; Sly et al., 2014). Increasing temperature of thermal treatment appeared to reduce the capacity of zein viscoelastic masses to stretch and orient with the stress direction, with the masses becoming tougher. Treatments at temperatures of 180°C and above resulted in very tough masses that were extremely cohesive.

Texture profile analyses were used to quantify the changes in viscoelasticity of the zein masses. In general, thermal treatment of zein increased cohesiveness, springiness, and Young's moduli of hydrated viscoelastic masses (**Figure 25B**). Springiness indicates the ability of a material to restore its shape after deformation, cohesiveness denotes its capacity to withstand stress, and Young's modulus describes material stiffness. Greatest increases in these parameters was observed in samples containing zein thermally treated at 190°C. Treatment at 200°C reduced Springiness and cohesiveness while causing no change in Young's modulus. Furthermore, Z-V-200 mixed poorly with water and formed an inhomogeneous mass with a brittle structure. Increases in Young’s moduli of zein viscoelastic masses were only apparent at treatment temperatures of 170°C and above.

Enhanced viscoelasticity in hydrated zein masses can be explained by thermally induced changes in the apparent molecular size of zein and by changes in secondary structure. Increased Young’s moduli between 170ºC and 190ºC coincided with increased molecular sizes observed by gel electrophoresis (**Figure 25**) and indicated a relation between strong intermolecular bonds and modulus development. This agrees with previous studies showing increased Young’s moduli of zein fibers subjected to chemical cross-linking (Selling, Woods, & Biswas, 2012), as well as after extrusion with increasing barrel temperatures up to 180°C (Selling, 2010). Infrared spectra also demonstrated an increase in the β-sheet structure content for hydrated zein that was thermally treated (**Figure 24B**). Previous studies underlined the importance of β-sheet formation and their role in intermolecular interactions for the development of zein based, gluten-free doughs (Erickson et al., 2012). Intermolecular bonds are important for generating material viscoelasticity, which is evidenced by the role of disulfide bonds in promoting the viscoelasticity wheat doughs (P. Shewry & Tatham, 1997). Unlike poorly interacting molecules of small dimensions that are readily pulled apart, large molecular assemblies act as entropic springs and generate elastic behavior when extended (Fantner et al., 2006; B. L. Smith et al., 1999). Increased interactions among zein molecules due to thermal treatment could then readily explain the improved viscoelastic behavior of zein.

5.4.4 Dough Extension Properties

In order to obtain high quality bread, a dough needs to possess three important rheological characteristics: 1) optimal consistency, 2) high extensibility, and 3) strain hardening behavior (Kokelaar et al., 1996). An example of rheological behavior of wheat dough with optimal consistency is shown in **figure 26**. Extensibility and strain hardening behavior in traditional bread doughs is provided by gluten, whereas gluten-free doughs lack these key properties and produce bread of poor quality. Characterization of hydrated zein masses demonstrated improvements in the dough viscoelastic properties when zein was thermally treated (**Figure 27**), and thermal treatment of zein could also improve its function in gluten-free doughs. Accordingly, extension tests were performed on dough prepared with zein, starch, and water to determine the effect of zein’s prior thermal treatment (**Figure 29**).

Doughs prepared with unheated zein quickly reached a peak stress during extension up to ~2% strain and then displayed significant strain thinning behavior (**Figure 27A**). This matched prior observations for untreated zein-based doughs (Federici et al., 2020). Doughs prepared with thermally treated zein showed similar behavior with many of the samples failing at a lower strain of ~1.5%. Only doughs prepared with zein thermally treated at 160°C displayed pronounced strain hardening behavior beyond 2% strain. The improved viscoelastic behavior can be attributed to the formation of a fraction of cross-linked zein at high molecular weight (**Figure 23A**), similar to what was described for doughs prepared with extruded zein (Federici et al., 2020). These results are also in agreement with what was observed by Lawton (J. W. Lawton, 1992a), where a more viscoelastic behavior was observed after zein was cross-linked by formaldehyde treatments, corroborating the hypothesis that larger assemblies generate viscoelastic behavior in dough systems. On the other hand, the inability of zein thermally treated at 170-190ºC to improve strain hardening of doughs was surprising given the observed increase in protein size (**Figure 23**) and a greater elasticity (measured by the Young Modulus) among hydrated zein masses (**Figure 25B**). However, this could be attributed to the importance of starch in defining the rheological properties of doughs and modifying the role of the protein components.

A dough generally possesses a partially elastic behavior and can store energy during mixing operations. For this reason, dough samples are typically allowed to rest for at least 15 minutes before rheological analyses. However, differences in dough consistency were observed over time when handling doughs made with Z-V-160. To qualify the impact of resting on extension for doughs made with zein, analyses were also performed without a resting time (**Figure 27B**). Doughs prepared with unheated zein exhibited similar stress-strain behaviors independent of resting time, with smaller resistance to extension in unrested dough. On the other hand, measurements of doughs prepared with Z-V-160 showed a significant increase in strain hardening behaviors above 2% strain when the resting period was omitted. This revealed a pronounced capacity of zein to store mixing energy after it had been thermally treated at 160ºC.

To further improve dough rheological behavior, higher zein powder contents (20% and 30% of flour weight) were tested using the same water content on the dough preparation. While greater quantities of unheated zein decreased dough consistency (**Figure 27C**), increasing the content of Z-V-160 to 30% led to an increased resistance to extension and a more pronounced strain hardening behavior. While it required an increase of the protein content, this study shows that it is possible to form a gluten-free dough with zein that is highly extensible and at the same time viscoelastic.

5.4.5 Dough electron microscopy

To gather information on the microstructure of the dough during extension, dough samples were collected after the rheological tests and immediately prepared for scanning electron microscopy analyses. **Figure 28** shows the micrographs of doughs containing zein, Z-V-160 and wheat gluten at increasing magnifications. A direct comparison of the amount of nanometer-scale fibrils structures between doughs at the same magnification revealed very similar morphologis. At larger length scales, dough samples with zein and Z-V-160 possessed larger stretched fibrous structures concentrated in discrete regions between starch granules (**Figure 28**, bottom row). Such fibers were of larger diameter for samples with Z-V-160, suggesting that thermal treatment enhances zein aggregation into those larger structures. Thick stretched fibrous material such as these were not observed in gluten-containing doughs. A prior study with zein resins found similar thick fibrils after stretching (Mattice & Marangoni, 2020a). Networks of agglomerated fibrous strands have also been proposed as the structure responsible for viscoelastic functionality of zein dough (J. W. Lawton, 1992b; Mejia et al., 2007). This is based on prior observations of zein fibrils within doughs when mixed above the glass transition temperature or with addition of organic acids, which were also the conditions at which zein doughs displayed enhanced viscoelasticity (J. W. Lawton, 1992b; Sly et al., 2014).

Despite mixing at 35ºC, doughs incorporating zein treated at temperature higher than 160℃ did not form fibrils after mixing and stretching but instead formed spheroidal protein bodies dispersed within the starch matrix (170 ℃ shown in **Figure 29**). These protein bodies were attributed to agglomerated zein. The apparent lack of interactivity between the protein bodies and absence of any apparent continuity of protein matrix explained the reduced viscoelasticity of doughs containing zein thermally treated at higher temperatures (**Figure 25**). Increased Young's modulus potentially impaired zein dispersion among starch granules, resulting in a non-extensible continuous phase dominated by starch intermolecular interactions. Furthermore, high temperature treatments may have negatively affected zein's capacity to form fibrils by promoting excessive chemical and physical changes, such as irreversible bond formation and aggregation into dense structures. Similar contraction of zein into discrete spheroidal bodies was observed in doughs mixed at temperatures lower than zein's glass transition or without added organic acids (J. W. Lawton, 1992b; Sly et al., 2014). Aggregation of zein particles into strands (fibrils/fibers) is considered a critical step in dough formation (Schober et al., 2010). Inability of the zein to form these fibrils after heat treatment above 160ºC could be attributed to increased cross-linking and excessive structural changes (**Figure 23**). Further research will be necessary to improve dispersion of thermally treated zein in gluten-free doughs and understand what factors favor fibril formation and extensibility in thermally treated zein rather than dense aggregate formation inside the dough.

5.5 Conclusion

This study indicated that thermal treatment of zein can be an effective strategy to improve zein based gluten-free dough breadmaking potential. Through the formation of cross-linked zein, dough showed a more pronounced strain hardening behavior, an essential property for dough gas retention, similar to what was previously reported when zein was treated with extrusion. The present study further corroborates the hypothesis that association of protein into larger strands and with the capacity to form interconnected networks is necessary to promote viscoelasticity in doughs. Excessive thermal treatment above 160 °C caused a greater degree of zein aggregation that formed discrete spheroidal protein bodies providing large increase of viscoelasticity within hydrated zein resins but that negatively impacted starch-rich doughs. More investigation is necessary to reveal which specific changes of the chemical and physical structure of zein confer the desirable formation of extensible fibril networks in doughs. This study showed that it is possible to form an extensible dough possessing a strain hardening behavior starting from gluten-free cereal storage proteins by use of controlled thermal treatments, potentially leading to high quality gluten-free bread. Studies on physical treatments or allowed chemical treatments that provide similar improvements to zein structure could be followed to further enhance their breadmaking potential.

