FUNCTIONALITY OF CORN AND SORGHUM PROTEINS IN VISCO-ELASTIC DOUGH SYSTEMS

by

BRENNAN M. SMITH

B.S., University of Idaho, 2006
M.S., Kansas State University, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science

KANSAS STATE UNIVERSITY
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Abstract

Zein, the storage protein of corn, has been shown to form a wheat-like dough; however the exact mechanism is unknown since zein lacks the large polymeric proteins found in wheat. To understand how zein forms a dough, different reagents were added during mixing of zein. Salts from the Hofmeister series were used to determine how hydrophobic interactions influence zein's dough forming ability. In addition, urea, ethanol, and beta mercaptoethanol (β-ME) were also tested to evaluate the effects of protein denaturation and disulfide bonds on zein dough formation and bread quality. Kosmotropic salts had a negative effect on zein dough formation indicating that increasing hydrophobic interactions prevented dough formation. Surface hydrophobicity was found to decrease significantly ($p < 0.05$) when zein was exposed to 1M or 2M of the kosmotropic salts. Conversely, chaotropic salts had a slight positive effect on zein dough formation as did urea and ethanol. Interestingly, β-ME had little effect on zein dough formation demonstrating that disulfide bonds played no role in zein dough development, and that large disulfide linked polymeric protein complexes were not present as found in wheat dough. Specific volumes of zein-starch bread increased as NaCl content in the bread formula decreased. Likewise, including 5% ethanol (v/v) in the bread formula was found to increase bread quality. Experiments were also conducted to compare the functionality of isolated sorghum proteins (kafirins) to commercially available zein produced during wet milling of corn. The effect of steeping, the first step in wet milling, on kafirin functionality was investigated. Sorghum flour was steeped for 0, 72, or 96 hours. Unsteeped sorghum flour was digested with Alcalase for 90 min at 50 °C. After steeping or digestion with Alcalase, kafirins were isolated from the remaining material. Both groups of Kafirins had the ability to form a zein-like visco-

elastic resin when mixed with warm water by hand. This is the first time that kafirin has been reported to form a visco-elastic resin using only water as a plasticizer.

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Zein, the storage protein of corn, has been shown to form a wheat-like dough; however the exact mechanism is unknown since zein lacks the large polymeric proteins found in wheat. To understand how zein forms a dough, different reagents were added during mixing of zein. Salts from the Hofmeister series were used to determine how hydrophobic interactions influence zein's dough forming ability. In addition, urea, ethanol, and beta mercaptoethanol (β-ME) were also tested to evaluate the effects of protein denaturation and disulfide bonds on zein dough formation and bread quality. Kosmotropic salts had a negative effect on zein dough formation indicating that increasing hydrophobic interactions prevented dough formation. Surface hydrophobicity was found to decrease significantly ($p < 0.05$) when zein was exposed to 1M or 2M of the kosmotropic salts. Conversely, chaotropic salts had a slight positive effect on zein dough formation as did urea and ethanol. Interestingly, β-ME had little effect on zein dough formation demonstrating that disulfide bonds played no role in zein dough development, and that large disulfide linked polymeric protein complexes were not present as found in wheat dough. Specific volumes of zein-starch bread increased as NaCl content in the bread formula decreased. Likewise, including 5% ethanol (v/v) in the bread formula was found to increase bread quality. Experiments were also conducted to compare the functionality of isolated sorghum proteins (kafirins) to commercially available zein produced during wet milling of corn. The effect of steeping, the first step in wet milling, on kafirin functionality was investigated. Sorghum flour was steeped for 0, 72, or 96 hours. Unsteeped sorghum flour was digested with Alcalase for 90 min at 50 °C. After steeping or digestion with Alcalase, kafirins were isolated from the remaining material. Both groups of Kafirins had the ability to form a zein-like visco-

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Chapter 1: Literature Review

Introduction

Celiac disease is a genetically controlled autoimmune disorder (Leeds et al 2008). It is estimated that celiac disease afflicts ~1% of the world's population, making it the most prevalent genetically controlled disease in humans (Fasano and Catassi 2001; Van Heel and West 2005). When celiac sufferers consume wheat, barley, or rye proteins an autoimmune response is triggered in the upper regions of the small intestine. This reaction causes a histamine response and a subsequent loss of intestinal villi. This response and loss of villi are associated with bloating, diarrhea, various forms of malnutrition, and other symptoms commonly attributed to celiac disease (Godkin and Jewel 1998; Green and Cellier 2007; Fasano and Catassi 2008; Weiser and Koehler 2008). To alleviate symptoms and lead a normal life, celiac patents must consume a diet devoid of wheat, barley, and rye (Fasano and Catassi 2001; Weiser and Koehler 2008).

Gluten-free breads are traditionally made from a batter rather than a dough and are typically of lower quality and have a shorter storage life compared to their wheat-based counter parts (Cornish et al. 2006; Arendt et al. 2008; Schober et al. 2008). It has been hypothesized by many that incorporation of proteins capable of forming networks into gluten-free bread could improve quality and shelf life (Cornish et al. 2006; Arendt et al. 2008; Schober et al. 2008). While some progress has been made, for example with the use of egg proteins, such gluten-free formulations still do not form dough. Gluten-free breads made from a batter system can only take the shape of the pan that the batter was poured in. Research has demonstrated that gluten-free bread produced from a true wheat-like dough utilizing carob germ proteins could greatly improve the quality of gluten-free bread while increasing

handleability (Bienenstock et al. 1935; Smith 2009; Smith et al. 2010, 2012). Schober et al. (2008, 2010, 2011) demonstrated that gluten-free bread could be made from a dough utilizing zein proteins isolated from maize. However the work done by Schober et al. (2008, 2010, 2011) did not identify the key components critical to zein functionality.

Sorghum and corn are both celiac safe grains with nearly identical storage proteins. Kafirin, the storage protein of sorghum, has not been shown to be able to produce a gluten-free dough or even a protein resin using only water as a plasticizer, while zein does (Lawton 1992; Oom et al. 2008; Schober et al. 2008, 2010, 2011). A few papers have addressed this issue by comparing kafirin with commercially available zein (Oom et al. 2008; Schober et al. 2011). While these papers addressed the physical and chemical nature of commercially available zein proteins, they did not identify the factors necessary to produce functional laboratory extracted zein isolates nor identify the critical aspects of zein functionality in a dough system (Lawton 1992; Oom et al. 2008; Schober et al. 2008, 2010, 2011). In order to gain understanding of the various aspects of the storage proteins of maize and sorghum, zein and kafirin will both be examined throughout this review.

Corn Production

Corn/maize is the most widely produced cereal crop worldwide and in the United States. World production for 2010 and 2011 was ~819 million metric tons (USDA-FAS 2012). Of this, the United States produced ~333 million metric tons (USDA-FAS 2012). The most common variety of corn produced in the United States is yellow dent corn (Leath 2003). Yellow dent corn is predominately used for ethanol production, animal feed, starch for various applications, oil, and human consumption (Duensing et al. 2003; Hobbs et al. 2003; Johnson and May 2003; Loy

and wright 2003; Maisch 2003; Mauro et al. 2003; Orthoefer et al. 2003; Rooney and Serna-Salvdivar 2003) . It is grown throughout the United States and is predominately grown in Iowa, Indiana, Nebraska, Minnesota, and Illinois (Fig 1 and Table 1).

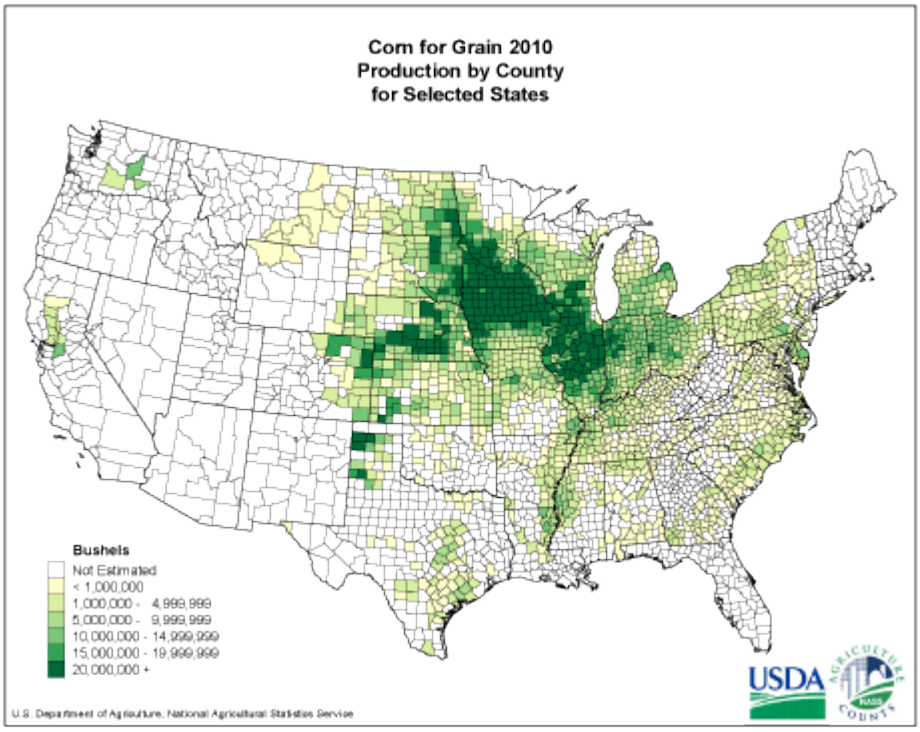

Figure 1: Corn for grain 2010: Production by county for selected states (Taken from USDA-NASS 2010)

Table 1: Corn yield and production by state (2007-2009) (Taken from USDA-NASS 2009).

Corn Yield and Production by State 2007–2009

State	Yield (per acre)			Production		
	2007 Bushels	2008 Bushels	2009 Bushels	2007 1,000 Bushels	2008 1,000 Bushels	2009 1,000 Bushels
South Dakota	121	133	153	542,080	585,200	719,100
Iowa	171	171	182	2,376,900	2,188,800	2,438,800
Illinois	175	179	175	2,283,750	2,130,100	2,065,000
Nebraska	160	163	178	1,472,000	1,393,650	1,575,300
Minnesota	146	164	175	1,146,100	1,180,800	1,251,250
Indiana	154	160	171	980,980	873,600	933,660

Source: USDA Crop Production Summary 2009

Corn can be grown in a variety of conditions and soils. In general, corn plants will not survive heavy frost and will grow at temperatures between 9 °C and 40 °C, where ~25 – 30 °C is considered optimum growing temperature. Growing seasons can vary based on a number of factors, but in general corn will require between 100 -140 days from germination to harvest (Farnham et al. 2003; Darrah et al. 2003).

Sorghum Production

Sorghum is an important crop throughout the world. It provides fodder, shelter, tools, sugar, and grain (House et al. 2000). Sorghum is considered to be the fifth most important cereal crop in the world and in the United States it is ranked third for cereal crop production (USDA-FAS 2012b). Worldwide sorghum production has been reported as ~65 million tons, of which, Africa and Asia accounted for ~55% and the United States for ~29 (USDA-FAS 2012b). Of the ~19 million metric tons grown in the United States, ~90% is grown in Kansas, Texas, Nebraska, Oklahoma, and Missouri (Fig 2 &3) (Rooney 2000).

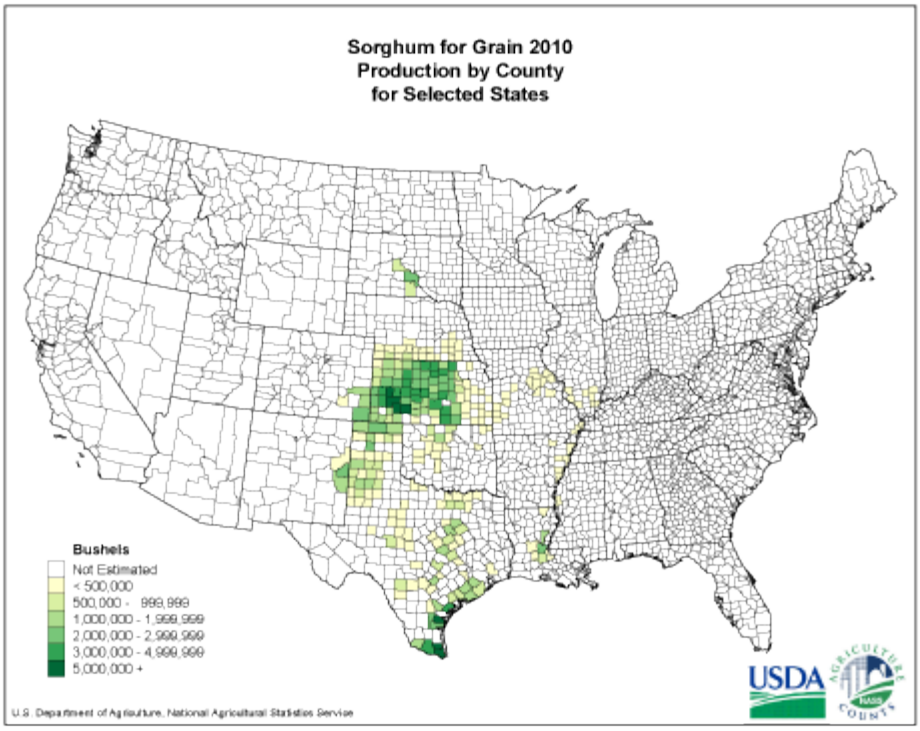

Figure 2: Sorghum for grain 2010: Production by county for selected states (Taken from USDA-NASS 2010b).

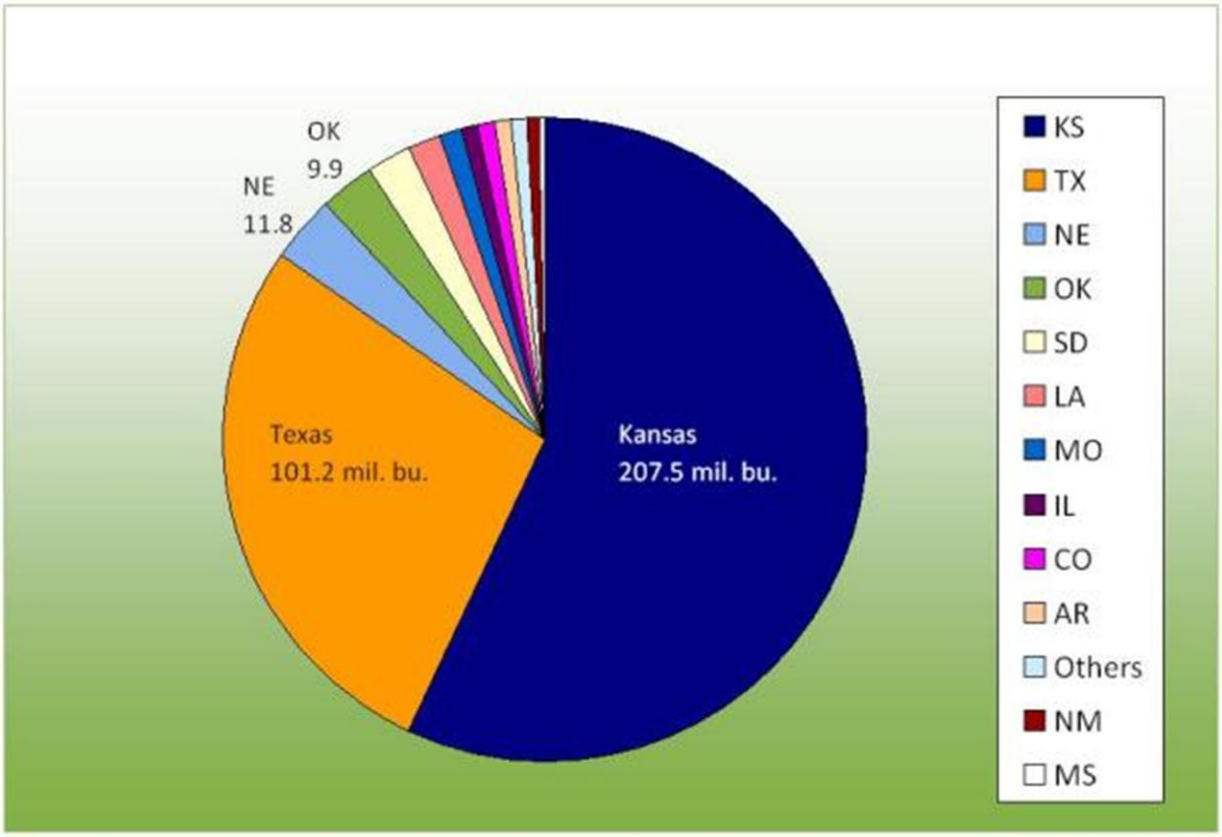

Figure 3: Sorghum production by state in millions of bushels (Taken from Wisner 2009).

Sorghum is grown throughout the world in a wide array of conditions. It is a drought hardy plant that withstands temperatures that range from ~10 – 35 °C but a light frost can be lethal to the crop (Kimber 2000). For this reason it is typically planted in early summer in the United States (Gardner et al. 1985). Sorghum can be grown in most types of soils: acidic, basic, heavy in clay, or light in clay (Kimber 2000). Altitudes up to 3000 m have been reported to be able to support sorghum (Kimber 2000). Therefore, sorghum can be grown almost anywhere where temperature, day length, and minimal moisture requirements are met.

Sorghum is planted in late May through early June in the United States. In other equatorial regions of the world, multiple sorghum corps can be grown annually. This is because

sorghum is a short-day plant and will only flower when the photo period is shorter than the critical maximum (Gardner et al. 1985). Some varieties of sorghum can be grown as a perennial in equatorial areas (Smith 2000). Temperatures in these regions are also conducive for sorghum growth (Smith 2000). Sorghum varieties grown in areas away from the equator, including the United States, have been selected to a have decreased photosensitivity and will flower to produce grain when days are long in summer months (Gardner et al. 1985).

Native Kafirin Structure

Protein is found throughout the caryopsis and is present in ratios of ~80% in the endosperm, ~16% in the germ, and 3% in the pericarp for sorghum (Rooney and Serna-Saldivar 2000). Of these proteins, about half are made of alcohol soluble proteins known as prolamin. This protein fraction is given its name due to its high levels of proline and glutamine. The prolamin fraction of sorghum is referred to as kafirin. Kafirin is higher in glutamic acid and aspartic acid when compared to zein (Rooney and Serna-Saldivar 2000). Kafirins are hydrophobic proteins located primarily in tightly packed protein bodies found in both the hard and soft endosperm (Fig 4) (Gardner et al. 1985).

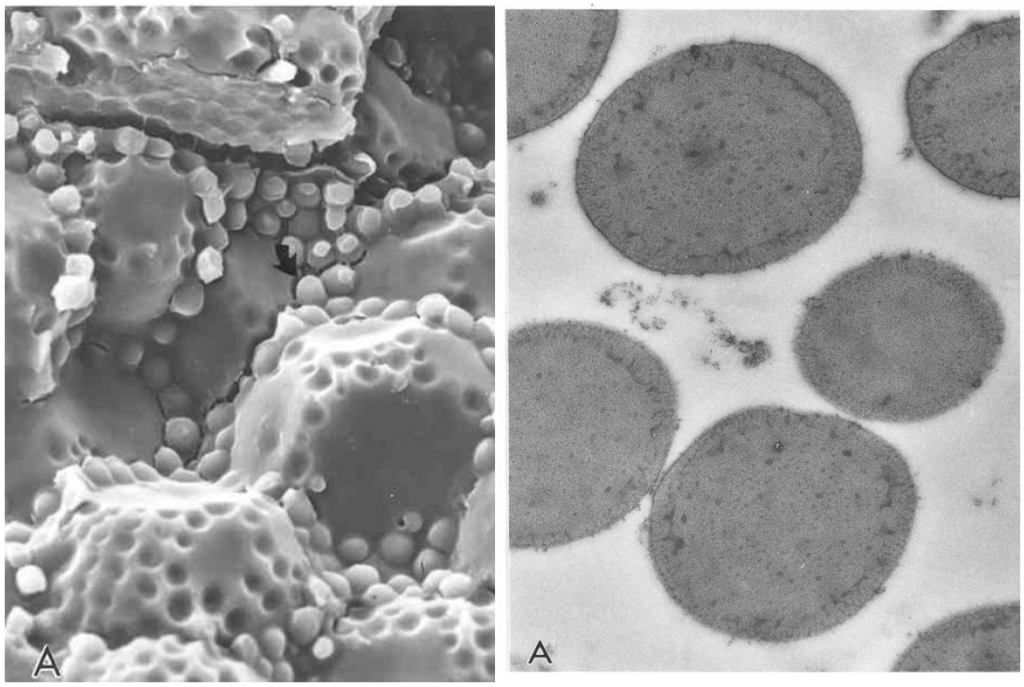

Figure 4: Kafirin proteins bodies with starch as seen by scanning electron microscopy (left). Kafirin protein bodies as seen by transmission electron microscopy (right) (Taken from Hamaker and Bugusu 2003).

Kafirins are made up of three fractions known as α – kafirin, β – kafirin, and γ – kafirin. The α – kafirin is the most prevalent form and is found in the innermost regions of the protein body. β – kafirin and γ – kafirin contain high levels of cysteine that form disulfide bonds. When covalently bound, these proteins form the dense web of protein material that makes up a majority of the outermost layer of the protein body (Fig 5) (Rooney and Serna-Saldivar 2000).

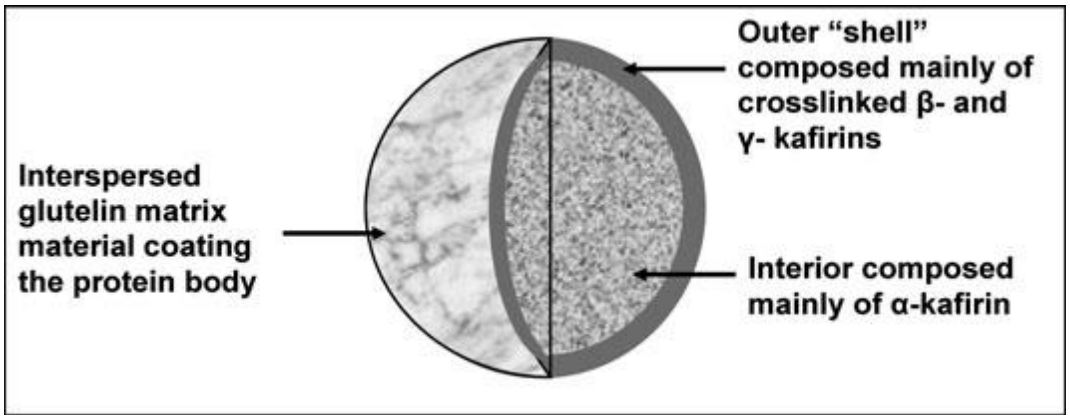

Figure 5: Artist's interpretation of a kafirin protein body with compositional components labeled (Taken from de Mesa-Stonestreet et al. 2010).

Other proteins of sorghum are in the form of glutelins, albumins, and globulins that make up enzymes, cell material, and other proteins needed for seed structure and plant development. Like most cereal grains, sorghum's limiting amino acid is lysine. Lysine is only present at ~45% of the FAO/WHO recommended levels (Rooney and Serna-Saldivar 2000).

Native Zein Structure

Native zein proteins are nearly identical to those of kafirins from sorghum. Like kafirin, zein is found throughout the endosperm of the caryopsis and is present in the greatest quantities in the vitreous endosperm. Zeins are found in protein bodies identical in appearance and organization to kafirins (Fig 4 & 5). The ratios of α, β, and γ protein fractions of kafirin and zein are the primary differences between the two proteins. Kafirin contains ~ 65-80% α-kafirin, ~7-8% β-kafirin, and 9-12% γ-kafirin, and zein contains ~70% α-zein, ~5% β-zein, and 20% γ-zein (Esen 1987; Shull et al. 1991; Watterson et al. 1993; Hamaker et al. 1995; Lawton and Wilson 2003; Belton et al. 2006; Schober and Bean 2008).

Zein Isolates: Structure and Uses

Historically, zein has played a vital role in many industrial applications. Zein is extracted from corn gluten meal, a byproduct of industrial corn starch isolation, and is almost pure α-zein. In the late 1940's and throughout the 1950'5, zein was used for the production of fibers (Shukla and Cheryan 2001; Lawton 2002). These fibers were produced by spinning alkaline zein solutions and curing them with acids, salts, and formaldehyde. Zein fibers were used for a variety of purposes including furniture padding and textile production. However, due to the expensive nature of the process and environmental issues that arose, fiber production was stopped (Shukla and Cheryan 2001; Lawton 2002).

In more recent times, the need for renewable resources has led to zein's use in food coatings that not only help with appearance of coated foods, but also aid in protecting product quality (Shukla and Cheryan 2001; Lawton 2002). Biodegradable films made from zein have also recently been successful for uses in bags and packaging (Shukla and Cheryan 2001; Lawton 2002). Yet another major use of zein has been for the production of controlled or sustained release of pharmaceuticals (Shukla and Cheryan 2001; Lawton 2002).

While there have been many uses of zein throughout the last century, the structure of zein proteins and how it relates to functionality is not well known. Argos et al. (1982) were the first to propose a structure for isolated zein proteins based on data from circular dichroism of zein dissolved in 70% aqueous ethanol. Here it was proposed that nine homologous repeating units made up of 18 residue helical wheels (Fig 6) arranged in anti-parallel positions and stabilized by hydrogen bonds (Fig 7) between pairs of amino acids in the polar regions of the protein (Fig 7). This led to the proposed nine helical structures (Fig 8), which led to the model

for the possible arrangement of zein in a plane and for stacking of molecular planes (Fig 9). Matsushima et al. (1993) later proposed a structure for reduced α-zein that indicated that zein exists as an asymmetrical particle of ~13 nm in length with an elongated molecular structure and an axial ratio of 6:1.

Using published physical measurements, computational algorithms, and structural modeling, Momany et al. (2006) proposed a different three-dimensional structure of α-zein in aqueous methanol solutions. In this model, coiled-coil triple superhelix structures were proposed (Fig 10). When coupled together, these triple superhelix structures were thought to form larger structures with lutein at the core (Fig 11). Lutein is naturally occurring in zein and difficult to remove (Momany et al. 2006).

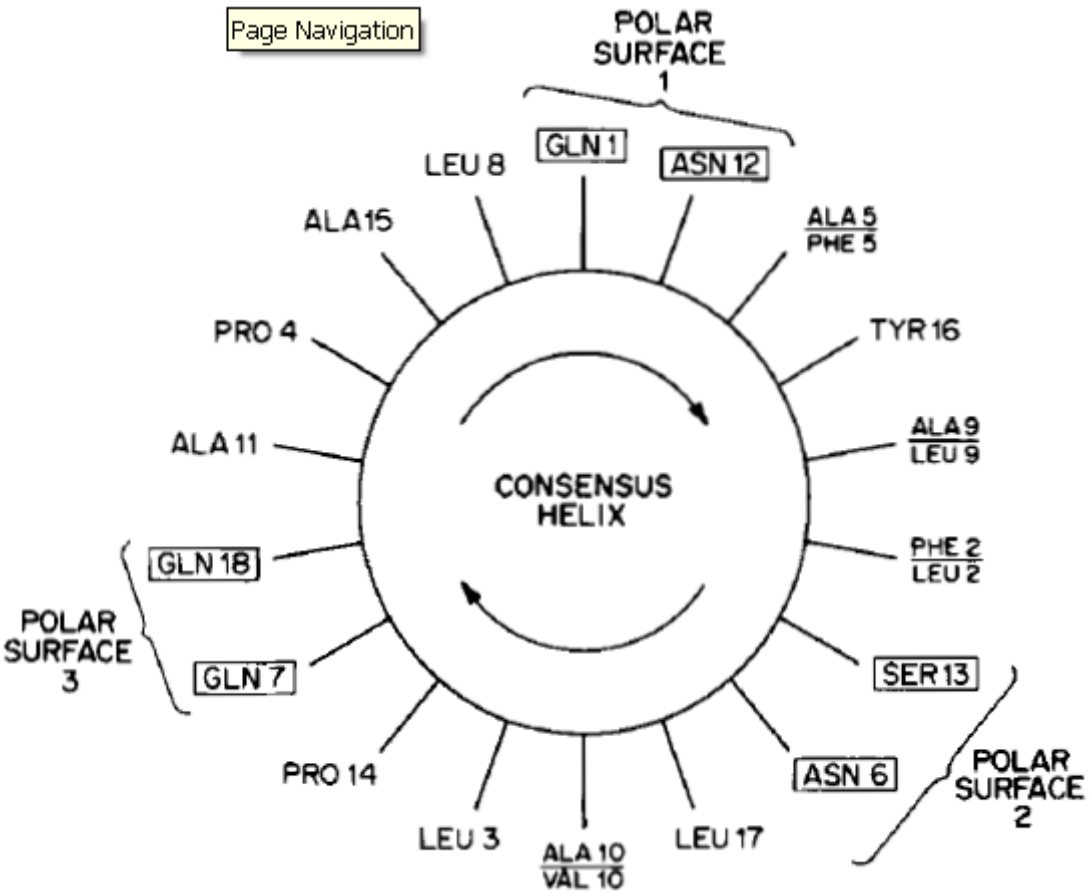

Figure 6: Helical wheel model for the 18 amino acid residue repeat sequence for α-zein (Taken from Agros et al. 1982).

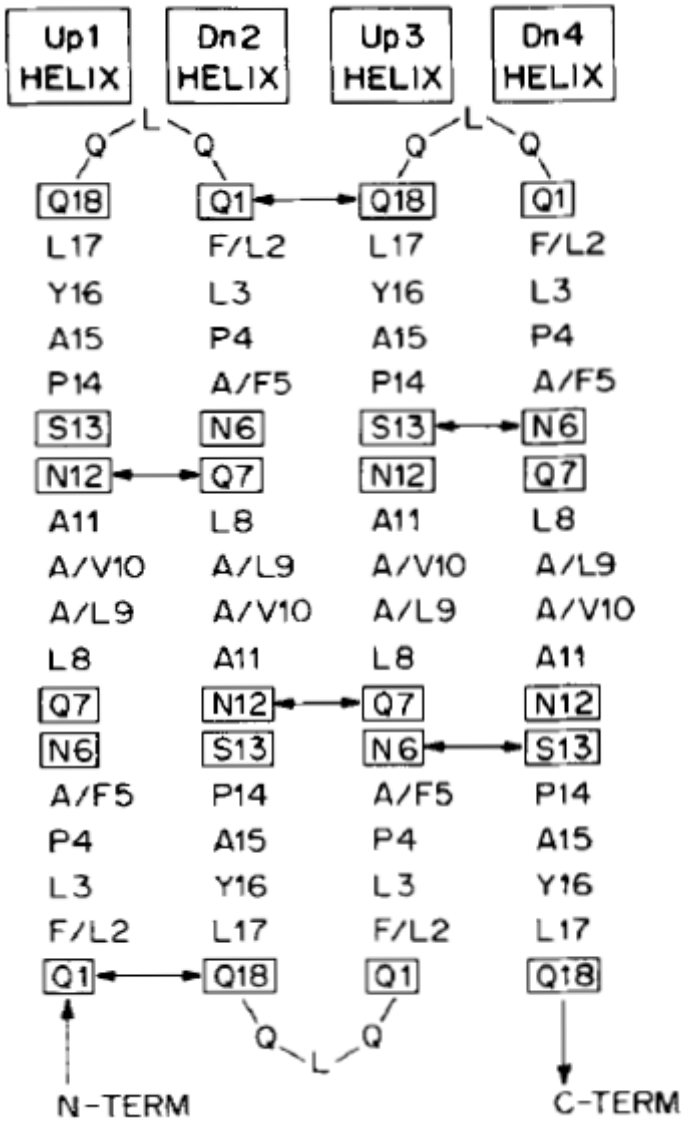

Figure 7: Possible hydrogen bonding interactions between polar amino acids of adjacent anti-parallel consensus helices (Taken from Argos et al. 1987).

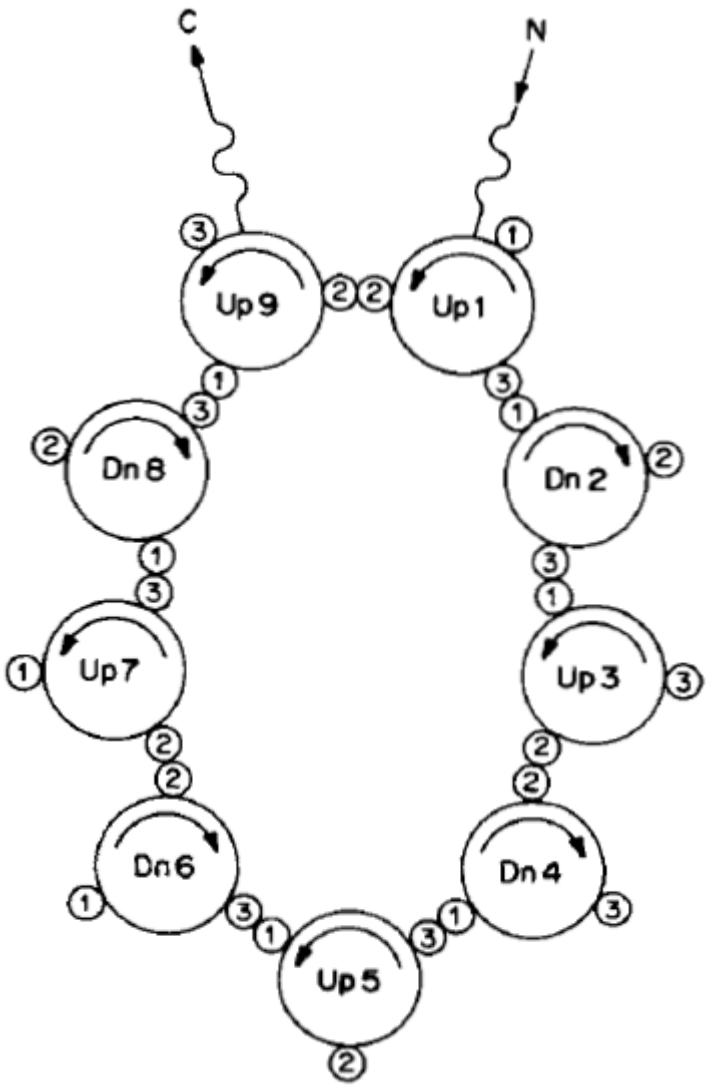

Figure 8: Proposed nine helical structure of α-zein in 70% methanol (Taken from Argos et al. 1982).

