Improvement of zein dough characteristics using dilute organic acids

by

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DECLARATION

I declare that the dissertation herewith submitted for the degree MSc Food Science at the University of Pretoria, has not previously been submitted for a degree at any other university or institution of higher education.

Alexandra Claire Sly
June 2013

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ABSTRACT

Improvement of zein dough characteristics using dilute organic acids

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The only treatment for coeliac disease, a common autoimmune disorder, is life-long adherence to a gluten-free diet. However, the replacement of wheat gluten, a key structural and functional component in bread, poses a major technological challenge for food scientists. The use of non-wheat cereal proteins, as alternatives to gluten, shows much promise in gluten-free bread making. Literature has shown that when zein, the maize prolamin protein, is subjected to wet heat above its glass transition temperature (T_g), the protein becomes viscoelastic, rubbery and dough-like. Gluten-like fibrils are visible, which form complex protein networks similar to those found in wheat dough. The resulting zein dough has viscoelastic characteristics and can be successfully used with hydrocolloids to produce gluten-free bread.

This project examined the influence of wet heat treatment and dilute organic acids (lactic acid and acetic acid) on the dough-making quality of non-wheat cereal proteins, such as kafirin and zein. Zein was the only non-wheat cereal protein to show any physical change when it was subjected to wet heat treatments, forming a dough-like substance. Acidification of the zein dough prepared at 40°C with concentrations of 0.7, 1.3 and 5.4% (v/v) organic acid in distilled water solutions, showed that the higher the concentration of acid used, the greater its effect on the dough's rheological properties. Tensile tests using a Keifer rig on zein dough showed that as the concentration of organic acid was increased from 0.7 to 1.3 and to 5.4% (v/v) the dough become softer and increasingly more extensible. The dough also exhibited less resistance to extension and reduced elasticity. CLSM revealed that the zein doughs contained a protein network, made up of fine protein fibrils, which became smoother and more homogenous as the concentration of acid was increased. Although SDS-PAGE revealed that no oligomerization took place with acid addition,

FTIR showed that zein dough prepared with distilled water at 40°C had elevated levels of β-sheets. When organic acids were added in increasing levels, corresponding increases in the quantities of α-helices in the protein were observed. Alveography showed that zein-based doughs prepared with dilute organic acids retained gases well and that the concentration of dilute organic acids influenced dough distensibility (biaxial extensibility) and stability (the ability of the dough to retain gas). Low concentration of acids (0.7 and 1.3%) increased dough stability to levels similar to that of strong wheat flour, 103 mm H_2O, but higher concentrations of acids (5.4%) led to a marked reduction in dough stability. Thus, by increasing zein dough functionality to such an extent, the apparent usefulness of the doughs and their ability to retain gases produced during fermentation is reduced. Simple distensibility tests on zein doughs showed that added organic acids promoted 'clumping' of the fine protein fibrils in the dough network into pronounced fibres. This would account for the decreased dough stability when high levels (5.4%) of organic acids were used. Baking trials with zein doughs were not successful as adequate leavening was impossible without an acid-tolerant leavening agent.

It is believed that dilute organic acids influence the rheological properties of zein dough by creating a positively charged environment, in which the protein is partially solubilized. The higher the level of organic acid used, the greater the positive net charge and the more pronounced the effect on the protein network structure. Organic acids could also improve fluidity of the zein dough by acting as plasticizers.

From this work it can be seen that although a protein network is present in all zein-based doughs, the ability of this network to retain gases is dependant on the level of organic acids present. The functional properties of zein-doughs made with low levels of organic acids (0.7 and 1.3%) shows potential in the production of gluten-free bread for individuals suffering from coeliac disease.

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1. INTRODUCTION

The shelves of health food shops and supermarkets in the West display increasing numbers of gluten-free products. This is due to a perceived need to follow gluten-free diets by consumers who are becoming increasingly aware of coeliac disease and of gluten intolerance, which can only be alleviated by adherence to a strict gluten-free diet (Ciacci, Maiuri, Caporaso, Bucci, Giudice, Massardo, Pontieri, Fonzo, Bean, Loergerf & Londei, 2007). The incidence of coeliac disease in South Africa has yet to be determined, but in the USA and Europe it has been estimated to be as high as one in every 133 individuals (Fasano, Berti, Gerarduzzi, Not, Colletti, Drago, Elitsur, Green, Guandalini, Hill, Pietazak, Ventura, Thorpe, Kryszak, Fornaroli, Wasserman, Murray & Horrath, 2003).