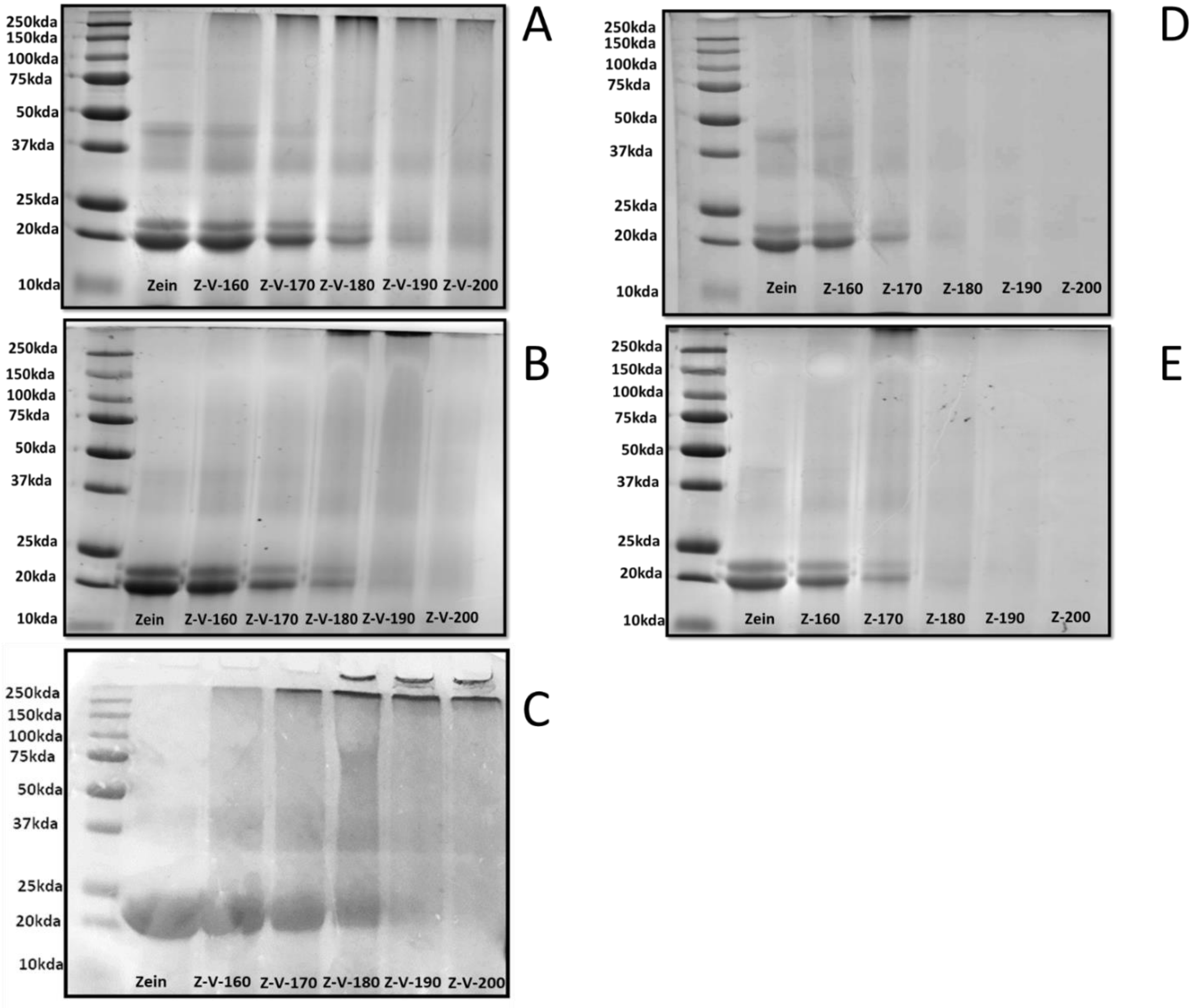


Figure 23 Gel electrophoresis of zein thermally treated in a vacuum oven (A-C) or standard oven (D-E) prepared with the following solvents: (A,D) Laemmli buffer, (B,E) Laemmli buffer and 5% V/V 2-Mercaptoethanol, (C) 6M urea and 5%V/V 2-mercaptoethanol

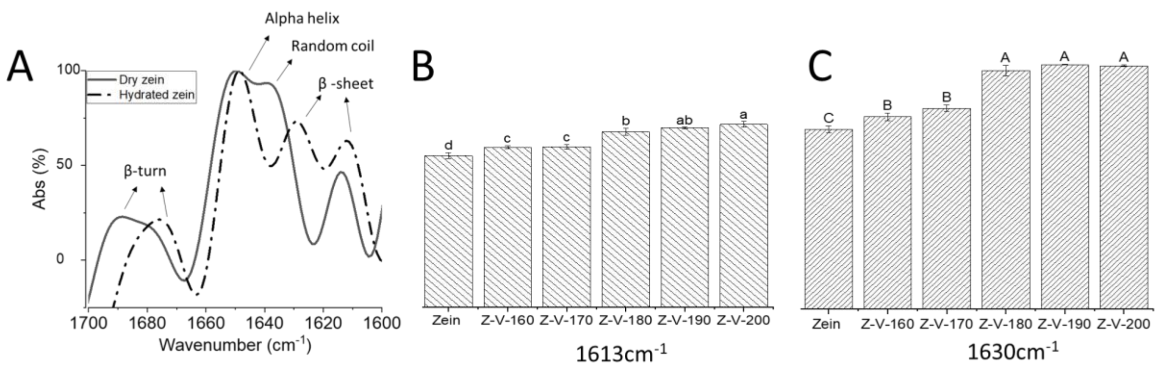


Figure 24 Fourier-deconvoluted infrared spectra of zein: (A) comparison of dry zein and zein hydrated with 5% lactic acid solution; (B) effect of vacuum-oven treatments on zein subsequently hydrated with 5% lactic acid solution at 1612cm-1 (C) effect of vacuum-ove

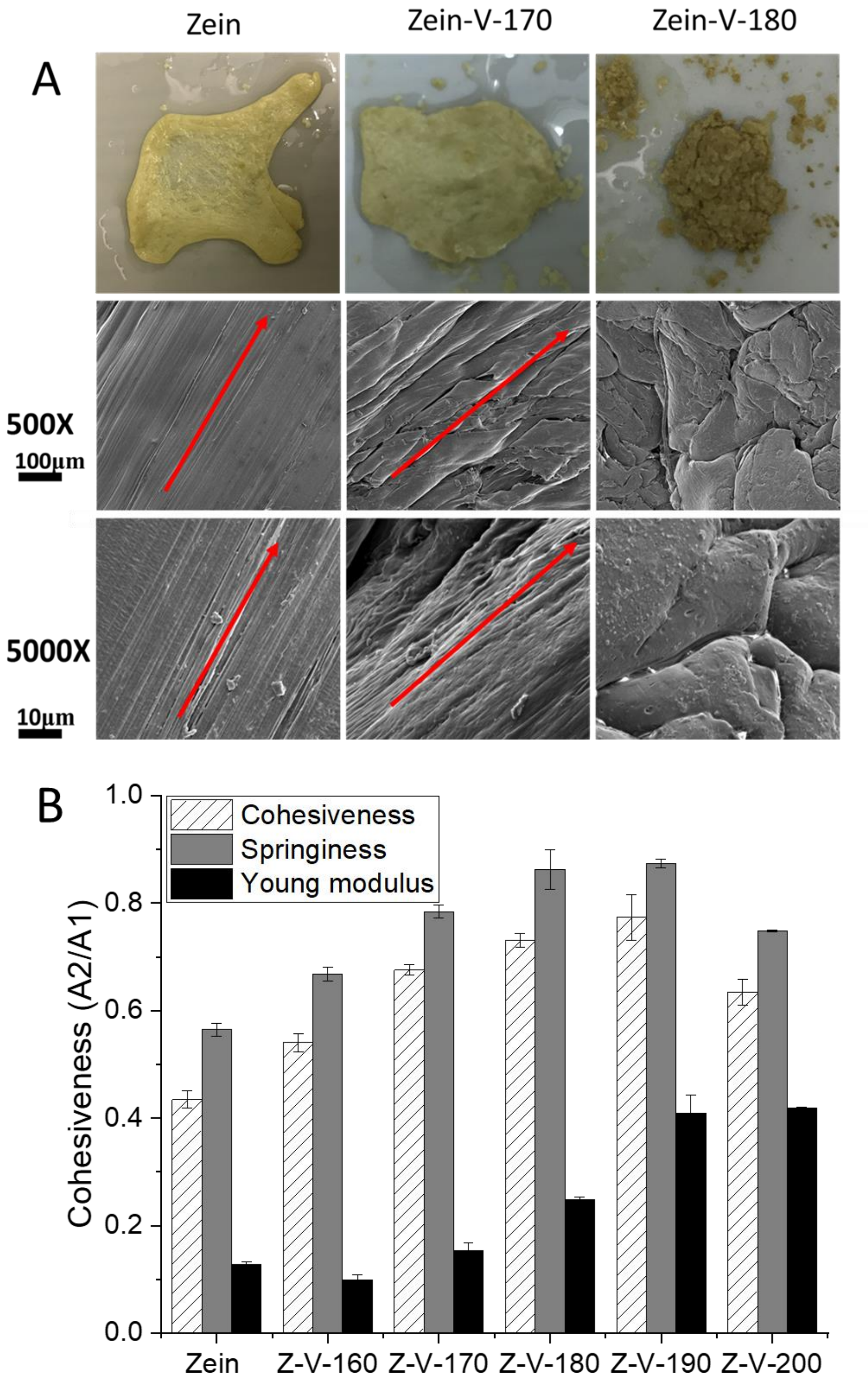


Figure 25 Effect of thermal treatment on hydrated zein: (A) photographs (top row) and scanning electron micrographs of hydrated zein surfaces after manual stretching, with arrows indicating direction of orientation; (B) results from texture profile analysis

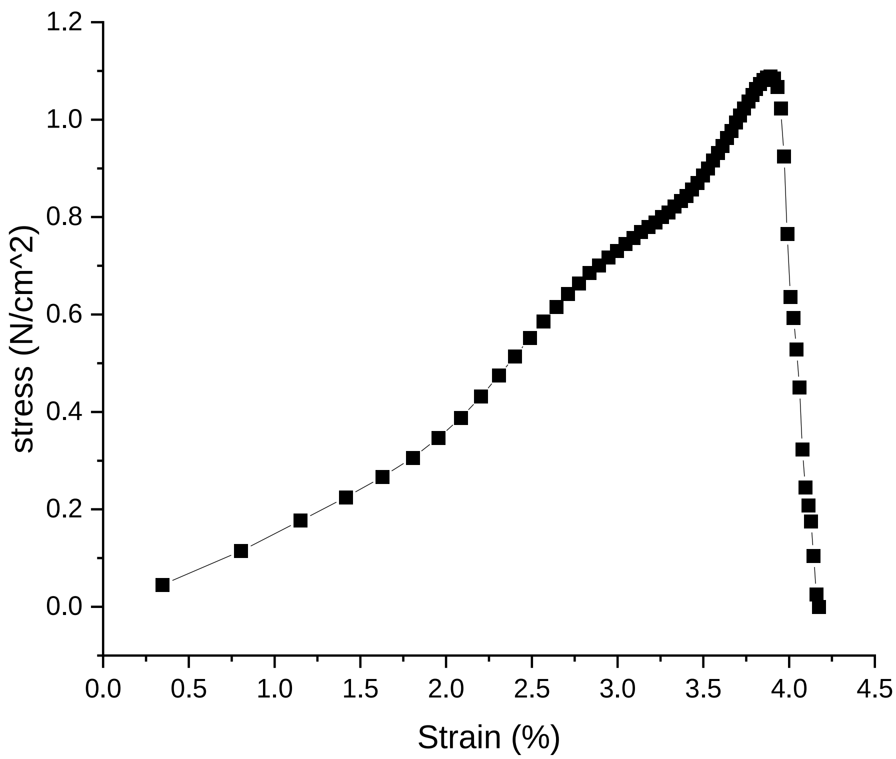


Figure 26 Extension tests of wheat doughs with an optimal consistency of 500 Brabender unit.