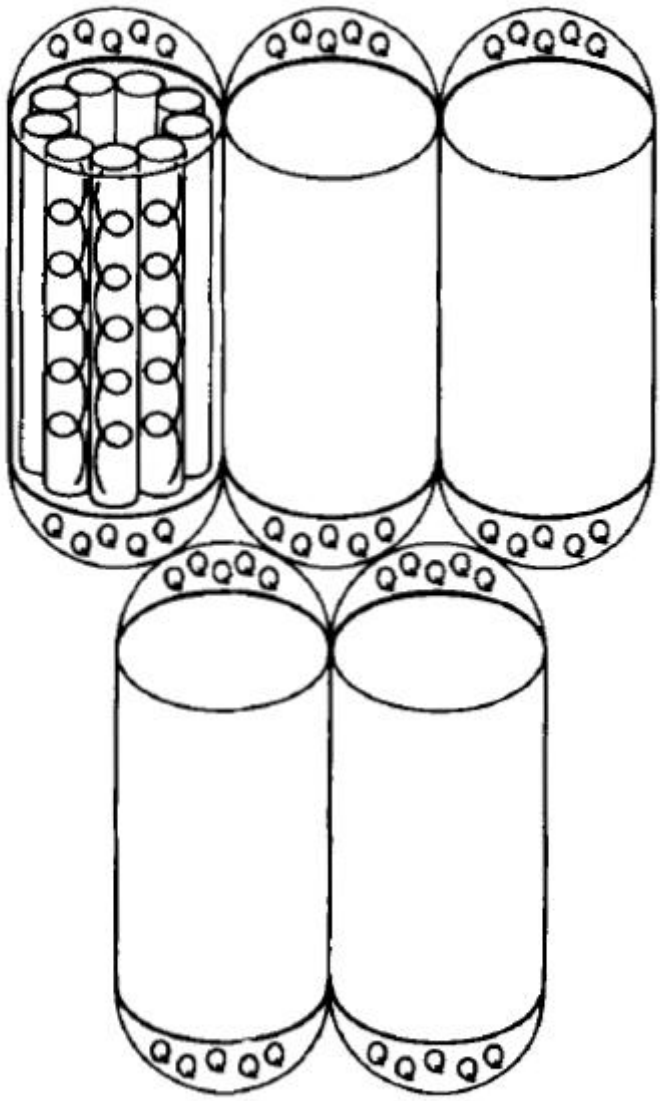

Figure 9: Possible arrangement of α-zein 9 helical structure in a plane and for structure of stacking of the molecular planes (Taken from Argos et al. 1982).

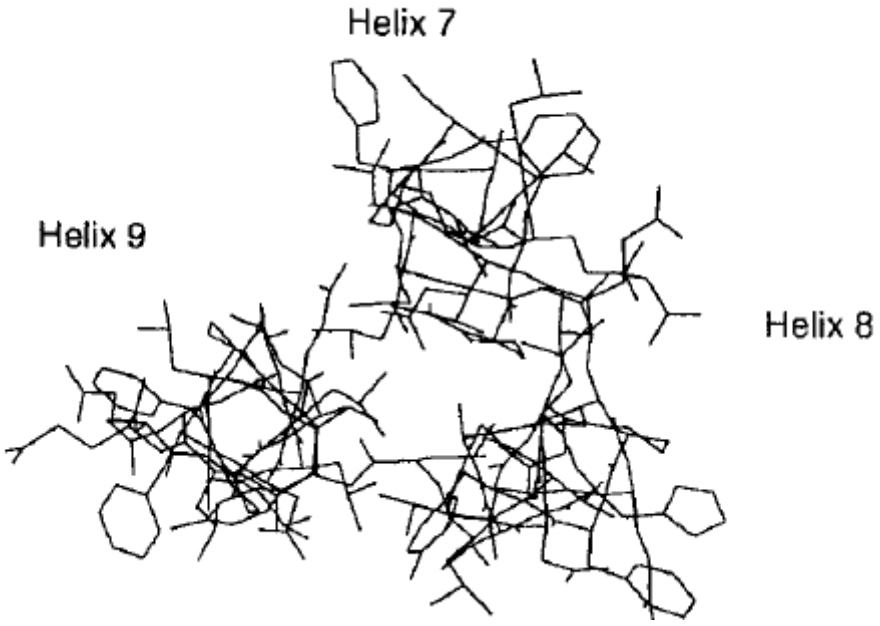

Figure 10: Stick model representation of the proposed coiled coil triple superhelix looking down the axis of the superhelix axis (Taken from Momany et al. 2006).

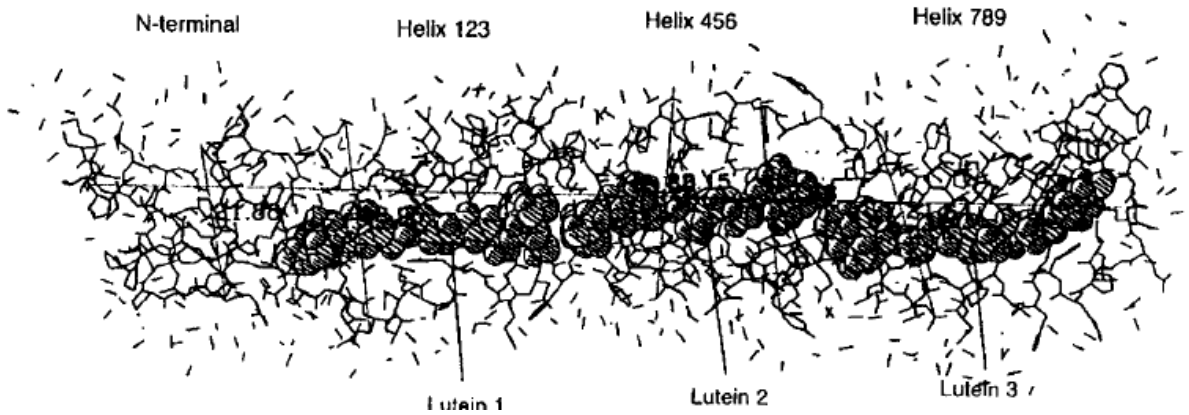

Figure 11: A stick model representation of the complete structure of α-zein in 70% methonal with coupling of the coiled coil triple superhelix structures around a lutien core (Taken from Momany et al. 2006).

While this research does propose possible structures of isolated α-zein in solutions, it gives little information into the structure of zein in a dry state or when hydrated with water Structural models based on the use of isolated zeins would also not be comparable to the

native structure of zeins as they exist in flour. It has not been possible to experimentally determine the structure of isolated zein using techniques such as x-ray crystallography or NMR.

Zein and Kafirin Isolates Functionality and Structure

Wheat is a unique cereal grain in that its storage proteins are able to form a visco-elastic network in the presence of water and energy input. Visco-elastic materials are materials that respond to an applied force or displacement by exhibiting either elastic or viscous behavior, or through a combination of these. This ability to form visco-elastic protein networks (i.e. gluten networks) is what allows wheat to form moldable, handleable dough and high quality baked goods. Because of the ability of wheat gluten to form dough and high quality baked goods, it is difficult to find substitutes that will function similarly to wheat gluten and provide a quality food source for those with wheat allergies and gluten intolerances. Currently, only a few non-wheat proteins that have some degree of visco-elastic properties have been identified. Proteins of the carob germ and isolated maize proteins have both been found to have gluten-like properties (Lawton 1992; Smith et al. 2010). Kafirins have nearly identical physical and chemical characteristics as zein protein, the predominant storage protein of maize. Due to the similarities between kafirin and zein, it is plausible that similar functional traits can be obtained from kafirin.

Because of the parallels between kafirin and zein and the more extensive research completed on zein, zein functionality will be reviewed and used as a model to discuss both zein and kafirin functionality. One of the earliest attempts to produce dough from zein protein was completed by Lawton (1992). In these experiments isolated zein was mixed with starch in a ratio of 10% zein to 90% maize starch to simulate the two predominant fractions of wheat flour

(protein and starch). When mixed with water above zein's glass transition temperature (T_g), zein-starch composite flours were able to produce wheat-like visco-elastic dough. It was found that the visco-elasticity of zein was dependent on the T_g of zein (i.e. temperature and water content). When mixed in a farinograph, dough mixed at 35 °C as opposed to 25 and 30 °C produce the most desirable or visco-elastic dough. Lawton also determined that like wheat dough, formation of visco-elastic zein-starch dough was dependent on the development of a network of protein fibers. It was also concluded that below zein's T_g no protein fibers were formed and therefore a visco-elastic dough was not developed.

To follow up on Lawton's work that defined the conditions necessary to form visco-elastic dough from zein and starch, Mejia et al. (2007) determined the required secondary structures of zein proteins needed to form visco-elastic dough. Like Lawton (1992), zein was mixed with maize starch in a ratio of 10% zein to 90% maize starch. The zein-starch composite flour was then mixed with water in a mixograph at 35 °C. It was found that substantial changes occurred to zein during mixing and dough formation. Before mixing, the zein was determined to contain ~65% α-helixes and ~30% β-sheets as established by Fourier transform infrared (FT-IR) spectroscopy. In a hydrated visco-elastic dough form at 35 °C, the ratio of α-helixes to β-sheets shifted to ~30% and ~48% respectfully. This demonstrated that the development of a visco-elastic dough from zein-starch composite flour was dependent on temperature, as determined by Lawton (1992), and shear from mixing. A temperature above zein's T_g coupled with shear in the form of mixing is the causative factor necessary for the increase of β-sheets and decrease in α-helixes needed to form a true wheat-like dough. However, it was found that the β-sheet structures were not stable when compared to those of wheat gluten. As the zein dough was

cooled there was a subsequent loss in β-sheet structures and visco-elasticity. Not only was it determined that β-sheets were necessary for zein-starch visco-elastic dough formation, but the quantity of these structures was directly related to dough quality in terms of resistance to mixing as determined by mixograph.

The first research to compare the functionality of kafirin to zein was completed by Oom et al. (2008). Kafirin was able to form resins similar to that of zein when plasticized with oleic acid at a level of 50% kafirin (w/w). Although the kafirins in this experiment were able to form similar resins to zein, the proteins were unable to form visco-elastic dough as described by Lawton (1992). Instead, the kafirin remained as discrete particles in the starch-water matrix. The researchers attributed this to the fact that the commercially purchased zein contained only α-zeins as determined by SDS-PAGE. Kafirins however, were found to contain both α-kafirin and γ-kafirin fractions in the laboratory extracted protein isolate. The researchers postulated that the γ-protein fraction, found in both zein and kafirin, which may be more hydrophobic in nature when compared to the α-protein fractions did not allow for appropriate hydration of the isolated proteins and subsequent plasticizing when mixed with water and starch. However, data on the hydrophobicity of the various kafirin subclasses is not clear cut. For example, α-kafirins elute much later than other kafirin sub-classes on RP-HPLC (Bean et al. 2000) and are soluble in more non-polar solvents than γ-kafirins (Shull et al. 1991), suggesting that the α-kafirins are more hydrophobic than the γ-kafirins. Another hypothesis made by the researchers is that the cysteine rich γ-kafirin was cross-linking to form disulfide bonds between cysteine residues. This could explain why the resins formed by kafirin were reported to be stiffer and more resistant to extension. It may also help to explain why the kafirin isolates used in the experiment were

unable to form visco-elastic dough. The formation of disulfide bonds between and within the proteins may not allow for the appropriate structural changes that were described by Mejia et al. (2007) to occur.

The first breads produced from visco-elastic zein-starch dough were completed by Schober et al. (2008). To accomplish this, modifications had to be made from the original dough formulation described by Lawton (1992) and used by others (Mejia et al. 2007; Oom et al. 2008). Flour composition for the zein-starch dough and bread was 20% zein-80% maize starch. Hydroxypropyl methylcellulose (HPMC) was also added as a functional ingredient. Other ingredients included water, yeast as a leavening agent, salt, and sugar. The dough was mixed at 40 °C instead of 35 °C. The modifications were made due to the inability of Lawton's (1992) zein-starch dough formulation to retain enough gas to produce satisfactory bread under the conditions of these experiments. HPMC is a surface active hydrocolloid that not only assisted with gas retention, but also allowed for zein fiber production within the restraints of the experiment. In previous work where zein fibers were identified as being crucial for visco-elastic dough formation, farinographs and mixographs were used to apply shear to the zein-starch water mixture in the form of mixing to create zein fibers (Lawton 1992; Mejia et al. 2007). Schober et al. (2008) mixed the zein-starch mixtures by hand, which would have imparted less energy into the zein-starch mixtures used in that study than in the work of Lawton (1992) and Mejia et al. (2007). The effect of energy input into zein-starch dough systems has not yet been studied, but it is possible that mixing conditions may influence the amount of β-sheet formed and influence formation of a protein network of fibers as identified by Mejia et al. (2007). Regardless, Schober et al. (2008) did demonstrate that HPMC could be used to increase the

functionality of zein in terms of protein fiber formation and gas retention in dough which was the primary intention of the research. In a latter study by Schober et al. (2010) the role of lipids in zein-starch dough formation was addressed. The partial removal of lipid from the surface of zein particles was attributed to stronger dough and higher quality bread by possibly allowing increased protein-protein interaction.

All of the previous research discussed above, utilized commercially available zein. While a bulk of the zein isolation process is well known, the proprietary nature of the product leaves many aspects of processing procedure unknown. While the commercially purchased zein definitely does function in the manner described in the various publications reviewed above, it is only indicative of how this particular commercially produced zein functions. For this reason, comparing laboratory extracted kafirins to the commercial available zein protein in terms of functionality should be conducted with caution as the two materials are not prepared in the same manner. Ideally, kafirins and zein should be extracted in an identical manner in order to draw conclusions on differences in their functionality. The commercially available zein should instead be used as a model in identifying the causative factors of both zein and kafirin functionality, so that a reproducibly functional protein isolate can be made in a laboratory setting. Realizing this, Schober et al. (2011) identified how different isolation procedures impacted zein and kafirin functionality. Relatively pure α-zein isolated in the lab was found to have some properties similar to commercial zein. However, laboratory isolated kafirins did not have the same functionality. One hypothesis put forward by Schober et al. (2011) was that α-kafirins were more difficult to isolate in a pure form than the α-zeins possibly due to similarities in hydrophobicity between α and β-kafirins. In RP-HPLC separations, the α and β zeins differed

substantially in their elution times, suggesting differences in surface hydrophobicity at minimum. It is possible then, that it is easier to isolate α-zeins from β-zeins by controlling the polarity of the extraction solvent. In contrast, α and β-kafirins elute close together in RP-HPLC suggesting that in sorghum these two kafirin types are more similar in surface hydrophobicity making it more difficult to extract α-kafirins not contaminated with β-kafirins.

Although the experimentally isolated zein was able to aggregate in water this research did not specify if it was able to form visco-elastic dough. The near absence of cysteine in the isolated zein implies that disulfide cross-linking is not responsible for zein resin and dough formation as it is with wheat gluten, but through other interactions. It was suggested that hydrophobic interactions were responsible for zein protein functionality (Schober et al. 2011).

In order to make kafirin function as commercially available zein, a full understanding of why commercially available zeins become visco-elastic is needed. It is clear that not all isolated zein is equal in terms of its functionality. Commercially isolated zein from corn gluten meal has been subjected to various processes including exposure to organic solvents, alkaline pH, and high temperatures (Lawton 2002). Any of these steps could produce modifications to the proteins that are responsible for the functionality of zein. By understanding the cause and mechanisms behind the functionality of isolated zein, they can be applied and modified to form visco-elastic kafirin-starch dough. This could be important for gluten-free food markets and in areas where sorghum proteins could be isolated from flour milling residues (da Silva and Taylor 2004) or dried distillers grains from the bio-ethanol industry (Wang et al. 2009).

In addition to the work described previously targeting visco-elastic dough formation, research has been done on modifying isolated kafirins to alter their functionality by creating

protein-polysaccharide and protein-protein complexes (Babiker and Kato 1998). A major limitation of this study was in the protein isolation methods used. Proteins were extracted from sorghum using a 0.03 M Tris-HCl buffer, pH 8, containing a reducing agent. By definition, the proteins isolated with this extraction procedure were not prolamins and were therefore not kafirins. The proteins that were extracted and used for conjugating polysaccharides and protein onto were most likely a mixture of albumins, globulins and perhaps γ-kafirins, which are known to be water soluble once reduced (Belton et al. 2006). Although no protein yields were reported by Babiker and Kato (1998), kafirin accounts for ~68-73% of the total protein found in sorghum grain (Hamaker et al. 1995), hence only a small fraction of the total proteins in sorghum could have been extracted. However, the isolation and modification of kafirins or zeins is an interesting concept and kafirins or zeins conjugated to polysaccharides or other proteins could have a pronounced change in functional properties and could lead to improved uses of sorghum and corn proteins.

The Effects of Hofmeister salts and Urea on Protein Structure and Function

Hofmeister or lyotropic salts are a series of salts assigned a ranking based on their ability to salt in or salt out proteins (Melander and Horvath 1977; Collins 2006; Peterson and Saykally 2006; Jungwirth and Winter 2008; Zhang and Cremer 2010). While the exact mechanism of the salting in or salting out effect is not well known, the addition of these salts is known to change the 3D structure of the proteins via disruption or promotion of hydrophobic or hydrophilic interactions within a protein depending on the salt used and the protein in question (Melander and Horvath 1977; Collins 2006; Peterson and Saykally 2006; Jungwirth and Winter 2008; Zhang

and Cremer 2010). In general, the anions have a greater effect on salting in or out than do cations (Zhang and Cremer 2010). The general order to which anions salt out proteins is:

$$CO_3^{2-} > SO_4^{2-} > H_2PO_4^- > Cl^- > Br^- > I^- > ClO_4^- > SCN^-$$

Where SCN^- has a salting in effect and Cl^- is considered to be the dividing point for salting in and salting out. The general order to which cations salt out proteins is:

$$NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$$

Traditionally these salts have been classified as kosmotropic (ions that salt out proteins) and chaotropic (ions that salt in proteins) in nature (Zhang and Cremer 2010). It was originally thought that kosmotropic ions strengthened the hydrogen bonding of water, providing a more ordered network of hydrogen bonded water (Zhang and Cremer 2010). This in turn, promoted the shielding of the hydrophobic regions of the protein via an increase in protein folding. This increase in folding also was thought to result in the precipitation of proteins in solution. Chaotropic salts on the other hand were thought to weaken the hydrogen bonding of bulk water allowing for unfolding of proteins and exposure of the hydrophobic regions of proteins (Zhang and Cremer 2010). Recent studies however, have shown that this hypothesis probably does not hold true (Omta et al. 2003, 2003b; Kropman and Bakker 2003; Batcherlor et al. 2004; Zhang and Cremer 2010). Kosmotropic and choatropic salts were found to have no effect on increasing or decreasing the hydrogen bonding of bulk water in solutions (Batcherlor et al. 2004; Zhang and Cremer 2010). Instead, the effects that lyotropic salts have on protein in uncharged systems seem to be dependent on the interaction that ions have with protein macromolecules and their surrounding hydration shells. This in turn can alter hydration enthalpies, surface tension increments, and ability of the ions to bind proteins (Zhang and

Cremer 2010). On a positively charged system, low concentrations of salts seem to have an inverse Hofmeister effect, which is dependent on electrostatic interactions based on size and hydration properties of the ions. In higher salt concentrations, the ions will neutralize the charge and the salts will begin to follow the normal salting in and salting out effects of the Hofmeister series. At higher salt concentrations, protein folding and solubility seem to be affected by interfacial tension at the protein water interface (Zhang and Cremer 2010).

Like the Hofmeister series, recent research has brought new insight into how osmolytes like urea can change protein structure and solubility. Traditionally, urea has been used as a protein denaturant. It has been generally accepted that urea acted as a protein denaturant by hydrogen bonding to polar side chains of amino acids. Current studies acknowledge that these interactions do occur, but several other mechanisms may also be occurring to denature proteins. With a 4 M solution of urea ~ 76% of the water is in contact with urea, leaving ~24% bulk water to contact proteins (Bennion and Dagget 2003; Zhang and Cremer 2010). This means that high concentrations of urea promote the unfolding of proteins because it is thermodynamically favorable due to the lack of bulk water in contact with the protein. It has also been shown that urea is capable of interactions with the peptide backbones of proteins (Auton and Bolen 2005; Street et al. 2006; Zhang and Cremer 2010). Furthermore, the structural changes made to water by urea may also affect the hydration properties of the protein and affect hydrophobic interactions (Zhang and Cremer 2010).

While little to no known work has been conducted on the zein and kafirin to examine the effects of lyotropic salts and osmolytes on their structure and function, several studies have investigated the effects of different salts and solvents on wheat gluten (Preston 1981; Preston

1984; Preston 1989; Butow et al. 2002; Wellner et al. 2003; Melnyk et al. 2011). In these studies, the predominant findings were that Hofmeister salts can affect mixing properties and qualities of dough from different wheat varieties. At lower concentrations (< 0.10M) salts classified as Kosmotrops were beneficial to wheat gluten functionality as seen by increased extensibility and resistance to breakdown (Preston 1989; Melnyk et al. 20011). Lower concentrations of choatropic salts had a negative effect on dough quality by shortening the time to breakdown and reduced extensibility (Preston 1989; Melnyk et al. 20011). The effects of Kosmotropic salts were largely attributed to electrostatic shielding of charged amino acids on the surface of gluten (Preston 1989). With higher concentrations of salts (> 0.50M) the choatropic salts had a beneficial affect on wheat gluten by disrupting inter-protein hydrophobic interactions (Preston 1989; Melnyk et al. 20011). This, in turn, changed protein conformation and increased dough extensibility and time to breakdown (Preston 1989; Melnyk et al. 20011). Higher concentrations of kosmotropic salts were found to have a negative effect on gluten and reduced dough quality. Since the Hofmeister salts have been found to have an impact on gluten functionality, it is likely that they will also have an impact on the zein functionality. The impact of Hofmeister salts should be investigated to further the utilization of zein by the possible increase in functionality due to disruption of hydrophobic interactions.

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Chapter 2: Role of Non-Covalent Interactions in the Production of Visco-Elastic Resins from Zein

Abstract

Zein has been used in the production of a wide variety of materials during the last century. One of the more intriguing developments in zein utilization has been the discovery that zein can be made to form a visco-elastic dough. Although significant research has been conducted to determine the functional properties of zein and how to modify these properties, little work has been done to determine how zein forms a visco-elastic dough. To investigate the role of various types of protein-protein interactions in zein's ability to form a visco-elastic material when mixed with water, several different reagents were added to zein while it was being mixed above its glass transition temperature. The role of hydrophobic interactions was evaluated through the addition of various salts from the Hofmeister series. In addition to urea, ethanol, and beta mercaptoethanol (β-ME) were also tested to evaluate the effects of protein denaturation and disulfide bonds on zein dough formation. Kosmotropic salts had a negative effect on zein dough formation indicating that increasing hydrophobic interactions prevented dough formation. Surface hydrophobicity was found to decrease significantly ($p < 0.05$) when zein was exposed to 1M or 2M of the kosmotropic salts. Conversely, chaotropic salts had a slight positive effect on zein dough formation as did urea and ethanol. Interestingly, β-ME had little effect on zein dough formation demonstrating that disulfide bonds played no role in zein dough development and that large disulfide linked polymeric protein complexes were not present as found in wheat and carob germ flour dough.

Introduction

Zein has played a vital role in many industrial applications throughout the last century. Commercial zein is typically isolated from corn gluten meal, a byproduct of industrial corn starch isolation, and is almost pure α-zein. In the late 1940's and throughout the 1950'5, zein was used for the production of fibers used for a variety of purposes including furniture padding and textile production (Shukla and Cheryan 2001; Lawton 2002). More recently , the need for renewable resources has led to zein's use in food coatings that not only help with appearance of foods, but also aid in protecting product quality (Shukla and Cheryan 2001; Lawton 2002). Biodegradable films and production of controlled or sustained release of pharmaceuticals made from zein have also recently been successful (Shukla and Cheryan 2001; Lawton 2002). Another potential use for zein is in the production of wheat-free breads for persons with celiac disease. Zein has been shown to produce a wheat-like dough and has been successfully used to produce bread when mixed with starch (Lawton 1992; Mejia et al. 2007; Schober et al. 2008, 2010, and 2011; Erickson et al. 2011)

While there have been many uses of zein throughout the last century, the structure of zein protein isolates and how it relates to functionality is not well known. As mentioned previously, commercial zein isolates are comprised almost entirely of α-zeins. This fraction of zein is known to contain low levels of cysteine (~0.8 %) (Shukla and Cheryan 2001). Cysteine is an amino acid capable of forming disulfide bridges with other cysteine residues, covalently linking two proteins into a larger protein complex or linking cysteine residues within a protein which in turns plays a role in the structure of that protein. It is well known that a major reason gluten from wheat achieves its visco-elastic behavior is through very high molecular weight

disulfide linked proteins. When the disulfide bonds in gluten are cleaved, wheat gluten loses its functionality. How zein, a protein known to have some function like wheat gluten, forms wheat-like dough with little to no cysteine residues is not currently known. Very little research has been conducted to determine how zein can be mixed into a visco-elastic material.

Because commercial zein contains little to no cysteine residues and thus no covalently linked disulfide bonded protein polymers, large gluten like polymeric protein complexes are not the causes of zein being able to form a visco-elastic material. Commercial zein isolates alone are capable of forming a visco-elastic material (or resin) when mixed with water above the glass transition of the proteins without starch present. Thus, functionality is due to protein-protein interactions which give zein the ability to form a visco-elastic resin. As large covalently linked protein complexes are not present in commercial zein isolates, non-covalent protein interactions likely play a key role in zein's visco-elastic functionality. There are various forms of non-covalent interactions that can occur within and between proteins. Of these, hydrogen bonding and hydrophobic interactions are the most widely studied. Traditionally, urea and ethanol have been used to exam the effect of hydrogen bonding in proteins (Mitchell and Littman 2000; Zhang and Cremer 2010). However, high concentrations of urea have been shown to promote the unfolding of proteins because it is thermodynamically favorable due to the lack of bulk water in contact with the protein and urea is capable of direct interactions with the peptide backbones of proteins (Bennion and Dagget 2003; Auton and Bolen 2005; Street et al. 2006; Zhang and Cremer 2010).

One method for studying the hydrophobic interactions of proteins has been through the use of salts ranked on their ability to salt in or salt out proteins. This ranking or series of salts is

known as the Hofmeister series (Melander and Horvath 1977; Collins 2006; Peterson and Saykally 2006; Jungworth and Winter 2008; Zhang and Cremer 2010). While the exact mechanism of the salting in or salting out is not well known, the addition of these salts is known to change the 3D structure of the proteins via disruption or promotion of hydrophobic or hydrophilic interactions within a protein depending on the salt used and the protein in question (Melander and Horvath 1977; Collins 2006; Peterson and Saykally 2006; Jungwirth and Winter 2008; Zhang and Cremer 2010).

In order to determine why zein is able to form visco-elastic materials similar to wheat gluten, the role of non-covalent interactions in commercial zein functionality was investigated. Zein's ability to form a visco-elastic resin was evaluated by mixing commercial zein isolates in a farinograph at elevated temperatures. Reagents such as ethanol and urea were added during mixing to determine the effect of denaturants on zein mixing properties. Similarly, to determine the role of hydrophobic interactions in zein's functionality, salts from the entire spectrum of the Hofmeister series were added to zein while being mixed in a farinograph at elevated temperatures. The results of this research should provide greater insight into how and why zein functions the way it does and allow for future manipulation of zein to increase its use in wheat-free food products.

Materials and Methods

Materials

Zein isolate was purchased from Sigma-Aldrich, Co., (St. Louis, MO). Vital wheat gluten was purchased from Midwest Grain Products (Atchinson, KS). Na_2SO_4, NaCl, NaI, NaSCN, urea, and beta mercaptoethanol (β-ME) was a purchased from Sigma-Aldrich, Co., (St. Louis, MO).

Ethanol under the trade name CHROMASOLV (95% ethanol+5% isopropanol) was purchased from Sigma-Aldrich, Co., (St. Louis, MO). Molecular weight standards lysozyme from chicken egg white (14 kDa), carbonic anhydrase (29 kDa), and bovine serum albumin (66 kDa) was purchased from Sigma-Aldrich, Co., (St. Louis, MO).

Zein Resin Formation

To determine the role of non-covalent interactions during zein mixing 1M and 2M solutions of Na_2SO_4, NaCl, NaI, and NaSCN were added to zein-water mixtures during mixing in a farinograph at a temperature above the glass transition of zein. These salts were selected to be representative of the Hofmeister series, where salts are ranked on their ability to "salt in" or "salt out" proteins. The addition of Hofmeister salts is known to change the secondary and tertiary structure of the proteins by disrupting or promoting of hydrophobic interactions within a protein depending on the salt used and the protein in question (Melander and Horvath 1977; Collins 2006; Peterson and Saykally 2006; Jungwirth and Winter 2008; Zhang and Cremer 2010). Anions of the Hofmeister series are known to have a greater affect on proteins than cations. The typical order of anions to salt out proteins is:

$$CO_3^{2-} > SO_4^{2-} > H_2PO_4^- > Cl^- > Br^- > I^- > ClO_4^- > SCN^-$$

Sulfate is considered to be on the extreme end of salting out whereas iodine and thiocyanate are on the extreme end for salting in proteins. Chlorine is considered to be in the middle of the spectrum of the Hofmeister series base on its ability to salt in or salt out proteins. While cations have a lesser affect on proteins than anions, they still have an effect on protein structure and solubility. Based on the salts' ability to salt out proteins, cations follow the general order:

$$NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$$

Sodium is considered to be in the middle of the Hofmeister series for the cations' ability to salt in or out proteins. For this reason sodium was chosen as the cation for all experiments used in this project.

In order to determine the importance of the different bonding interactions on zein resin formation, zein was mixed by a Farinograph-AT (Duisburg, Germany) at 73 rpm for 20 min at 40 °C. For all mixing experiments, 40 g of zein was placed in the farinograph's 50 g mixing bowl. After one minute of calibration time 20 mL of de-ionized (DI) water or solution of interest was added and mixing continued. Treatments included Na_2SO_4, NaCl, NaI, and NaSCN at concentrations of 0 to 2M, urea at concentrations 0 to 4M, and ethanol at concentrations of 5 - 15% % (v/v). In addition, 2% (β-ME) (v/v) was also used as a treatment. Vital wheat gluten was examined under the same conditions as zein, except gluten was run at 30 °C and 30 mL of water.

After mixing in the farinograph for 20 min zein resins were immediately frozen in liquid nitrogen, stored at -80 °C, and lyophilized. Lyophilized resin was ground via mortar and pestle and stored at – 20 °C on desiccant for subsequent analysis. For visual representation of the materials formed during mixing, in some cases zein and gluten were immediately removed from the farinograph and photographed.

Size Exclusion Chromatography

To determine if there were any changes to the molecular weight distribution of the zein proteins during mixing, lyophilized samples collected from the farinograph-AT were solublized at a concentration of 4 mg sample to 1 ml of a 50 mM Tris-borate pH 10.0 buffer containing 2%

SDS for 30 min with continuous vortexing. Samples were centrifuged for 5 min at 9,300 *g* and the aliquots were collected and heated for 5 min at 95 °C. Molecular weight standards were extracted and analyzed in the same manner and used to indicate molecular distribution of zein samples. To determine if any disulfide linked polymers were present, 2% β-ME (v/v) was added to an aliquot of the solubilized proteins. Samples were analyzed via size exclusion (SE) HPLC using an Agilent 1100 HPLC system equipped with a Biosep-3000 column (Phenominx, Torrance, CA) and guard column using 50 mM sodium phosphate, pH 7.0 buffer containing 1% SDS (w/v) as a mobile phase (Bean and Lookhart 2001). Proteins were detected at 214 nm with a UV detector over a 30 min span with a flow rate of 1 mL/min and an injection volume of 20 µL. Column temperature was fixed at 40°C.

Solubility

To determine how the different reagents mixed with zein affected zein solubility, 5 mg of zein was vortexed for 30 min in 1 mL of a given treatment solution and centrifuged at 9,300 *g* for 5 min. The supernatant was collected and analyzed via SE-HPLC as described above. Salt treatments solutions included Na_2SO_4, NaCl, NaI, and NaSCN at a concentration of 2M. Other treatments included ethanol at a concentration of 15% (v/v). Urea was also analyzed at concentrations of 1M, 2M, and 4M. Controls included a negative control consisting of DI H_2O and a positive control of 50 mM Tris-borate pH 10.0 buffer containing 2% SDS. As zein is readily soluble in this latter solution it was expected that all the zein was soluble in this solvent. Treatments were compared by integrating the total peak area from the SE-HPLC chromatograms. Solvent blanks were analyzed for each treatment to insure that no sample matrix peaks were included in the integration of results.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) backscattering electron (BSE) imaging was completed using a Nova Nano SEM 430 (FEI Company, Hillsboro, Oregon) in low vacuum mode (0.98 torr) equipped with a low voltage high contrast detector (vCD) with 15.0 KV used for analysis. Elemental mapping was completing using an energy-dispersive detector (EDS). For each map, 500, 000 scans were completed at each location to determine locations of elements of interest.

Surface hydrophobicity

Surface hydrophobicity was determined as described by Chelh et al. (2006) with modifications. Zein (5mg protein) was vortexed for 30 min at 40°C in 20mM Na-Phos buffer pH 6.0 with 2M concentrations of Na_2SO_4, NaCl, and NaSCN. A control mixed only with the 20mM Na-Phos buffer was also measured. After vortexing, samples were allowed to cool to room temperature and 200 µL of 1 mg/mL bromophenol blue (BPB) in DI H_2O was added. Samples plus BPB were then vortexed for 10 min. After vortexing the samples were centrifuged for 15 min at 2,000 *g*. Next, 900 µL of 20 mM Na-Phos buffer pH 6.0 was added to 100 µL of the supernatant (1:10 dilution). The diluted samples were vortexed and read at an absorbance of 595 nm on a Beckman DU 530 Life Science UV/Vis Spectrophotometer (Beckman, Coulter, CA). The instrument was blanked with 20 mM Na-Phos buffer pH 6.0. Samples were compared against their corresponding controls that contained only the sample matrix (i.e. the salts) at the appropriate concentration. Surface hydrophobicity was calculated using the equation:

$$\text{BPB bound } (\mu g) = 200\ \mu g\ (\text{Abs control} - \text{Abs sample}) / \text{Abs control}$$

Experimental Design

Samples were run in triplicates for the solubility and surface hydrophobicity tests and analysis of variance was completed with a $P < 0.05$ using Statistical Analysis Software (SAS 9.1, SAS Institute Inc. Cary, NC). A comparison of means using Tukey's studentized range test was used to determine differences in hydrophobicity.