Gluten imparts significant structural qualities to baked products such as bread, and its replacement in food poses a major technological challenge (Gallagher, Gormley & Arendt, 2004) for food scientists. Gluten-free alternatives lack the sensory properties of their gluten-containing counterparts. These products are often inferior in terms of physical characteristics and quality. They have a dense, unleavened appearance, as well as a poor texture and mouth-feel. This is particularly so in the case of gluten-free bread, which makes it exceptionally difficult for consumers to follow a strict gluten-free diet. Currently, the approach to creating gluten-free foods of a high quality is to make use of various functional ingredients that improve structure, mouth-feel, acceptability and even shelf-life. These functional ingredients include alternative starches, hydrocolloids, gums, dairy products and other non-gluten proteins such as eggs (Gallagher et al., 2004). However, these functional ingredients and other additives are costly, may pose the risk of allergens and are regarded with suspicion by consumers who are influenced by a growing trend towards ‘natural’ products. There is, therefore, a need to produce a gluten-free, cost-effective alternative that has similar characteristics to gluten in baked goods, is nutritionally sound and that consumers consider both safe and tasty.

A possible source for this alternative is to be found in non-wheat cereal proteins. Africa is fortunate to have access to many local cereal grains such as sorghum, millets and maize, which contain proteins that have considerable potential for use in gluten-

free bread formulations (Gallagher et al., 2004; Taylor, Schober & Bean, 2006). An advantage of using local cereals is that they are nutrient dense, readily available and do not have to be imported. Currently, to keep up with the demand for wheat bread and other wheat-based products, sub-Saharan Africa relies heavily on imports of wheat, as this temperate cereal is not well suited for cultivation in hot arid climates (Taylor, 2004).

The focus of this dissertation is to investigate ways to improve the functional properties of indigenous non-wheat cereal-based doughs without the use of conventional additives such as hydrocolloids and enzymes. This would provide a substitute for gluten in gluten-free products. One means of doing this is through the use of organic acids, which are some of the natural products of sourdough fermentations. Acidification, by sourdough fermentation has been found to impact positively on the structure-forming components of dough, including proteins (Arendt, Ryan & Dal Bello, 2007). However, to date there is minimal scientific work on the effect of acidification on the structure-forming components of gluten-free non-wheat cereal based doughs.

2. LITERATURE REVIEW

The following literature review gives a brief overview of coeliac disease and its prevalence, as well as the biochemistry and physiology of the disease. The challenges facing food technologists in the replacement of gluten in foods is covered, and the current actions taken to improve the quality of gluten-free foods through the use of other functional ingredients and proteins are also discussed.

2.1 Coeliac disease

Coeliac disease is a common, autoimmune disorder that has genetic, environmental and immunologic components (Alaedini & Green, 2005). It is T-cell mediated (Gianfrani, Auricchio & Troncone, 2005) and occurs when genetically predisposed individuals suffer autoimmune reactions to the prolamin proteins found in gluten (Ciacci et al., 2007). Gliadin derived peptides that are either in their native or deamidated form, activate lamina propria infiltrating T-lymphocytes, which release pro-inflammatory cytokines such as γ-interferon. This in turn leads to profound tissue remodelling. Gliadin also contains peptides that are able to activate a non T-mediated response (Gianfrani et al., 2005).