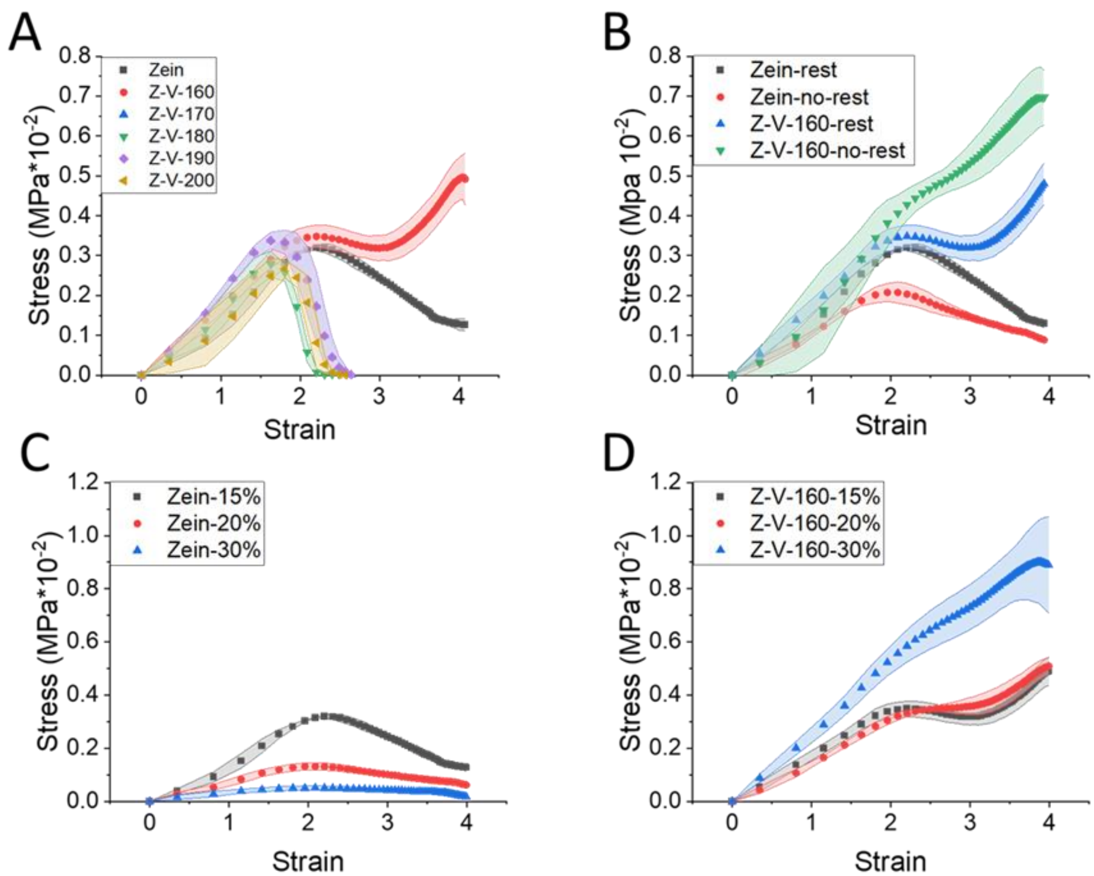


Figure 27 Extension tests of doughs at 35℃ containing zein with or without prior thermal treatment: (A) effect of thermal treatment temperature, (B) effect of a 15-minute rest period, (C) effect of zein content (no thermal treatment), (D) effect of thermally-treated-zein (Z-V-160) content

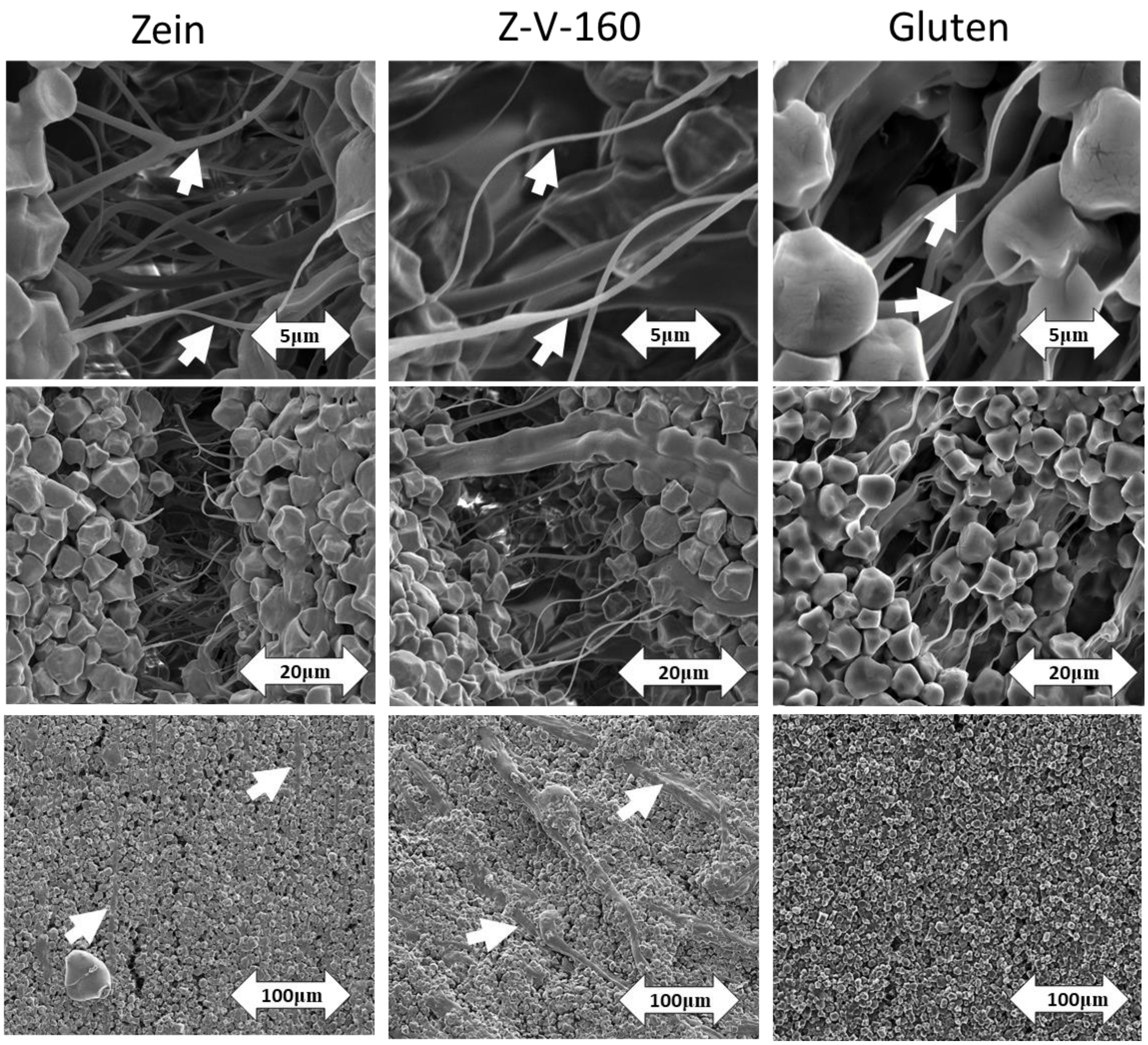


Figure 28 Images of dough samples containing starch with zein, Z-V-160, gluten. White arrows indicate relevant structure discussed in the text.

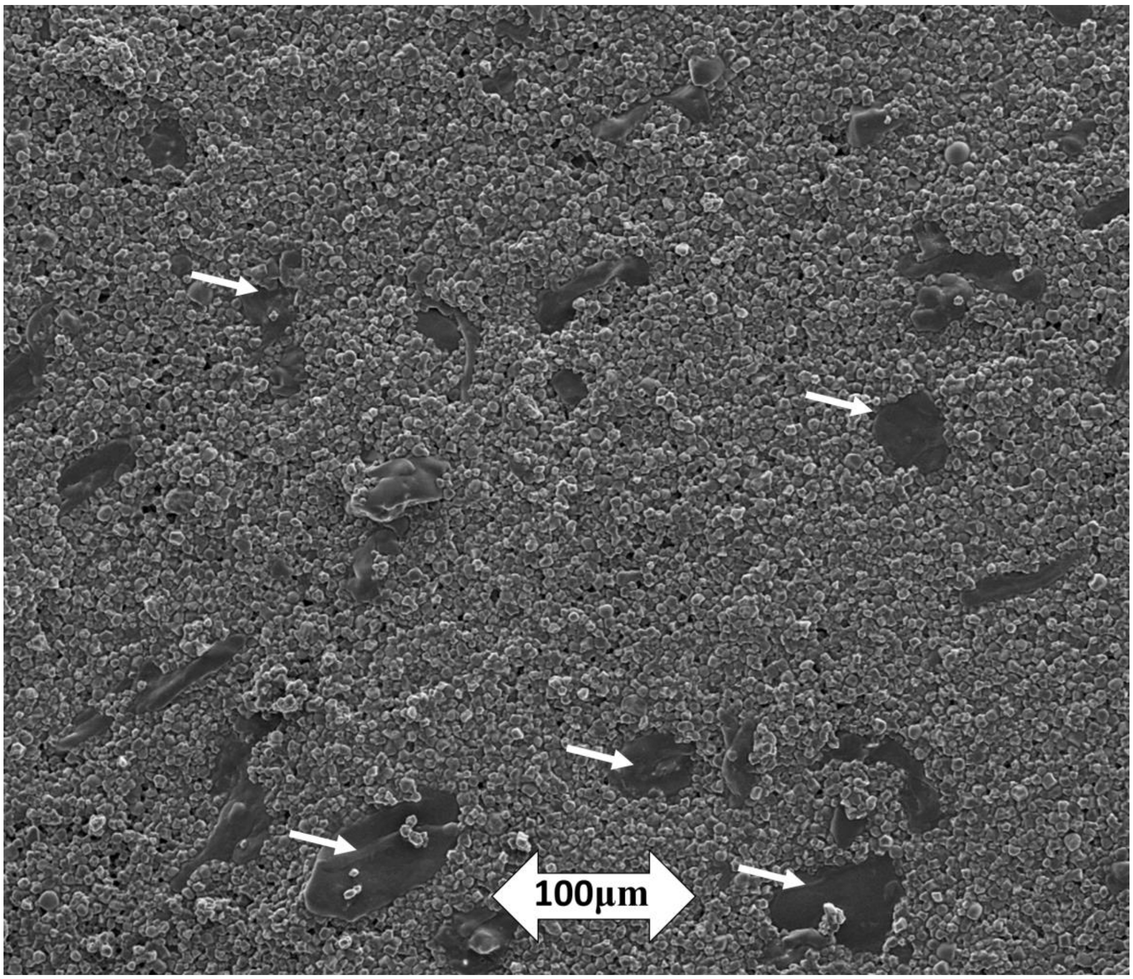


Figure 29 Images of dough samples containing starch with Z-V-170. Arrows indicates zein particles.

Table 9 Abbreviations for samples of zein thermally treated for 60 minutes at the specified temperatures within a vacuum oven or oven at standard pressure

Nomenclature	Temperature (°C)	Treatment
Zein	-	-
Z-V-160	160	Vacuum
Z-V-170	170	Vacuum
Z-V-180	180	Vacuum
Z-V-190	190	Vacuum
Z-V-200	200	Vacuum
Z-160	160	No-Vacuum
Z-170	170	No-Vacuum
Z-180	180	No-Vacuum
Z-190	190	No-Vacuum
Z-200	200	No-Vacuum

CHAPTER 6. EFFECT OF EXTRUDED AND THERMALLY TREATED ZEIN ON BREAD QUALITY

6.1 Abstract

Zein is an attractive alternative for gluten free breads because of its unique viscoelastic properties when wetted, yet it is use in breadmaking remains limited. The main purpose of this study was to investigate the use of zein and treated zein for bread production purposes. A farinograph and a Rheofermentometer were used to study the mixing properties and gas retention ability of zein doughs, showing low mixing tolerance and limited ability to retain gas, highlighting the need of improvement of zein doughs rheological properties. Despite that, zein bread was produced showing specific volume and hardness like a wheat bread but higher bread bubble size. Finally, the quality of bread with the incorporation of thermally treated zein and extruded zein was also investigated, showing a negative effect on bread bubble size despite the improved dough rheological properties. Poorer gas retention is likely due to the tendency of treated zein to aggregate, resulting in a not homogeneous coverage of starch granules, and thus larger bread bubbles. This study will help in the improvement of zein based bread and in the design of treatments to better functionalize zein to obtain high quality bread.