Results and Discussion

Resin Formations

The addition of salts from the Hofmeister series to zein resins mixed at 40 °C was found to have a profound effect on zein resin formation. At the extreme end of the kosmotropic salts (salting out), Na_2SO_4 completely disrupted zein's ability to form a visco-elastic resin (Fig 1). This is evident by the complete lack of resistance to mixing on the Y axis of the farinograms (Fig 1) for both the 1M and 2M treatments. It should be noted that these concentrations were chosen based on past research that has reported that below 1M, salts most likely impact electrostatic interactions in proteins and are not at high enough levels to alter the water surrounding the proteins and impact hydrophobic interactions (Preston 1989; Melnyk et al. 20011). The control farinogram, zein and water only, shows a farinogram curve similar to what one would expect from gluten except zein does not seem to break down like gluten. Mixing zein in the presence of NaCl produced similar results (Fig 2), i.e. no visco-elastic resin could be formed despite NaCl being in the middle of the Hofmeister series. Visually, when compared to a control zein resin mixed with only water, the zein mixed with either Na_2SO_4 or NaCl was easily distinguished and remained in discrete particles and would not form a resin (Fig 3). The effect of Na_2SO_4 follows the general order of the Hofmeister series well, in that Na_2SO_4 prevented zein resin formation

by increasing protein aggregation through hydrophobic interactions. In the presence of kosmotropic salts such as Na_2SO_4, the salts are excluded from the outer hydration shell of the proteins forcing the proteins into a purely aqueous environment (Temasheff and Arakawa 1995). This, in turn, forced the proteins to bury hydrophobic regions and further exclude water from the interior of the proteins. Such interactions resulted in the formation of dry looking discrete zein particles and lack of resin formation (Fig 1-3). Note that increasing the amount of water during mixing zein in the presence of Na_2SO_4 did not change these results, the zein would not absorb additional water and excess water simply ran out of the farinograph mixing bowl.

Interestingly, zein was very sensitive to the effects of the kosmotropic salts as NaCl is considered to be neutral in the Hofmeister series for most proteins. However, zein is a very hydrophobic protein (Osborne 1908; Argos et al. 1982; Shukla and Cheryam 2001; Lawton 2002; Schober et al. 2010 and 2011) and the NaCl may be skewed to the salting out end of the Hofmeister series when dealing with zein. This may be why NaCl had similar effects as Na_2SO_4 on zein resin formation.

In contrast to the Na_2SO_4 and NaCl, the addition of 1M and 2M solutions of NaI and was found to have a slight softening effect on the resin (Fig 4). This is evident by the overall decrease in farino units over the course of the mixing. The farinograms for NaI also show a decrease in deviation from the mean mixing force (fig 4). The zein mixed with 1M NaI took longer to hydrate than that of 2M NaI (Fig 4). The reasoning behind this is unknown.

Overall, mixing zein in the presence of NaSCN produced similar results to that of NaI (Fig 5). As with NaI, mixing zein with NaSCN produced a resin at lower peak farino units. Thus, both NaI and NaSCN made the resin softer and slightly more extensible. Although subjective, the

softening of zein resins during mixing with NaI and NaSCN was readily apparent when resins were stretched by hand and compared to the control resin mixed with DI H_2O. From the farinograph data and the subjective observations from stretching the resins by hand after mixing, these results show that chaotropic salts of the Hofmeister series had some beneficial effect on zein resin formation, possibly by decreasing intra-protein hydrophobic interactions and promoting the unfolding of zein proteins. This unfolding of proteins, typically seen with the addition of chaotropic salts, may allow for increased interaction between proteins by exposing regions of the protein that were previously buried. Such non-covalent protein-protein interactions may be one reason why zein is capable of forming a visco-elastic resin. The strong negative effects of Na_2SO_4 and NaCl contrasted against the slight positive effects of NaI and NaSCN show that zein resin formation is sensitive to non-covalent interactions.

To further investigate the roles of non-covalent interactions on zein isolate functionality, the addition of urea and ethanol during mixing was evaluated. While urea and ethanol are known to impact protein structure, the exact mechanism of urea and ethanol's ability to change protein functionality is not well understood. Traditionally urea was thought to disrupt hydrogen bonding between hydrophobic amino acids. More recently urea has been identified to bind directly to the peptide backbone altering protein solubility. Urea also changes the amount of bulk water coming into contact with proteins (Bennion and Dagget 2003; Auton and Bolen 2005; Street et al. 2006; Zhang and Cremer 2010). The structural changes made to water by urea may also affect the hydration properties of the protein and effect hydrophobic interactions (Zhang and Cremer 2010). Regardless of the specific reasons, both urea and ethanol had an effect on zein resin formation. As concentrations of urea (Fig 6) and ethanol (Fig

7) added during mixing increased, the zein resins resistance to mixing decreased. This is indicative of a softer resin and it was noted that these resins could be extended farther by hand before breaking when compared to the control. Interestingly, the shape and resistance (Y axis) of the farinograms were nearly identical between 4M urea and 15% ethanol (v/v), 2M urea and 10% ethanol (v/v), and 1M urea and 5% ethanol (v/v) (compare Fig 6 to Fig 7).

While the above results show that non-covalent interactions play a role in the formation of visco-elastic zein resins, they provide no information on the role of covalent disulfide bonds. In order to determine if disulfide bonds are important for zein resin formation, a solution of 2% β-ME was added to zein during mixing in the farinograph. Figure 8 shows that the addition of 2% β-ME did not disrupt zein's ability to form a resin when mixed at 40 °C. Commercially isolated zein is comprised almost entirely of α-zein that is known to have very little cysteine (Lawton 2002). Thus, the small amount of disulfide bonding present does not influence zein's functionality. Addition of 2% β-ME did slightly decrease mixing resistance and minimize deviation from the mean mixing force (Fig 8). This may have been an effect of the cleaving of some intra-disulfide bonds that may have been present (Cabra et al. 2008) or may have been due to 2% β-ME having a similar affect as the chaotropic salts of the Hofmeister series. There was no observable difference between β-ME treated resins and the control resin when pulled apart by hand (Fig 9).

It is widely accepted that the functionality of wheat gluten is dependent on the formation of large polymeric protein complexes held together via disulfide bonding between cysteine residues (Carceller and Aussenac 1998; Stevenson et al. 2003). In the presence of β-ME these disulfide bonds are cleaved, rendering gluten unable to form a visco-elastic substance. To

demonstrate this, 2% β-ME was added to a commercially available wheat gluten isolate and mixed in the farinograph. It was found that cleavage of disulfide bonds did indeed prevent gluten from forming a visco-elastic material (Fig 10). When comparing the 2% β-ME treated wheat gluten to the β-ME treated zein, the key differences to note are that zein was able to produce a resin that maintained its integrity throughout mixing, while gluten produced a sticky paste that had little resistance to mixing (compare farinograms from Figure 8 to Figure 10 and photos in Figure 9 to those in Figure 11). This is significant in that few proteins are capable of forming a visco-elastic substance like wheat gluten. Zein from corn and caroubin from carob germ flour are the only other reported proteins known to behave like gluten (Bienenstock et al. 1935; Lawton 1992; Mejia et al. 2007; Oom et al. 2008; Schober et al. 2008, 2010, and 2011; Smith 2009; Smith et al. 2010). Gluten's functionality is dependent on very high molecular weight proteins formed through disulfide bonds. Gluten has been studied extensively over the past century and many believe that high molecular weight proteins formed through covalent bonding are the only way to form visco-elastic doughs and gluten-like materials from proteins. Caroubin, a non-wheat protein was found to function for identical reasons as wheat gluten (i.e. high M_w disulfide bonded proteins) (Smith 2009; Smith et al. 2010). This makes zein unique in that it is capable of forming a gluten-like resin from relatively small molecular weight proteins (~21-22 kDa) that are not dependant on disulfide linkages like caroubin and wheat gluten (Carceller and Aussenac 1998; Bean and Lookhart 2001; Lawton 2002; Stevenson et al. 2003; Smith 2009; Smith et al. 2010).

Size Exclusion Chromatography

To determine if changes to the molecular weight distribution of zein occurred during mixing with the various treatments SE-HPLC was used. The commercial zein used in this work did not contain significant amounts of disulfide bonded protein complexes (Fig 12). If disulfide bonds were present a shift to smaller molecular weight proteins would have been observed when comparing the non-reduced to reduced sample. This would have been evident by chromatogram peaks shifting to a latter elution time from non-reduced zein to reduced zein. This further solidifies the theory that commercial zein isolate does not form a resin due to disulfide bonded proteins. Furthermore, none of the treatments had an effect on covalent interactions of zein over the course of mixing, as no changes to the molecular weight distribution occurred during mixing (Fig 13). All peaks eluted at the same time with no shifts in peak areas for any treatment. Because changes in covalent interactions would cause shifts in peaks indicative of changes to molecular weight distribution, zein does not form resins due to changes in covalent interactions (i.e. the formation of larger disulfide bonded polymeric proteins) during mixing. Note that these samples were dissolved and analyzed in the presence of SDS, a strong protein denaturant. Any protein complexes held together via non-covalent interactions would be expected to be disrupted by the SDS.

SEM and Elemental Mapping

In order to gain further insight into zein's ability to form visco-elastic resins, SEM coupled with elemental mapping was used to examine and compare zein isolates mixed in a farinograph and treated with 2M concentrations of Na_2SO_4, NaCl, NaI, and NaSCN. Imaging and

elemental mapping allowed for observations of zein resin structure to be made and to determine how the salt treatments were interacting with zein resins on a microscopic level.

In general, treatments and controls that were able to form a resin, produced a dense protein mass. This mass appeared to be comprised of stacked layers. This is apparent in the control resin (mixed with water only) by the overall elongation and linear organization of voids and the fact that the outermost layers of the resin can be seen flaking off the surface (Fig 14).

SEM imaging and elemental mapping overlays further confirm the effects of the Hofmeister series on zein resin formation. For zein treated with Na_2SO_4 and NaCl, where no resin was formed, SEM imaging and elemental map overlays showed discrete particles of zein coated with salt (Fig 15 and Fig 16 respectively). This indicates that the majority of the salts were not dispersed into the protein. The exclusion of the kosmotropic salts from the outer hydration spheres of the zein would result in salts being present mainly at the exterior of the protein particles. On the chaotropic side of the Hofmeister series, shown earlier to promote zein resin formation, SEM imaging and elemental map overlays showed dense resins with the salt elements dispersed throughout the resin (Fig 17 and Fig 18 respectively). This was consistent with the ability of the chaotropic salts to penetrate the hydration layer around the proteins and directly interact with the proteins, preventing the “hydrophobic effect” where proteins bury their hydrophobic regions. This was significant in that it shows kosmotropic salts remain on the surface of the protein promoting intra-protein hydrophobic interactions, while chaotropic salts are dispersed within the resin, signifying the promotion of resin formation and disruption of intra-protein hydrophobic interactions.

Solubility

The Hofmeister series is commonly used to salt in or salt out proteins. This means that the salts are used to change protein solubility to either promote flocculation or solubilization. In order to determine if the effects of the salts, urea, and ethanol on zein protein functionality were due solely to changes in non-covalent interaction or if changes to solubility played a role as well, the changes to zein solubility with the various treatments was examined. Effects on zein solubility were examined at 2M concentration for each salt, 15% (v/v) concentration for ethanol, and 1M, 2M, and 4M urea. These treatments were compared against a negative control (DI H_2O) and a positive control (50 mM Tris-borate pH 10.0 buffer containing 2% SDS), which solublized all of the protein. It was found that none of treatments had significant differences ($p < 0.05$) in solubilizing the zein isolate except for 4M urea compared to the positive control (Fig 19). Thus, the overall softening effects of the 4 M urea treatment on zein seen with the farinograms (Fig 6) may have been due to both an increase in zein solubility and changes to non-covalent protein interactions. The effects of all other treatments can be attributed to changes in non-covalent interactions and not changes to zein solubility. It was surprising that the chaotropic salt treatments did not have any effect on zein solubility since salts have been shown to solubilize some gluten (Preston 1981). This was probably due to the hydrophobic nature of zein. With zein, the disruption of hydrophobic interactions by the chaotrops promoted the interaction of zein with water only enough to promote zein resin formation (Fig 4-5) but not enough to solubilize zein.

Surface Hydrophobicity

To confirm changes to zein isolate protein structure in the presence of the Hofmeister salts used in this study, surface hydrophobicity was determined by the proteins ability to react with BPB. Previous work with BPB has been shown to bind to the hydrophobic side chains of amino acids (Chelh et al. 2006). More importantly, the use of BPB allowed the surface hydrophobicity of proteins to be determined without the need to solubilize the proteins first. More widely used surface hydrophobicity assays require the proteins to be solubilized. Zein can only be solubilized in the presence of high levels of detergents such as SDS or aqueous alcohols both of which would alter the tertiary structure of the protein and thus alter surface hydrophobicity.

With the addition of kosmotropic salts to zein, it was expected that there should be an increase in hydrophobic interactions within the protein. The strengthening of these hydrophobic interactions promotes protein folding because the hydrophobic regions of the protein are burying themselves to escape water. This mechanism in turn, buries the hydrophobic amino acids into the core of the protein leaving more hydrophilic regions of zein exposed to water. This in turn would restrict access of BPB from binding zein. On the other end of the Hofmeister series, chaotropic salts (NaI and NaSCN) would reduce the effects of hydrophobic interactions and results in an unfolding of proteins. The unfolding of zein and subsequent exposure of hydrophobic amino acids would allow for greater BPB binding. In general this was the case when BPB was reacted with zein. Figure 20 shows that in the presence of 1M and 2M concentrations of Na_2SO_4 and NaCl, zein had significantly less ($p < 0.05$) surface hydrophobicity. These results suggest that increased protein aggregation is why zein was

unable to form a resin and remained as discrete particles when mixed via farinograph (Fig 1-3). At a concentration of 0.5 M, Na_2SO_4 and NaCl were not significantly different when compared to the control. This is probably because low concentrations of salts seem to have an inverse Hofmeister effect, which is dependent on electrostatic interactions based on size and hydration properties of the ions (Zhang and Cremer 2010). Only when the salts have neutralized the overall charge distribution of the protein will they begin to have a Hofmeister effect (Zhang and Cremer 2010). The naturalization of charge distribution is also dependent on the protein and the salt used (Zhang and Cremer 2010). Only the 0.5 M treatment of NaSCN had significantly different hydrophobicity than the control, which again may have been due to changes in electrostatic interactions rather than hydrophobicity. Both the 1M and 2M concentrations of NaSCN did not significantly change surface hydrophobicity relative to the control. This means that there was not a significant increase in hydrophobic amino acids at the surface of the zein proteins when zein was exposed to the NaSCN. The changes to zein resin formation in the presence of NaSCN relative to the control may have just been due to changes in protein secondary structure by disrupting hydrophobic interactions. This may have allowed for increased hydration and softening of the zein resin. Such changes may not be detected by overall surface hydrophobicity. It should be noted that NaI could not be used in this experiment because the BPB assay is a photometric assay. Solutions of NaI turn brown in color with time, so the subtraction of absorbance of the treated zein from the salt control would change as readings were being taken.

Conclusions

Zein proteins are known to form a visco-elastic resin in the presence of water when mixed above zein's glass transition temperature. Addition of β-ME to cleave disulfide bonds did not change functionality of zein as it did with wheat gluten. SE-HPLC confirmed that no changes to covalent interactions, resulting in changes to molecular weight distribution, had occurred during mixing for all treatments. The addition of higher concentrations of kosmotropic salts (1M and 2M) had a profound negative effect on zein's ability to form a resin. This disruption of zein's ability to form a resin can be attributed to an increase in intra-protein hydrophobic interactions. This was evident by significant decreases in surface hydrophobicity which signifies a burying of hydrophobic amino acids within zein. On the chaotropic side of the Hofmeister series, results were less clear. An increase in resin forming ability was observed when compared to a control. However, the surface hydrophobicity was not found to be different for the control. This means there was not an increase in hydrophobic amino acids at the surface of zein. The differences seen in zein functionality may have been due to a loosening of protein structure rather than a complete unfolding. The knowledge gained form this research shows that zein functionality is not dependent on changes to covalent interactions or disulfide bonding. Instead, unlike wheat gluten, zein is heavily reliant on non-covalent interactions between small molecular weight proteins for functionality. This research will aid in the future utilization of zein in food and other industrial applications.

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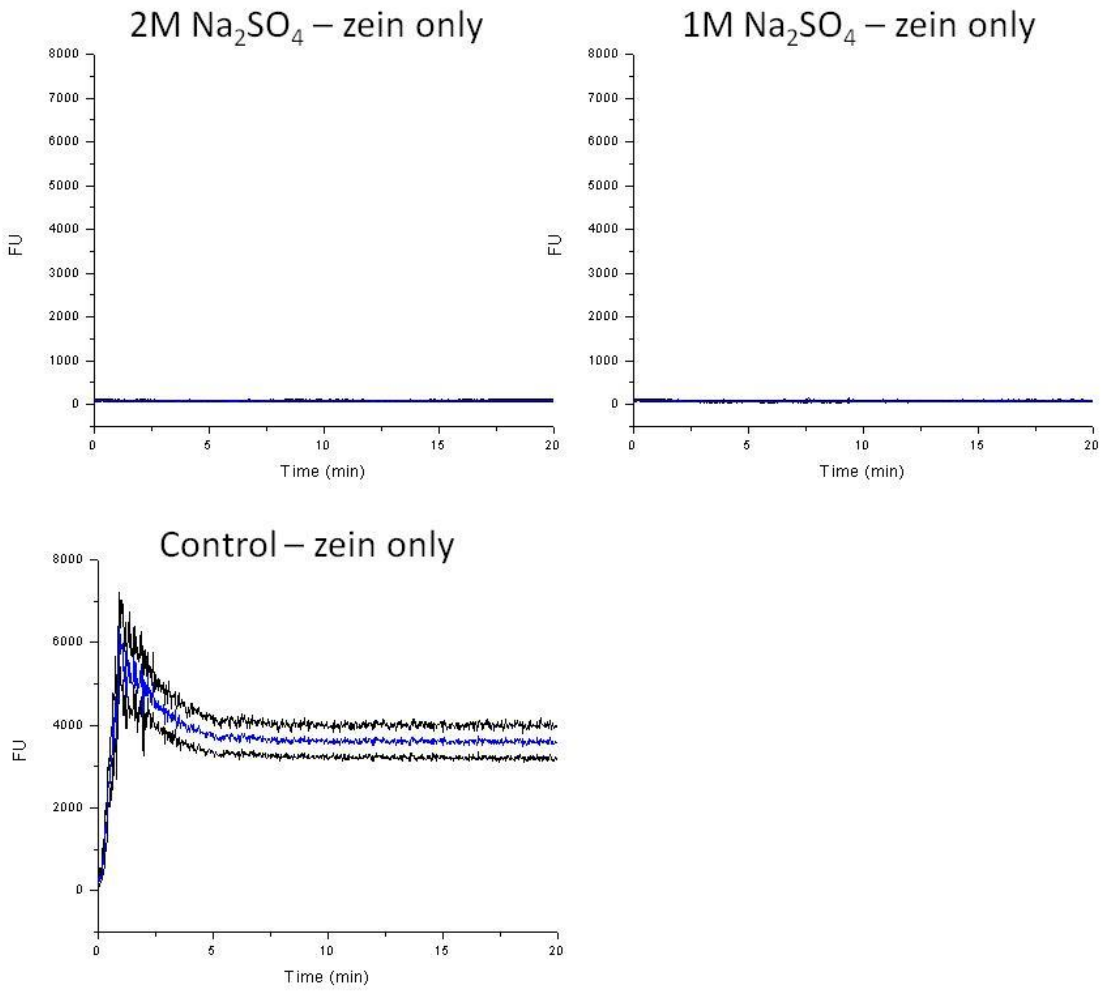

Figure 1: Farinograms from: 2M Na_2SO_4 treated zein mixed for 20 min (top left), 1M Na_2SO_4 treated zein mixed for 20 min (top right), and control zein mixed for 20 min with DI water (bottom left). The blue line represents the mean resistance from mixing. The black lines represent the minimum and maximum deviations from the mean mixing line during mixing.

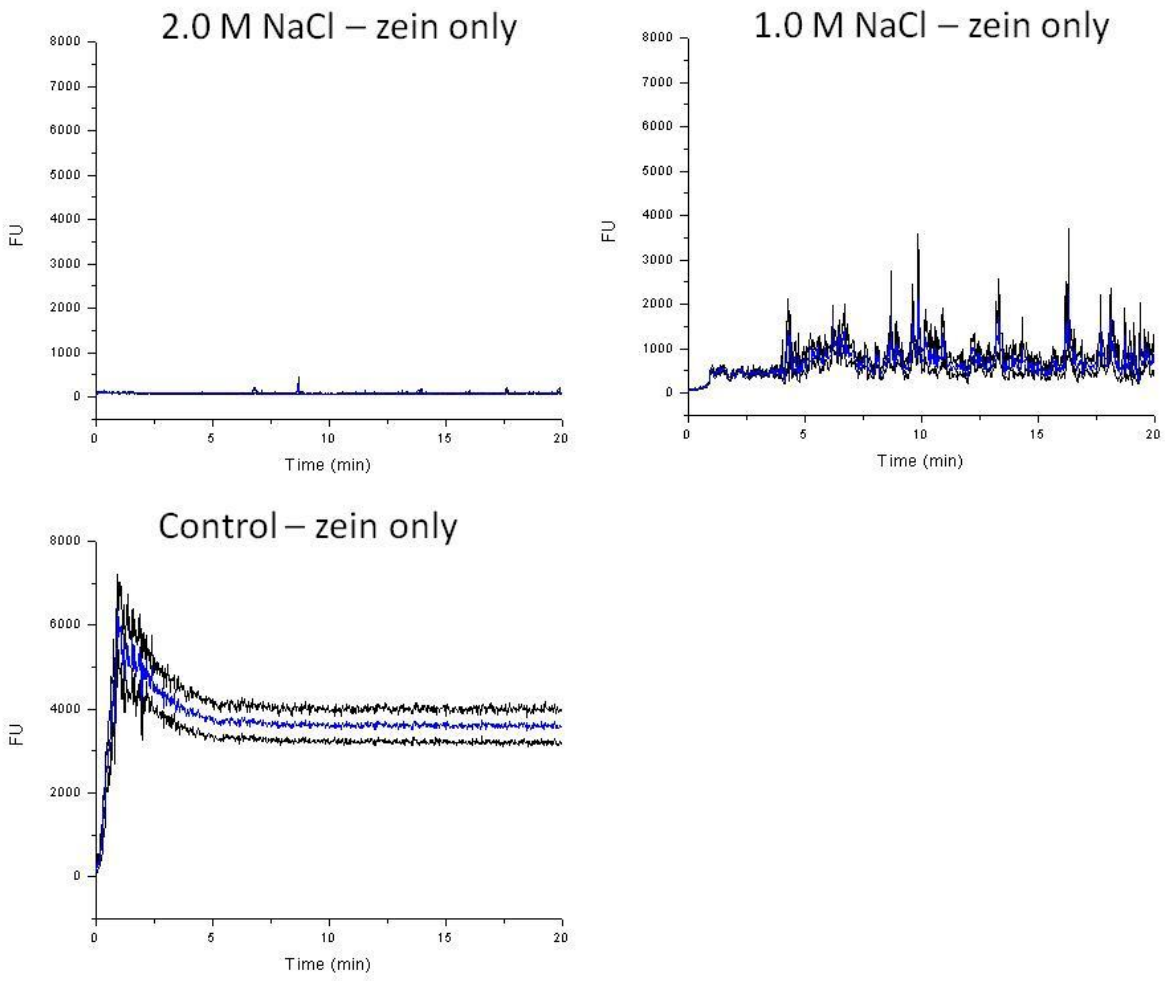

Figure 2: Farinograms from: 2M NaCl treated zein mixed for 20 min (top left), 1M NaCl treated zein mixed for 20 min (top right), and control zein mixed for 20 min with DI water (bottom left). The blue line represents the mean resistance from mixing. The black lines represent the minimum and maximum deviations from the mean mixing line during mixing.

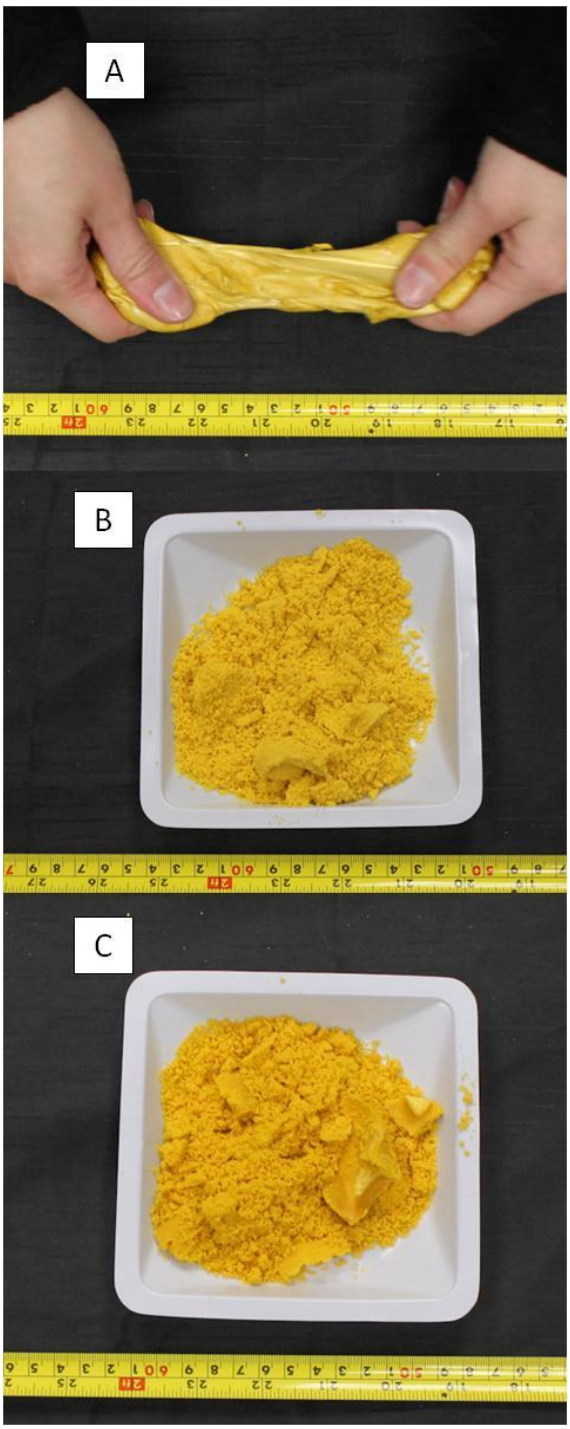

Figure 3: Visual representation of control zein resins (A), zein that could not form a resin when mixed in the farinograph with 2M Na_2SO_4 (B), and zein that could not form a resin when mixed in the farinograph with 2M NaCl (C).

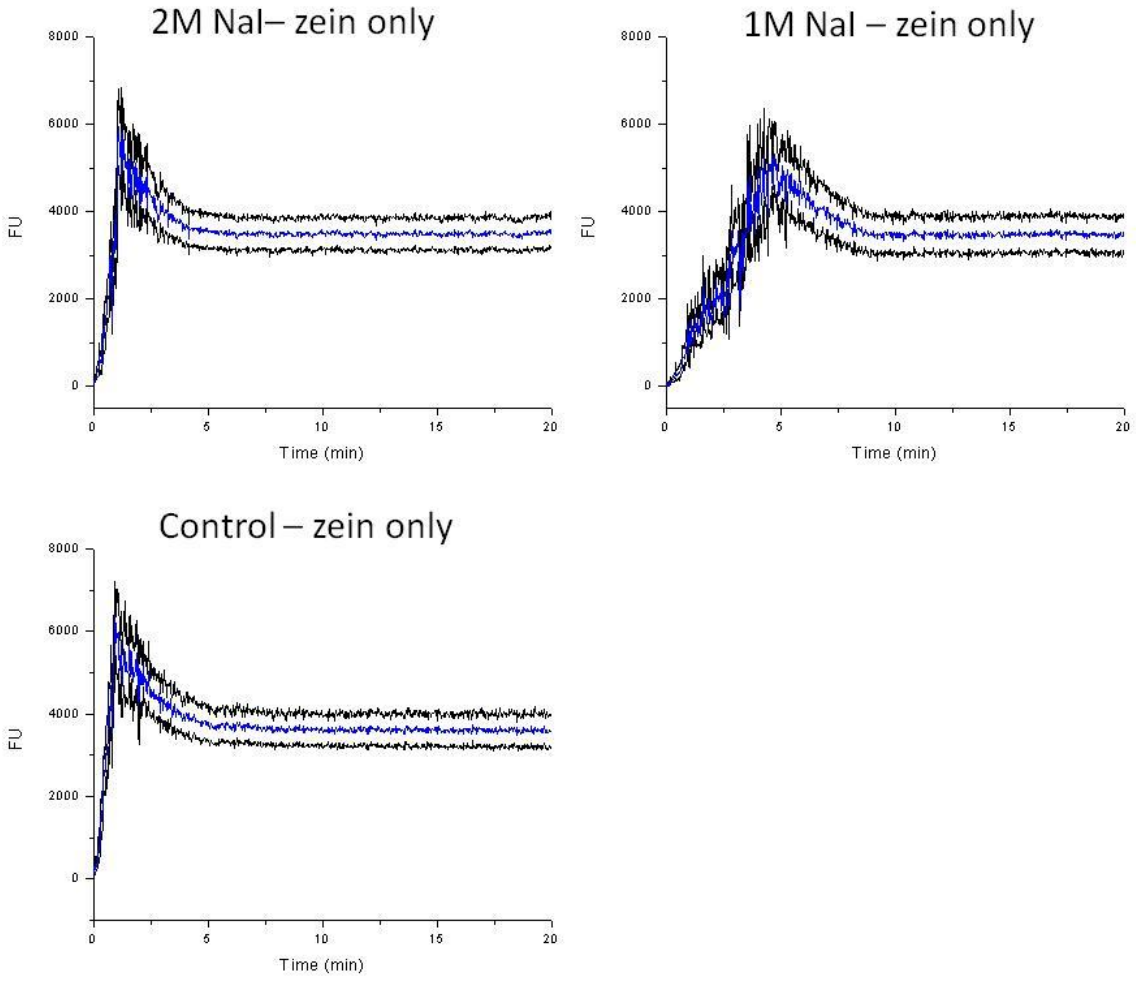

Figure 4: Farinograms from: 2M NaI treated zein mixed for 20 min (top left), 1M NaI treated zein mixed for 20 min (top right), and control zein mixed for 20 min with DI water (bottom left). The blue line represents the mean resistance from mixing. The black lines represent the minimum and maximum deviations from the mean mixing line during mixing.

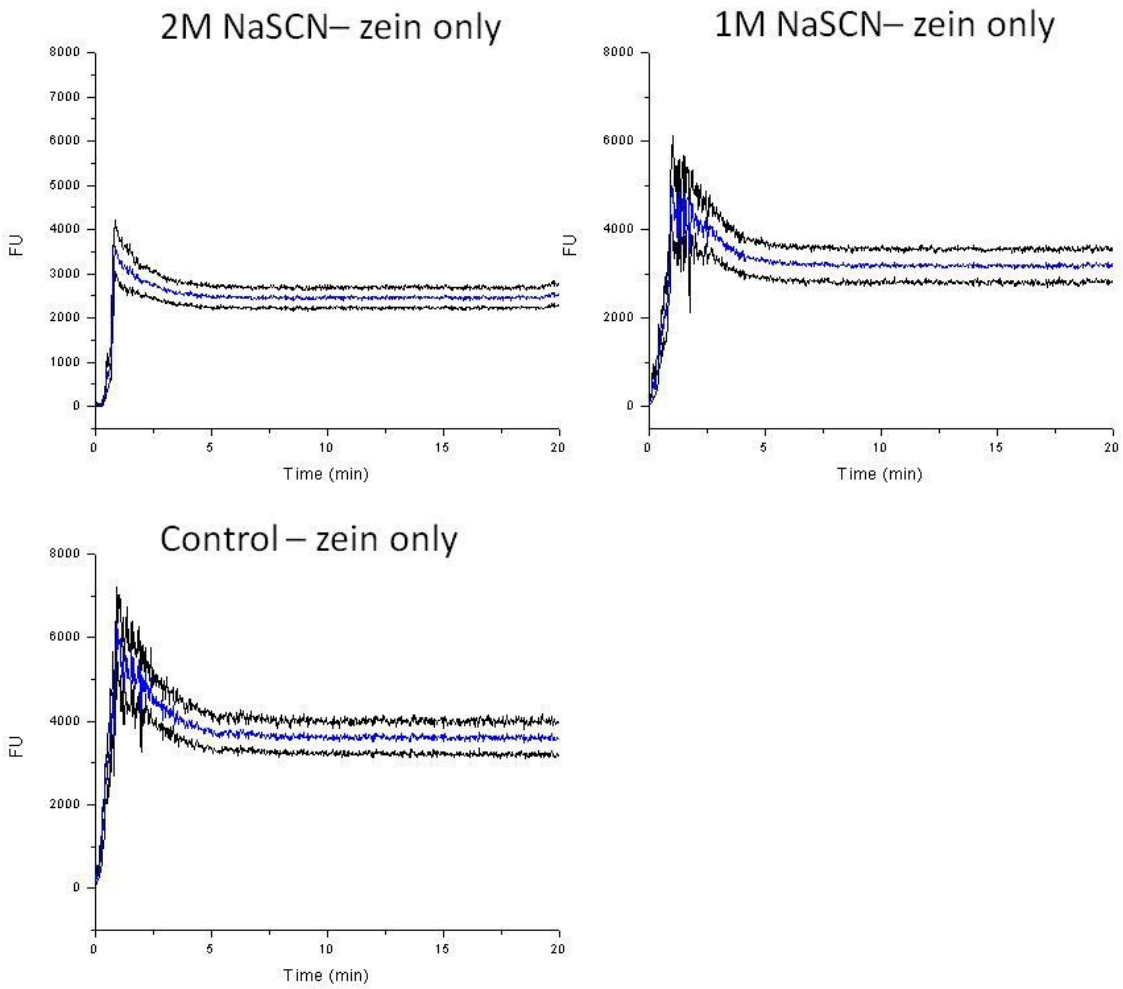

Figure 5: Farinograms from: 2M NaSCN treated zein mixed for 20 min (top left), 1M NaSCN treated zein mixed for 20 min (top right), and control zein mixed for 20 min with DI water (bottom left). The blue line represents the mean resistance from mixing. The black lines represent the minimum and maximum deviations from the mean mixing line during mixing.