Inflammation of the small bowel mucosa, with varying degrees of intestinal villous atrophy, crypt hyperplasia and an increased number of intraepithelial lymphocytes are characteristic of this disease (Achour, Thabet, Sakly, Mankai, Sakly, Ayadi, Sfar, Amri, Harbi, Essoussi, Krifa, Ajmi & Ghedira, 2010). This loss of absorptive villi (Dieterich, Ehnis, Bauer, Donner, Volta, Riecken, & Schuppan, 1997; Achour et al., 2010) affects the efficiency of nutrient absorption of any foods eaten (Ciacci et al., 2007). Apart from malabsorption being a direct consequence of the disease, individuals suffering from coeliac disease also follow a strict gluten-free diet and eliminate entire food groups that are often nutrient dense (Ciacci et al., 2007). Nutrients that are potentially lacking from the diet of a person with coeliac disease include iron, folic acid, calcium and fat-soluble vitamins (Feighery, 1999).

The symptoms associated with coeliac disease include nausea and diarrhoea, as well as weight loss and growth problems in children (Denery-Papini, Nicolas & Popineau, 1999). In many cases, patients have minimal symptoms, and often gastrointestinal symptoms are absent altogether (Feighery, 1999). The formation of autoantibodies can also contribute to other gluten induced autoimmune diseases. Thus, coeliac disease should also be considered in a wide range of clinical situations including anaemia and osteoporosis, and in patients with a range of associated disorders such as type 1 diabetes (Feighery, 1999). The symptoms of coeliac disease, including gastrointestinal, non-gastrointestinal and any biochemical abnormalities are alleviated once gluten is eliminated from the diet (Ciacci et al., 2007). Clinical and mucosal recovery after following a gluten-free diet is objective evidence that the enteropathy of the disease is gluten induced (Feighery, 1999).

Although therapeutic alternatives to treating coeliac disease have been explored (Sollid & Khosla, 2005), the only realistic and practical treatment for coeliac disease is a lifelong adherence to a gluten-free diet (Ciacci et al., 2007). Gluten type proteins are found in all Triticeae cereal species, a tribe within the Pooideae subfamily of grasses (Alm, Fang, Busso, Devos, Vollan, Grieg & Rognli, 2003), which includes durum wheat, spelt wheat, kamut, einkorn, triticale and closely related cereal types like barley and rye. Foods containing components from these grains should be avoided, as well as any of their by-products (Gallagher et al., 2004). This includes bread, biscuits, pasta and cereals, which represent dietary staples for a large portion of the world population (Arendt, Morrissey, Moore & Dal Bello, 2008). Other foods that should also be avoided are processed foods such as hot dogs, salad dressings, canned and dried soup mixes, as well as processed cheese and cream sauces, as these may contain wheat and gluten-derivatives as thickeners and fillers (Gallagher et al., 2004). These authors also noted that certain pharmaceuticals and medications use gluten as tablet binders.

2.2 Prevalence of coeliac disease

Until the late 1970s, coeliac disease was considered rare, with a worldwide prevalence of 0.03%; its diagnosis was based on three clinical symptoms: diarrhoea,

malabsorption and weight loss (Lohi, Mustalahti, Kaukinen, Laurila, Collin, Rissanen, Lohi, Bravi, Gasparin, Reunanen & Mäki, 2007). In the late 1980s the prevalence rates were estimated to be one in a thousand individuals or lower (Feighery, 1999). However, the prevalence of this disease has increased, and it is now estimated (as reviewed by Erickson, Campanella & Hamaker, 2011; Crockett, Ie, & Vodovotz, 2011) that one in every 133 people in the United States and Europe suffers from this disease. Gallagher et al. (2004) stated that recent epidemiological studies have shown that the prevalence of this disease has been significantly underestimated. According to Gallagher et al. (2004) and Feighery (1999), the epidemiology of coeliac disease can be explained through the use of an iceberg model (Figure 2.1). This model explains that only a small number of individuals has been clinically proven to suffer from the disease (A), whereas a larger number of individuals has undiagnosed coeliac disease and, since they have no prominent symptoms, they suffer from the disease in its silent form (B). A certain proportion of individuals in a population are also predisposed to develop coeliac disease later in life (C).

From this model one can see that the effect of coeliac disease on a country as a whole is often underestimated. Increased rates of detection are due to heightened awareness of the disease and improved diagnostic techniques (Gallagher et al., 2004). The development of highly sensitive and specific serological tests such as anti-endomysial (EMA) and anti-tissue transglutaminase antibodies (anti-tTG) has led to increasingly more individuals being correctly diagnosed (Fasano & Catassi, 2001). This in turn has led to a change in the epidemiological pattern of coeliac disease, and an increase in the market for gluten-free products that are safe for consumption by coeliacs.