6.2 Introduction

Celiac disease (CD), as mentioned in chapter 2.3.1, is an autoimmune condition of genetically predisposed individual to the exposure of their small intestine to food containing gluten (Fasano, 2009). Considering studies from the United States and Europe, it is estimated that 3 to 13 individuals every 1000 habitant are affected by CD (Guandalini & Assiri, 2014). However, there is no therapy available at the moment and the only way to avoid the symptoms is the complete avoidance of gluten ingestion (Ciccocioppo & Corazza, 2005)

Nevertheless, producing high quality GF foods such as bread represent a challenge for food manufacturer (Morreale, Garzón, & Rosell, 2018). Consequently, the overall quality of gluten-free products tends to be significantly lower when compared to the gluten containing counterpart.

Gluten highly contributes to dough rheology and greatly affect crumb structure of bread; thus, its replacement is critical in achieving good quality GF bread products. Several gluten-free formulations have been reported in literature adopting a wide range of strategies. In the attempt of replacing gluten functionality, the addition of hydrocolloids, proteins of various origin, enzymes, and the use of sourdough have been used by the baking industry (Ngemakwe, Le Roes-Hill, & Jideani, 2015; Renzetti & Arendt, 2009; Ziobro et al., 2016). The most popular strategy involves the use of hydrocolloids such as HPMC, Guar gum, Psyllium Xanthan gum (Cappelli, Oliva, & Cini, 2020). Hydrocolloids improve dough breadmaking performance acting on the water phase of the dough, known as liquor, where they increase the viscosity and stabilize the gas cells from coalescence (Dickinson, 2010). Protein-rich ingredients such as soy, egg white, egg and pea are also often used for gluten replacement (Roman et al., 2019). The formation of a continuous protein matrix within the dough and the bread allow to obtain an adequate product. Nevertheless, the relative high hydrophilicity of the protein fraction in soy, eggs and pulses consisting mainly of albumins and globulins generates dough with different consistencies compared to flours containing large amounts of prolamins, such as wheat flour.

Prolamin-rich protein isolates from gluten-free cereals such as zein and kafirins raised interest in gluten-free breadmaking due to their unique viscoelastic properties. Zein was used to produce bread in combination with HPMC and corn starch in an early study from Schober (T. J. Schober, S. R. Bean, D. L. Boyle, & S. H. Park, 2008). The bread produced in this study had an acceptable specific volume (3.2ml/g) together with a regular, fine crumb grain, yet the presence of HPMC was crucial for the dough gas retention and bread quality. The influence of incorporation of salts on zein–starch dough systems and bread were evaluated by Smith (B. M. Smith, Bean, Selling, Sessa, & Aramouni, 2017). The presence of NaCl at concentration of 125mM or lower resulted in bread with good specific volumes (>3 ml/mg) without the addition of hydrocolloids. More recently, a few more studies investigated the incorporation of zein into gluten-free dough for breadmaking purposes (Akin, Bean, Smith, & Tilley, 2019; Berta, Koelewijn, Ohgren, & Stading, 2019; Khuzwayo et al., 2020). Despite encouraging results showed zein's breadmaking potential, physiochemical treatments to zein are necessary in order to make further improvements (Taylor, J. Taylor, et al., 2016)

It is known that during extrusion process, the molten polymer are exposed to temperature and shear which are able to affect the molecular structure of the processed material (Capone, Di Landro, Inzoli, Penco, & Sartore, 2007). Similarly, thermal treatment without involving shear could lead to substantial modification to zein structure. At 120°C some changes begin to occur, such as protein cross-linking, and gradual structural degradation as temperature increase (G. W. Selling, 2010). It was hypothesized that rearrangement and cross-linking during high-temperature extrusion operations and oven thermal treatments improves interconnectivity among the proteins and extension properties in bread doughs, translating to improved gas retention and loaf volume in the final bread. In this study, some key factors for breadmaking, such as water content and proofing time, will be optimized for zein breadmaking; then, bread will be prepared with dough incorporating zein treated as described in chapter 4 and 5.

6.3 Materials & Methods

6.3.1 Materials

Commercial all-purpose wheat flour (Great Value, Walmart) was purchased from a local market. Zein and starch were obtained as described in chapter 4.3. Commercial salt, commercial pure sugar (sucrose), and yeast from a local market. Hydroxypropyl methylcellulose (HPMC, E 464) and soy lecithin (E 322) were used. Distilled water was used for the bread preparation. Extruded zein and thermally treated zein were produced as described in chapters 4 and 5 of this dissertation.

6.3.2 Dough characterization

The optimization of hydration of dough was studied through the aid of a Farinograph-E (Brabender GmbH and Co KG, Duisburg, Germany). The equipment was installed with a 50 g mixing bowl, as described by the standard method ICC 115/1, setting the bowl temperature at 35 °C instead of 30 ° C. Dry ingredient consisted in a mixture of starch and zein or gluten in the ratio of 5.6:1. Three different starches were tested: corn, rice, and potato at different levels of hydration. Water was added at a temperature of 35°C.

The CO2 production and height of dough during proofing was studied with a Rheofermentometer F4 (Chopin Technologies, Villeneuve La Garenne Cedex, France). Dough samples were prepared in the Farinograph-E (Brabender GmbH and Co KG, Duisburg, Germany) equipped with the 300 g mixing bowl, with dried yeast (2 g/100 g of flour) and salt (NaCl; 1 g/100 g of flour). After the mixing was terminated about 315 g of dough were weighted inside the

Rheofermentometer cylinder and allocated in the chamber which was conditioned at a temperature of 30 °C for 2 h.

6.3.3 Breadmaking and characterization of bread incorporating thermally treated zein

The following gluten free dough formulation was used for bread samples: Water 102.76g, potato starch 48.68g, rice starch 48.68g, zein 16.23, sugar 5.41g, yeast 3.25g. Six different bread types were produced: five test bread made with thermally treated zein at 160ºC, 170ºC, 180ºC, 190ºC, 200ºC and one made of untreated zein. Two loaves of each type were produced. All the ingredients were preconditioned and mixed at 35°C for 5 min (KitchenAid Ultra Power KSM95WH – 300 W). The doughs were weighed in a greased steel pan (6-inch long). The pans were placed in a proofing cabinet (Metro C5 9 Series, InterMetro Industries Co., Wilkes-Barre, PA) for 30 minutes at 35°C and 85% RH to permit fermentation and rising of the dough. Baking was conducted at 165°C in a hot air convection ventilated rotating oven (Doyon Inc, Liniere, Quebec, Canada) for 30 minutes. Bread loaves were then allowed to rest for 90 minutes before being analyzed.

Bread volume was calculated according to the AACC 10-05.01 method and expressed as specific volume; the analysis has been repeated on 2 loaves for each bread type. The equipment used was a volumetric device, hourglass-shaped, composed of two chambers: one containing the test loaf, and the other one containing rapeseeds. The two chambers were connected by a transparent duct on which a scale was displayed.

Texture profile analysis (TPA) of bread crumb was performed according to the AACC 74-09 Standard method. Sample slices of bread had a height of 25 mm. The samples were pressed by a cylinder probe with a diameter of 36 mm to reach a 40% deformation. All data were analyzed with the software texture Exponent (Stable Micro Systems, England). The main texture parameter considered was hardness cohesiveness, and springiness.

6.3.4 Breadmaking and characterization of bread incorporating extruded zein

The following gluten free dough formulation was used for bread samples: Water 96g, potato starch 48.68g, rice starch 48.68g, zein 16.23, sugar 5.41g, yeast 3.25g, salt 2.16g, HPMC 2.16g, soy lecithin 2.16g. Five different bread types were produced: four test bread made with thermally treated zein at 90ºC, 120ºC, 140ºC, 160ºC, and one made of untreated zein. One bread using wheat flour was also produced with addition of 71.56g of water. Two loaves of each type were produced. All the ingredients were preconditioned and mixed at 35°C for 5 min (KitchenAid Ultra Power KSM95WH – 300 W). The doughs were weighed in a greased steel pan (6-inch long). The pans were placed in a proofing cabinet (Metro C5 9 Series, InterMetro Industries Co., Wilkes-Barre, PA) for 60 minutes at 35°C and 85% RH to permit fermentation and rising of the dough. Baking was conducted at 165°C in a hot air convection ventilated rotating oven (Doyon Inc, Liniere, Quebec, Canada) for 30 minutes. Loaves were then allowed to rest for 90 minutes before being analyzed.

Bread volume was calculated according to the AACC 10-05.01 and expressed as specific volume; the analysis has been repeated on 8 loaves for each bread type. The equipment used was a volumetric device, hourglass-shaped, composed of two chambers: one containing the test loaf, and the other one containing rapeseeds. The two chambers were connected by a transparent duct on which a scale was displayed.

Water content was determined by gravimetric analysis after drying using the official method (**AACCI 44-15.02)**. In brief, samples of bread crumb weighing ~1.5 g were excised from loaves and placed in a disposable aluminum pan with a diameter of 10 mm. Samples were then dried in a 105°C oven overnight. 3 replicates of bread crumb were analyzed for each bread type.

To evaluate the color of the crumb, pictures of the slices of each kind of bread were taken using a black box with a white background where the light was controlled. The analysis of crumb color in CIE L*a*b* system was performed using the Color Sample Tool of Photoshop, and for each different bread type, two points of the same image have been analyzed. Once obtained the L*a*b* coordinates for each point, a ΔE was calculated, using as reference the color coordinates of wheat bread.

To evaluate the bubbles areas two software were used: ImageJ (Abràmoff, Magalhães, & Ram, 2004)to scale and crop the image (40mmx40mm), and MATLAB (MathWorks, Massachusetts, USA) for bubble segmentation. For bubble segmentation, it has been used “Hybrid Active Contour Model” given in (Liu et al., 2014). This method is an Active Contour framework that depends on both intensity and edges.

Micrograph of the of bread were obtained with a FEI NOVA nano SEM Field Emission Scanning Electron Microscope (Hillsborough, Oregon, USA) attached with an Everhart Thornley detector. Sample preparation and analysis were performed as described in chapter 4.3.7.

Texture profile analysis of bread crumb was performed at day 0, 2, 5 and 8, on 8 loaves for each bread type, using a texture analyzer TA.HD plus (Stable Micro Systems, England), and the test was performed according to the **AACC 74-09** Standard method. Sample of bread crumb were taken from the center of the loaf and were given the same shape of the probe and a height of 25 mm. The samples were pressed by a cylinder probe with a diameter of 36 mm to reach a 40% deformation. All data were analyzed with the software texture Exponent (Stable Micro Systems, England). The parameters considered were hardness, cohesiveness, and springiness.