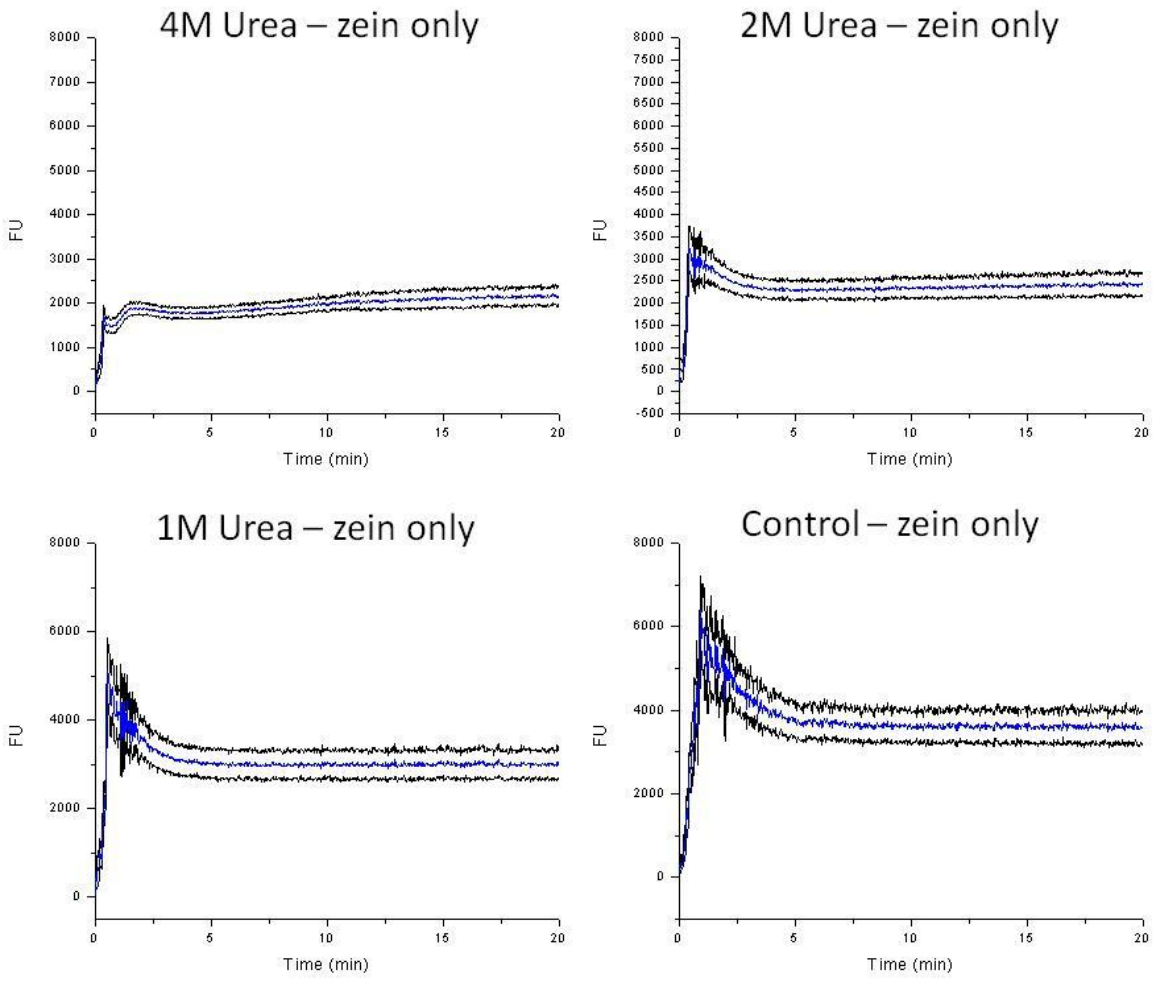

Figure 6: Farinograms from: 4M urea treated zein mixed for 20 min (top left), 2M urea treated zein mixed for 20 min (top right), 1M urea treated zein mixed for 20 min (bottom left) and control zein mixed for 20 min with DI water (bottom right). The blue line represents the mean resistance from mixing. The black lines represent the minimum and maximum deviations from the mean mixing line during mixing.

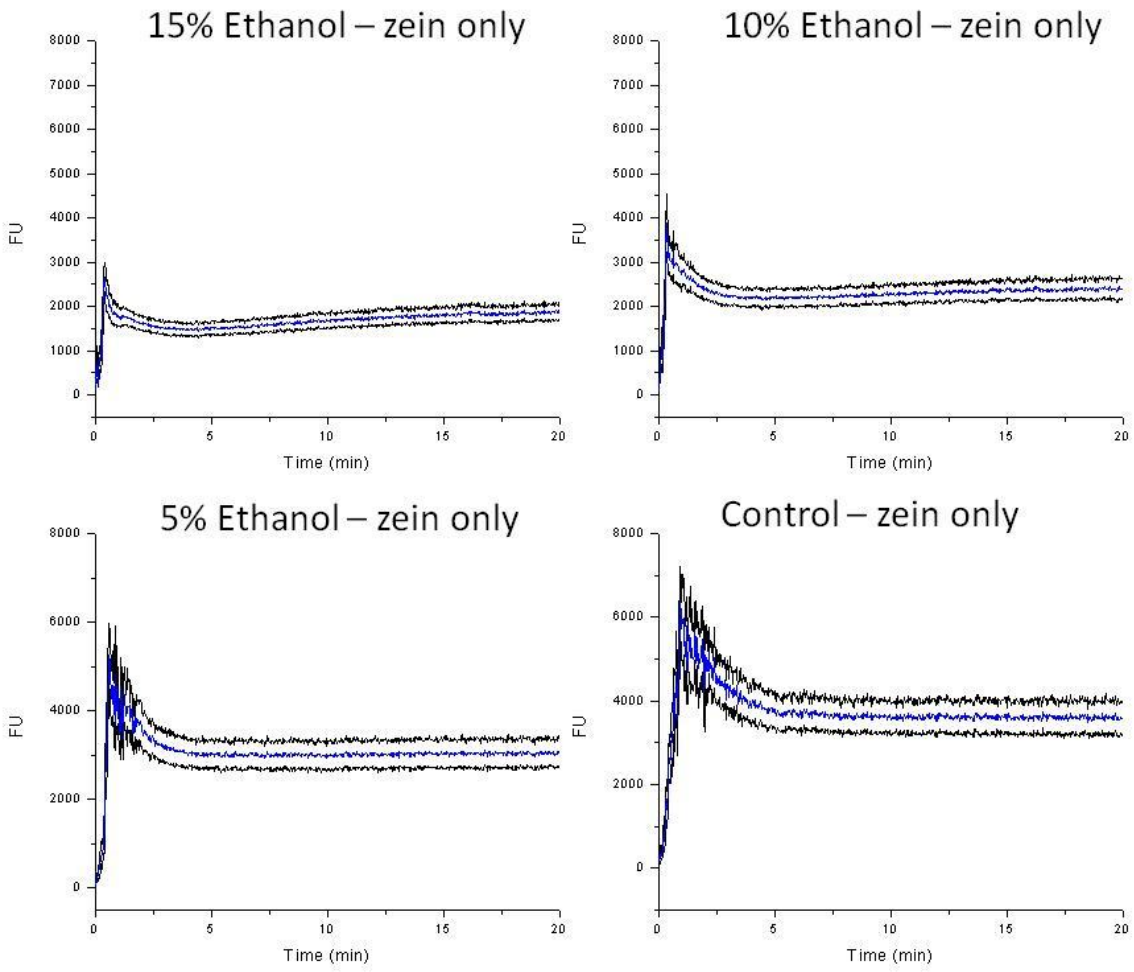

Figure 7: Farinograms from: 15% ethanol (v/v) treated zein mixed for 20 min (top left), 10% ethanol (v/v) treated zein mixed for 20 min (top right), 5% ethanol (v/v) treated zein mixed for 20 min (bottom left) and control zein mixed for 20 min with DI water (bottom right). The blue line represents the mean resistance from mixing. The black lines represent the minimum and maximum deviations from the mean mixing line during mixing.

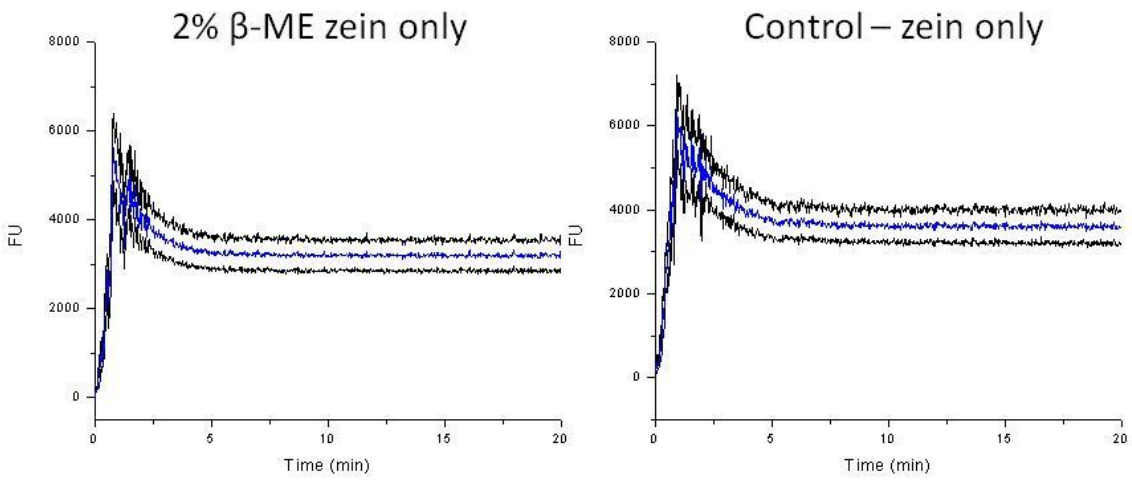

Figure 8: Farinograms from: 2% β-ME (v/v) treated zein mixed for 20 min (left), and control zein mixed for 20 min with DI water (right). The blue line represents the mean resistance from mixing. The black lines represent the minimum and maximum deviations from the mean mixing line during mixing.

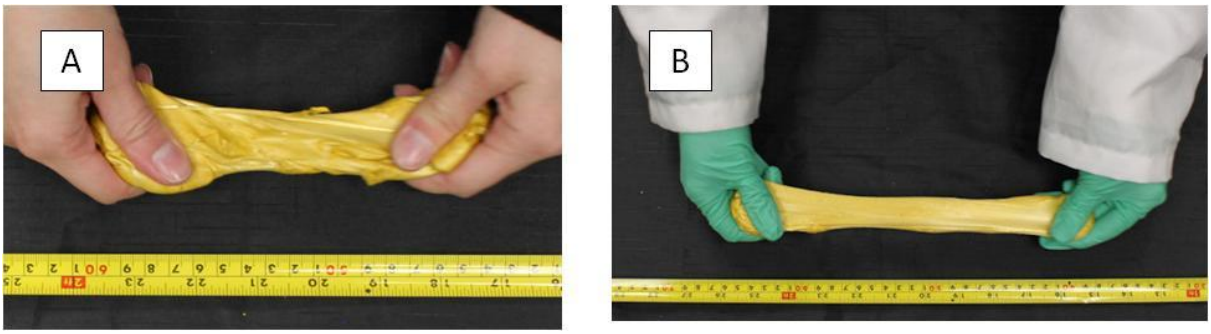

Figure 9: Visual representation of the control zein resin on the left (A) and zein resin that was formed under reducing conditions (2% β-ME) on the right (B).

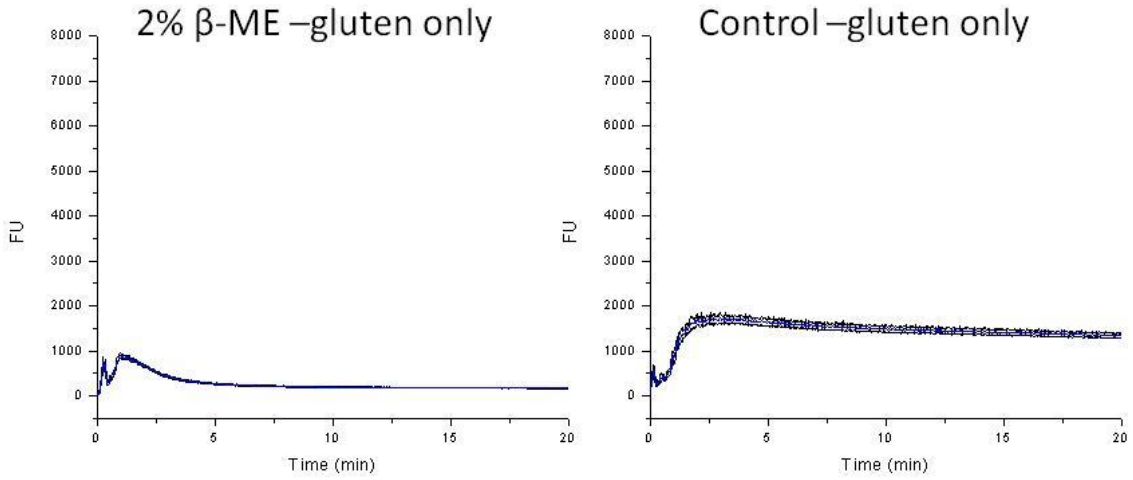

Figure 10: Farinograms from: 2% β-ME (v/v) treated wheat gluten mixed for 20 min (left), and control gluten mixed for 20 min with DI water (right). The blue line represents the mean resistance from mixing. The black lines represent the minimum and maximum deviations from the mean mixing line during mixing.

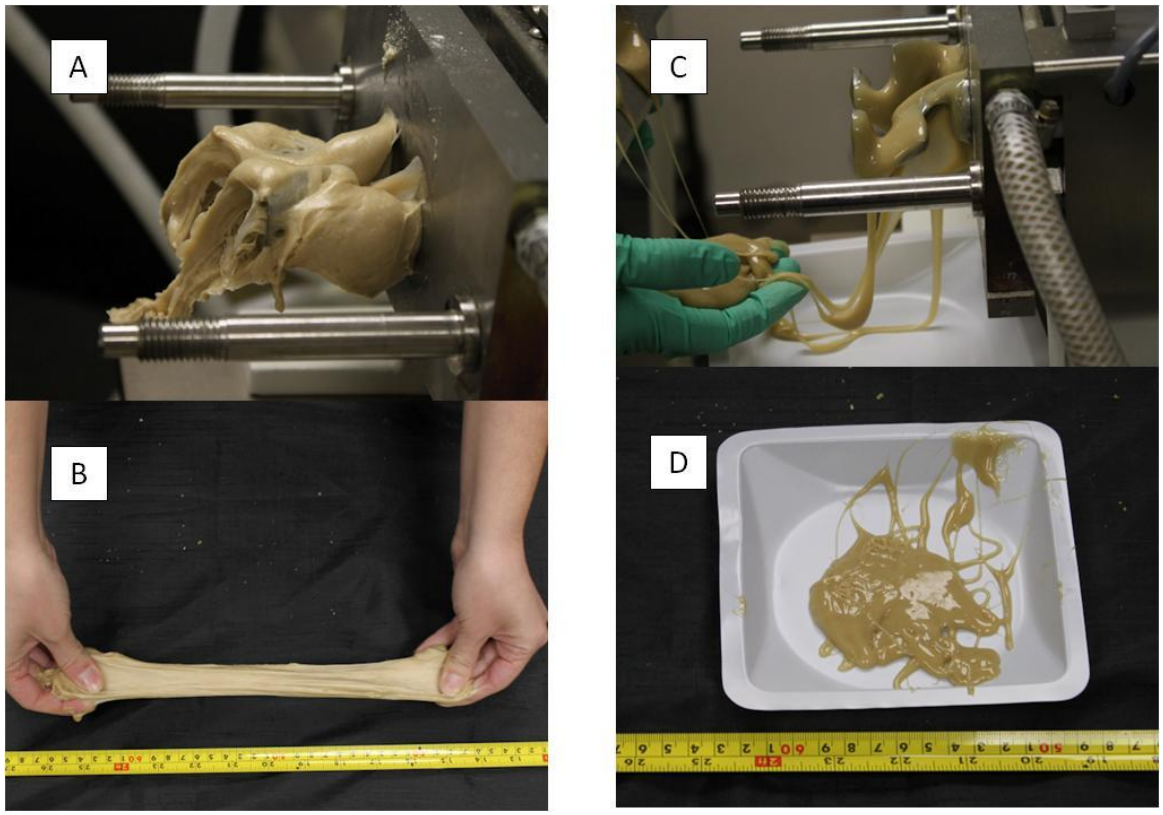

Figure 11: Visual representation of wheat gluten after mixing (A and B) and wheat gluten that was mixed under reducing conditions (2% β-ME) (C and D).

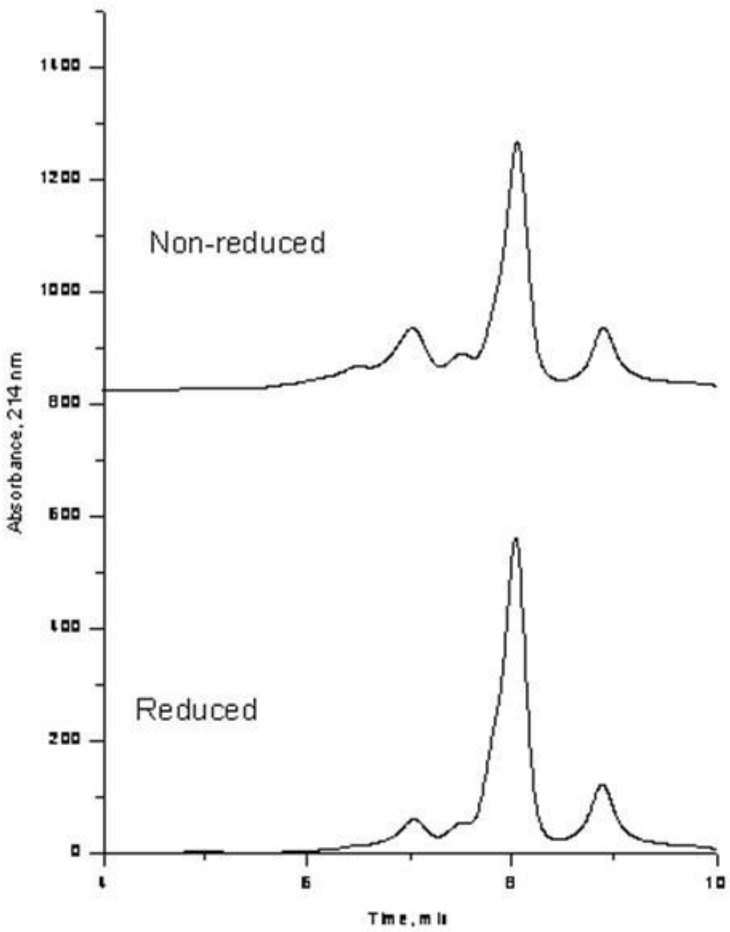

Figure 12: Size exclusion chromatograms of reduced (bottom) and non-reduced (top) solubilized commercial zein isolates.

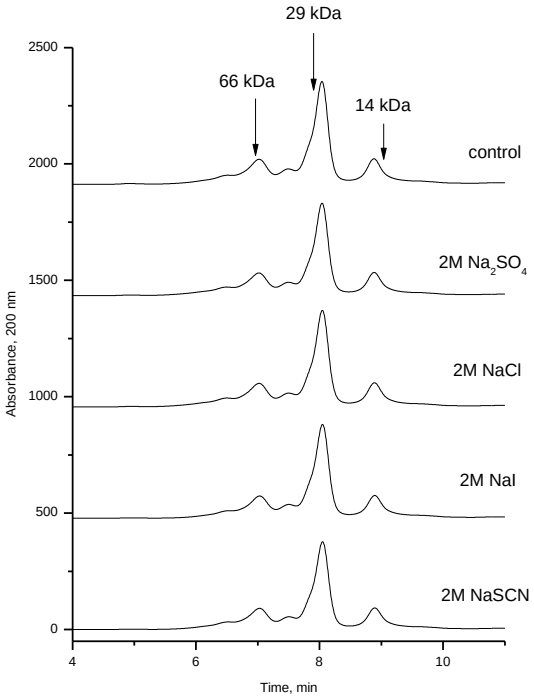

Figure 13: Size exclusion chromatograms of various treatments applied to zein. Treatments are labeled on the right side of size distribution plots.

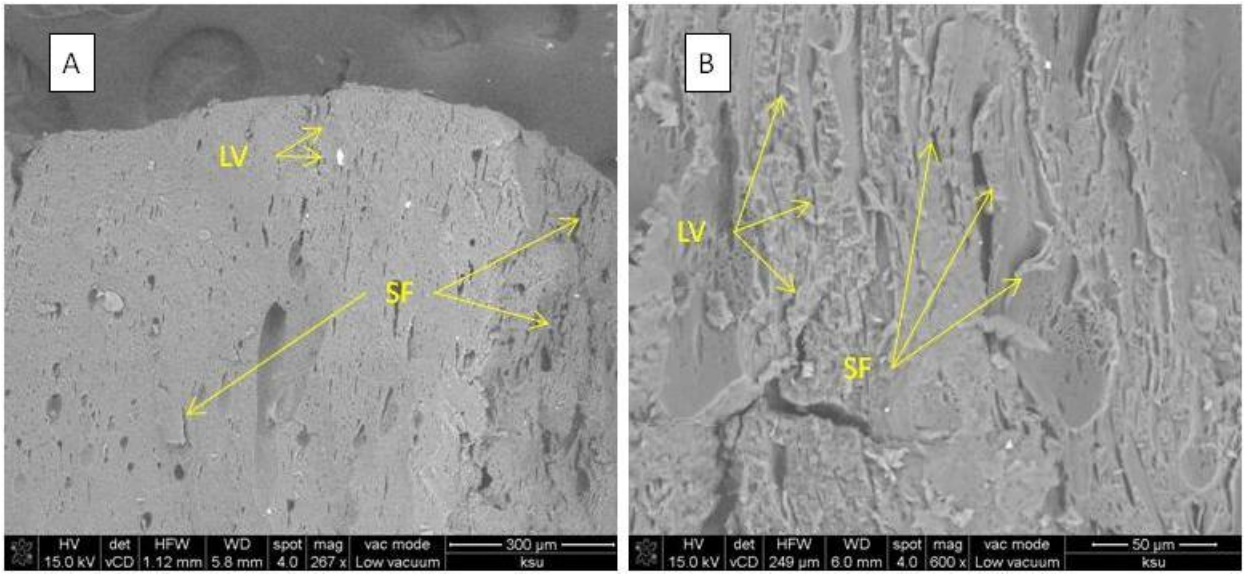

Figure 14: 267 X magnification SEM of zein mixed with DI water at 40°C for 20 min (A). 600 X magnification SEM of zein mixed with DI water at 40°C for 20 min (B). LV designates linear voids zein protein and SF designates surface flaking.

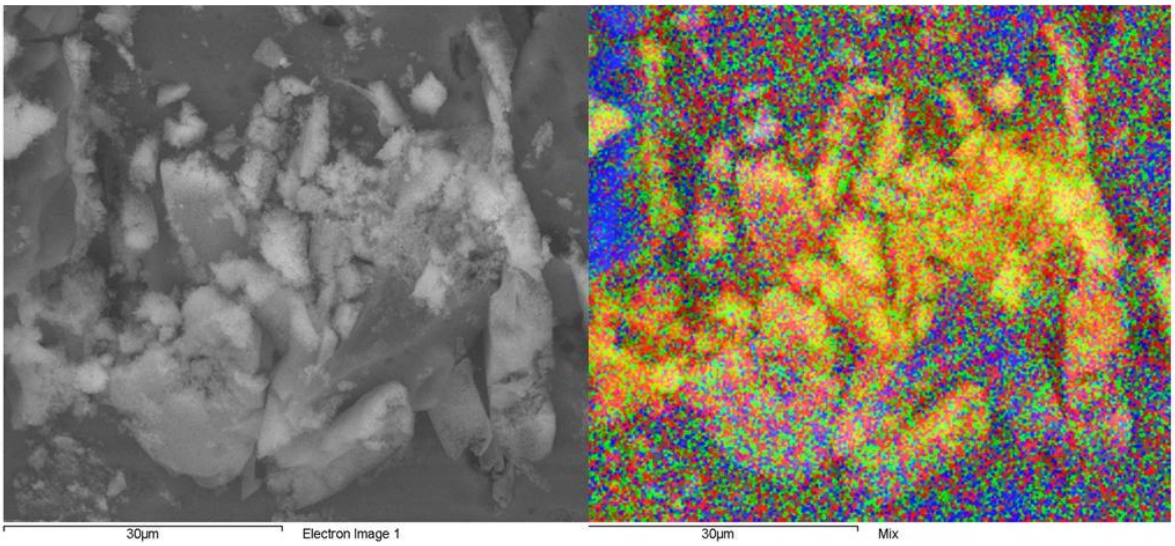

Figure 15: SEM of zein mixed with 2M treatment of Na_2SO_4 at 40°C for 20 min (left). Elemental map overlays of zein mixed with 2M treatment of Na_2SO_4 at 40°C for 20 min (right). Carbon=blue, sodium=red, and oxygen=green. Overlapping of sodium and oxygen resulted in yellow coloration and carbon overlapping with sodium or oxygen resulted in various shades of blue and purple.

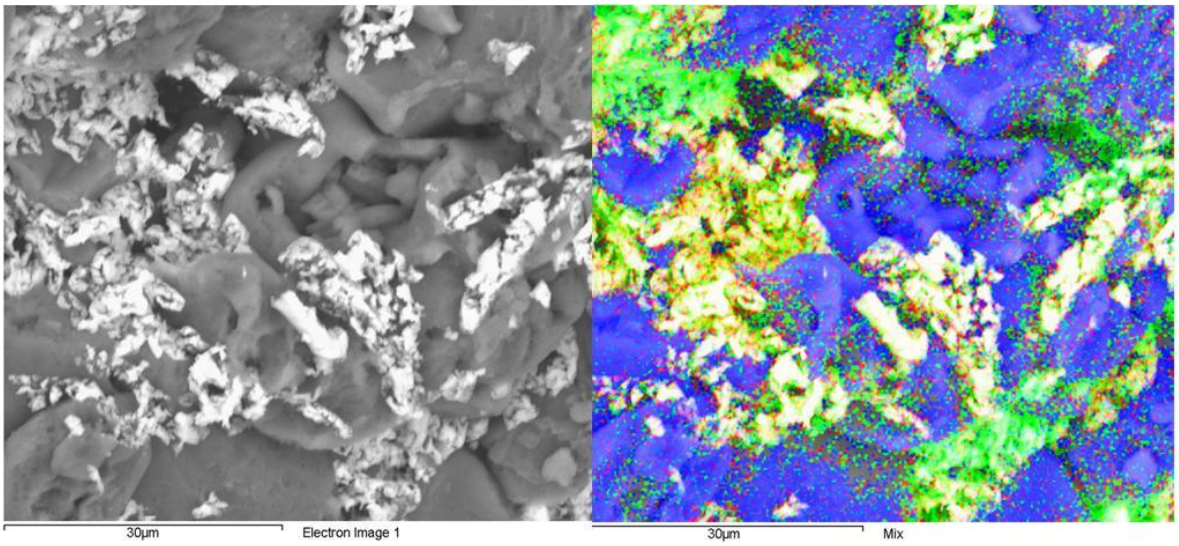

Figure 16: SEM of zein mixed with 2M treatment of NaCl at 40°C for 20 min (left). Elemental map overlays of zein mixed with 2M treatment of NaCl at 40°C for 20 min (right). Carbon=blue, sodium=red, and chlorine=green. Overlapping of sodium and oxygen resulted in yellow coloration and carbon overlapping with sodium and chlorine resulted in various shades of blue and purple.

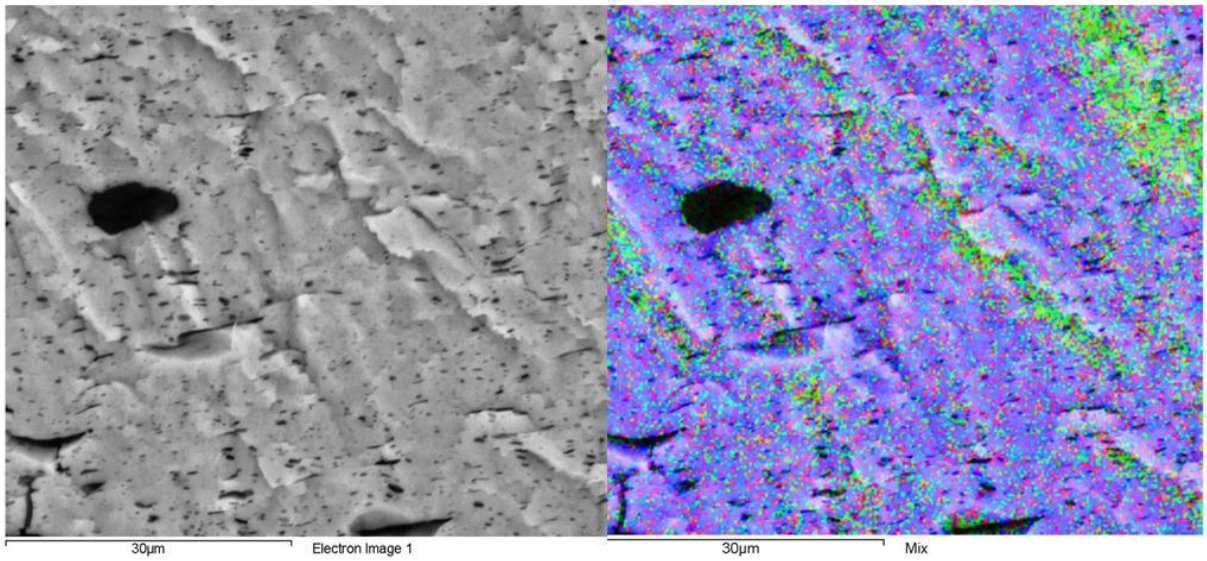

Figure 17: SEM of zein mixed with 2M treatment of NaI at 40°C for 20 min (left). Elemental map overlays of zein mixed with 2M treatment of NaI at 40°C for 20 min (right). Carbon=blue, sodium=red, and Iodine=green. Overlapping of sodium and iodine resulted in yellow coloration and carbon overlapping with sodium or iodine resulted in various shades of blue and purple.

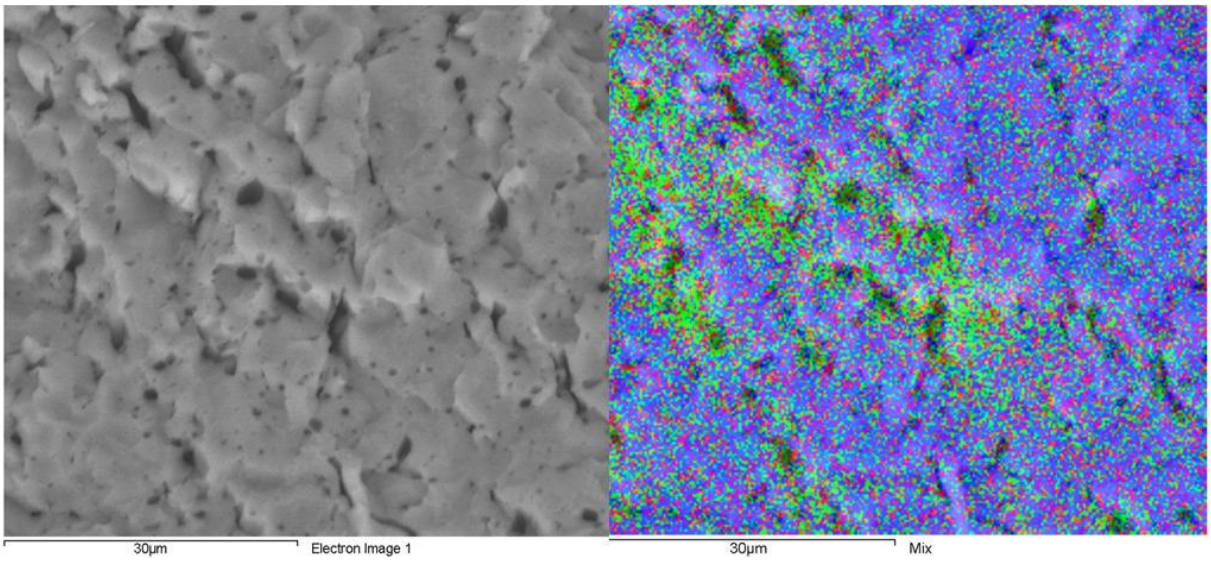

Figure 18: SEM of zein mixed with 2M treatment of NaSCN at 40°C for 20 min (left). Elemental map overlays of zein mixed with 2M treatment of NaSCN at 40°C for 20 min (right). Carbon=blue, sodium=red, and sulfur=green. Overlapping of sodium and sulfur resulted in yellow coloration and carbon overlapping with sodium or sulfur resulted in various shades of blue and purple.

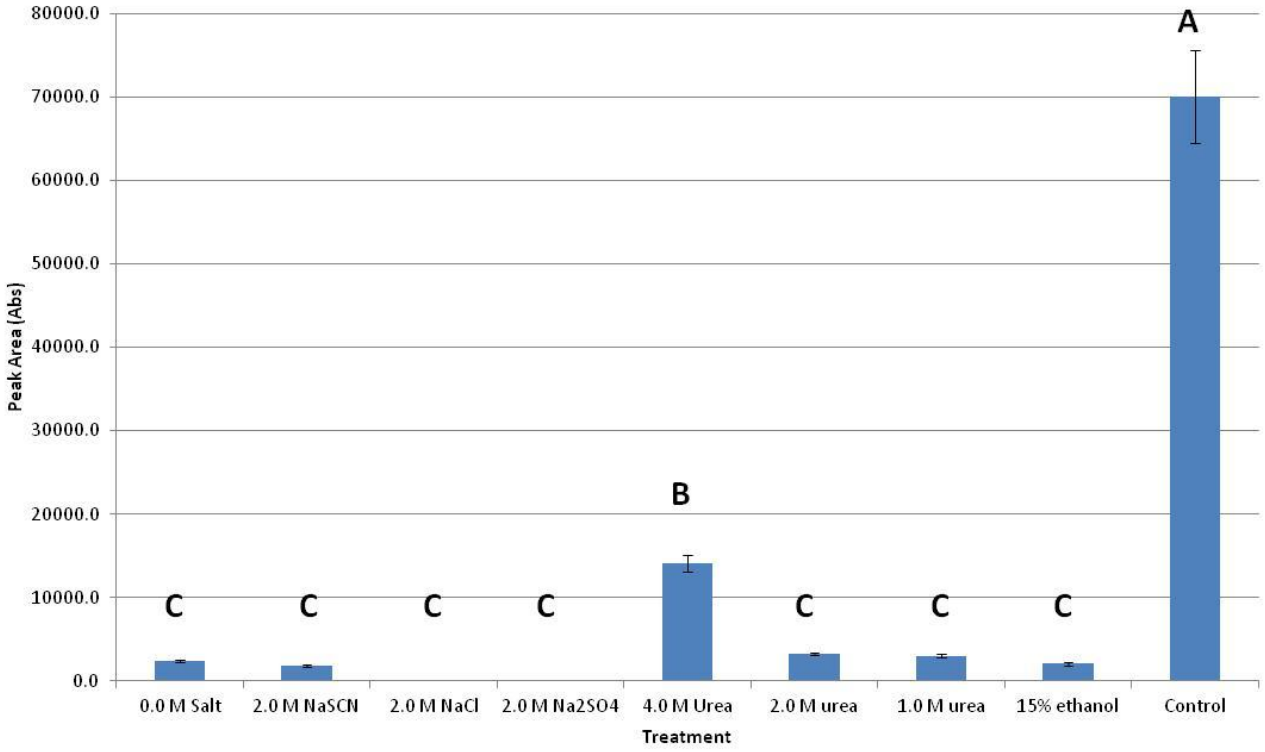

Figure 19: A graphical representation of zein solubility determined by taking the total area under SE-HPLC chromatogram peaks (y axis) for zein vortexed with the various treatments (x axis). The upper case letters represent differences of means where similar letters identify no significant differences between the means ($P < 0.05$).