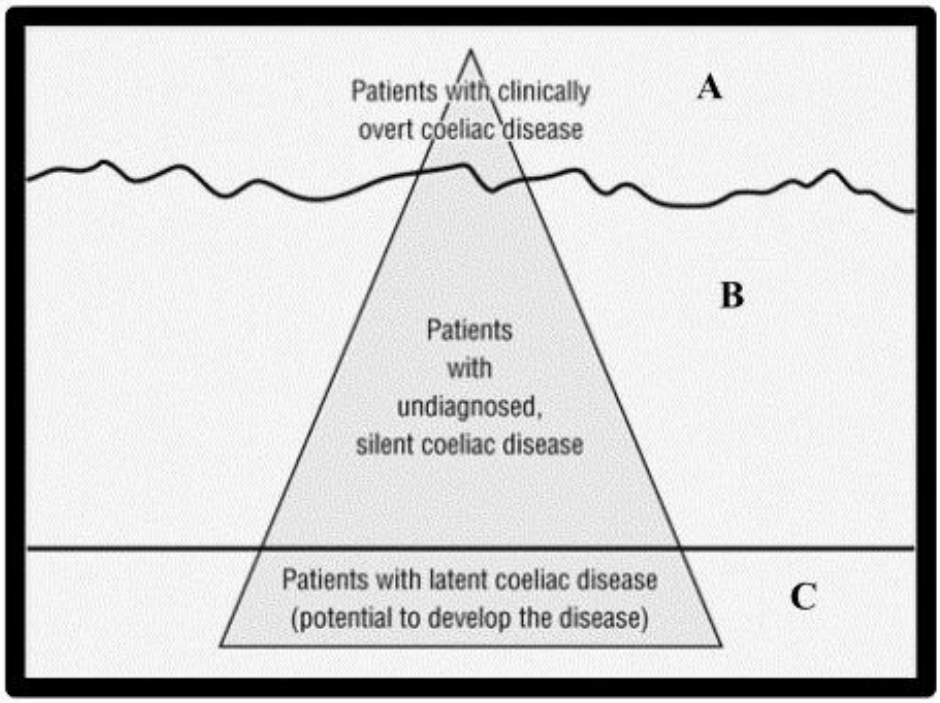

Figure 2.1: The iceberg model, which shows that the prevalence of coeliac disease is often drastically underestimated. (from Gallagher et al., 2004).

2.3 Biochemistry and physiology of coeliac disease

2.3.1 Prolamins

The proteins responsible for coeliac disease are the major cereal storage proteins, the prolamins (Denery-Papini et al., 1999). Prolamins are the main endosperm storage proteins in cereal grains. Exceptions to this are oats and rice. According to Duodu, Tang, Grant, Wellner, Belton and Taylor (2001), prolamins are hydrophobic proteins that contain large numbers of non-polar hydrophobic amino acids. They are insoluble in water but are soluble in aqueous alcohol. Prolamins are rich in proline (non-polar) and glutamine (polar), containing between 30 and 70% of these amino acids. (Shewry & Halford, 2002). The molecular weight of prolamins varies from about 10 000 to 100 000, which suggests a great variability in protein structure between prolamins present in the Triticeae and Panicoideae tribes (Shewry & Halford, 2002).

The Triticeae tribes include cereals such as wheat, barley and rye, while the Panicoideae include cereals such as maize, sorghum and millets (Kasarda, 2001; Shewry and Halford, 2002). The prolamins of the Triticeae can be classified into three main groups; the sulphur-rich, the sulphur- poor and the high molecular weight types (Shewry & Halford, 2002). However, these groups do not correspond directly to the glutenin and gliadin fractions of wheat, because both sulphur-rich and sulphur-poor forms of these prolamins occur. Gliadins are monomeric proteins and are classified as α-, ß-, γ- and ω-gliadins according to their mobility on electrophoresis at a low pH and their amino acid sequences. The glutenins are polymers. Important sequence homologies exist between these two fractions in wheat and the prolamins of other Triticeae cereals (Denery-Papini et al., 1999).