A shelf-life study on bread was conducted to study the change in texture and starch retrogradation. After cooling, bread was sealed in plastic bag and stored at room temperature for 0, 2, 5 and 8 days. At every day, 2 loaf of bread were analyzed. Texture properties were determined as reported in section 2.4.6. Thermal analysis of the bread crumb was performed to study the enthalpy changes that occur during melting of the starch with a differential scanning calorimeter Discovery DSC Q2000 (TA Instruments, New Castle), calibrated with indium standard. Tzero pans and hermetic lids were used. The analyses were performed on fresh bread and during storage at day 2, 5 and 8. For each kind of bread two repetitions were done. The sample weight was ≈ 20-22mg. Samples in the DSC were heated from 10°C to 160°C at a rate of 5°C/min. Enthalpy of retrogradation peaks was then calculated using the software TRIOS (TA Instruments, New Castle).

6.3.5 Statistics

All data were subjected to analysis of variance (ANOVA). The statistical software SAS 9.4 was used for the evaluation of statistical significance of the differences between mean values by Tukey’s test.

6.4 Results & discussion

To obtain good quality bread, dough consistency needs to be optimized by adjusting the amount of water added with the dry ingredients. A large number of empirical tests are available to optimize the dough hydration (B. J. Dobraszczyk & M. P. Morgenstern, 2003) among which, one of the most popular is the farinograph. Farinograph tests are reported in Figure 30. Doughs made with potato starch and corn starch could not form a dough with an optimal consistency of 500 BU without observing phase separation as shown in Figure 30. On the other hand, rice starch dough showed an optimal consistency when the ratio between flour and water was 1:1. To compare zein behavior with gluten, a dough made with rice starch and gluten instead of zein was prepared and water was adjusted for optimal consistency. Farinograph of rice starch and gluten doughs are shown in Figure 30. Zein dough showed a very low stability, with consistency dropping steadily during the 20 min mixing time. This behavior is typical of weak flour, where glutenin fraction is not sufficient to stabilize dough rheological properties (Bonilla et al., 2020). Zein was previously reported to behave similarly to gliadin when incorporated into a dough without the presence of high molecular weight glutenin (Fevzioglu et al., 2012). In contrast, dough produced with rice starch and gluten showed good stability to mixing.

Another important processing parameter to optimize and obtain high quality bread is the proofing time. To assess the optimal proofing time of zein-based dough, the samples were tested with a Rheofermentometer. Dough development and direct and indirect pressure are reported in Figure 31. Dough development of dough containing zein and gluten showed a similar trend, with a rapid development observed in the first 30 min, followed by a sharp decline for zein dough and a more gradual decline for gluten. These results indicated poor gas retention of the dough, showing low heights and an early height maximum compared to Rheofermentometer tests performed on wheat dough (Gao, Tay, Koh, & Zhou, 2017). Similarly, direct and indirect pressure increased rapidly until 20 min of proofing, after which direct and indirect pressure rapidly decreased; this behavior is symptomatic of dough permeability to CO2 and instability of gas cells. Thus, a short proofing time of zein doughs obtained bread with good specific volumes above 3.25 cm^3/g (B. M. Smith et al., 2017), while other studies using long proofing times obtained low specific volumes in the range of 1.9-2.2 cm^3/g when an additive was not incorporated (Ozturk & Mert, 2018). Zein-based doughs are characterized by a low mixing stability and a limited ability to retain gas compared with dough formulated with wheat flour.

After optimizing the moisture content and the fermentation time, zein bread was produced and compared with a bread produced using wheat flour. A mixture of rice starch and potato starch was employed together with yeast and sugar. Bread values of specific volume, crumb hardness, springiness, and cohesiveness are reported in Table 10. Zein bread hardness and specific volume were not significantly different from wheat bread. On the other hand, springiness, and cohesiveness of zein bread were inferior to the wheat dough counterpart. Similar to crumb hardness, springiness and cohesiveness are related with consumer acceptance of bread (Stanley Cauvain, 2015). These results indicated that structural differences are present between zein bread and wheat, with the latter more elastic. Nevertheless, it is not possible here to determine unequivocally which factor was more important in determining differences in bread properties. It is well known that the amount of gas retained in bread has a direct consequence on bread texture (SP Cauvain, 2004). While product density was comparable, crumb porosity of zein bread was larger than wheat bread. Further investigation will be necessary to improve zein dough's gas retention and improve its breadmaking potential.

6.4.1 Incorporation of thermally treated zein

Breadmaking was performed to assess the effect of thermally treated zein on the quality of resulting bread. Loaf of bread obtained are shown in Figure 32. We can observe that bread made of zein, zein-160 and zein 170 were all able to retain CO2 produced during fermentation, even though they show clear differences in bubble formation. On the other hand, we can visualize that the sample baked with zein-180, zein-190 and zein-200 were not able to retain the gas during fermentation, showing a large hole in the center due to escape of CO2, a common defect in dough with low ability of retaining gas (S. P. Cauvain & Young, 2007). Quantitative information on bread quality is reported in Figure 32. Zein bread had a specific volume of 3.42±0.14, comparable to what observed by other authors (B. M. Smith et al., 2017). No significant difference in specific volume and hardness were observed with addition of thermally treated zein. On the other hand, while specific volume remained stable, the number of bubbles slightly decreased in zein treated at 160℃ and drastically dropped in zein treated at 170℃. This is an indication of weakening of the matrix and lower ability of zein to stabilize gas cells. However, no holes were detected suggesting the presence of a continuous protein matrix. In contrast, zein treated between 180 and 200℃ demonstrated a low ability to form a network able to retain gas and stabilize cells, resulting in the formation of a large hole in the center of the bread.

The protein matrix formed in between starch granules in a dough it is known to be essential for quality bread (Moore et al., 2004). Gradual loss of dough's ability to retain gas and the general increase in bubble size are related to the loss of viscoelasticity discussed in chapter 5. However, thermal treatments, while improved rheological properties of thermal treated zein at 160, showed the formation of larger bubbles compared to the bread with untreated zein. This unexpected behavior can be simply explained with the dough electron microscopy images shown in Figure 33. While thermal treatment improved dough rheological behavior, it tended to favor zein-zein interaction compared to zein-starch interactions. This resulted in fibers of larger size, as observed in Figure 33 and a minor amount of starch granules covered with zein leading to the formation of larger bubbles formation in bread. Finally, higher temperature treatments impaired zein's ability to form a continuous protein matrix so that bubble size increased dramatically, and the dough was not able to retain CO2 anymore. We can conclude that the ability of zein to surround starch granules is a fundamental feature for gas retention and the formation of good quality zein-bread.

Viscoelastic behavior of zein and gluten proteins is also dependent on their glass transition. For instance, as water is taken up during mixing, gluten goes through a glass transition that renders plastic and form a dough (Faubion & Hoseney, 1990). Similarly, Lawton reported that in order to develop an elastic dough, it is important to exceed zein glass transition to allow it's rearrangement in fibrils (J. W. Lawton, 1992b). It is known that cross-linking of biopolymers results in an increase of their glass transition temperature. Previous studies reported that cross-linking of gluten-fractions increase its glass transition temperature significantly (Hernández-Muñoz, Villalobos, & Chiralt, 2004). This led to the hypothesis that the rise in molecular weight due to thermal treatments potentially increased the glass transition temperature of zein. If zein is mixed below its glass transition, it will not possess enough molecular mobility to re-arrange its secondary structure and thus form a viscoelastic dough. Therefore, an increasing amount of crosslinking may have raised the glass transition temperature of Zein above 35ºC impairing its ability of forming a viscoelastic mass. Further investigation will be necessary to understand the reason of lack of aggregation of cross-linked zein.

6.4.2 Incorporation of extruded zein

Specific volumes of baked bread are reported in Table 11. The values of specific volume reported in our study are comparable to a previous study on zein bread (B. M. Smith et al., 2017). Apart from the zein extruded at 120℃ formulation, zein-starch breads possessed a lower specific volume than the wheat control bread. Bread made with zein extruded at 90℃ had the lowest specific volume among all formulations. This indicates that extrusion at lower temperature (<

120°C) reduces the capacity of the zein-starch system to retain gas, while extrusion at higher temperatures can restore or even improve gas retention properties. Protein networks generated by zein cross-linking could increase the strength of the dough and of the bread, leading to increased gas retention. Having said that, it is possible to state that zein extrusion has an effect on bread quality, since the process increased its specific volume.

Color of bread samples is reported in Table 11. After analyzing the different types of bread, a ΔE was then calculated for each bread to quantify the difference between color of the wheat bread and color of all the breads made with different zein. The major difference between the samples is constituted by the *b* value (yellowness) with the highest value reported for the zein sample and the lowest for wheat bread. A positive correlation between the extrusion temperature in the final stage and the *b* value has been found indicating that the extrusion process degrade the xanthophylls which are responsible for the yellow color of zein (Sessa and Palmquist, 2008). Carotenoids are known for being sensitive to heat treatments (Updike & Schwartz, 2003). It is possible to observe that, higher extrusion temperatures led to zein-starch breads with color attributes that were more like wheat bread. Within the fresh bread characterization, color analysis is of major importance, since the use of zein in food industry is limited by its inherent strong yellow color. Specifically, in bread, yellow color might be perceived as a negative attribute. Other methods are available to remove color from zein, however they generally involve the use of organic solvents (D. J. Sessa, Eller, Palmquist, & Lawton, 2003). Thus, extrusion can be a suitable method to improve color of zein bread.

Image analysis was performed to evaluate the area (mm^2) of the air bubbles of the different types of gluten free breads, which was then compared with the area of the air bubbles of wheat bread. Figure 34 displays the mean cross-sectional area of bubbles found in the crumb for each type of bread. Bubble areas were greatest for breads prepared with zein extruded at 140°C and smallest for breads prepared with wheat. In order to be defined as crumb cells in baked goods, size of air bubbles should be between 0,1 mm^2 and 4 mm^2 (Farrera-Rebollo et al., 2012). Holes with diameter smaller than 0,1 mm^2 might not be considered as crumb cells, and areas larger than 4 mm^2 could be considered as bread crumb defects. Gradual increase in extrusion temperature lead to the formation of larger bubbles in bread crumb as observed in figure 35. This indicated that overall, despite rheological improvement shown in chapter 4, zein extrusion did not help in improving gas retention, contrary, a negative effect was observed. Overall zein bread presented an average pore size larger than the wheat control, symptom of a inferior ability of retaining CO2 during proofing, despite the use of HPMC. In fact, in this class of bread, it is very common to have to face the problem of poor gas retention. During baking, the starch-protein network formed in the previous steps of mixing and proofing could not hold the gas produced by yeast. For this reason, bigger holes can be a common defect. Large pores are generally considered a symptom of poor gas retention, frequently caused by lack of dough elasticity (van Riemsdijk, van der Goot, Hamer, & Boom, 2011). Larger bubble size from images indicated that, in general, extrusion of zein does not improve the stability of smaller gas bubbles during baking of breads. Similar to thermally treated zein, further research will be necessary to favor zein-starch interaction to obtain a more homogeneous coverage of starch granules.