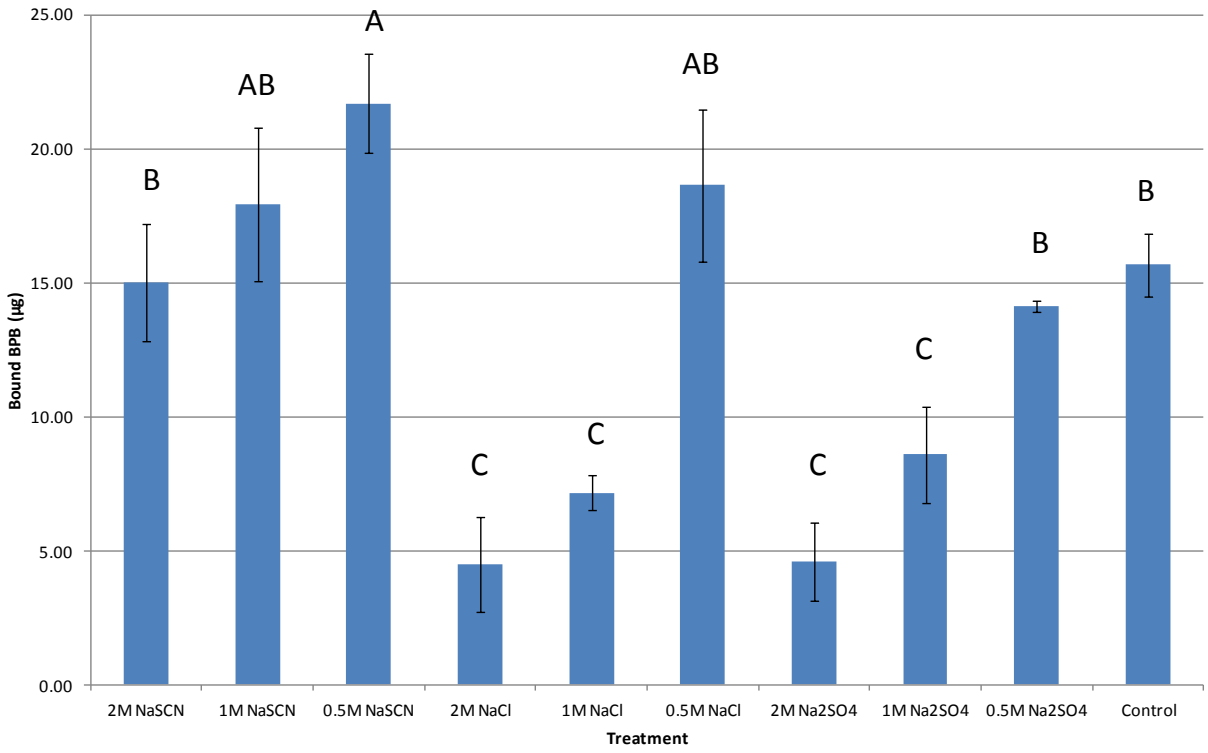

Figure 20: A graphical representation of zein's surface hydrophobicity determined by zein's ability to bind BPB (y axis). Zein was treated with NaSCN, NaCl, Na_2SO_4 at concentrations 2M, 1M, and 0.5M vortexed and compared against a control (x axis). The upper case letters represent differences of means where similar letters identify no significant differences between the means ($P < 0.05$).

Chapter 3: Practical Implications of Non-Covalent Interactions in Zein-Starch Dough and Bread Quality

Abstract

A major limitation in the production of wheat-free breads is the lack of protein functionality in non-wheat cereals. Breads made from non-wheat flours such as rice, maize, and sorghum must be made from thick batters and are of lower quality than wheat bread. The development of visco-elastic dough from non-wheat proteins would allow a wider range of gluten-free products to be made and would improve the quality of such foods. Zein has been shown to form wheat-like dough; however the mechanism of dough formation with zein is unknown. To identify the factors responsible for dough development in zein-starch mixtures and their influence on zein bread quality, a mixture of 20% zein-80% maize starch was mixed with water and different reagents known to alter zein protein-protein interactions. The salts, NaSCN, NaCl, and Na_2SO_4 were evaluated at concentrations from 0 – 2M for their influence on the properties of zein-starch dough systems. NaSCN at low concentrations made a softer more wheat-like dough. Urea and ethanol had similar affects on the zein-starch dough and produced softer more workable dough. With increasing concentrations of NaCl and Na_2SO_4 there was a coalescing of the proteins and no dough was formed. The use of the reducing agent β-ME had little effect on the mixing properties of zein-starch dough. Specific volumes of zein-starch bread increased as NaCl content in the bread formula decreased. Likewise, including 5% ethanol (v/v) in the bread formula was found to increase bread quality. This research demonstrated that unlike wheat, zein proteins are capable of forming visco-elastic dough due to non-covalent interactions rather than disulfide linked high M_w proteins.

Introduction

Celiac disease afflicts ~1% of the world's population and is a growing concern (Fasano and Catassi 2001; Van Heel and West 2005). Foods catering to celiac suffers must be devoid of wheat, barley, and rye (Godkin and Jewel 1998; Green and Cellier 2007; Fasano and Catassi 2008; Weiser and Koehler 2008). Gluten-free foods are known to be of poorer quality than foods that are produced with gluten, especially leavened bread products (Cornish et al. 2006; Arendt et al. 2008; Schober et al. 2008). It has been hypothesized that the lack of a protein network is one reason for the general poor quality of gluten-free foods, especially breads. Gluten protein networks provide elasticity and extensibility of wheat products and are what gives wheat its ability to form a handleable dough and retain CO_2 for leavening. In addition, the protein networks in wheat have been attributed to prolonged shelf life of bread and an increase in quality in terms of softness and volume (Cornish et al. 2006; Arendt et al. 2008; Schober et al. 2008).

The lack of dough formation is a limiting factor in gluten-free bread quality and diversity. Traditionally gluten-free breads are made from a thick paste like batter. This batter can only take the shape of the pan that it is baked in. The introduction of gluten-free breads made from a wheat-like dough would allow for the development of products that more closely resemble wheat breads. This would add a great deal of diversity to gluten-free breads by allowing for production of products that currently are not being made because they require a visco-elastic dough for production.

Unfortunately, there are few known proteins other than those of wheat capable of forming a visco-elastic dough. Other than wheat, zein proteins from corn and caroubin from

carob germ are the only proteins that have been physically and chemically defined in terms of their ability to form visco-elastic doughs (Bienenstock et al. 1935; Lawton 1992; Mejia et al. 2007; Oom et al. 2008; Schober et al. 2008, 2010, 2011; Smith 2009; Erickson et al. 2011; Smith et al. 2010, 2011. Caroubin was found to form a visco-elastic wheat-like dough due to networks of disulfide linked proteins of very high molecular weight, much like wheat (Bienenstock et al. 1935; Smith 2009; Smith et al. 2010, 2012). The exact chemical nature of zein's ability to form a visco-elastic dough and yeast leavened bake product has yet to be defined. Previous work on zein has primarily focused on product development and optimization of breads utilizing zein-starch mixtures (Schober et al. 2008, 2010, 2011). The use of hydroxypropyl methylcellulose (HPMC) in zein-starch breads was found to produce high quality gluten-free bread. This research demonstrated that HPMC coated the zein in a dough system and allowed for thinner protein fibers and subsequent softer more wheat-like doughs. Schober et al. (2010) also reported that partial defatting of zein furthered its ability to produce a more wheat-like dough, possibly by enhancing protein-protein interactions.

To fully take advantage of zein's ability to form dough when mixed with starch above its glass transition temperature, the factors responsible for zein dough formation must be identified and applied to baking systems. In the previous chapter, non-covalent interactions were found to play a critical role in protein-protein interactions during zein mixing. Altering the non-covalent interactions of the zein proteins could completely inhibit the ability of zein to be mixed into a visco-elastic material. How such protein-protein interactions function in a dough system where zein is mixed with starch is not known. Furthermore, how such interactions impact the final bread quality of zein-starch mixtures is also not known. Therefore, the

objectives of this research were to determine the chemical aspects of zein's ability to form a wheat-like dough when mixed with starch and apply this practically to baking systems.

Materials and Methods

Materials

Zein isolate was purchased from Sigma-Aldrich, Co., (St. Louis, MO). Unmodified native corn starch was purchased from Bob's Red Mill (Milwaukie, OR). Sucrose was purchased from the local grocery store (C&H Sugar Company, Crockett, CA) and NaCl was a purchased from Sigma-Aldrich, Co., (St. Louis, MO). Ethanol under the trade name CHROMASOLV (95% ethanol+5% isopropanol) was purchased from Sigma-Aldrich, Co., (St. Louis, MO). Instant dry yeast was obtained from Fleischmann's Yeast (AB Mauri Food, inc., Chesterfield, MO).

Dough Formation

The research reported in chapter 2 demonstrated that zein's ability to form visco-elastic materials (resins) was very sensitive to non-covalent interactions. However, that research was conducted on pure zein. To be able to isolate the effects to just protein, zein based breads must be made with zein-starch mixtures. In order to determine if non-covalent interactions impact zein-starch dough and bread quality, various salts from the Hofmeister series were added to zein-starch formulas during mixing.

In order to determine the importance of the different bonding interactions on zein-maize starch dough formation, dough was mixed by a Farinograph-AT (Duisburg, Germany) at 73 rpm for 20 min. For all dough mixing experiments, 40 g of a 20% zein-80% corn starch mixture was placed in the farinograph's 50 g mixing bowl. After one minute of calibration time 30 mL of de-ionized (DI) water or solution of interest was added and mixing continued for 20

min. Treatments included Na_2SO_4, NaCl, NaI, and NaSCN at concentrations of 0.0M, 0.5M, 1M, and 2M, 0-4M urea, and ethanol was evaluated at concentrations of 5%, 10%, and 15% (v/v). An aqueous solution containing 2% β-ME (v/v) was also used as a treatment.

After mixing in the farinograph, dough samples were immediately frozen in liquid nitrogen, stored at -80 °C and lyophilized. Lyophilized dough was ground via mortar and pestle and stored at – 20 °C on desiccant for subsequent SEM analysis.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) backscattering electron (BSE) imaging was completed using a Nova Nano SEM 430 (FEI Company, Hillsboro, Oregon) in low vacuum mode (0.98 torr) equipped with a voltage high contrast detector (vCD). 15.0 KV was used for analysis.

Baking Experiments

In order to investigate the effects of NaCl on zein based bread quality, bread was produced using NaCl concentrations from 0.0M to 2M.

To investigate the effects of Ethanol on zein based bread quality, concentrations of 0%, 5% or 10% ethanol (v/v) were used. The basic bread formula used for all baking experiments was as follows: 100 g flour (20% zein + 80% corn starch), 1 g sucrose, and 2 g active dry yeast. 75 mL of the salt solutions, ethanol, or DI water were added to the dry ingredients for all experiments.

Baking Procedure

Dry ingredients were blended to homogeneity except for NaCl. In experiments requiring NaCl or ethanol, the salt or ethanol was mixed with DI water to the desired concentration. All ingredients were then tempered to 40 °C prior to mixing for 12 hrs. A 100g pen mixer (National

MFG, Lincoln, NE.) was used to mix zein-starch mixtures for all experiments. The mixer was placed in a 40 °C environmental chamber and allowed to temper for 12 hrs prior to mixing. Dough was mixed for 30 seconds and scraped down from the sides of the mixing bowl to insure homogeneity. An additional 5 min of mixing was completed after the scraping of the bowl. Dough was then removed from the mixing bowl, hand kneaded to form a ~5 cm X ~7.5 cm cylindrical dough ball, and placed in an oiled bake pan. Bake pans were of the same dimensions described in AACCI method 10-10.03 for 100g loaves (Optimized Straight-Dough Bread-Making Method) (AACCI 2000). Pans containing dough were proofed at 40 °C for 30 min at a relative humidity of 90%. A standardized proof time was chosen over proofing to height due to the extremes in formulations. A time of 30 min was determined to be optimum during preliminary research. After proofing, dough was baked at 220°C for 20 min in a deck oven (1T2, Doyon, Linière, Qc, Canada). Breads were allowed to cool for 1.5 hrs prior to analysis.

Bread analysis

Bread volume was measured by rape seed displacement. Specific volume was obtained 1.5 hrs post baking by the equation:

Specific Volume = loaf volume/loaf weight

Bread loafs were sliced at a thickness of 2.5 cm with a cutting jig to ensure uniformity in surface and thickness between slices. Only slices from the center of the bread were taken for analysis to avoid irregularities between slices. Bread slice images were collected and analyzed via a CALIBRE C-Cell (CCFRA Technology Ltd., Appleton, Warrington, United Kingdom). For analysis, the greater the specific volume was considered most desirable, and C-Cell images were used to

account for undesirable traits such as large cell size, thickening of cell walls, and large holes that indicate crumb failure.

Experimental Design

Three breads corresponding to each treatment and control were baked. Analysis of variance was completed with a $P < 0.05$, using Statistical Analysis Software (SAS 9.1, SAS Institute Inc. Cary, NC). A comparison of means using Tukey's studentized range test was used to determine differences in quality. Here optimum quality was selected based on the criterion of the highest specific volume, and crumb structure.

Results and Discussion

Dough Formation

When zein-starch mixtures were mixed in the presence of Na_2SO_4 the zein-starch system was unable to form a dough (Fig 1). Zein remained in discrete particles dispersed throughout the zein-starch-Na_2SO_4-water mixture. At 0.5M concentration of Na_2SO_4, the flour mix showed little resistance to mixing and resulted in a thick paste that was not extensible when pulled apart by hand (Fig 2). These results were similar to that found when mixing zein only with Na_2SO_4 (i.e. the zein did not form a cohesive mass and only formed discrete particles). However, when zein-starch was mixed with NaCl, very weak dough was formed at 0.5M and 1M NaCl (Fig 3) The dough produced from the 0.5M NaCl treatment was stiff and had limited extensibility (Fig 4). This is probably why the farinogram shows a higher resistance to mixing when compared to the control (Fig 3). Furthermore, the farinogram representing zein-starch flour treated with a solution of 1M NaCl appears to have a peak mixing point and break down (Fig 3). This was not the case for any other treatment. The dough produced from the 1M NaCl

treatment was stiff and had little extensibility (Fig 4). Note, the large numeric difference between the minimum and maximum values of the farinogram demonstrated stiff dough that was not as extensible as the control (Fig 3). Zein-starch mixtures were unable to form a dough in the presence of 2M NaCl (Fig 3-4).

When compared to the control dough, the use of NaSCN at low concentrations appeared to soften the dough (Fig 5). This was probably due to the weakening of zein's non-covalent interactions, as demonstrated in chapter 2. However, at higher concentrations, NaSCN was able to gelatinize starch (Ahmad and Williams 2002) and thus created an entirely different system of zein mixed with gelatinized starch. The results of this were quite interesting and produced a substance that was more resistant to mixing (Fig 5) and very extensible when stretched by hand compared to the control (Fig 6). At 1M NaSCN, the zein-starch mixture had lower resistance to mixing than with the 2M concentration (Fig 5). Interestingly, the resistance to mixing increased during the course of mixing (Fig 5). If allowed to mix for longer periods of time (up to 3 hrs) the 1M NaSCN treated zein-starch flour had similar resistance to mixing as the 2M NaSCN treatment (data not shown). This was most likely due to the gradual gelatinization of starch during mixing with the 1M NaSCN treatment. Furthermore, the mixture remained plastic when cooled to room temperature, which may indicate that the zein-gelatinized starch mixture has a lower glass transition temperature than zein in water alone. Future work examining the effects of incorporating gelatinized starch into zein-based gluten-free breads would be valuable addition to this research. Substituting pre-gelatinized starch for part of the native starch in zein-starch mixtures may be able to improve zein's ability to produce a visco-elastic dough and improve bread quality.

In addition to the Hofmeister salts tested above, urea and ethanol were also tested for their impact on the properties of zein-starch dough systems. Both compounds were found to alter the visco-elastic properties of zein only mixtures in chapter 2. With the zein-starch mixtures, it was found that the addition of urea and ethanol had similar effects on dough properties. Urea at low concentrations seemed to soften the dough and make it more extensible when pulled apart by hand (Fig 7 and 8 respectively). As the concentration of urea increased, the resistance to mixing decreased, resulting in a very thin consistency and sticky dough with the 4M urea treatment (Fig 7 and 8). The stickiness observed in the urea treatments may have been due to partial gelatinization of starch. Urea has been previously shown to lower the gelatinization temperature of starch (Hebeish et al. 1981; Chiou et al. 2005; Kou and Wang 2006).

The incorporation of ethanol into the zein-starch mixture followed a similar trend as the urea (Fig 9) but did not produce a dough that was sticky when handled (Fig 10). Overall the urea and ethanol treatments followed the same trends seen in chapter 2. Because these treatments followed the same trends seen in chapter 2, the softening effect on the dough from ethanol and urea can be attributed largely to the softening of zein proteins. As mentioned in chapter 2, 4M urea was shown to partially solubilize zein. This solubilization may be one reason why the 4M urea treatment zein-starch mixtures produced a very weak dough that could not be extended a great distance before breaking.

In order to test the importance of disulfide bonding in zein-starch dough formations a 2% solution (v/v) of β-ME was added to the zein-starch mixtures. Disulfide bonds are a critical factor in wheat gluten and caroubin network formation (Smith 2009; Smith et al. 2010, 2012). .

Addition of β-ME had little effect on zein's ability to form a visco-elastic dough with only slight differences seen in the farinogram of the reduced sample relative to the control (Fig 11). Compared to the control β-ME had a slight decrease on mixing force, had a similar shaped mixing curve, and had a smoother mixing curve with less deviation from the average mixing force. No differences were observed between the β-ME treated and control zein-starch doughs when extended by hand (Fig 12). This was similar to what was seen when pure zein was mixed with β-ME in chapter 2 and may have been due to disruption of intra-molecular disulfide bonds in the zein (Cabra et al. 2008) or possibly due to changes in protein structure from the β-ME itself in a similar fashion as the Hofmeister salts impact zein.

Another interesting property of zein-starch dough was that it seemed to be very resistant to breakdown via shear from mixing. This may have been due to the fact that unlike wheat, zein doughs were not reliant on disulfide bonds between cysteine residues for functionality as shown by this research. It has been proposed that breakdown of wheat during mixing is due to changes in molecular weight distribution of the large polymeric protein complexes, either through disulfide interchange or simply breakage of the large polymeric proteins at disulfide bonds (Greenwood and Ewart 1979; Hamer and Vliet 2000).

It should be noted that in cases where no dough was formed, dough could not be formed by simply adding more water. Excess water was simply excluded from the mixture. This also means that the water level could not be optimized based on the mixing curves from the farinograph. The zein-starch mixtures had a finite amount of water that could be held before they began to exclude water. Adding water to lower mixing resistance would have only resulted in excess water excluded from the mixture with little affect of the mixing curve.

Scanning Electron Microscopy

In order to obtain a visual representation of how zein-starch mixtures form dough, scanning electron microscopy was used. For the SEM work, only the 2M salt treatments were compared to the control. It was found that the control dough had distinct fiber-like networks interspersed throughout the starch and in some instances the protein was found encompassing the starch granules (Fig 13) much like that seen in some of the classic imaging done on wheat dough, where gluten is observed in fibers encompassing starch granules (Hoseney 1998). With both the Na_2SO_4 and NaCl treatments, the zein protein remained as discrete particles dispersed throughout the starch (Fig 14 and Fig 15 respectively). The salts can also be observed as a white crust like material on the surface of the proteins and starch (Fig 16). SEM of the 2M NaSCN treatment confirmed that some starch was gelatinized with this treatment when compared to the control that was mixed (Fig 13 and 17). As can be seen in Figure 17, only a few starch granules can be observed and a thick solid layer can be seen in which the remaining starch granules are embedded. The control has large amounts of starch granules that account for much of what can be seen in the image (Fig 13). An image of the zein-starch mixture that was not mixed in water was also included as a reference to better observe differences occurring with treatments (Fig 18). Distinct starch granules and zein fragments can be seen in Figure 18. Compared to the control that was mixed in the farinograph (Fig 13) the development of zein protein fibrils during mixing can easily been seen. This signifies a change from discrete zein particles to zein fibers dispersed throughout the starch when mixed with water at 40 °C.

Protein network formation may be a determining factor in forming visco-elastic dough networks. However, the traditional hypothesis has been that the only route to form a visco-

elastic protein based dough was through the formation of large protein complexes, composed of covalent interactions between proteins, as found in wheat. Because some proteins (i.e. wheat and caroubin) are capable of forming networks through covalent interactions between proteins, research by others have attempted to use other means of promoting protein cross-linking such as with the use of transglutaminase (Gujral and Russell 2004; Moore et al. 2006; Renzetti et al. 2008). The results of this research demonstrate that covalent cross-linking of proteins play only a minor role, if any, in the production of dough from zein-starch mixtures. Since visco-elastic dough can be formed by means other than covalent cross-linking, perhaps many other proteins sources would be capable of forming visco-elastic doughs by promoting or diminishing hydrophobic or hydrophilic interactions and hydrogen bonding. This may be a promising new area of research in food protein functionality that has the potential to greatly impact the quality of gluten-free foods.

Effect of NaCl Addition on Zein-Starch Bread Quality

NaCl is a commonly used baking ingredient. However, as covered previously in this research it appears to have a detrimental effect on zein-starch dough development. Because of this, breads were baked using concentrations of NaCl from 0M to 2M. As the concentration of NaCl in the bread formulas increased, the specific volume of the zein-starch bread decreased (Fig 19). Breads made with various levels of NaCl are shown in Figure 20. However, in the lower concentrations of salts, large holes were observed in the crumb (Fig 21). Thus, the specific loaf volume data should be viewed with caution and only interpreted as an indicator of what happens as NaCl is changed in the zein-starch bread formula and not as a means to predict differences between individual treatments. In previous publications hydroxypropyl

methylcellulose (HPMC) was used to improve zein-starch bread quality (Schober et al. 2008, 2010, 2011). However, in order to see the direct effects of NaCl on zein-starch breads, HPMC was omitted from the current research. A major reason for this was that the Hofmeister effect also influences HPMC (Liu et al. 2008). In Schober et al. (2010) a formulation for optimized yeast leavened zein based bread contained 5% sucrose, 1 %, NaCl, and 2 % HPMC (on a flour basis). The bread production process in this study used a 20 min fermentation fallowed by hand kneading and 5 min of rest. After this the bread proofed for 35 min and was baked. The 1% NaCl may have been necessary for adequate yeast fermentation during the 60 min of total fermentation time or may have had a positive effect on HPMC functionality (Liu et al. 2008). The total affects of HPMC on the Schober et al. (2008, 2010, and 2011) system are not known, but overall were positive. What is shown by looking at the research reported in this chapter is that salt is a critical factor in zein-starch dough development and subsequent bread quality. However, certain formulations and baking techniques may require some NaCl or other salts. In these cases, it is critical that the minimum amount of NaCl necessary to produce the optimum bread in terms of overall quality be determined.

Effect of Ethanol on Zein-Starch Bread Quality

Based on research from chapter 1, the use of chaotropic salts improved zein's ability to form visco-elastic materials. One hypothesis for the current chapter was then that such materials would also improve zein-starch dough properties. However, as chaotropes partially gelatinized starch, their use complicates dough formation. From a practical standpoint, the use of such chaotropes in bread production is not an option as these compounds are not generally regarded as safe (GRAS). However, the use of low levels of ethanol also altered the properties

of zein when mixed (chapter 2). Ethanol also altered the properties of zein-starch dough when mixed, making the dough softer and more extensible (Figures 9 and 10 respectively). When breads were baked with different levels of ethanol, it was found that the addition of 5% ethanol resulted in a slight increase in specific loaf volume (Fig 22 and 23). This was interesting given that the 10% ethanol produced a poorer quality of bread, as it produced a softer more wheat-like dough. This may have been due to inhibition of the yeast (*Saccharomyces cerevisiae*) to produce CO_2 in the presence of 10% ethanol. The use of 5% ethanol produced a more homogenous crumb structure devoid of large holes when compared to the control (Fig 24). The use of low levels of ethanol could therefore be used to improve the quality of zein-starch breads. As with the experiments baking bread with various levels of NaCl, HPMC was omitted from the bread formulas using ethanol. This was so the effect of the ethanol addition on the zein-starch mixtures could be directly observed. Using ethanol with HPMC in the bread formula could alter the functionality of HPMC and the interaction between HPMC and zein. Future research investigating the combined effects of NaCl, HPMC, and ethanol should be conducted to fully optimized zein-starch breads.

Conclusions

Salts of the Hofmeister series known to salt out proteins made zein in zein-starch mixtures remain as discrete particles. NaCl a salt known to be in the middle point of salting in or salting out proteins also has a negative effect on zein protein network formation. At lower concentrations NaCl did not disrupt zein's ability to form dough. Practically, this can be applied to baking situations, so that salt is present at minimum levels. Additionally, to enhance the functionality of zein in dough systems, low levels of ethanol can be added. It would also be

interesting to find GRAS substances capable of changing hydrophobic interactions in a similar manner to NaSCN. This research provides a means to further enhance zeins utility in gluten-free food systems.

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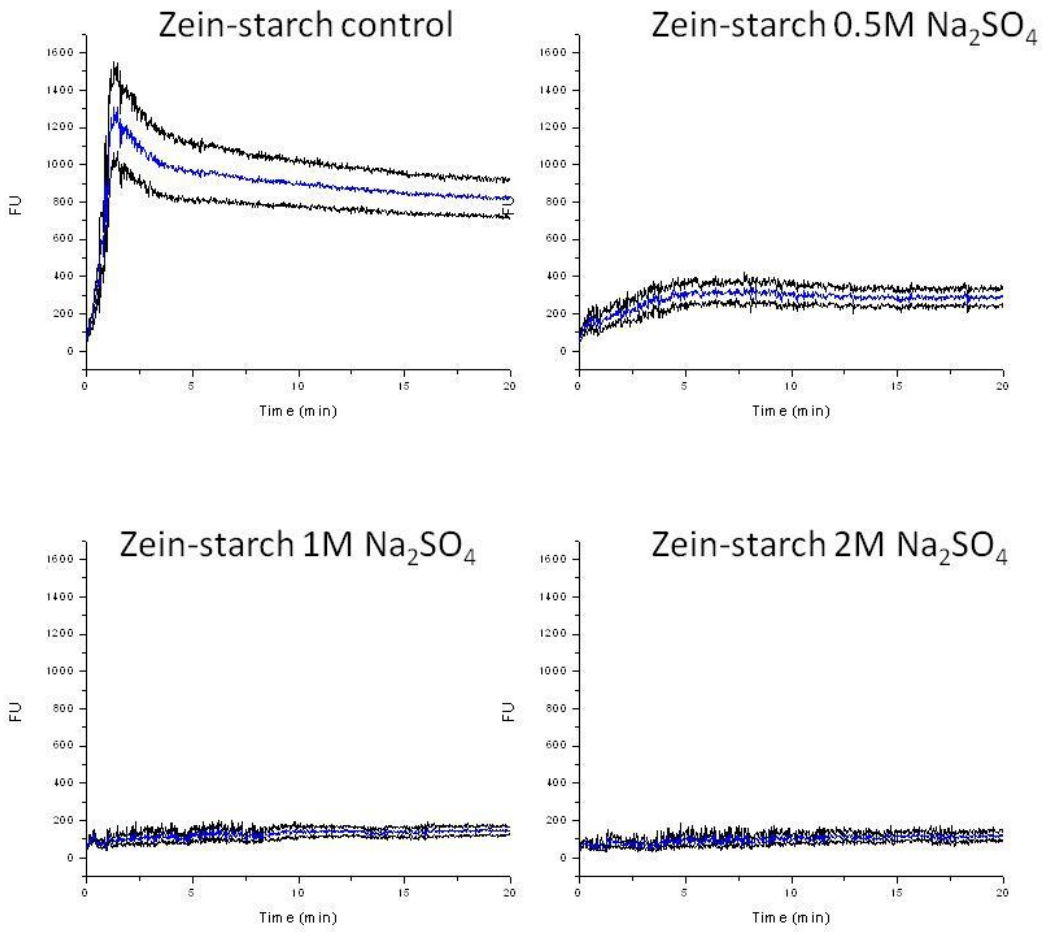

Figure 1: Farinograms of zein-starch mixtures from: Control mixed for 20 min with DI water (top left), 0.5M Na_2SO_4 treatment mixed for 20 min (top right), 1M Na_2SO_4 treatment mixed for 20 min (bottom left), and 2M Na_2SO_4 treatment mixed for 20 min (bottom right).

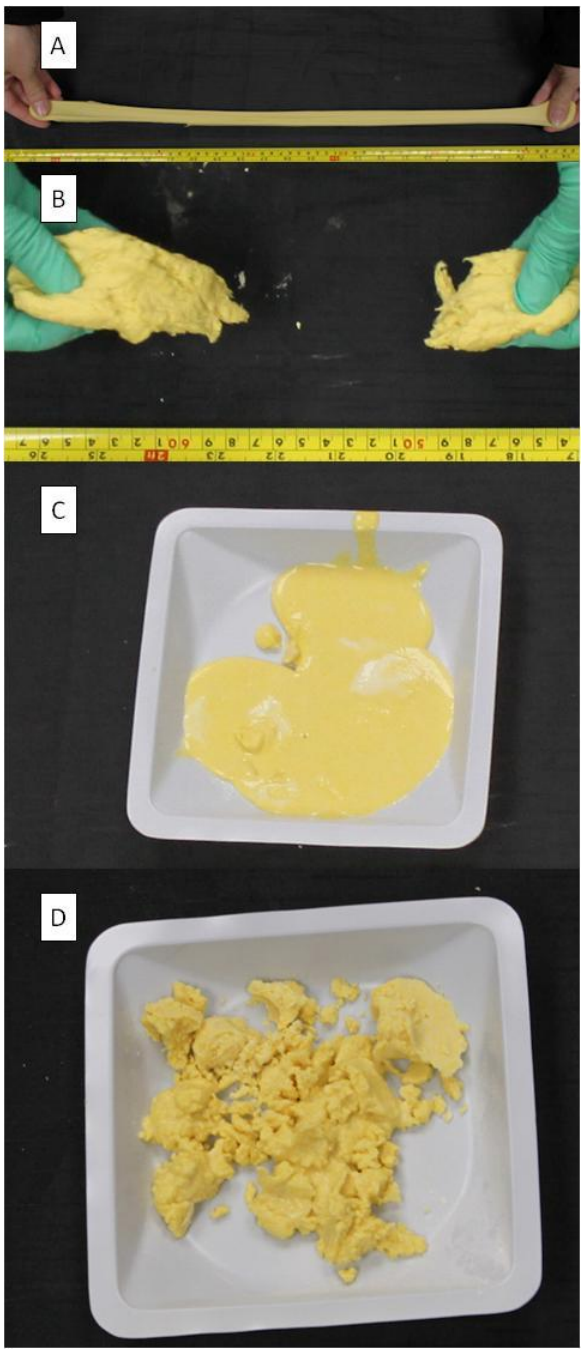

Figure 2: Visual representations of: control zein-starch dough mixed for 20 min with DI water (A), 0.5M Na_2SO_4 treatment mixed for 20 min (B), 1M Na_2SO_4 treatment mixed for 20 min (C), and 2M Na_2SO_4 treatment mixed for 20 min (D).

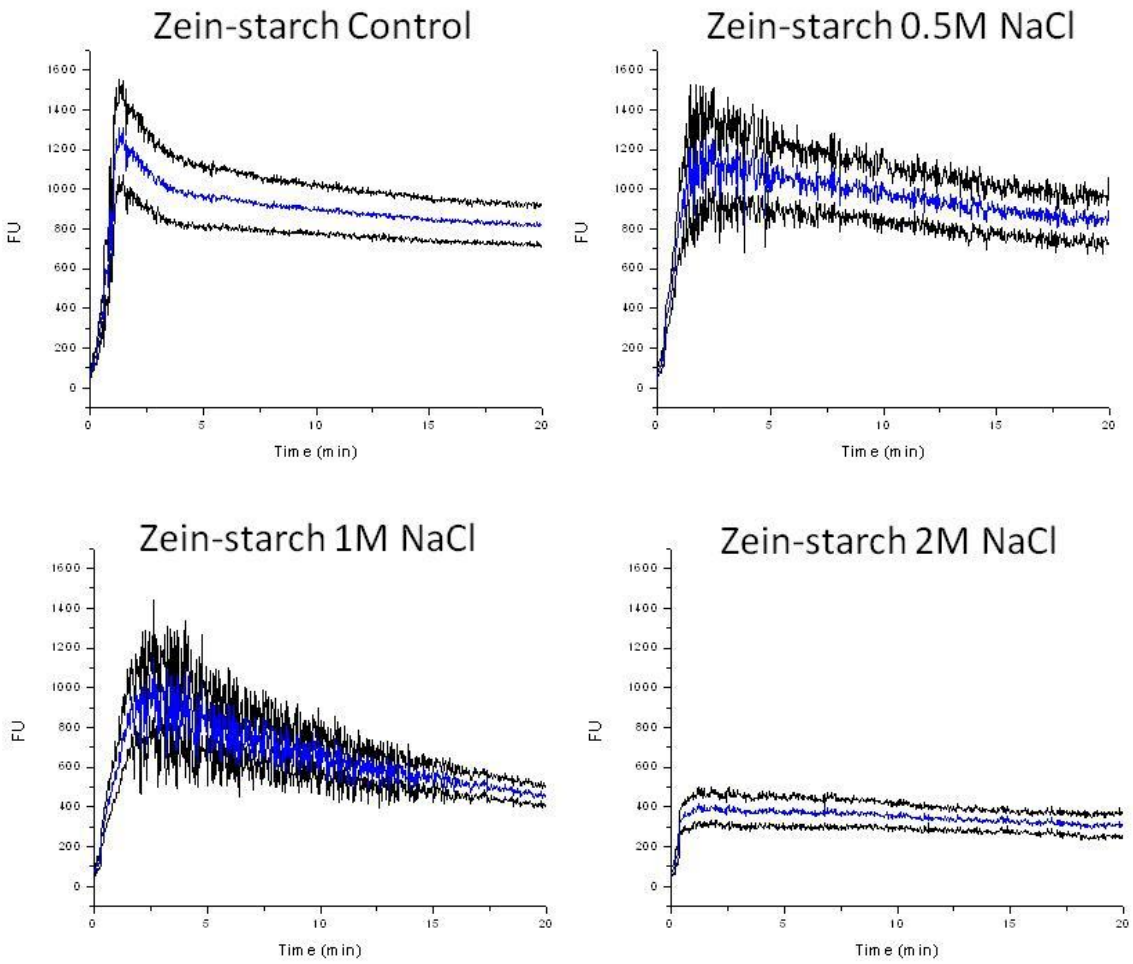

Figure 3: Farinograms of zein-starch mixtures from: Control mixed for 20 min with DI water (top left), 0.5M NaCl treatment mixed for 20 min (top right), 1M NaCl treatment mixed for 20 min (bottom left), and 2M NaCl treatment mixed for 20 min (bottom right).