2.3.2 Peptides responsible

Gliadins and glutenins have been shown to contain specific peptide sequences that are not tolerated by coeliacs. Although the toxicity of gliadins is well known, the toxicity of glutenin is uncertain (Denery-Papini et al., 1998). Tests on the toxicity of specific peptides have been conducted using *in vitro* and *in vivo* techniques. *In vitro* studies have implicated both the N-terminal and far C-terminal domains of one of the wheat prolamins, α-gliadin. Peptides in this region may act as epitopes that trigger immune events leading to enteropathy (Sturgess, Day, Ellis, Lundin, Gjertsen, Kontakou & Ciclitria, 1994). Gliadin peptides specifically induced early phosphorylation of protein in epithelial cells (Ciacci et al., 2007).

According to Denery-Papini et al. (1999) there is a general consensus surrounding the toxicity of the 31-49 sequence (LGQQQPFPPQQPYPQPQPF) of α-gliadins as measured by both *in vivo* and *in vitro* techniques. Other sequences, such as the 3-24 or 1-30 peptides of the α-gliadins, are apparently only toxic *in vitro*. The homologous peptide 206-216 (sequence SGQGSFQPSQQ), which is found on the C-terminal domain of two α-giadin variants, stimulated T-cell clones during *in vivo* testing. Sturgess et al. (1994) found that peptides containing the putatively active tetrapeptide motifs QQQP and/or PSQQ, which contained the potentially antigenic β-reverse turns, are proposed models for peptide toxicity in coeliac disease.

Thus, it has been proposed that a good immunochemical test for potential toxins should not focus on specific individual peptides but should focus on the prolamins from all cereals that are not tolerated by coeliac sufferers. Denery-Papini et al. (1999) maintain that this test should not produce cross-reactions between prolamins from cereals that are tolerated by these individuals either.

2.4 The functional role of gluten in wheat based food products

Coultate (2009) stated that only flour made from wheat can be made into loaves with a leavened, open crumb structure. This is due to the protein gluten that is found in wheat. Gluten is responsible for providing many of the structural and functional properties characteristic of a wide range of food products, especially bread. When wheat flour is hydrated with water and kneaded, cohesive and extensible dough (Gallagher et al., 2004) with a viscoelastic network is formed. The two fractions of gluten, gliadins and glutenins, both contribute to the viscoelastic properties of this network (reviewed by Uthayakumaran, Gras, Stoddard & Bekes, 1999). Gliadins generally contribute towards the dough network's viscosity, while glutenins contribute towards elasticity. Lindsay and Skerritt (1999) believe that it is the interactions between these proteins, particularly the disulphide bonded glutenin macropolymer, that lead to the unique physical properties of gluten dough. The viscoelastic properties of the gluten network are crucial for the water-holding capacity of the dough, as well as gas retention during fermentation (Arendt et al., 2008). The absence of this network results in reduced gas retention and structure formation (Hager & Arendt, 2013) that leads to dense unleavened baked goods. Thus, the gluten network is a major contributor to the important rheological properties of dough (Lazaridou, Duta, Papageorgiou, Belc & Biliaderis, 2007) and the final baked product.

The viscoelastic system of gluten is very complex. It involves a combination of covalent and non-covalent bonds. As previously stated, the high molecular weight (HMW) glutenin subunits are believed to be chiefly responsible for the elastic properties of gluten (Andersson, Öhgren, Johansson, Kniola & Stading, 2011). Belton