The different types of bread were analyzed using a Scanning Electron Microscope (SEM) to verify the possible difference in the microscopic structure of the breads (Figures 35). It is possible to see the starch granules swollen and with irregular shapes, but still retaining their granular identity. The protein phase surrounds the starch granules. Details of residual starch matrix can be observed in Figure 35a. Starch is deeply interconnected with the protein phase with apparently strong links. However, despite protein contribution it's generally the starch fraction which is now the continuous phase which dictate the mechanical properties in bread (Hug-Iten, Handschin, Conde-Petit, & Escher, 1999). The authors also stated that gluten could be substituted by other structure which can interact with starch such as hydrocolloids or pregelatinized starches. In breads made with zein extruded at 120, 140 and 160°C some elongated, fibrous structures were observed (Figures 35d-f) that were like fibrous structures observed in wheat samples (Figure 35a). Fibrous structures were relatively rare and often found as broken fragments (Figure 35d). More investigation will be necessary to fully understand if fibrous structures affect the texture of bread as well. Furthermore, more research will be necessary to understand the reason of the formation of those structures in cereals protein like gluten and zein.

6.4.3 Shelf-life study of bread incorporating extruded zein.

Figure 36 shows the selected texture parameters of bread crumb: hardness, cohesiveness, and springiness, and their trend during bread aging (fresh bread, day 2, day 5, day 8). At day 0, wheat bread resulted harder compared to all other gluten-free breads. As expected, hardness constantly increases for all the baked breads during their shelf-life storage, reaching maximum hardness after 8 days. At the end of shelf life, difference in hardness resulted analogue to day zero, where wheat bread resulted harder compared to all other formulation. Little difference has been observed between bread incorporating extruded zein at different temperatures. The higher hardness of wheat bread can be explained by the fact that gluten proteins are able to form a strong network, which contains a wide number of small cells. In bread made with zein, extruded or not, the zein-starch network formed was weaker and the air bubbles were less in number and bigger in size than in wheat. Moreover, the presence of different starches between wheat bread and gluten free bread, wheat, and rice-potato respectively, could influence the texture.

When the bread was fresh, at day 0, the most cohesive samples were those made with unmodified zein and zein extruded at 160°C. Also, wheat bread showed good cohesiveness, while bread with zein extruded at 120°C, 140°C and 90°C had the lowest ability to recover their shape once compressed. At the last day of shelf-life, wheat control, bread with unmodified zein, zein extruded at 120°C, 140°C and 160°C showed the highest values of cohesiveness. Previous studies found an inverse relationship between hardness and cohesiveness, the softer the bread, the easier it will recover from a stress (Armero & Collar, 1997). The results of this work are partially in line with this statement. It is interesting to see how the situation is different at the beginning and at the end of shelf life. At day 0, in fact, gluten-free breads do not follow this behavior except for the case of unmodified zein. At day 8, hardness and cohesiveness of all gluten-free breads were inversely proportional. This behavior could be caused by the fact that at the beginning of shelf life the hardness might depend only on the structure of the bread based upon the network formed in the previous steps. State of the starch would be less important because it was still predominately in the amorphous state, which can influence the value of hardness. Instead, during bread aging the amount of retrograded starch is the major responsible of the increased hardness of bread (Miyazaki, Maeda, & Morita, 2005). For wheat bread the situation was different. Its behavior could be due to the ability of gluten to form a strong network in which proteins and starch are well bonded. Resulting bread was then harder but at the same time more cohesive thanks to the elasticity that gluten and especially HMW glutenin confers to it.

Regarding the springiness, at day 0, there was no significant difference between the springiness values of all gluten-free bread, while wheat bread showed the highest capability to spring back. Differences in springiness diminished at the end of the shelf-life study so that gluten-free breads and wheat bread displayed comparable values. The exception to this was bread prepared with zein extruded at 90°C, which apparently has a very low ability to spring back after the first compression. Figure 36 show the starch gelatinization enthalpy of different formulation, showing a lower value for wheat bread compared to all the other gluten free formulation. During storage, all the formulations register a similar increase in enthalpy during time. At 8 days, like day 0, enthalpy of wheat bread resulted lower compared to all the zein bread tested. These results suggest that the starch in the different breads retrogrades in a similar way. Thus, the different type of proteins added as gluten substitute had no effect on starch retrogradation rate. Among gluten-free leavened products, the rate of aging is very fast and the shelf-life of bread inevitably shorter. In fact, the lack of gluten in gluten-free products is critical, because gluten plays a role in the formation of gas cell structure and prevention of staling(Ahlborn, Pike, Hendrix, Hess, & Huber, 2005)

6.5 Conclusion

This study shows that zein, similarly to gliadin, possess low stability during mixing and poor ability to retain gas. Nevertheless, after optimization of processing, zein bread showed a hardness and specific volume comparable to a wheat bread, while lower springiness and cohesiveness were observed. Despite the improvement of elasticity observed with extrusion and thermal treatments, the use of treated zein did not lead to major improvement in breadmaking performance. The main reason for failure in treated zein to improved bread quality can be found in the effect of treatments on zein aggregation, which lead to the formation of zein large aggregate/fibrils instead of covering large portions of starch granules. Further investigation on zein plasticization and zein-starch interactions will be necessary to further improve the rheological behavior of zein based dough and consequently zein bread. Favoring interactions with starch while improving its elasticity could allow the formation of doughs with viscoelastic properties analogues to gluten.

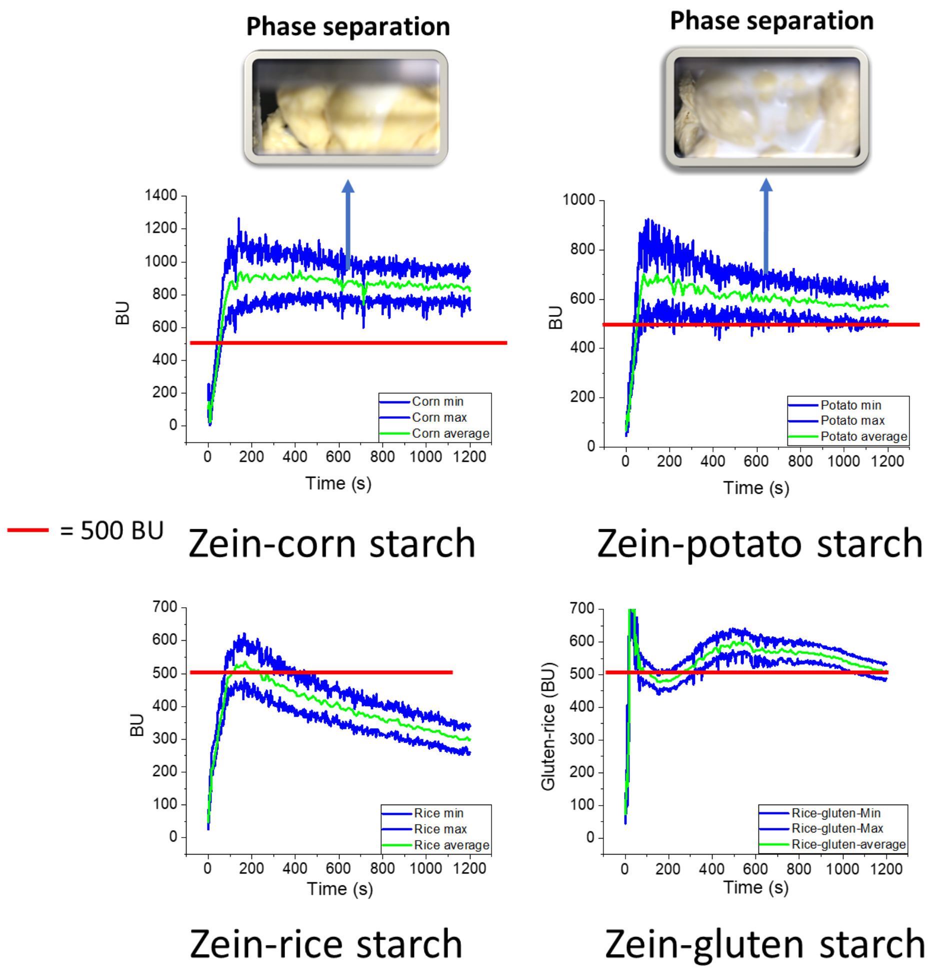


Figure 30 Farinographs of different dough systems. Analysis were performed at 35℃.

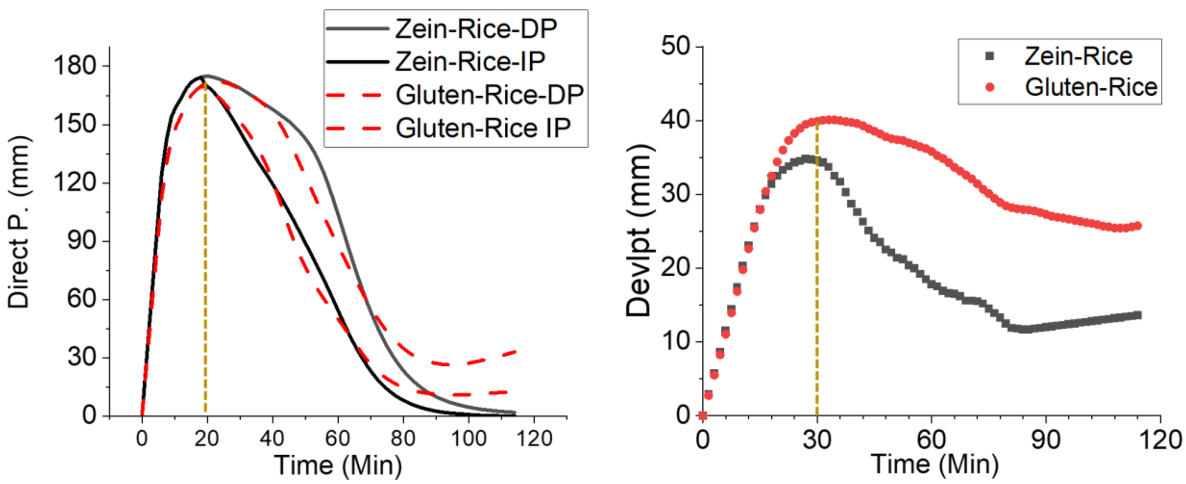
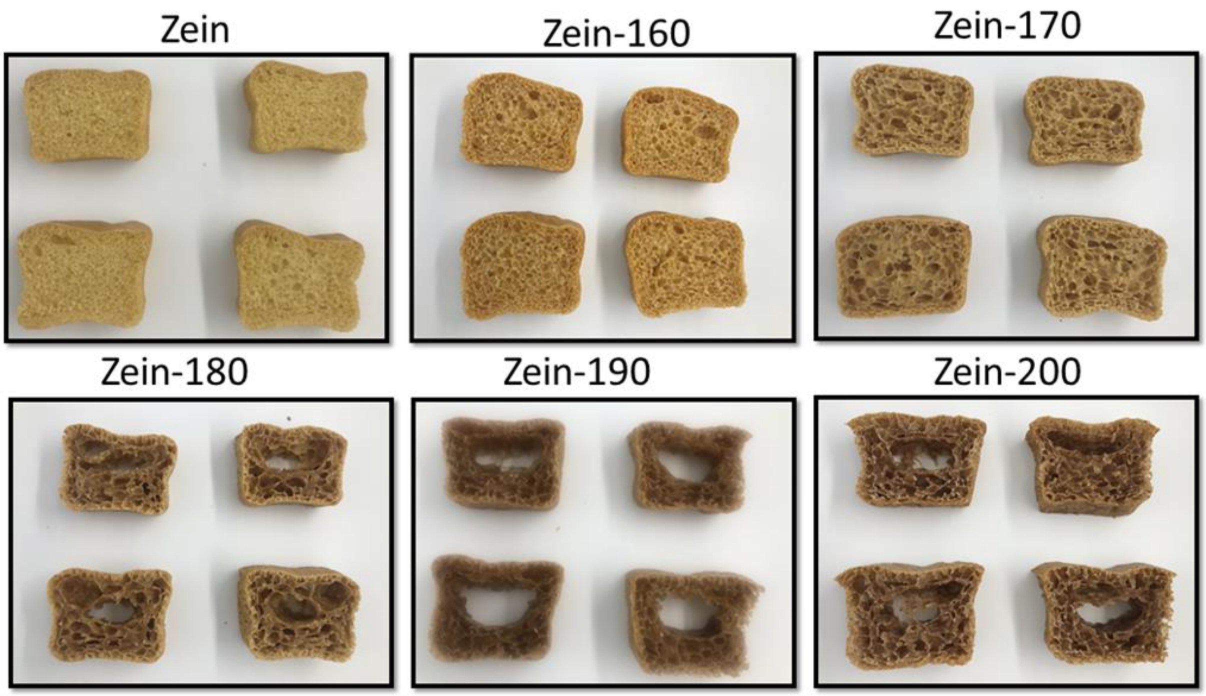


Figure 31 Rheofermentometer parameters of zein-rice and gluten rice doughs.