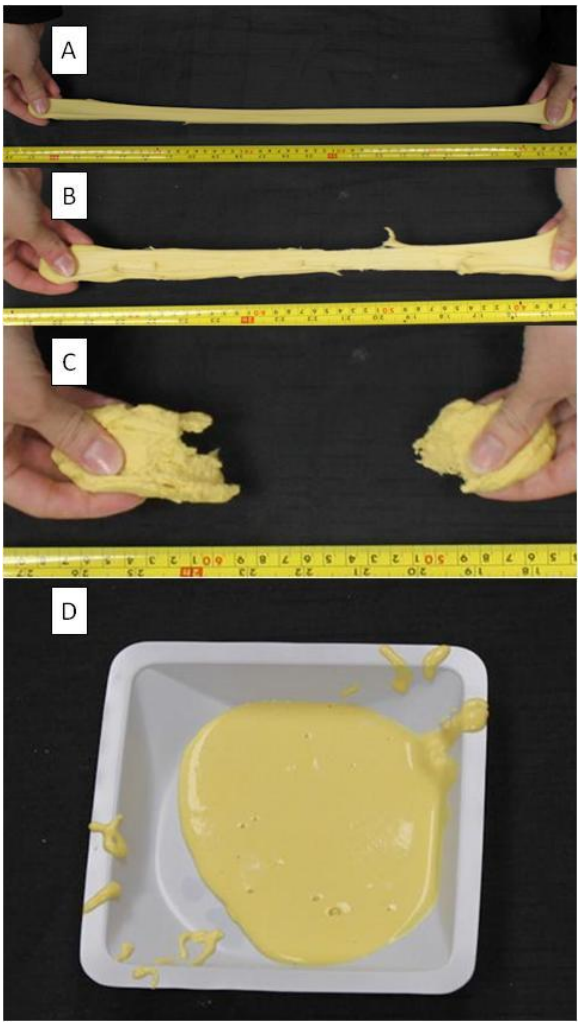

Figure 4: Visual representations of: control zein-starch dough mixed for 20 min with DI water (A), 0.5M NaCl treatment mixed for 20 min (B), 1M NaCl treatment mixed for 20 min (C), and 2M NaCl treatment mixed for 20 min (D).

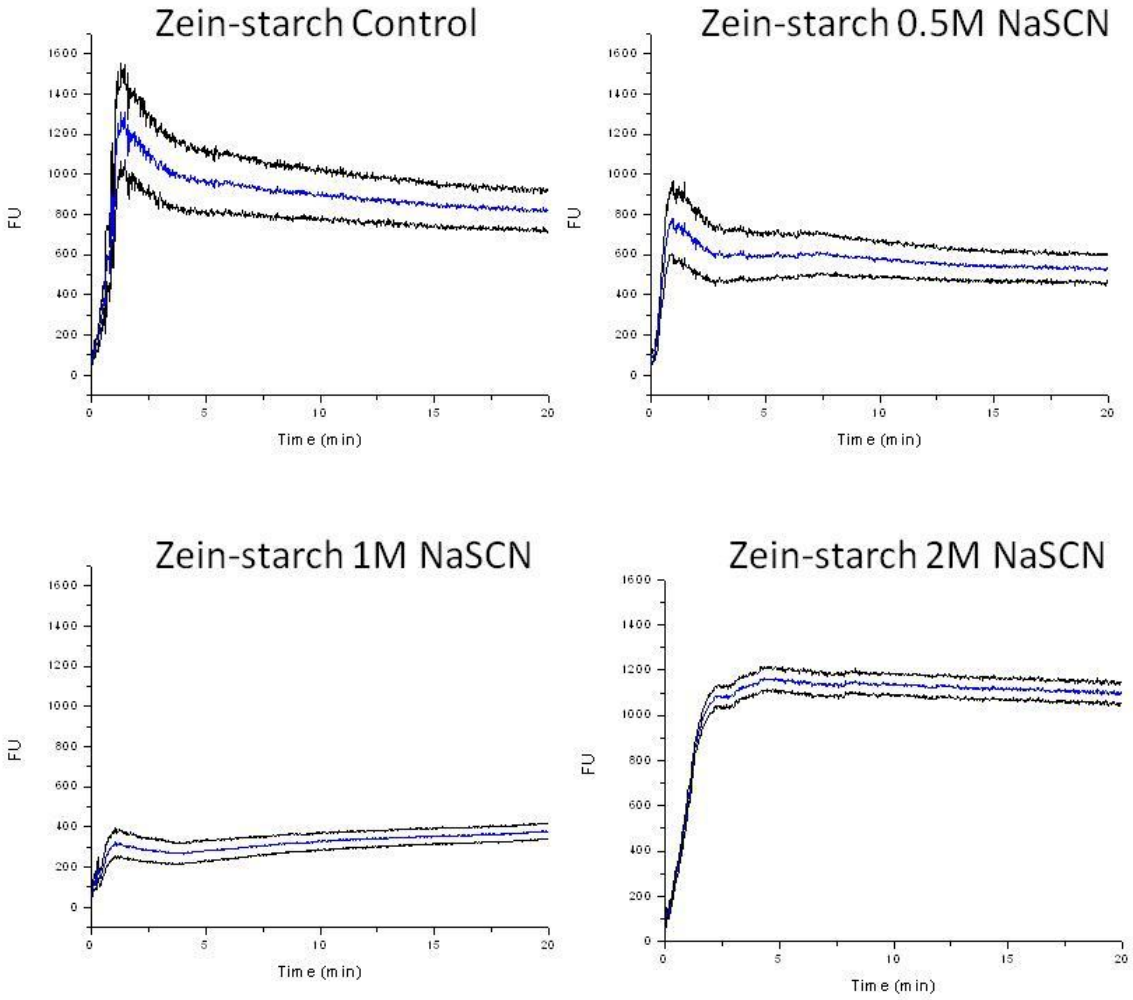

Figure 5: Farinograms of zein-starch mixtures from: Control mixed for 20 min with DI water (top left), 0.5M NaSCN treatment mixed for 20 min (top right), 1M NaSCN treatment mixed for 20 min (bottom left), and 2M NaSCN treatment mixed for 20 min (bottom right).

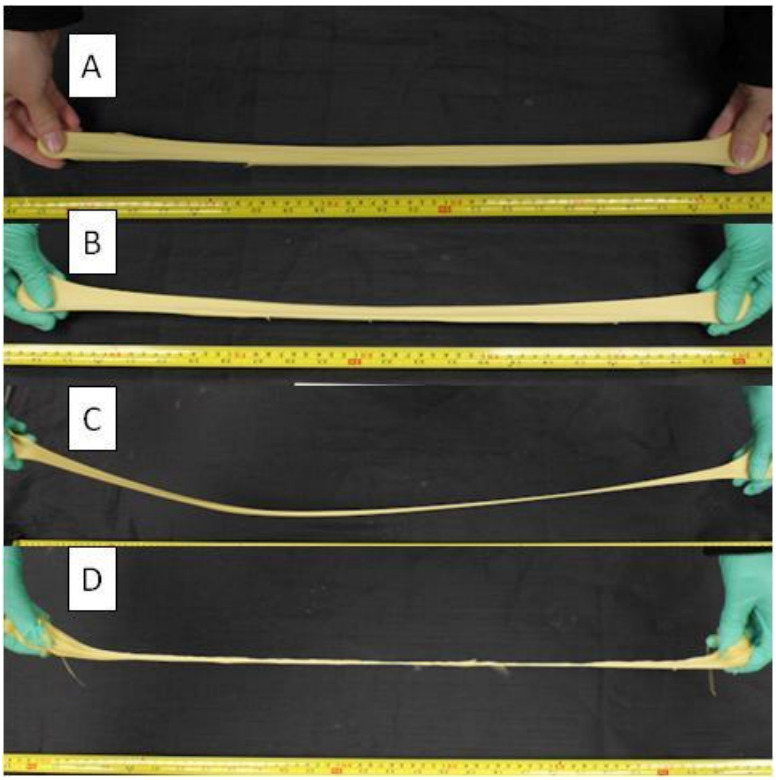

Figure 6: Visual representations of: control zein-starch dough mixed for 20 min with DI water (A), 0.5M NaSCN treatment mixed for 20 min (B), 1M NaSCN treatment mixed for 20 min (C), and 2M NaSCN treatment mixed for 20 min (D).

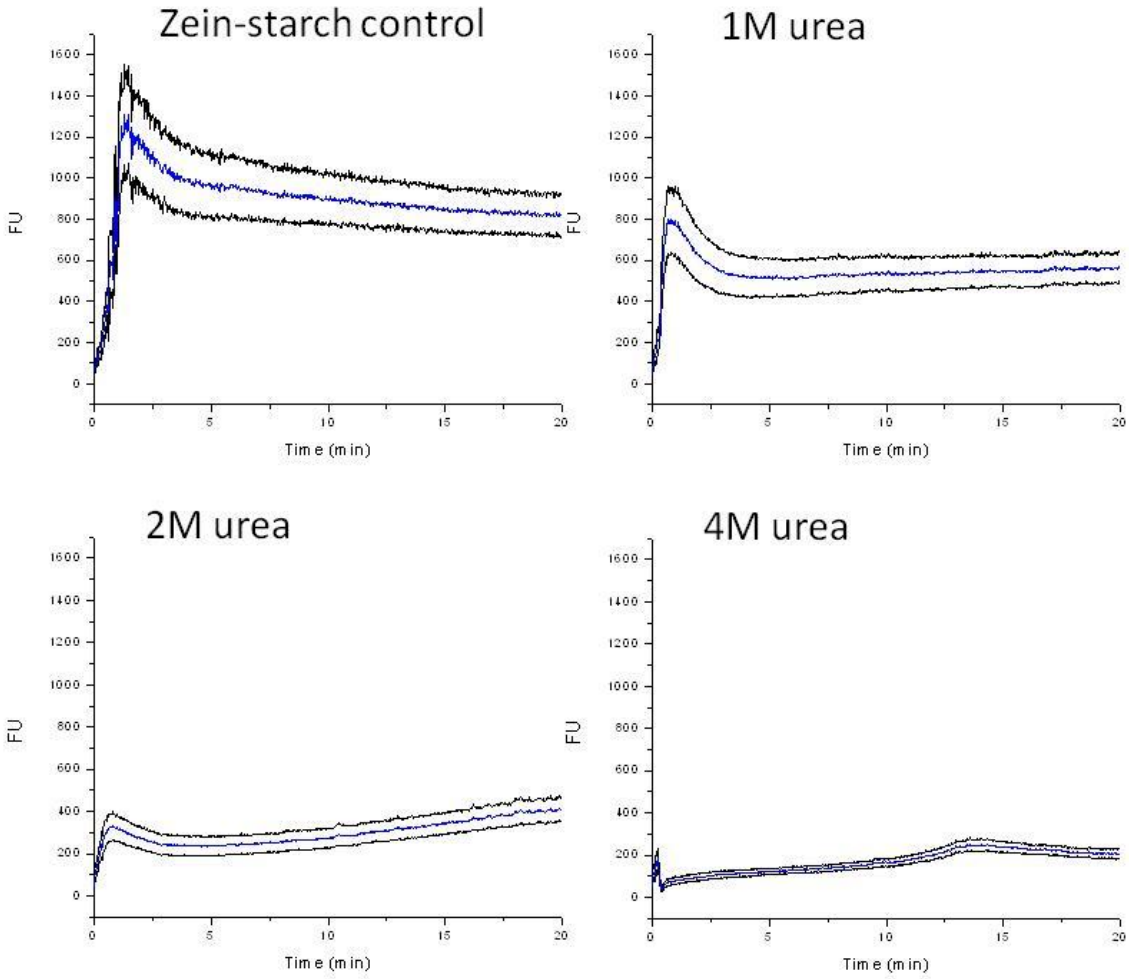

Figure 7: Farinograms of zein-starch mixtures from: Control mixed for 20 min with DI water (top left), 1M urea treatment mixed for 20 min (top right), 2M urea treatment mixed for 20 min (bottom left), and 4M urea treatment mixed for 20 min (bottom right).

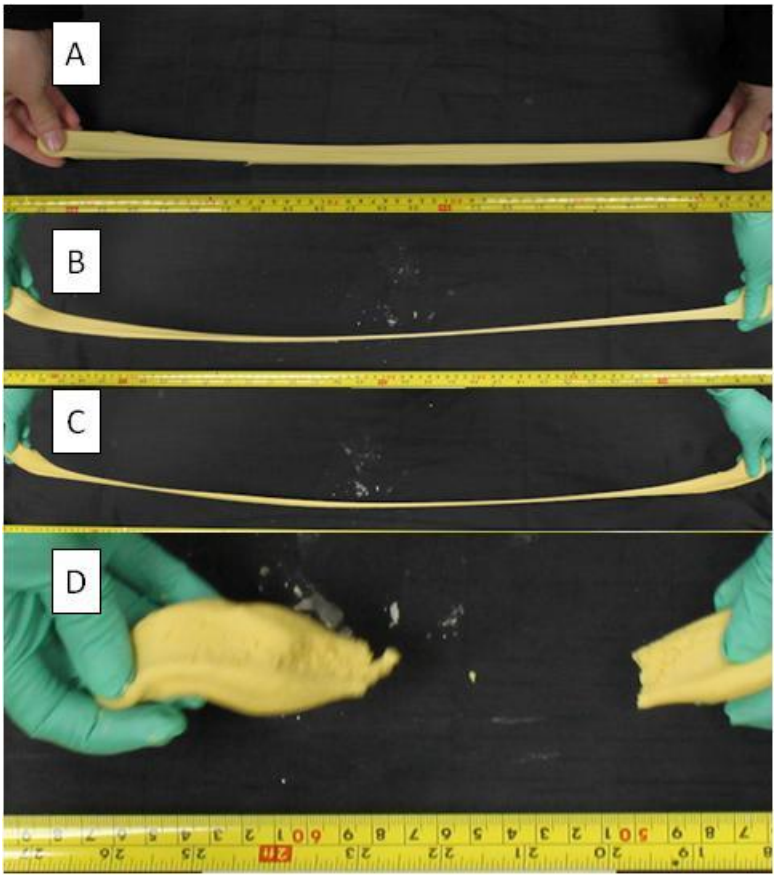

Figure 8: Visual representations of: control zein-starch dough mixed for 20 min with DI water (A), 1M urea treatment mixed for 20 min (B), 2M urea treatment mixed for 20 min (C), and 4M urea treatment mixed for 20 min (D).

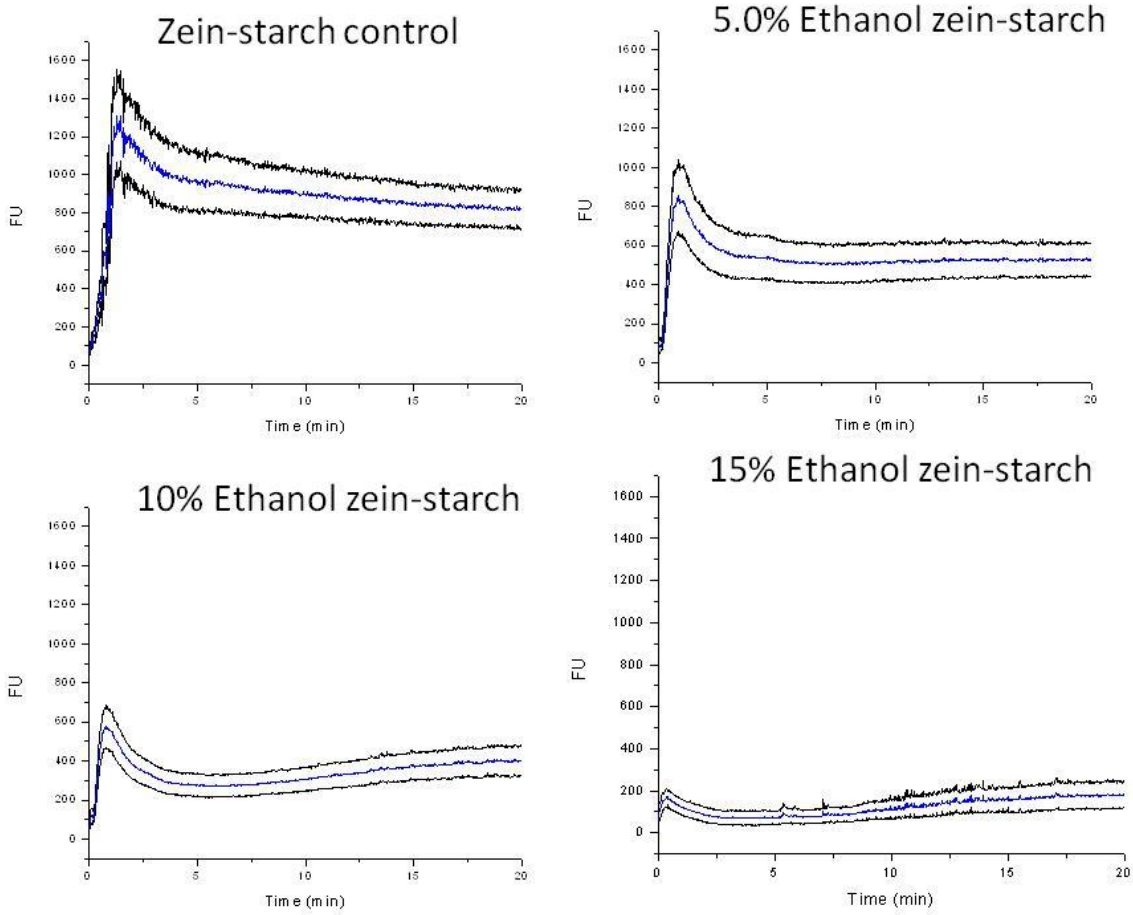

Figure 9: Farinograms of zein-starch mixtures from: Control mixed for 20 min with DI water (top left), 5% ethanol (v/v) treatment mixed for 20 min (top right), 10% ethanol (v/v) treatment mixed for 20 min (bottom left), and 15% ethanol (v/v) treatment mixed for 20 min (bottom right).

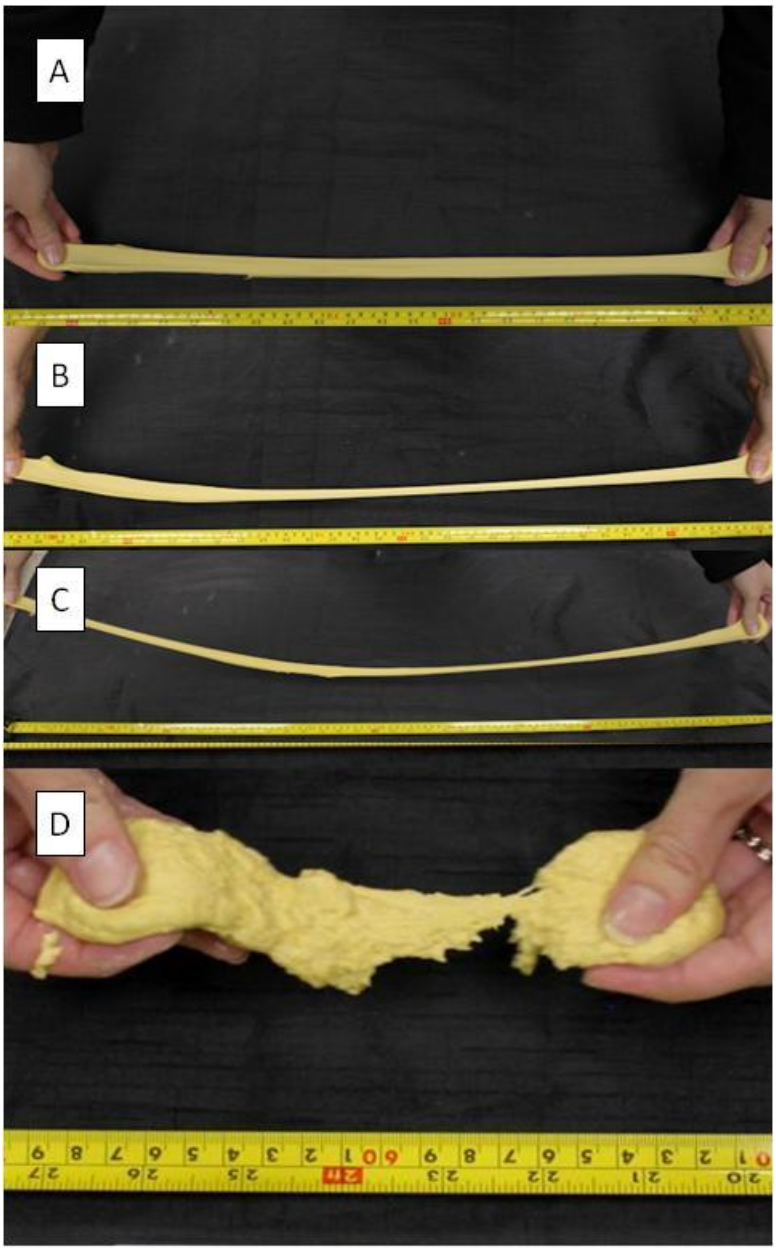

Figure 10: Visual representations of: control zein-starch dough mixed for 20 min with DI water (A), 5% ethanol (v/v) treatment mixed for 20 min (B), 10% ethanol (v/v) treatment mixed for 20 min (C), and 15% ethanol (v/v) treatment mixed for 20 min (D).

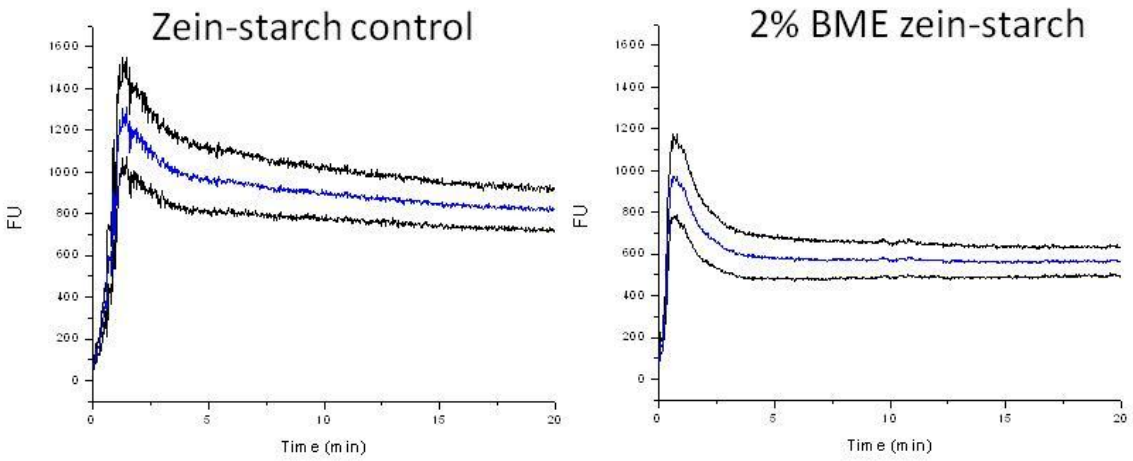

Figure 11: Farinograms of zein-starch control mixed for 20 min with DI water (left) and zein-starch mixed with 2% β-ME (v/v) (right) for 20 min.

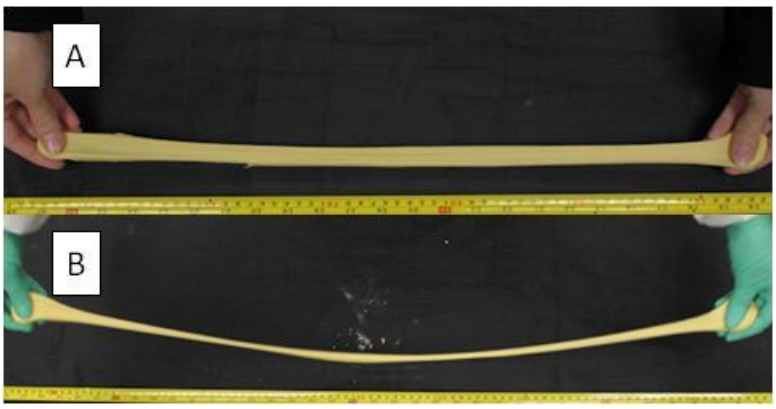

Figure 12: Visual representations of: control zein-starch dough mixed for 20 min with DI water (A), 2% β-ME (v/v) treatment mixed for 20 min (B).

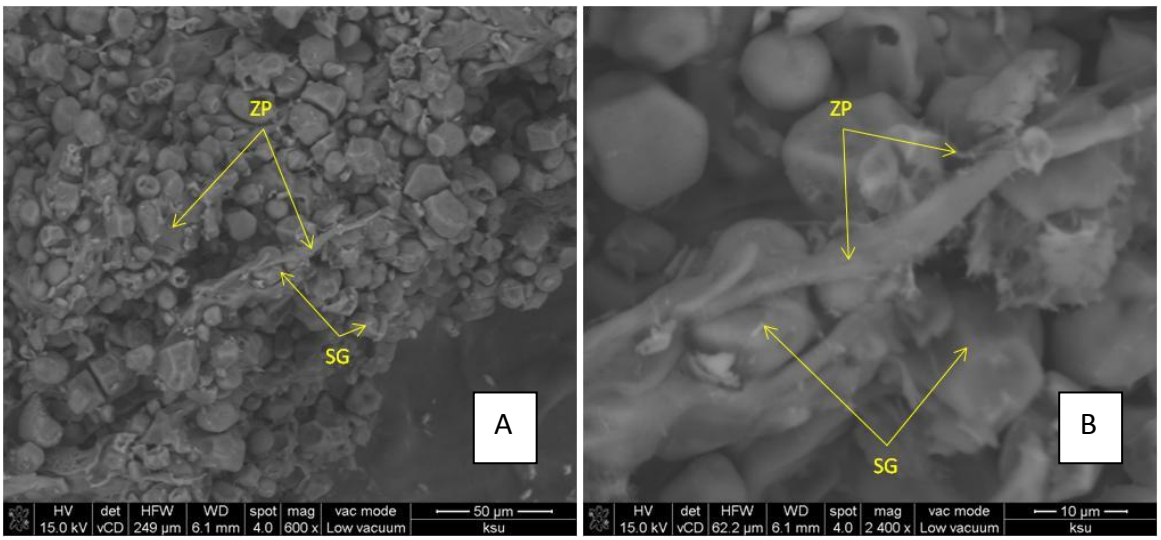

Figure 13: 600 X magnification SEM (A) and 2,400 X magnification SEM (B) of zein-starch flour mixed with DI water at 40°C for 20 min (control with mixing). ZP designates zein protein and SG designates starch granules.

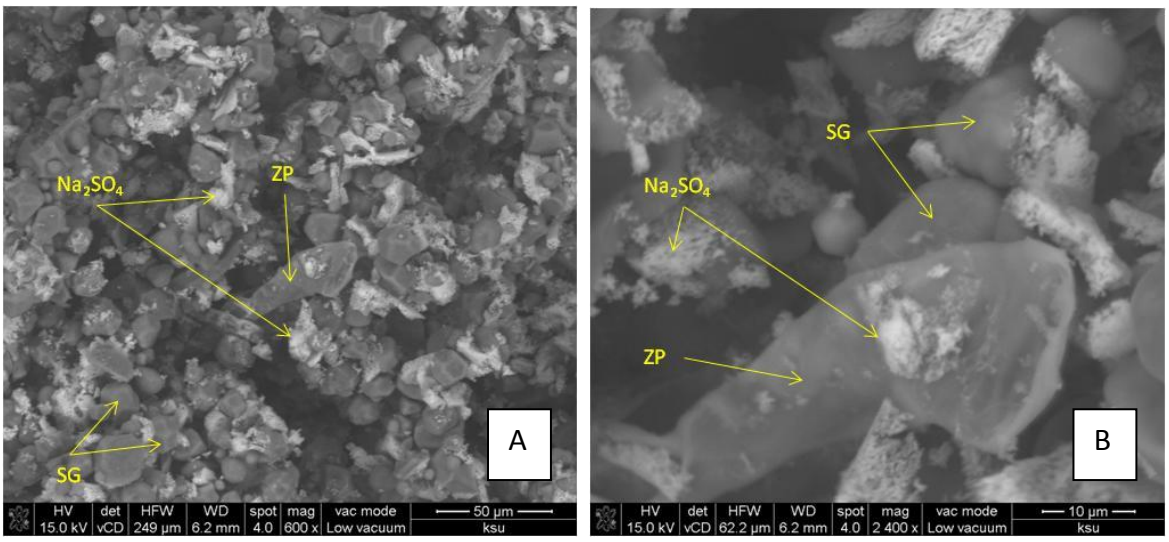

Figure 14: 600 X magnification SEM (A) and 2,400 X magnification SEM (B) of zein-starch flour mixed with a solution of 2M Na_2SO_4 at 40°C for 20 min (control with mixing). ZP designates zein protein, SG designates starch granules and Na_2SO_4 represents areas with large amounts of Na_2SO_4.

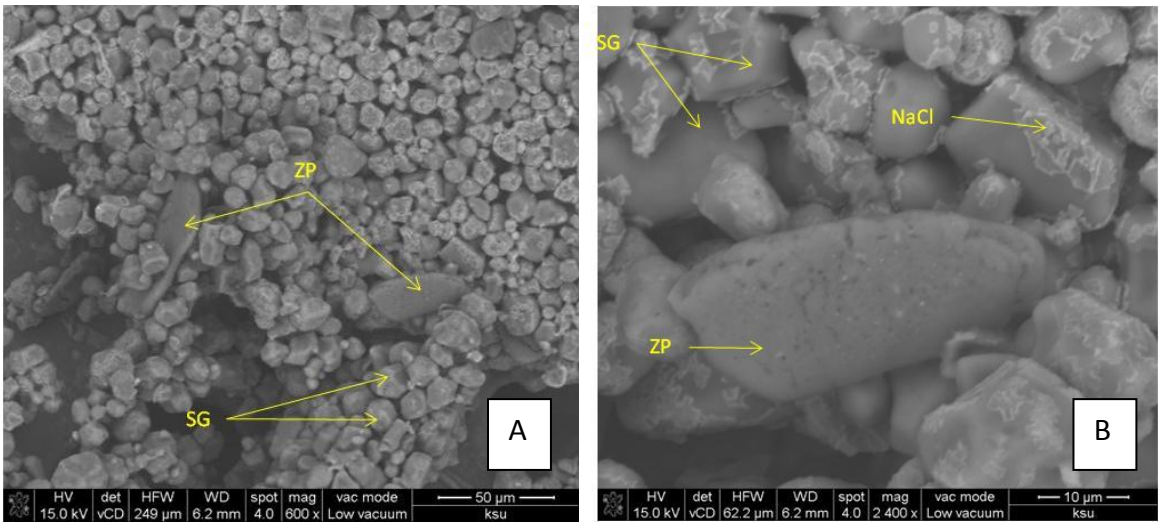

Figure 15: 600 X magnification SEM (A) and 2,400 X magnification SEM (B) of zein-starch flour mixed with a solution of 2M NaCl at 40°C for 20 min (control with mixing). ZP designates zein protein and SG designates starch granules.

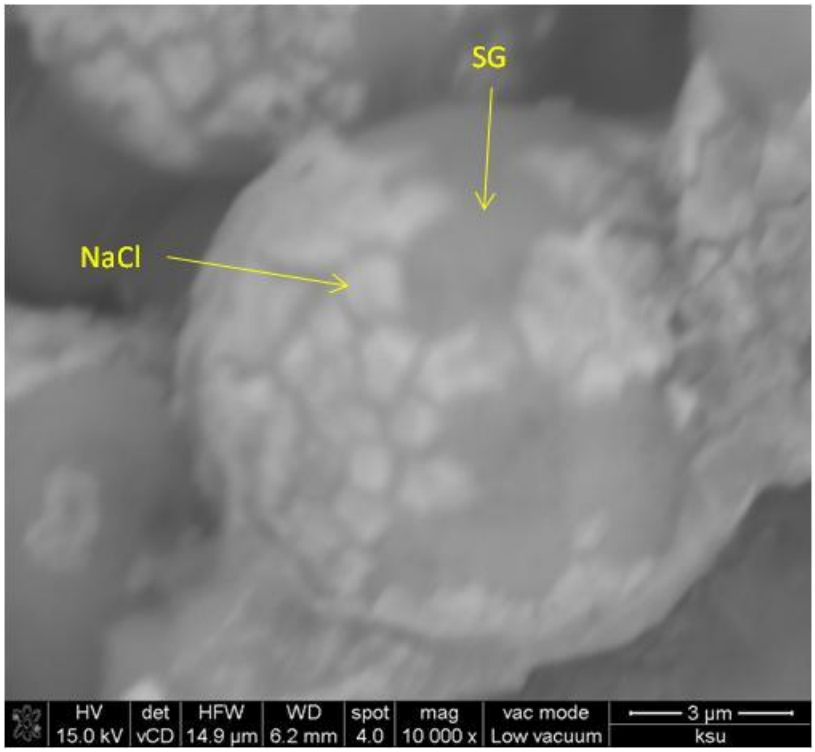

Figure 16: 10,000 X magnification SEM of zein-starch flour mixed with a solution of 2M NaCl at 40°C for 20 min (control with mixing). SG designates starch granules and NaCl represents areas with large amounts of NaCl.

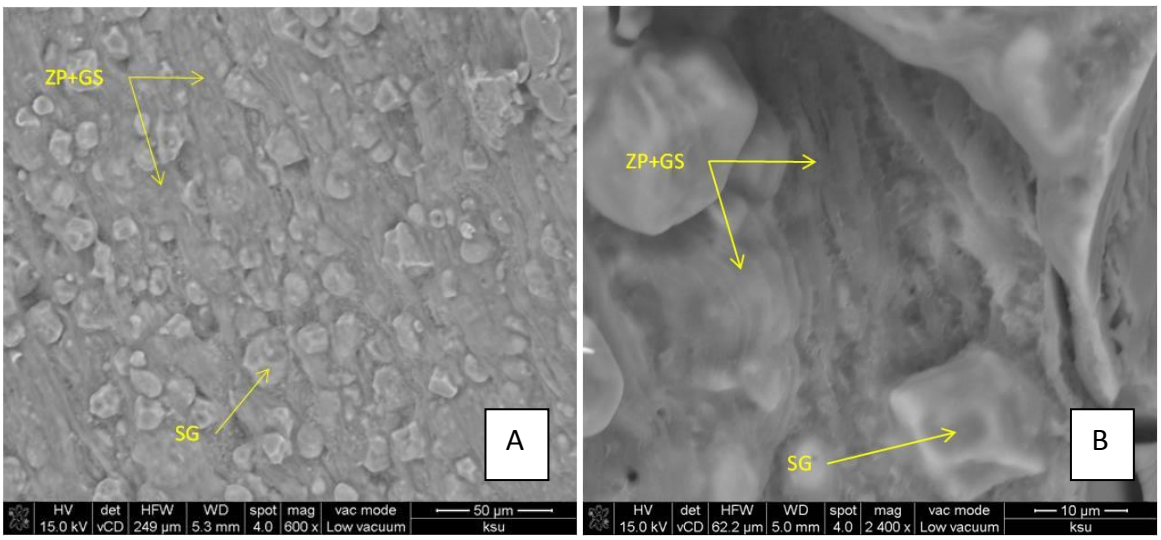

Figure 17: 600 X magnification SEM (A) and 2,400 X magnification SEM (B) of zein-starch flour mixed with a solution of 2 M NaSCN at 40°C for 20 min (control with mixing). ZP+GS designates a mixture of zein protein gelatinized starch and SG designates starch granules or starch remnants.