Sample	Specific volume (ml/g)	Bubble count/loaf (n)	Hardness (N)
Zein	3.42±0.14	266[a]±24	3.93±0.43
Zein-V-160	3.65±0.11	236[a]±38	3.42±0.29
Zein-V-170	3.83±0.15	135[b]±21	4.16±0.92
Zein-V-180	3.43±0.10	N/A	N/A
Zein-V-190	3.65±0.17	N/A	N/A
Zein-V-200	3.55±0.12	N/A	N/A

Figure 32 Images of thermally treated bread (top), Table with bread quality parameters (bottom)

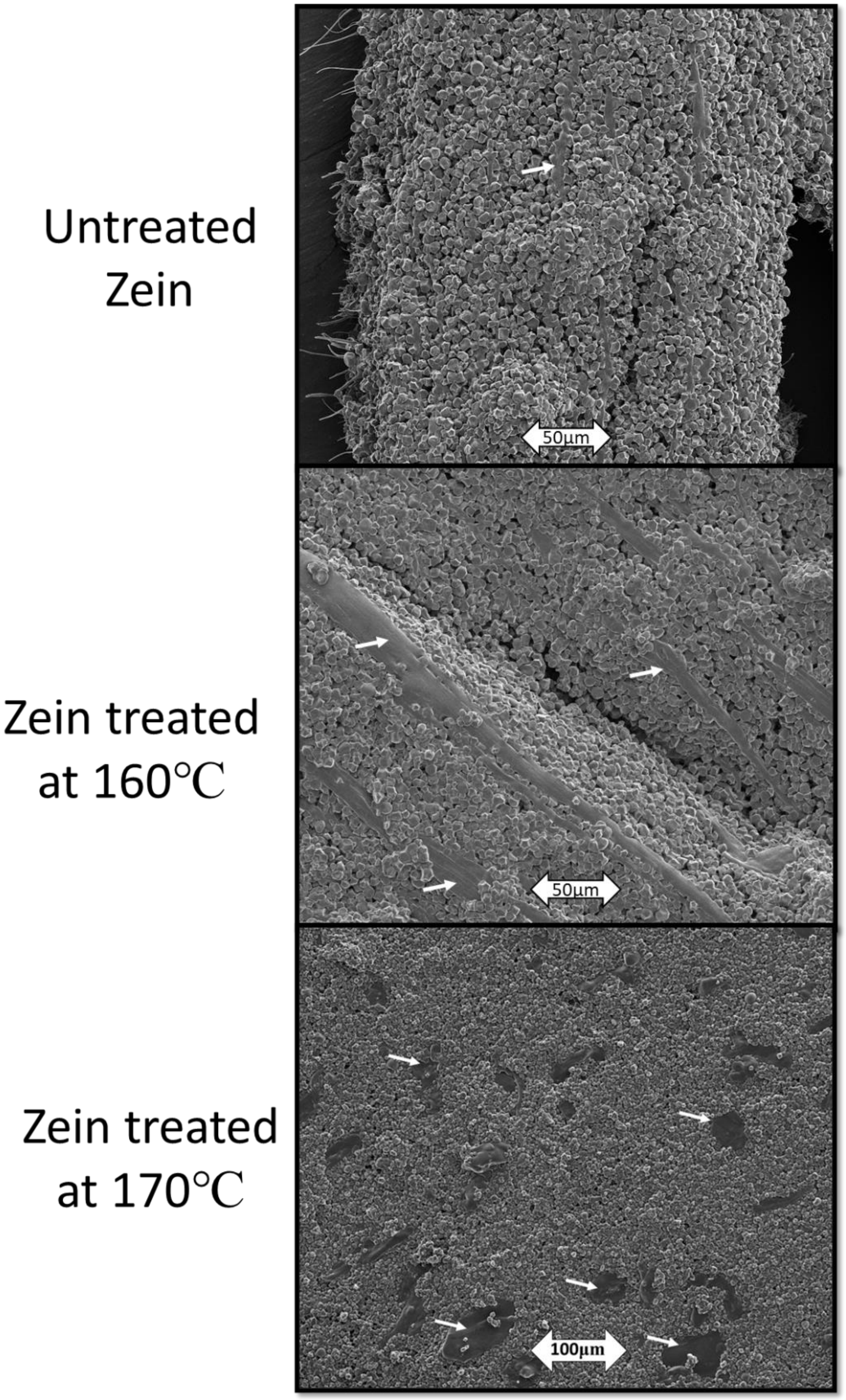


Figure 33 Electron micrograph of dough incorporating thermally treated zein

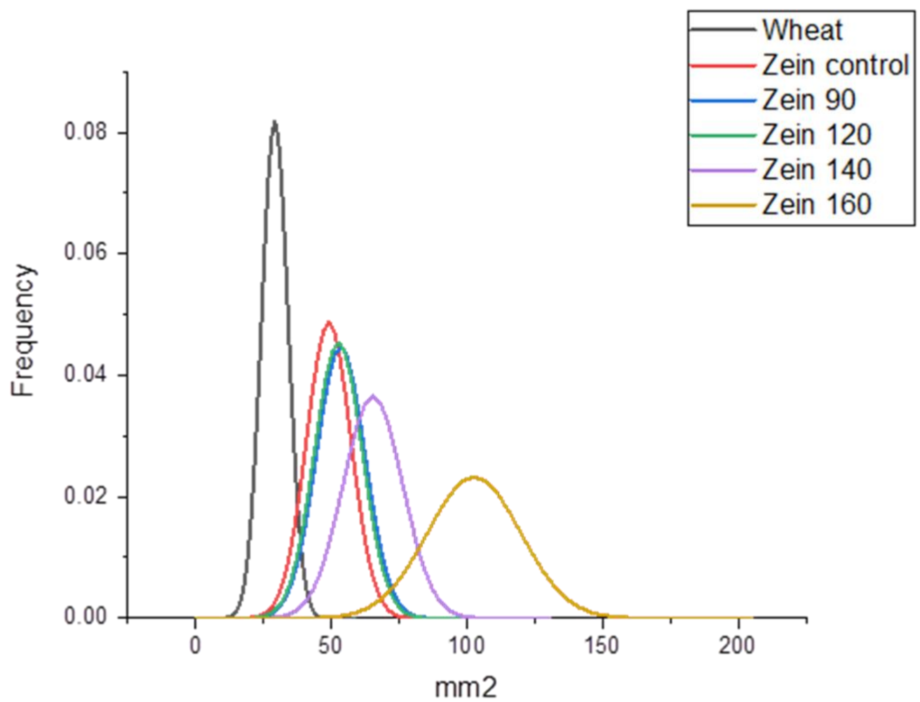


Figure 34 Bread bubble size distribution

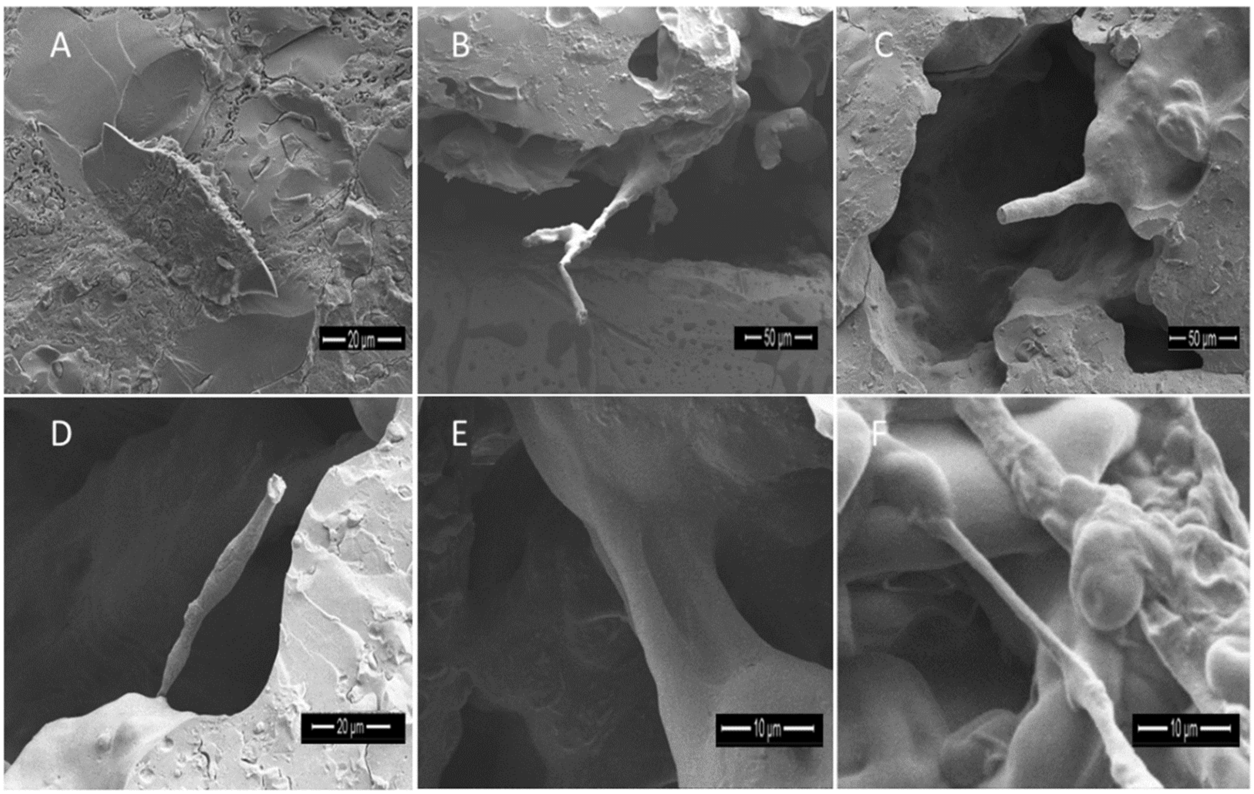


Figure 35 Micrographs of wheat bread (a), bread made with zein (b), Zein extruded at 90°C (c), Zein extruded at 120°C (d), Zein extruded at 140°C (e), Zein extruded at 160°C (f)