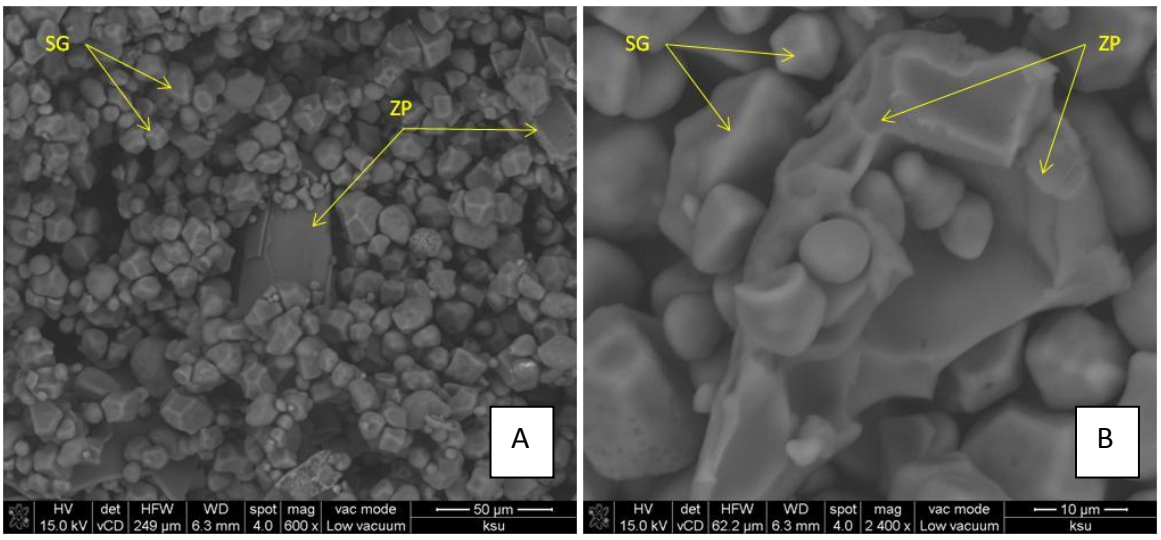

Figure 18: 600 X magnification SEM (A) and 2,400 X magnification SEM (B) of zein-starch flour mix (control without mixing). ZP designates zein protein and SG designates starch granules.

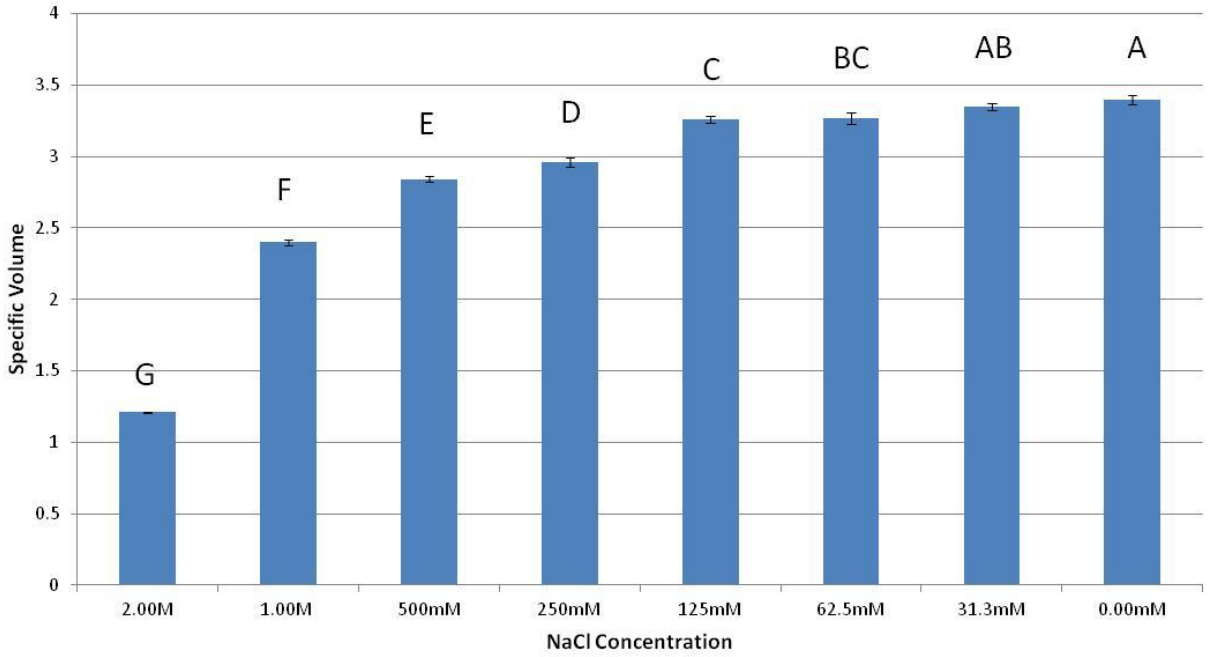

Figure 19: A graphical representation of specific volumes (y axis) for breads baked with NaCl treatments at concentrations of 0.000M, 31.3mM, 62.5mM, 125mM, 250mM, 500mM, 1.00M, and 2.00M (x axis). The upper case letters represent differences of means where similar letters identify no significant differences between the means ($P < 0.05$).

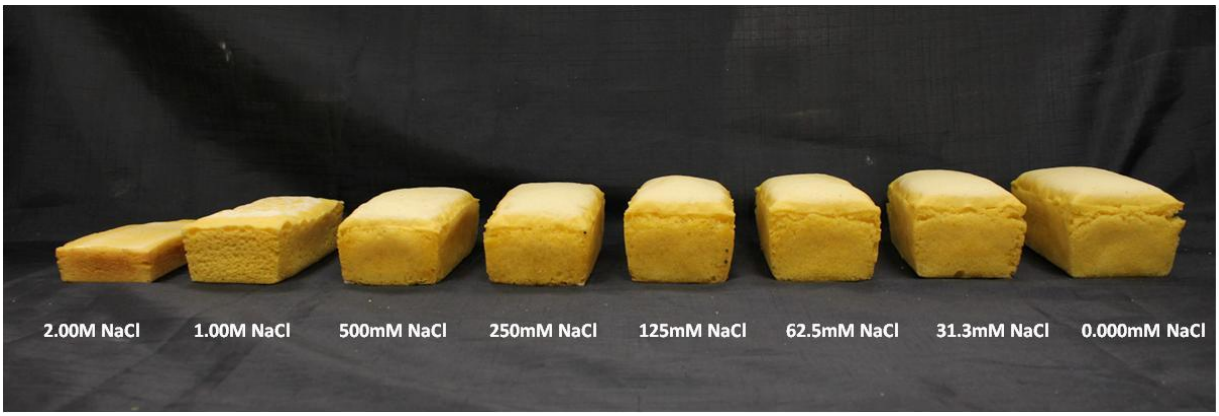

Figure 20: Comparison of bread loaves treated with varying NaCl concentrations. From left to right: 2.00M NaCl, 1.00M NaCl, 500mM NaCl, 250mM NaCl, 125mM NaCl, 62.5mM NaCl, 31.3mM NaCl, 0.000mM NaCl. Breads represent loaves made from 100 g of flour.

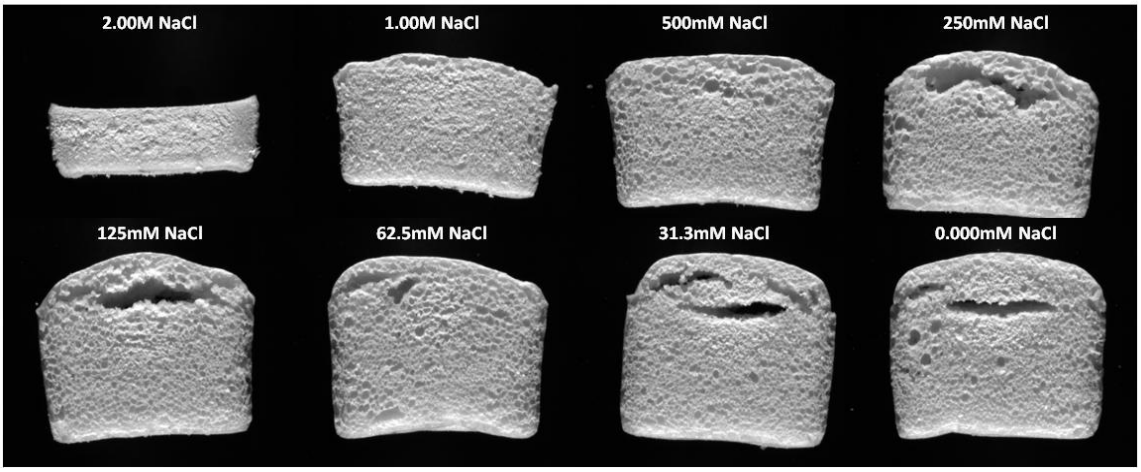

Figure 21: Comparison of slices of bread made with varying concentrations of NaCl. From left to right top: 2.00M NaCl, 1.00M NaCl, 500mM NaCl, and 250mM NaCl. From left to right bottom: 125mM NaCl, 62.5mM NaCl, 31.3mM NaCl, 0.000mM NaCl. Breads slices represent loaves made from 100 g of flour.

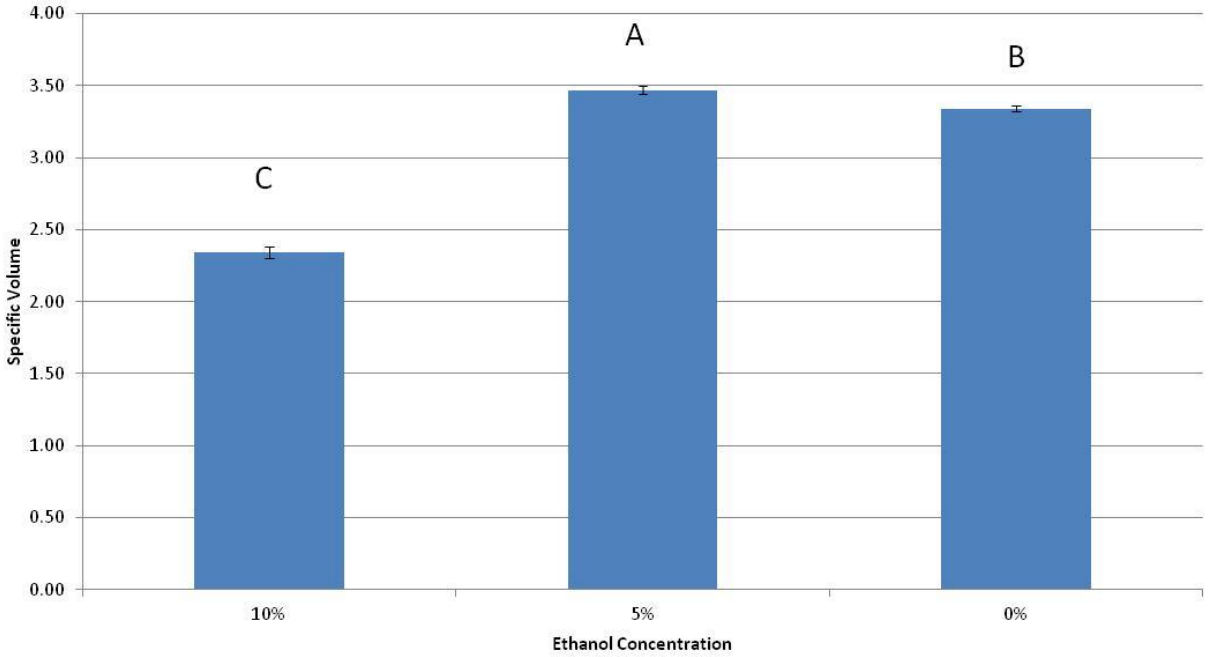

Figure 22: A graphical representation of specific volumes (y axis) for breads baked with ethanol treatments at concentrations of 0.0%, 5.0%, and 10% (x axis). The upper case letters represent differences of means where similar letters identify no significant differences between the means ($P < 0.05$).

Figure 23: Comparison of bread loaves treated with varying ethanol concentrations. From left to right: 10%ethanol, 5.0% ethanol, 0.0% ethanol. Breads represent loaves made from 100 g of flour.

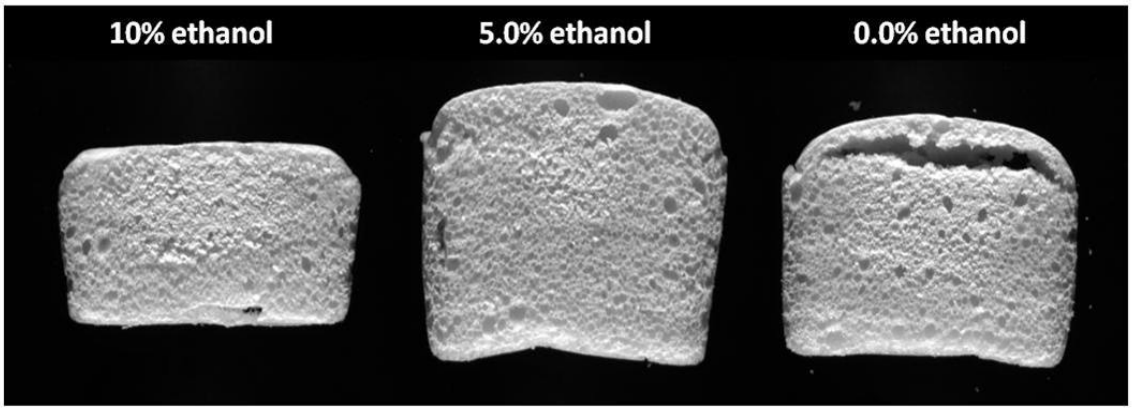

Figure 24: Comparison of slices of bread made with varying concentrations of ethanol. From left to right top: 10%ethanol, 5.0% ethanol, 0.0% ethanol. Breads slices represent loaves made from 100 g of flour.

Chapter 4: Effect of Proteolysis on Kafirin Functionality

Abstract

Kafirins and zeins have nearly identical chemical properties. However, to date kafirin has not been found to have the ability to form a visco-elastic resin. In publications where kafirin functionality was investigated, laboratory extracted kafirin was compared to commercially isolated zein. Commercial zein is subjected to many conditions during production that laboratory isolated kafirin is not. One reason for the difference in functionality between commercial zein isolates and laboratory extracted kafirin may lie in how commercial zein is produced. To begin to evaluate the effects of commercial zein extraction procedures on kafirin functionality, one of the first steps of commercial zein production, grain steeping, was investigated. To see what effect steeping had on kafirin functionality, sorghum flour was steeped for 72 and 96 hrs at 50 °C. Unsteeped sorghum flour was digested with Alcalase for 90 min at 50 °C. After steeping or digestion with Alcalase, kafirins were isolated from the remaining material. Both groups of kafirins had the ability to form a zein-like visco-elastic resin when mixed with warm water by hand. Furthermore, analysis by RP-HPLC showed that digestion of kafirin was occurring. This is the first time that kafirin has been reported to form a visco-elastic resin using only water as a plasticizer.

Introduction

Native zein proteins are nearly identical to those of kafirin from sorghum. The ratios of α, β, γ protein fractions of kafirin and zein are the primary differences between the two proteins. Kafirin contains ~ 65-80% α-kafirin, ~7-8% β-kafirin, and 9-12% γ-kafirin, and zein contains ~70% α-zein, ~5% β-zein, and 20% γ-zein (Esen 1987; Shull et al. 1991; Watterson et al. 1993; Hamaker et al. 1995; Lawton and Wilson 2003; Belton et al. 2006; Schober and Bean 2008). Although these similarities are known, there have been no successful attempts at producing a kafirin isolate that functions similarly to a commercially available zein isolate. (Oom et al. 2008) were able to get kafirin to form a zein like visco-elastic resin, but oleic acid had to be added as a plasticizer at a rate of 50% (w/w) to induce functionality. Schober et al. (2011) hypothesized that contamination of α-kafirins by cysteine rich β-kafirins prevented functionality by cross-linking the isolated proteins via disulfide bonds. However, Schober et al. (2011) were not able to get kafirins to form a resin in the presence of a reducing agent. This suggests that disulfide cross-linking by the β-kafirins may not have been the cause for kafirin's inability to form visco-elastic resins. However, kafirin isolates that contain a large amount of cysteine from β-kafirins may also have disulfide bonds buried where they are inaccessible to reducing agents. Schober et al. (2011) also reported that laboratory extracted zein appeared to have better functionality if the zein isolates contained pure α-zein. Such laboratory isolates were able to coalesce into aggregates but did not have the visco-elastic properties of commercially available zein isolates. Thus, having pure α-zein (or α-kafirin) may improve the functionality of corn or sorghum protein isolates, but simply having pure α-zein or α-kafirin will not result in functional protein isolates.

The commercial production of zein isolates includes many steps in the extraction, isolation, and drying process that could have an effect on protein structure and function (Johnson and May 2003). For this study, steeping, the first major step that could change protein structure and function in zein isolation was examined. Zein is extracted from corn gluten meal, a byproduct of corn starch isolation, of which steeping is the first step. Corn is typically steeped for ~48 hours prior to wet milling. Steeping allows for a purer starch to be isolated by loosening the proteins from the starch granules via reduction of disulfide bonds and digestion (Wall and Paulis 1978; Johnson and May 2003) both of which could impact protein functionality. However, as discussed above, and as shown in chapter 2 of this dissertation, simple reduction of disulfide bonds alone does not appear to play a role in functionality of zein. For that reason, it was the objective of this preliminary study to find out if steeping and subsequent proteolysis resulted in changes to the ability of kafirins to form a zein-like visco-elastic resin.

Materials and Methods

Sample Preparation

The sorghum hybrid used in this study was Fontenelle 1000 (F-1000) from Lane county, KS, from the 2008 growing season. Sorghum grain was milled with a Cyclone Sample Mill (UDY Corporation, Fort Collins, CO.) equipped with a 0.5 mm screen. The resulting flour was stored at room temperature until use.

Steeping

For steeping, 350 mL de-ionized (DI) water containing 1.05g of sodium metabisulfite (SMB) was added to 100 g milled F-1000 flour. Temperature during steeping was maintained at 50 °C for 0, 72, or 96 hrs. After steeping the flour water mixture was centrifuged at 2,200 *g* for

10 min and the supernatant discarded. The residue remaining after centrifugation was then re-dispersed in 350 mL of DI H_2O in a washing step. The mixture was centrifuged again under at 2,200 *g* for 10 min and the supernatant discarded. The remaining pellet was then air dried in a glass drying dish overnight. The dried pellet was then ground in an electric coffee grinder for subsequent extraction.

Alcalase Digestion

Digestion was carried out on 100 g of sorghum flour. Here, 2 ml of ≥ 0.8 U/g protease from *Bacillus licheniformis* (Alcalase) (Sigma St. Louis, MO) was brought to 350 mL with 50 °C DI H_2O and added to the 100 g of flour. This mixture was kept at 50 °C for 90 min and stirred every 15 min. After digestion the flour water mixture was centrifuged at 2,200 *g* for 10 min. The supernatant was discarded. The pellet was then re-dispersed in 350 mL of DI H_2O in a washing step. The mixture was centrifuged again under at 2,200 *g* for 10 min. Washing and centrifuging was repeated twice. The remaining pellet was then air dried in a glass drying dish overnight. The dried pellet was then ground in an electric coffee grinder for subsequent extraction.

Extraction

The steeped and dried flour was extracted using 350 mL of 70% aqueous ethanol (CHROMASOLV 95% ethanol+5% isopropanol, Sigma-Aldrich, Co., St. Louis, MO) containing 1.05 g SMB. The kafirins were extracted for 4 hrs at room temperature with continuous stirring. After the 4 hrs, the flour ethanol solution was centrifuged at 2,200 *g* for 10 min. The supernatant was carefully decanted and then air dried in a glass drying dish overnight. After drying, the protein was scraped from the glass dish and stored in plastic tubes for subsequent analysis.

RP-HPLC

Prior to analysis, isolated kafirins and zeins were dissolved as described by Bean et al. (2011). Briefly, 5 mg of kafirin or zein isolate was re-dissolved using 1 mL of 60% *t*-butanol (v/v) containing 0.5% sodium acetate (w/v) and 2% β-mercaptoethanol (β-ME) (v/v) for 15 min with continuous vortexing. For the control kafirin and zein extract, 100mg of sorghum or corn flour was extracted using the above solvent as described in Bean et al. (2011).

Proteins were analyzed via RP-HPLC as described in Bean et al. (2011) using an Agilent 1100 HPLC system equipped with a Poroshell 300 SB (2.1 × 75 mm) C18 column (Agilent, Palo Alto, CA) and guard column. Separations were made using a gradient as follows: 0 min, 20% acetonitrile + 0.07% trifluoracetic acid (TFA) (w/v) (solvent B); 5 min 40% solvent B; 15 min 60% solvent B. Solvent A was de-ionized water + 0.1% TFA (w/v)A flow rate of 0.7 mL/min and a column temperature of 50 °C was used for all separations. Sample detection was by UV at 214 nm and 10 µL of sample was injected for all samples.

Resin Forming Ability

To test the digested kafirin isolates' ability to form a zein like resin, kafirin isolates were moistened with warm DI water and kneaded between the index finger and thumb for 5 min. The ability of the isolates to form a resin was judged based on their ability to for a smooth homogeneous dough-like substance that was extensible when pulled apart by hand.

Results and Discussion

RP-HPLC

RP-HPLC analysis of the steeped samples revealed that during steeping some proteolysis was occurring. Kafirins extracted from flours steeped for 72 and 96 hours had major changes

when compared to a kafirins extracted from unsteeped flour (Figure 1). The peaks shape and size changes as steep time increased. There was also a shift in peak area to an earlier elution time as steep time increased (10-12 min). This is indicative of a decrease in protein hydrophobicity as a result of proteolysis. In general when proteins are cleaved into small peptides they become more soluble. The changes to kafirins that occurred during steeping shown in Fig 1 can be attributed to the partial digestion of kafirins by enzymes native to the sorghum or enzymes produced via bacterial growth during steeping. It should also be noted that initial tests showed that steeping intact grain did not result in heavily digested kafirins (data not shown). In the wet milling process, corn grain is usually steeped for ~48 hrs at elevated temperatures. Zein protein bodies have less γ and β fractions than kafirin (Esen 1987; Shull et al. 1991; Watterson et al. 1993; Hamaker et al. 1995; Lawton and Wilson 2003; Belton et al. 2006; Schober and Bean 2008). Because γ and β fractions are largely found on the outside of protein bodies in corn and sorghum, many studies have proposed that the increase in cysteine from the higher levels of γ and β fractions in kafirin increases the amount of cross-linking on the outer layers of the protein bodies. This higher level of cross-linking found with kafirin protein bodies when compared to zein protein bodies has been attributed to the lower levels of protein digestibility in sorghum than in corn (Hamaker et al. 1995; Oria et al. 1995; Duodo et al 2001, 2002, and 2003; Belton et al. 2006). For comparison, zein from corn flour and commercial zein isolates were also analyzed via RP-HPLC (Fig 2). The commercially isolated zein had a different chromatogram relative to the zeins extracted from corn flour, most notably in the early regions of the chromatogram (~4-9 min) which is known to be where the γ and β fractions elute (Bean et al 2000). The later region (~10-14 min) also showed signs of

degradation when compared to a control zein extracted fresh from corn flour, most notably in the loss of resolution (Fig 2). Commercial zein has also had been extracted under harsh conditions that may also have an effect on the structure of the proteins (Lawton 2002; Johnson and May 3003).

Since steeping appeared to result in the degradation of sorghum proteins, then it can be hypothesized that the use of proteases to digest the flour rather than steeping could result in kafirin isolates with similar functionality as those obtained from steeped flour. Based on previous studies, Alcalase was chosen for this project as it can digest proteins under neutral conditions and has been shown to work on cereal proteins (Treimo et al 2008). It was found that a digestion time of 90 min at 50 °C appeared to slightly affect the kafirins as seen by slight changes to the peak patterns in the 10-11 min range (Fig 3). This suggests that limited degradation of the kafirins occurred during the Alcalase digestion. Sorghum proteins are known to be resistant to digestion and future work aimed at optimizing digestion conditions and exploring additional proteases is warranted.

Resin Formation

Steeping resulted in kafirins capable of forming a resin. Kafirins extracted from the flour steeped for 72 hrs produced a smoother, lighter colored resin than the kafirins extracted from flour steeped for 96 hr (Fig 4). The 96 hour treatment produced a resin that had a yellow color and had dark colored particles dispersed throughout (Fig 4). Although digestion conditions were not optimized, 90 min of Alcalase digestion did result in kafirin being able to form a resin when hand kneaded in warm water (Fig 4). However, this resin was not very extensible when compared to the steeped treatments. This may be due to the very limited proteolysis seen in

figure 3. This demonstrates that one aspect of producing functional kafirin may be from a partial proteolysis. This may also explain why laboratory extracted zein does not produce resins like the commercially available zein does (Schober et al. 2011).

Another aspect of kafirin functionality that needs to be explored is the removal of β-kafirins from the α-kafirin fraction. It was hypothesized by Schober et al. (2011) that α-kafirins were more difficult to isolate in a pure form than α-zein due to similarities in hydrophobicity between α and β-kafirins. In RP-HPLC separations, α and β zein differed substantially in their elution times, suggesting differences in surface hydrophobicity at minimum (Bean et al 2000). It is possible then, that it is easier to isolate α-zein from β-zein by controlling the polarity of the extraction solvent. In contrast, α and β-kafirins elute close together in RP-HPLC (Bean et al 2000) suggesting that in sorghum these two kafirin types are more similar in surface hydrophobicity making it more difficult to extract α-kafirins not contaminated with β-kafirins (Schober et al. 2011). However, if possible, removal of β-kafirins from kafirin isolates may improve kafirin functionality, not due to increases in disulfide bonding as postulated by Schober et al. (2011) but rather due to changes in non-covalent interactions between proteins. The proteolysis that occurs during steeping may also change the nature of non-covalent interactions in isolated zein and kafirin.

The research presented in this chapter was of necessity limited in scope and only preliminary in nature. Future work needs to address more quantitative methods to evaluate the functionality of small amounts of isolated proteins (using the farinograph would require at minimum 30g of isolated protein which is not practical at the lab scale). Testing protein functionality by simply stretching the isolated proteins by hand gives good indications of

functionality and can help narrow down experimental conditions, but is not quantitative. In the future the use of the farinograph equipped with a 10g mixing bowl would allow for assessment of development curves that might provide more information.

Conclusions

Partial proteolysis of kafirin appeared to play a role in the production of visco-elastic resins. RP-HPLC showed that a steeped or Alcalase treated kafirins did undergo a partial proteolysis. These digested proteins were then able to form a visco-elastic resin similar to that of a commercially available zein isolate. This is the first time that kafirin has been reported to form a visco-elastic resin using only water as a plasticizer.

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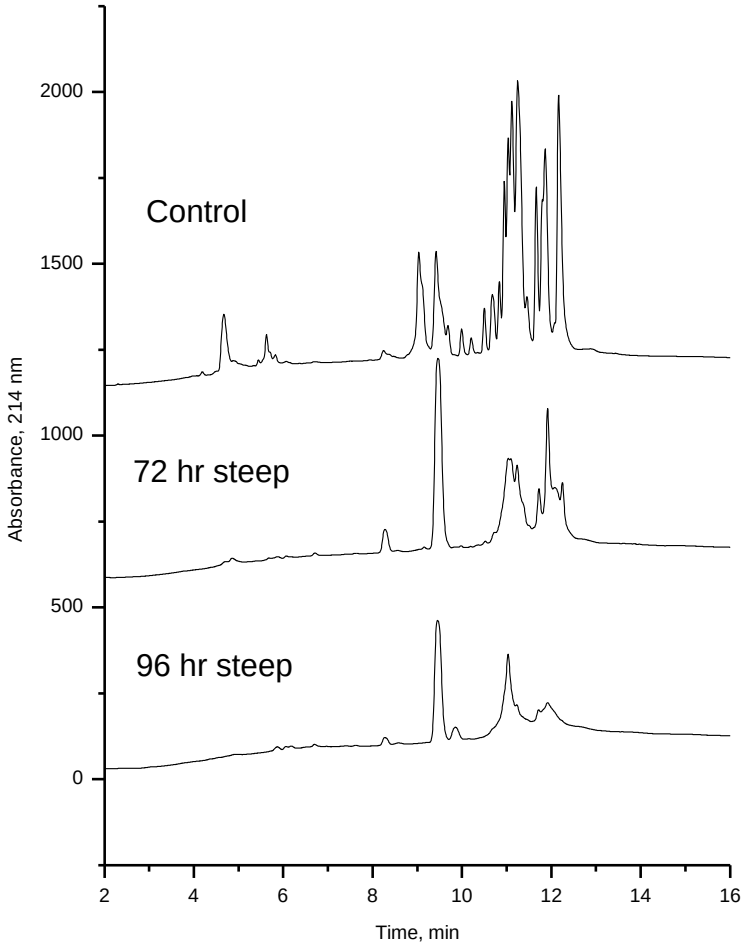

Figure 1: RP-HPLC chromatograms of kafirins extracted from untreated sorghum flour (top), sorghum flour steeped for 72 hrs (middle), and sorghum flour steeped for 96 hrs (bottom).

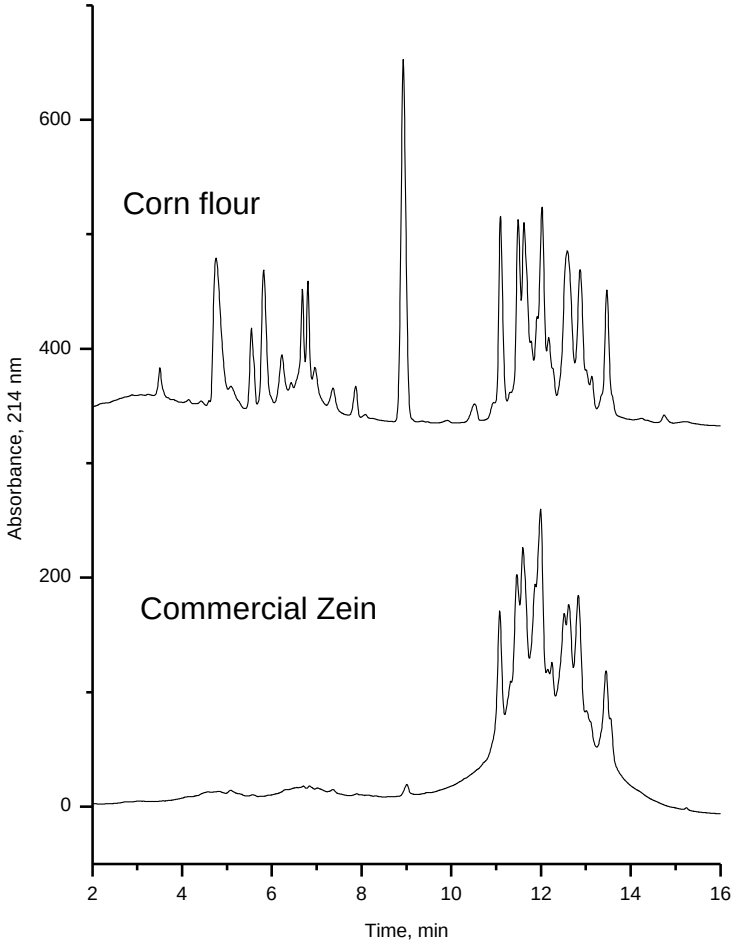

Figure 2: RP-HPLC chromatograms of zeins extracted from untreated maize flour (top) and commercially available zein isolate (bottom).

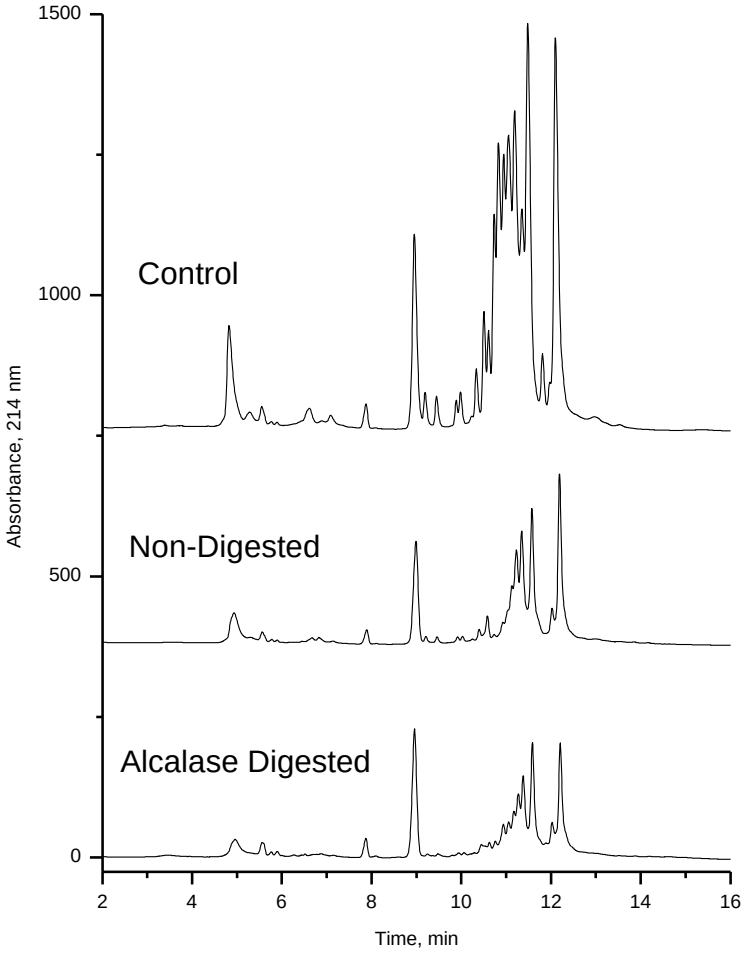

Figure 3: RP-HPLC chromatograms of kafirins extracted from untreated sorghum flour (top), kafirins extracted from non-digested sorghum flour following the same procedure as the digested sorghum flour (middle), and kafirins extracted from sorghum flour digested with Alcalase (bottom).

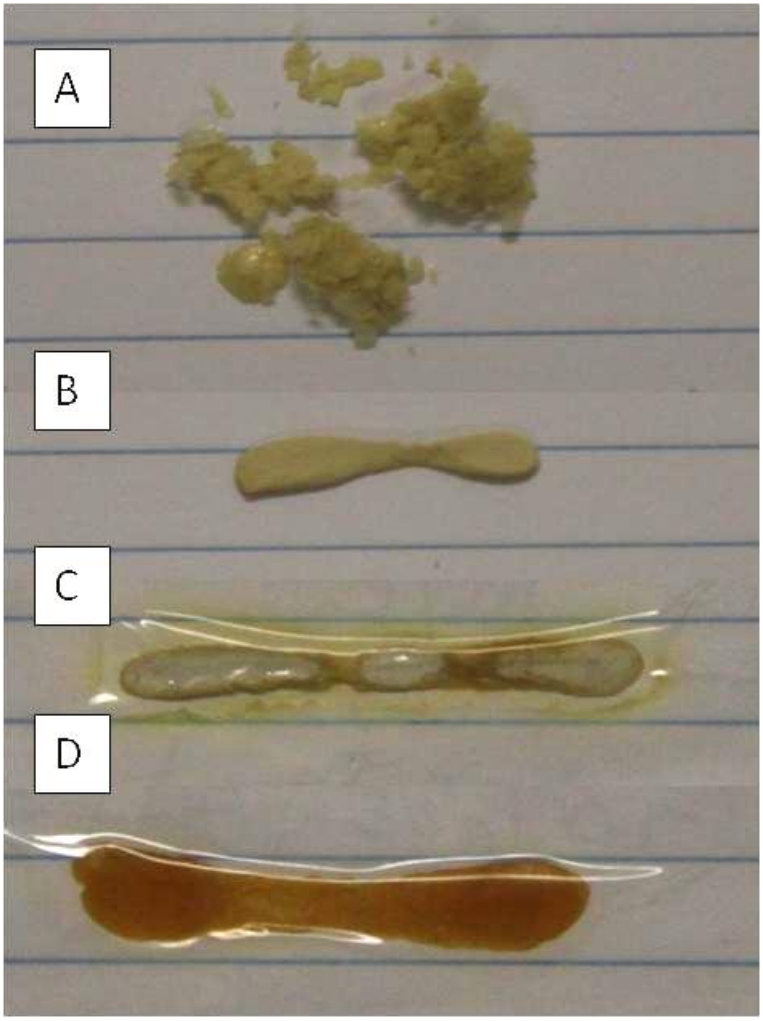

Figure 4: Visual representation of kafirins extracted from the various treatments and hand kneaded in the presence of warm water. From top to bottom: Kafirin extracted without treatment (A), kafirin digested with Alcalase (B), kafirin extracted from flour steeped for 72 hrs (C), and Kafirins extracted from flour steeped for 96 hrs (D).

Chapter 5: Effect of HPMC on the Quality of Wheat-Free Bread Made from Carob Germ Flour-Starch Mixtures[1]

[1]This chapter has been accepted for publication in the Journal of Food Science

Smith B. M., Bean S. R., Herald T. J., Aramouni F. M. 2012. Effect of HPMC on the Quality of Wheat-Free Bread Made from Carob Germ Flour-Starch Mixtures. J. Food Sci. (in Press)

Abstract

Carob germ proteins have been shown to have functional properties similar to wheat gluten enabling formulation and production of yeast leavened gluten-free baked goods from a true dough rather than a stiff batter. The purpose of this research was to optimize the production of wheat-free bread containing carob germ flour, corn starch, NaCl, sucrose, hydroxypropyl methylcellulose (HPMC), and H_2O. A key criterion was to formulate viscoelastic dough similar to wheat dough. To that end, response surface methodology (RSM) was used to determine optimal levels of carob germ flour, H_2O, and HPMC. Components varied as follows: 4.94 – 15.05% for carob germ flour, 0.05 – 3.75% HPMC, and 65.25 – 83.75% H_2O (percents are on a flour basis, where carob germ flour in combination with maize starch equal 100%). Sucrose, NaCl, and yeast were held constant at 2%. Bread parameters evaluated were specific volume and crumb hardness, where the largest specific volume and the lowest value for crumb hardness were considered most desirable. The optimum formula as determined by RSM consisted of 7% carob germ flour, 93% maize starch, 2% HPMC, and 80% H_2O with predicted crumb hardness of ~200 g of force and a specific volume of ~3.5 cm^3/g. When proof time was optimized, a specific volume of ~5.6 ml/g and crumb hardness value of ~156 g of force was observed. Carob germ flour may be used as an alternative to wheat flour in formulating viscoelastic dough and high quality gluten-free bread.

Introduction

Wheat-free foods developed for people with wheat intolerances are typically of poor quality when compared to their wheat based counterparts. One reason for this may be the lack of a protein network, which gluten provides for wheat products (Schober et al. 2008). In wheat products, gluten provides elasticity and extensibility. Elasticity and extensibility are the unique functional attributes that allow for dough formation and gas retention in wheat products. Gluten has also been attributed to soft crumb structures and prolonged freshness of wheat based foods (Cornish et al. 2006). The lack of protein networks may be one contributing factor to the lack of quality and rapid staling associated with gluten-free foods (Arendt et al. 2008; Schober et al. 2008).

The formation of dough via gluten networks allows for easier handleability and processing of baked goods. Gluten-free bread products are typically produced from batters resembling that of a traditional cake batter (McCarthy et al. 2005; Schober et al. 2007, 2008) which limits the types of baked products that can be produced from a batter system. Batter based baked goods can only take the shape of the pans they are baked in, whereas dough on the other can be molded into different shapes resulting in numerous different types of products.

Both carob germ flour and zein have been shown to form viscoelastic dough with properties similar to that of wheat. Zein, however has a glass transition temperature above room temperature which makes bread production somewhat problematic (Mejia et al. 2007; Oom et al. 2008; Schober et al. 2008, 2010, 2011). Carob germ flour is able to form viscoelastic dough when mixed with starch and water at room temperature. Caroubin, the gluten-like

proteins found in carob germ flour, were first identified to have gluten-like properties by Bienenstock et al. (1935). While caroubin is not the same as wheat gluten, caroubin does function in a similar fashion. Caroubin has been reported to form wheat-like dough due to disulfide bonded high molecular weight proteins (Bengoechea et al. 2008; Smith et al. 2010). Mixtures of 30% carob germ flour and isolated starches have been used to produce wheat-free breads (Bienstock et al. 1935; Smith 2009). Wheat-free breads containing carob germ flour had reduced effects of staling when compared to other gluten-free breads. However, breads made from 30% carob germ flour/70% maize starch mixtures was dense (specific volumes of ~2.5 mL/g) and had high crumb hardness values. This was probably due to the fact that the amount of carob germ flour required to hold gas produced tough dough resulting in lower quality bread. For this reason it was hypothesized that reducing the amount of carob germ flour while adding a hydrocolloid (HPMC) would soften the dough while allowing greater gas retention. This in turn should result in greater bread quality in terms of specific volume and crumb hardness.

Thus the objectives of this study were to optimize a formula of gluten-free bread containing carob germ flour and HPMC. To accomplish this objective, response surface methodology (RSM) was used to determine which levels of carob germ flour, HPMC, and water produced the highest quality bread. The optimum gluten-free bread formulation was then proofed for varying times to determine the optimum formulation's greatest potential quality.

Materials and Methods

Baking Ingredients

Carob germ flour was generously donated by Danisco USA, inc. (New Century, KS). Unmodified native corn starch was purchased from Bob's Red Mill (Milwaukie, OR). Methocell

K4M food grade modified cellulose was kindly donated by The Dow Chemical Company (Midland, MI). Sucrose was a white food grade table sugar purchased from the local grocery store (C&H Sugar Company, Crockett, CA). NaCl was a white non-iodized table salt (Morton International, inc. Chicago, IL). Instant dry yeast was obtained from Fleischmann's Yeast (AB Mauri Food, inc., Chesterfield, MO).

RSM Design

Response surface methodology was used as previously described (McCarthy et al. 2005; Schober et al. 2005), with modifications to determine the effects of HPMC on carob germ flour in a gluten-free bread system. Ingredient factors carob germ flour, HPMC, and water were used to optimize the gluten free bread formula. A central composite design was employed using the following ingredient levels; carob germ flour levels were 4.94% - 15.05% (starch + carob germ flour = 100 % flour), water levels were 65.25% - 83.75% (flour basis) and HPMC levels were 0.05%-3.75% (flour basis). This design consisted of five levels of each factor and a total of 20 different combinations (Table I). Carob germ flour, water and HPMC levels were determined by extensive preliminary research. A key criterion for determining the maximum and minimum values of the factors was the dough's viscoelastic properties. Viscoelastic dough was defined by the ability of the dough to be sheeted using a commercial sheeter (National MFG. Co., Lincoln, NE) with rollers gapped at ¼ of an inch. The dough was required to produce free standing bread without the aid of bake pans, and be easily worked by hands (moldable without sticking to hands or other surfaces). Model selection and optimization were performed as described by Schober et al. (2005), using carob germ flour, HPMC, and water levels as the factors. Other ingredients in the bread formulation, i.e. salt, sucrose, and yeast were held constant at 2%

(flour basis). Statistical analysis of the RSM was completed with Design Expert 6.0.01 (Stat-Ease Corporation, Minneapolis, MN). Model quality is shown in Table II.

Carob Germ Flour Bread production

Gluten-free bread was produced from 150 g of flour (starch + carob germ flour) by the formula described above. Dry ingredients were blended to homogeneity except for the yeast. Yeast was prehydrated for 2 min prior to dough mixing in the amount of 32°C water needed for bread production. After yeast hydration, the water-yeast mixture was added to the dry ingredients and mixed for 30 sec with a 300 W Kitchen Aid Mixer (Ultra Power, St Joseph, MI). The mixer was equipped with a flat beater attachment and dough was mixed on the lowest speed for 30 sec. Dough was scraped down from the sides of the mixing bowl after the initial mixing. An additional 2 min of mixing was completed at a mixing speed of four. Dough was then removed from the mixing bowl and hand kneaded for 1 min to remove large pockets of air. A portion of dough was removed equal to 100 g of flour, rounded, and placed in an oiled bake pan. Bake pans were of the same dimensions described by AACCI method 10-10.03 for 100g loaves (Optimized Straight-Dough Bread-Making Method) (AACCI 2000). Pans containing dough were proofed at 32°C for 45 min at a relative humidity of 85%. A standardized proof time was chosen over proofing to height due to the extremes in formulations dictated by the RSM design (Table I). A time of 45 min was determined to be optimum during preliminary research. After proofing, dough was baked at 210°C for 20 min in a deck oven (1T2, Doyon, Linière, Qc, Canada). Breads were allowed to cool for 2 hr prior to analysis.

Wheat Bread Production

For comparison to the carob germ flour based bread, wheat bread (100g loaf) was prepared using the optimized straight dough baking method 10-10.03 (AACCI 2000).

Bread analysis

Bread volumes were measured by rape seed displacement. Specific volume was obtained 2 hr post baking by the equation:

Specific Volume = loaf volume/loaf weight

Crumb hardness was determined by texture profile analysis (TPA) as described by Schober and others (2007). TPA was performed with a TA.XT plus (Stable Micro Systems Ltd., Godalming, Surrey, United Kingdom). A 25 mm diameter cylindrical plastic probe attached to a 30 kg load cell was used. A pre-test, test, and post-test speed of 2.0 mm/sec was used with a trigger force of 5.0 g to compress the center of the crumb a distance of 40% of the slice thickness (2.5 cm). Rest time between cycles was 5.0 sec. Slices were analyzed 2 hr post baking. Lower hardness values are considered more desirable. Bread was sliced to a uniform thickness of 2.5 cm for analysis.

Optimization of Proof Time

One of the major limitations of the RSM optimization of carob germ flour based gluten-free bread system was the proof time of 45 min for all variable combinations. This was necessary to accommodate for extremes in variable combinations. Therefore, the optimum formulation was produced again under the same conditions covered previously, but with the use of multiple proof times to observe the optimum formulations maximum bread quality potential. Here, proof times of 45, 50, 55, 60, 65, 70, 75, and 80 min were used. Breads were

analyzed for specific volume and hardness as mentioned previously. A visual inspection of the crumb was conducted on the breads, Crumb failure in the form of voids in the bread was considered unacceptable. The proof times were chosen so that bread would be taken beyond the point of crumb failure. An optimum proof time was selected based on the criterion of the lowest hardness value, highest specific volume, and the absence of crumb failure. Three breads corresponding to each proof time were baked. Analysis of variance was completed with a $P < 0.05$, using Statistical Analysis Software (SAS 9.1, SAS Institute Inc. Cary, NC). A comparison of means using Tukey's studentized range test was used to determine differences in quality.

Results and Discussion

RSM/Bread Optimization

One indication of bread quality is specific volume. High specific volumes are often associated with a softer crumb and higher overall quality (McCarthy et al. 2005). One of the issues associated with gluten-free breads is depreciated specific volumes when compared to similar sized wheat breads. A loaf of wheat bread made by the AACCI method 10-10.03 (Optimized Straight-Dough Bread-Making Method) (AACCI 2000) will generally have specific volumes ranging from ~5.5-7.0 ml/g depending on the source of wheat. Gluten-free breads typically have a specific volume ranging between ~1.5-4.0 ml/g (McCarthy et al. 2005; Lazaridou et al. 2007; Schober et al. 2005, 2007; Schober 2009; Smith 2009). Loaf specific volume clearly increased by increasing water level and decreasing carob germ flour levels (Fig. 1). Both factors had a significant ($P < 0.001$) linear effect on specific loaf volume (Fig 1). The effects of HPMC were less clear and were dependent on the amount of carob germ flour and water present in the formulation.

Another indication of bread quality is crumb hardness, where lower hardness values correspond to higher quality bread (McCarthy 2005). For this research the bread quality in terms of crumb hardness followed the same trends as specific volume analysis. Here, hardness values decreased by increasing water level and decreasing carob germ flour levels (Fig. 2). Both factors had a significant ($P < 0.001$) quadratic effect.

The predicted optimum values for carob germ flour, water, and HPMC were 7%, 80%, and 3% respectfully. This formulation was predicted to result in bread with a specific volume of 3.46 mL/g and a hardness of 216.53 g. To confirm these numbers the predicted optimum formulation was baked three times resulting in bread with specific volumes of 3.56 ± 0.12 mL/g and hardness values of 200.28 ± 50 g which confirmed the predicted optimum formulation. The results of the RSM fit well with the initial hypothesis stated that addition of the hydrocolloid HPMC and the reduction in the amount of carob germ flour would result in higher quality bread than that described by Smith (2009). The carob dough produced from the optimum formulation was easily sheeted, able to produce a free standing roll, and was easily worked by hand (Fig. 3). During mixing, dough at this formulation resembled a thick paste that did not easily stick to hands or bench tops. Upon kneading by hand, the dough resembled something similar to wheat dough.

Optimization of Proof Time

The optimization of proof time showed that bread quality could be improved by increasing the proof time of the optimum formulation determined by RSM. As proof time increased, specific loaf volume increased (Fig. 4) and crumb hardness values decreased (Fig. 5). The optimum time was determined by the presence or absence of crumb failure, evident by

separation of crust and crumb, or large holes in the crumb. A proof time of 70 min the gluten-free breads had no signs of crumb failure or separation of crust and crumb. At 75 min there was a slight separation of crust and crumb and at a proof time of 80 min there were large holes present (data not shown). A proof time of 70 min under the constraints of this research produced bread with a specific volume of 5.62 ±0.10 ml/g and a crumb harness value of 156.25 ± 19.02 g. These values far exceed that of most l gluten-free breads (McCarthy et al. 2005; Lazaridou et al. 2007; Schober et al. 2005, 2007; Schober 2009; Smith 2009). In fact the specific volume of ~5.6 ml/g and hardness of ~156 from the optimized carob germ flour bread were very close to what is typically seen in similar sized wheat breads (Smith 2009). Furthermore, the crumb structure and slice dimensions are very similar to that of a wheat bread baked from a similar sized loaf of wheat flour (Figure 6).

Conclusions

High quality gluten-free bread was produced using carob germ flour and HPMC. To the best of our knowledge this is the first time that values of crumb hardness and specific volume of gluten-free bread have approached quality attributes similar to that of wheat bread. This gluten-free bread formulation is unique in that a true dough was formed. The ability to make gluten-free breads from true dough will provide the means to diversify gluten-free products by allowing for divergence away from the traditional constraints of batter based gluten-free breads.

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Table I: Coded variable levels for carob germ flour, HPMC, and water for the experimental RSM design.

Table I
Experimental Design

	Coded Levels[a]		
Treatment	**Carob Germ Flour**	**Water**	**HPMC**
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	0
13	-1.682	0	0
14	+1.682	0	0
15	0	-1.682	0
16	0	+1.682	0
17	0	0	-1.682
18	0	0	+1.682
19	0	0	0
20	0	0	0

[a]Coded levels (flour bases): Carob Germ Flour: -1.682 = 4.94%, -1 = 7%, 0 = 10%, +1 = 13%, +1.682 = 15.05%; Water: -1.682 = 65.25%, -1 = 69%, 0 = 74.5%, +1 = 80%, +1.682 = 83.75%; HPMC: -1.682 = 0.05%, -1 = 0.8%, 0 = 1.9%, +1 =3%, +1.682 = 3.75%.

Table II: Model quality of the RSM design.

Parameter	Model	Model Quality		
		SMSS	Lack-of-Fit	R^2
Specific volume (ml/g)	Linear	$P < 0.05$	$P > 0.05$	0.76
Hardness (g of force)	Quadratic	$P < 0.05$	$P > 0.05$	0.68

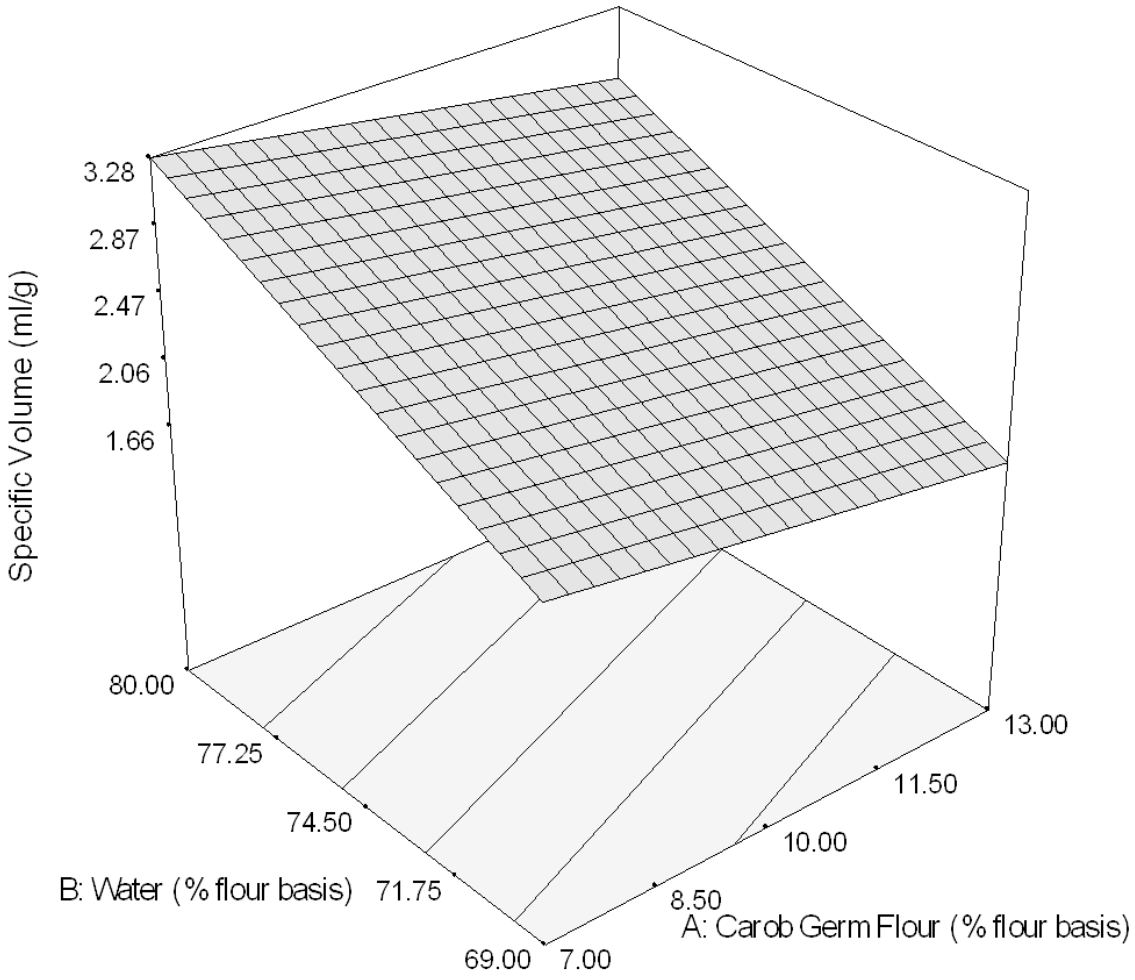

Figure 1: Effect of water (Y axis) and carob germ flour (X axis) level on specific volume (vertical axis) with hydroxypropyl methyl cellulose (HPMC) held constant at 3% (flour basis).

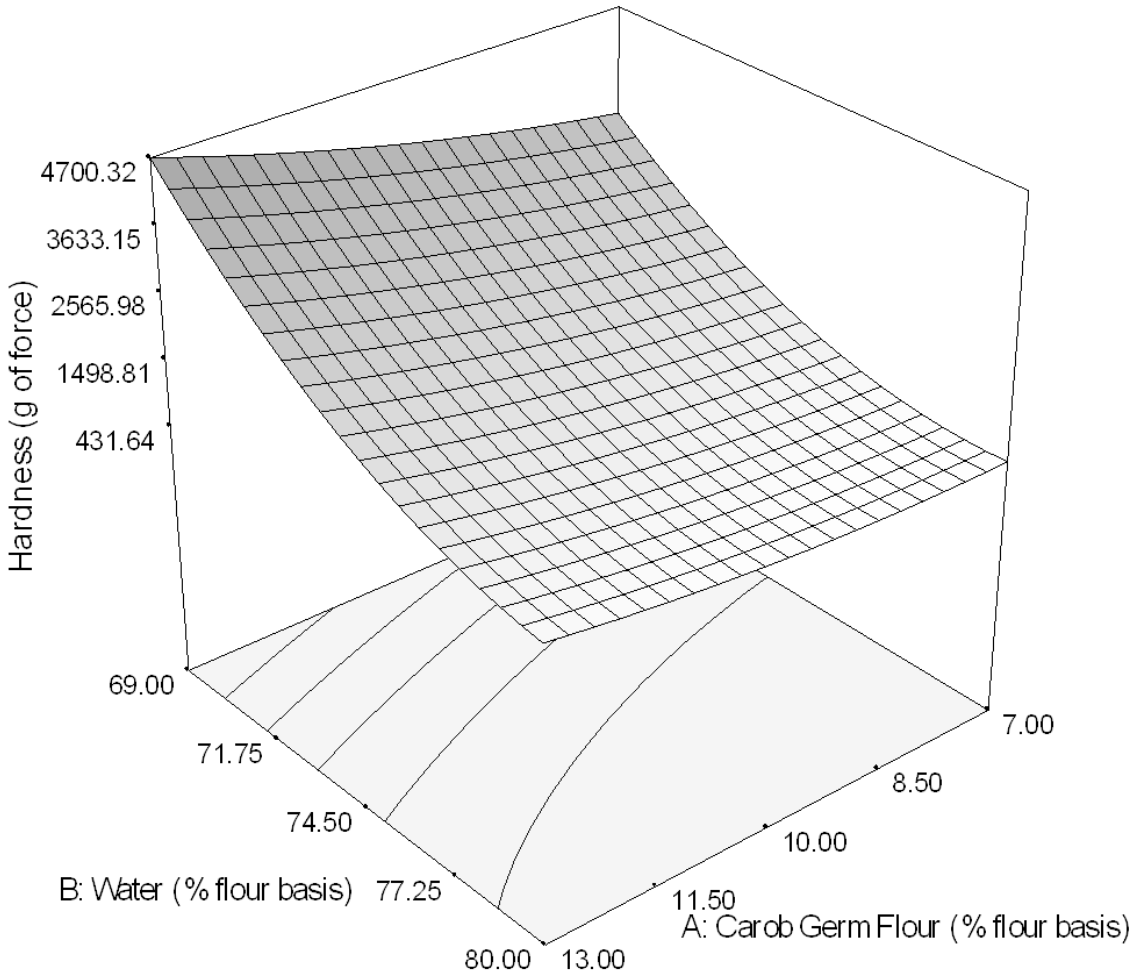

Figure 2: Effect of water (Y axis) and carob germ flour (X axis) on crumb hardness (vertical axis) with hydroxypropyl methyl cellulose (HPMC) held constant at 3% (flour basis).

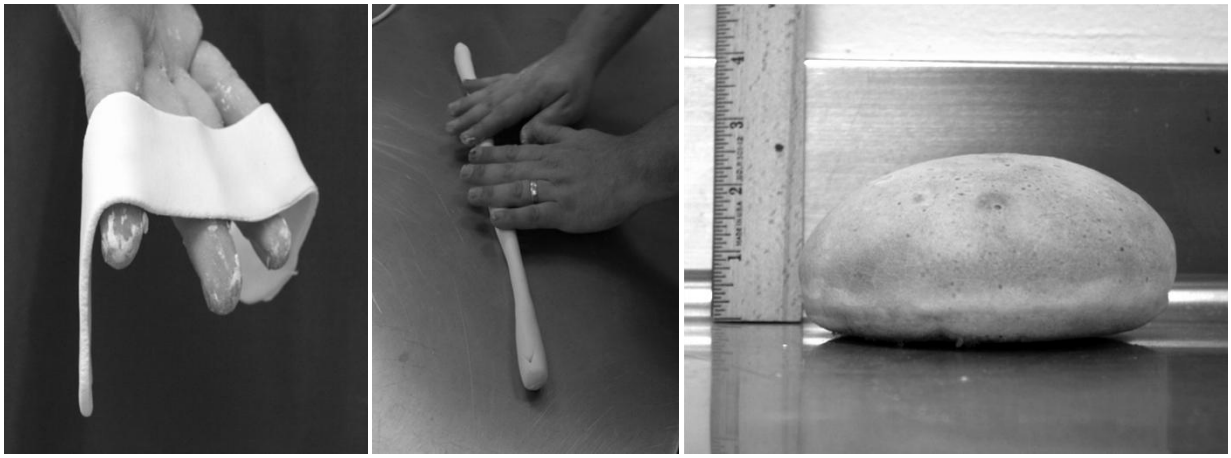

Figure 3: Pictorial representations of dough produced from the optimum formulation sheeted with a dough sheeter gaped at ¼ of an inch (left), rolled by hand (handleability) (middle), and a free standing roll produced without the aid of a bake pan (right).

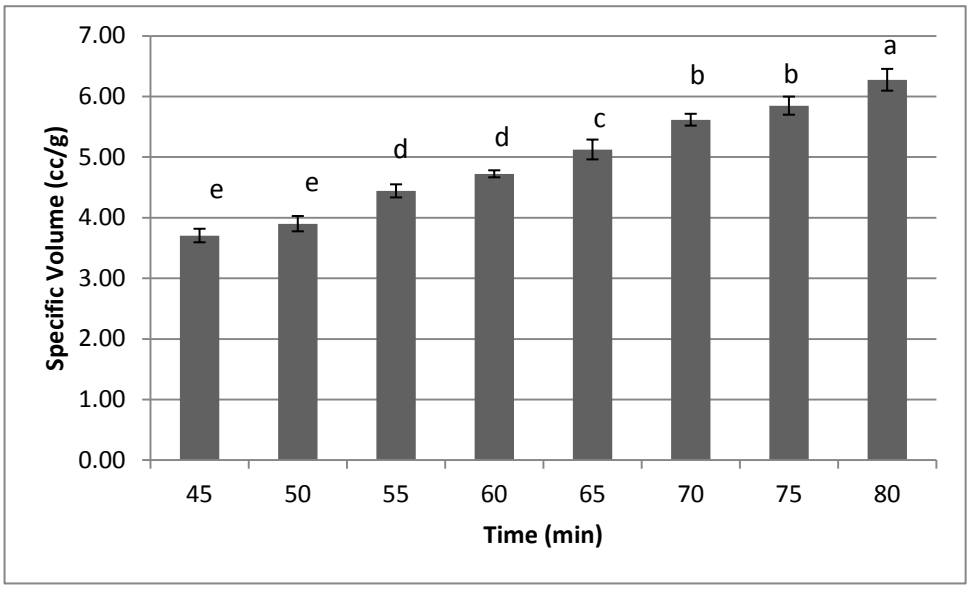

Figure 4: A graphical representation of specific volumes (y axis) for breads baked at times 45, 50, 55, 60, 65, 70, 75, 80 min (x axis). The lower case letters represent differences of means where similar letters identify no significant differences between the means ($P < 0.05$).

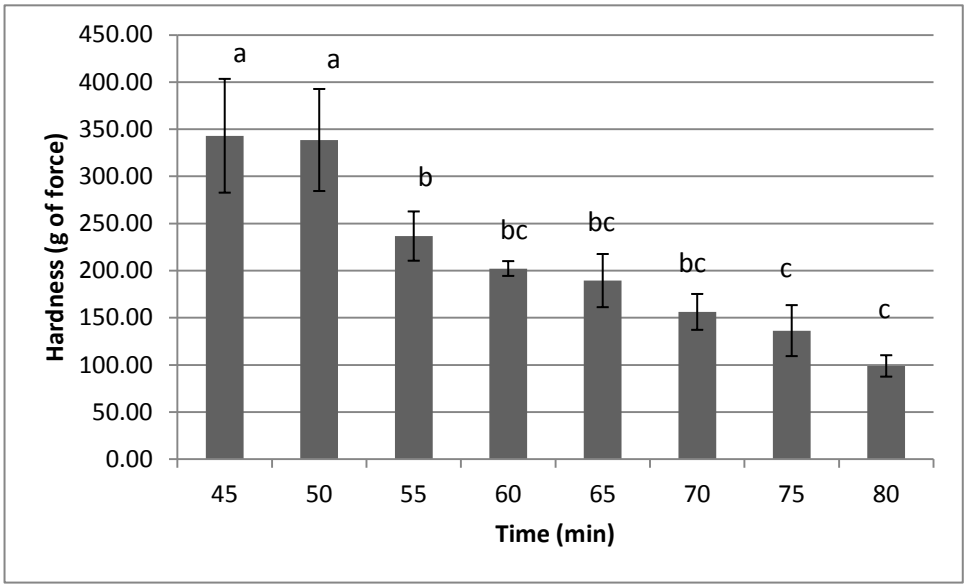

Figure 5: A representation of crumb hardness (y axis) for breads baked at times 45, 50, 55, 60, 65, 70, 75, 80 min (x axis). The lower case letters represent differences of means where similar letters identify no significant differences between the means ($P < 0.05$).

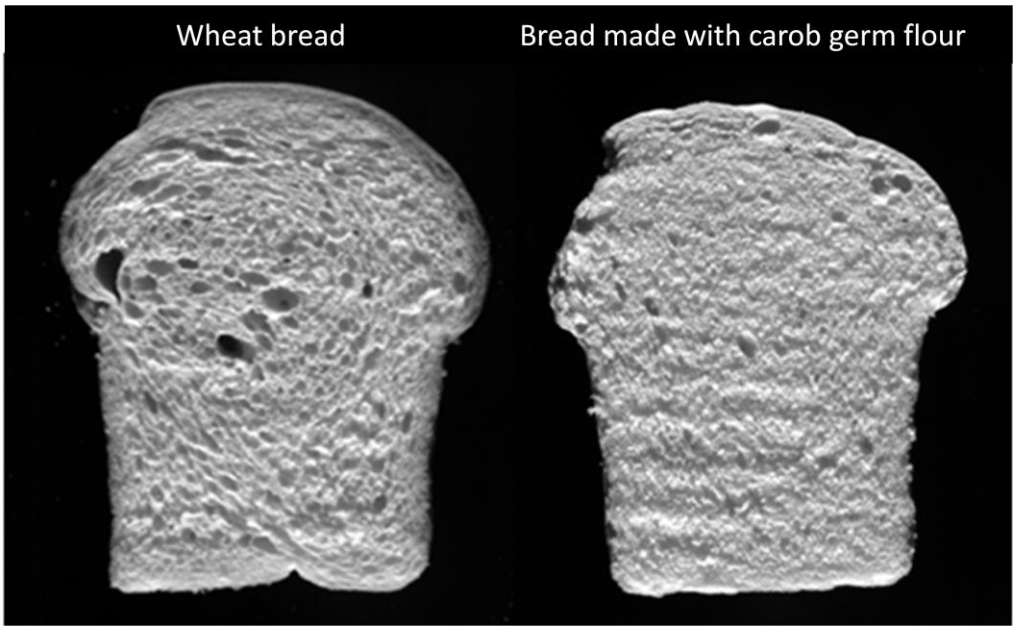

Figure 6: Comparison of a slice of wheat bread made in accordance to AACC method 10-10.03 (left) and bread containing the predicted optimums of Carob germ flour, water, and HPMC (right). Both breads represent loaves made from 100 g of flour.

Chapter 6: Future work

Future Work

The results of this dissertation provide insight into producing dough from non-wheat proteins and increasing the quality of gluten-free breads. There is significant research still to be conducted on dough formation using non-wheat proteins as well as with wheat proteins. The study of the functional proteins from zein, sorghum, and carob germ flour may also help provide some insight into the unique properties of gluten proteins.

With regards to the functionality of zein and kafirin, it would be interesting to characterize zein and kafirin secondary structures as they are forming resins or dough using techniques such as FTIR. This would give further insight to changes that need to occur to zein and kafirin to improve their functionality. It would also be worthwhile to investigate how the Hofmeister salts and other protein denaturants effect the glass transition of zein. Zein's glass transition being above room temperature was problematic throughout this research. If zein's glass transition could be lowered so that doughs and resins were plastic at room temperature then traditional dough quality tests such as aveleograph and extensograph could be preformed. Practically lowering the glass transition temperature of zein would make it easier to form products, i.e. dough would not have to be mixed and handled at elevated temperatures.

Because some of the treatments to zein resulted in zein forming a softer more visco-elastic dough, it would be beneficial to find chemicals generally regarded as safe that functioned similarly to the non-edible chemicals used. While some of the chemicals used in this research are not edible, the changes to zein reported could be used for industrial application, such as clays, resins, and bioplastics. Furthermore, numerous optimizations studies could be completed for the production of bread by addition or subtraction of gums, salts, and ethanol.

The proteolysis work completed on kafirin in this dissertation was intended only as a preliminary investigation into how processing of corn and sorghum proteins may impact their functionality. This work needs to be continued so that both kafirins and zeins could be made to functional like commercial zein when extracted in the laboratory and to shed light on how zein and kafirin could be processed specifically for food applications such as the production of non-wheat dough. The inability of laboratory zeins and kafirins is a major issue in determining why zein functions and how to improve upon zein and kafirin functionality.