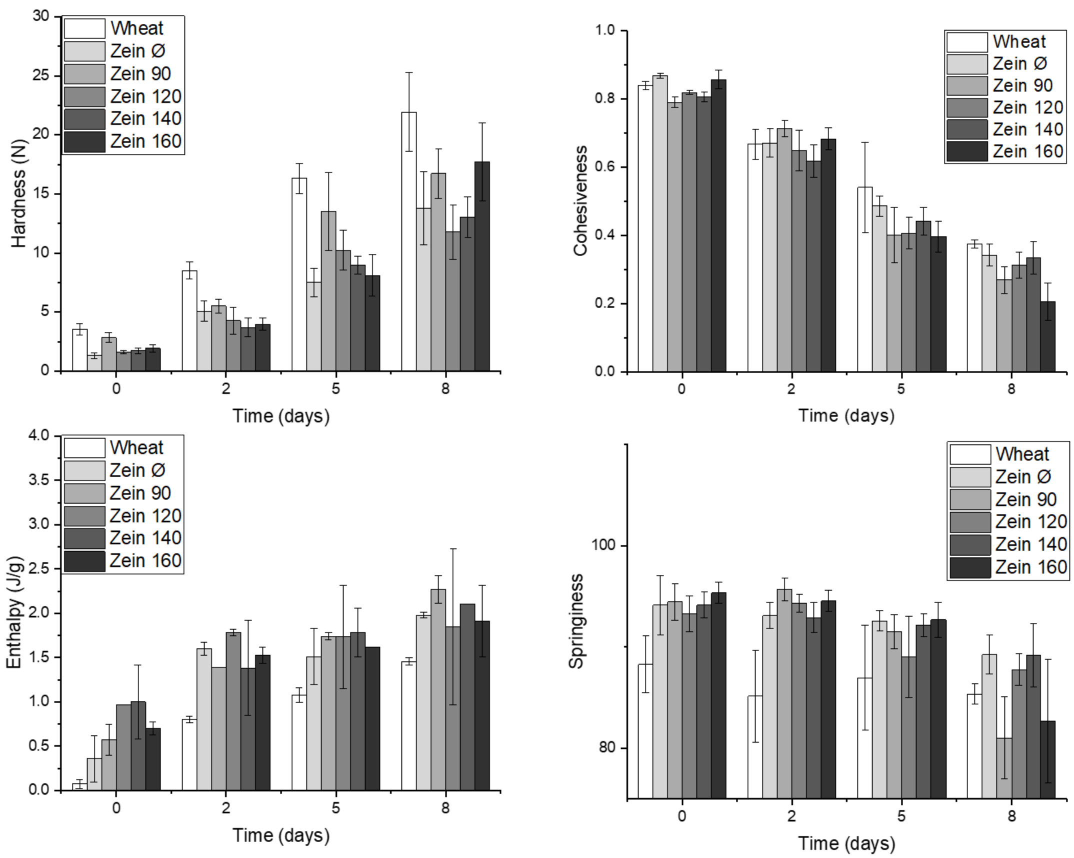


Figure 36 Texture profile analysis and DSC parameters of zein breads during shelf life

Table 10 Comparison of zein bread with wheat bread

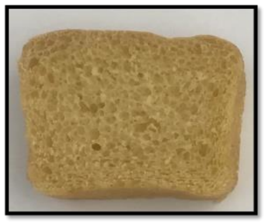
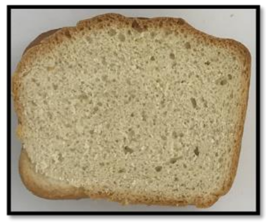
	Zein Bread	**Wheat bread**
Hardness (N)	3.96 ± 0.43	3.54 ± 0.45
Specific volume (cm^3/g)	3.43 ± 0.14	3.33 ± 0.05
Springiness	0.817 ± 0.044	0.882 ± 0.028
Cohesiveness	0.569 ± 0.025	0.840 ± 0.011
Image		

Table 11 Physical properties of bread incorporating extruded zein

	Specific volume	Water content	L*	a*	B*	Hardness	Cohesiveness	Springiness
Wheat	3.33 ± 0.11	43.95 ± 0.11	69.5± 2.1	-3 ± 1.4	22.75 ± 1.8	3.54 ± 0.45	0.84 ± 0.01	0.88 ±0.3
Zein	3.11 ± 0.04	46.12 ± 0.18	74.5 ± 3.5	0.5 ± 0.7	40.5 ± 13.4	1.3 ± 0.21	0.87 ± 0.01	0.94 ± 0.03
Zein 90°C	2.98 ± 0.24	46.62 ± 0.07	78 ± 1.4	0 ± 1.4	27 ± 9.9	2.84 ± 0.43	0.79 ± 0.01	0.94 ± 0.02
Zein 120°C	3.36 ± 0.05	46.46 ± 0.17	74.5 ± 7.8	1.5 ± 2.1	34 ± 11.3	1.61 ± 0.16	0.82 ± 0.01	0.93 ± 0.02
Zein 140°C	3.17 ± 0.09	47.59 ± 0.1	68.57 ± 2	1.5 ± 0.7	28.7 ± 5.2	1.73 ± 0.25	0.80 ± 0.01	0.94 ± 0.01
Zein 160°C	3.17 ± 0.12	45.96 ± 0.22	73.3 ± 3.8	0.3 ± 0.4	23 ± 8.5	1.94 ± 0.3	0.86 ± 0.03	0.95 ± 0.01

CHAPTER 7. CONCLUSION AND FUTURE RECOMMENDATIONS

In this dissertation, treatment to structure zein and improve its elastic response has been investigated. A good understanding of the generation of extensibility and elasticity in cereal proteins is necessary to produce dough with good viscoelastic properties. This study improved the current understanding of the generation of elasticity and strain hardening behavior in cereal proteins, with a focus on zein. While zein extensibility has been better explored through the study of the formation of fibrils, little is known on how to generate a better elastic behavior in dough without the use of gluten.

In Chapter 3 electrospun fibrils from zein were produced with the hypothesis that gluten elasticity is obtained through the formation of large semi-flexible fibrils, thus forcing zein to form similar fibrils could generate architectures able to generate an elastic response comparable to gluten. Nevertheless, experimental evidence did not show any supporting evidence of this hypothesis as incorporation of fibrils in dough did not result in any benefit in terms of dough rheological properties. Contrary, evidence was collected on the importance of chemical factors, such as the presence of organic acids such as acetic acid, lactic acid, and oleic acid on the stabilization of dough viscoelastic properties. These results suggest that the mere generation of large fibrils assemblies is not sufficient to grant a stable viscoelastic behavior if such structures are not stabilized by enough intermolecular interactions.

On the other hand, chapter 4 and 5 investigated the effect of thermal treatments on zein and their effect on dough rheological behavior. The main hypothesis behind the design of such treatments is that high temperature experienced during extrusion or thermal treatment can favor molecular modification leading to the formation of large molecule assemblies. Large molecules such as HMW glutenin in gluten are better suited to store elasticity as they can form a large network of non-covalent sacrificial bonds which can be broken when the molecule undergoes an external stress and spontaneously reformed through a favorable influence of entropy. Both extrusion and thermal treatment showed the generation of larger aggregate. Experimental evidence on mechanical properties on hydrated zein showed that the increasing presence of large aggregates lead into the dramatic rise of elasticity of zein. Furthermore, spectroscopic evidence indicated the increase of β-sheet formation at increasing thermal treatment, suggesting that high thermal treatment had a stabilizing effect on the formation of β-sheet structures. Increased β-sheet content was associated with increased elasticity, adding evidence on the role of β-sheet structure on the storage of energy and consequently elasticity, similarly to what was observed in gluten. Moreover, addition of treated zein to dough changed its rheological behavior, converting the typical strain thinning behavior of zein dough to strain hardening typical of dough containing gluten.

Evidence collected in chapter, 4, and 5 confirmed the central hypothesis stating that the generation of larger zein assemblies could lead to an improvement of strain hardening and elasticity in zein dough. On the other hand, information collected in chapter 3 indicated that only the formation of physical fibrils is not a sufficient element to generate a good elastic dough. In synthesis, only treatment favoring the formation of zein large assemblies at a molecular level such as extrusion or thermal treatment resulted effective in enhancing zein elasticity.

Despite encouraging results in the improvement of rheological properties of dough showed in chapter 4 and 5, the incorporation of extruded zein and thermal treatment did not lead to improvement of zein dough gas retention. This leaves the space for future investigation to better understand zein dough structures with the scope of improving gas retention. As temperature of treatments increased, elasticity of zein masses increased. However, higher treatment did not improve further dough rheological properties due to problem of zein dispersibility. Thus, future studies aimed to further improve zein dispersibility, focusing on enhancing zein interaction with starch and water, have potential to lead to the formation of dough with better breadmaking performances. Furthermore, a better understanding of zein self-assembling mechanism could favor the development of zein dough with enhanced viscoelastic properties.

Results in chapter 5 provided new information on treatment to enhance zein elasticity, however the exact mechanism at the base of the generation of elasticity in zein and in general in cereal proteins are still object of debate. Like the disulphide interchange model, the addition of covalent cross-linking between zein molecules lead to the formation of a more elastic dough, however, covalent bonds are not suited to directly store energy and thus generating elasticity. A potential explanation is the stabilization of β-sheet structure through covalent cross-linking, where ester bond formation due to processing conditions has an analogue role to disulfide bridges in gluten. New studies on zein at a molecular level could potentially shed light into the mechanism with which elasticity is generated and potentially improve the understanding of other cereal protein as well.

Finally, the results of this dissertation can potentially generate a direct impact on society, offering scalable tools to generate new ingredients from zein with enhanced elastic properties. For instance, higher elasticity could help to design formulation to generate high quality gluten-free baked goods. Furthermore, the improved elastic properties of zein could improve its ability to replace gluten in products different from baking good, as for instance in the production of meat analogues where gluten is a good structuring agent, but it is use is limited by a negative perception among consumers. Moreover, benefit imparted by extrusion and thermal treatment could favor application of zein in industries different than food, for instance it could find applications as a plastic biopolymer.

VITA

Enrico Federici earned his B.S degree in food science and technology in July 2014 from University of Parma. During his master's in food science and technologies at the University of Parma he joined Dr. Campanella's Lab as a visiting scholar for 6 Month where he worked on his thesis. In October 2016 earned his master's in food science and technologies. After that he worked for 6 months as a research assistant at the University of Parma. Joined Dr. Jones lab in August 2017 as a Ph.D. student. After terminating his doctoral program, Enrico will join Beyond Meat as a scientist in the innovation team in El Segundo, California.

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Federici, E., Selling, G. W., Campanella, O. H., & Jones, O. G. (2020). Incorporation of Plasticizers and Co-proteins in Zein Electrospun Fibers. Journal of Agricultural and Food Chemistry, 68(49), 14610-14619.

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