(1999) proposed the 'loop and train' theory (Figure 2.2) in which the linear HMW glutenin subunits are stabilized by intermolecular hydrogen bonds, forming aligned β-sheet structures; such structures are believed to contribute substantially to the elastic behaviour of gluten in wheat dough. Non-covalent bonds such as hydrogen bonds, ionic bonds and hydrophobic bonds are important for the aggregation of gliadins and glutenins; this aggregation greatly influences the structuring and the mechanical properties of the protein network (Wieser, 2007). It has been proposed that stretching of the gluten polymer results in deformation of the loops and then the trains, as non-covalent bonds are broken, thus causing the proteins to slide over one another (Belton, 1999). The elastic restoring force is provided by the re-establishment of the loop-train equilibrium (Lindsay & Skerritt, 1999). The process of working the dough favours the alignment of the linear glutenin polymers through the formation of end-to-end disulphide bonds (Belton, 1999). According to these authors, this increases the effective molecular weight of the subunit and, hence, the number of protein–protein interactions. Since each extended polymer will be able to interact with a greater part of the total matrix, the resistance to deformation and the restoring force after deformation will be increased (Belton, 1999).

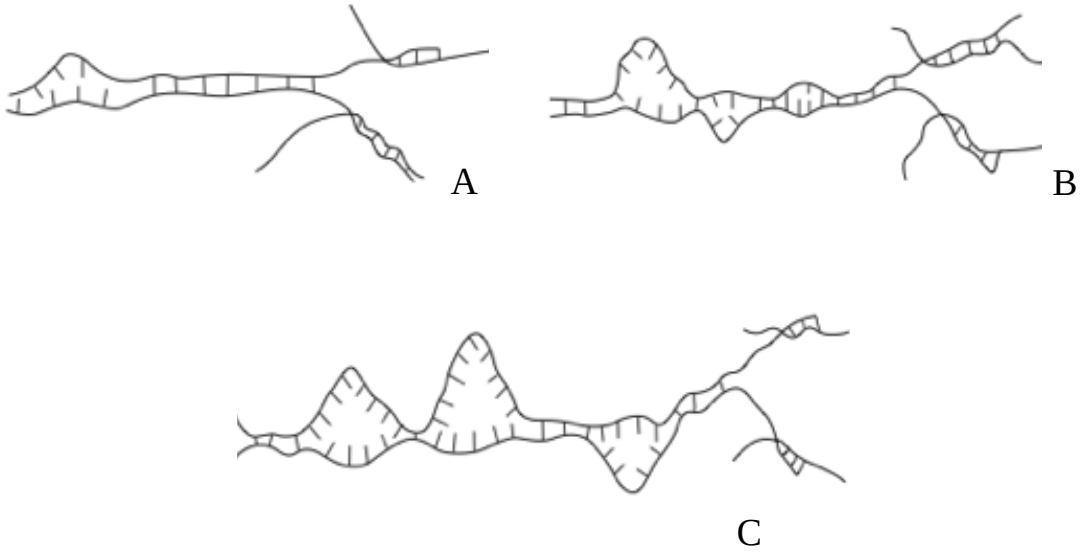

Figure 2.2: The effects of hydration on the loop and train behaviour of HMW subunits. For simplicity, interactions between two chains are shown. (A) Low levels of hydration – hydrogen bonds are mainly interchain. (B) Intermediate levels of hydration – some loops are formed. (C) High levels of hydration – the system contains many loops but sufficient interchain bonds exist to maintain interchain contacts (From Belton, 1999).

2.5 Current gluten-free products and research

As stated, the creation of gluten-free baked foods poses a major technological challenge to food scientists. It is very difficult to imitate the unique rheological properties of wheat dough, whilst also ensuring that the final product is nutritionally valuable and has acceptable sensory properties (Torbica, Hadnadev & Dapčević, 2010). This is particularly true for bread, which represents a dietary staple for a large portion of the population. Wheat flour is the preferred flour to use when baking, as it produces baked goods with a light, airy texture. Baked products containing no gluten lack the characteristic elasticity found with wheat dough, are smaller in volume and tend to be of a poorer quality (Schober, Bean & Boyle, 2007). The absence of gluten often results in a liquid batter rather than a dough (Gallagher et al., 2004; Schober, Moreau, Bean & Boyle, 2010; Erickson et al., 2011), and can result in baked bread with a crumbly texture, poor colour and other post-baking quality defects (Torbica et al., 2010). The form of breads made from a batter system is also limited as they can only take the shape of the pan into which the batter was poured (Smith, 2012). The shelf-life of gluten-free breads is relatively short (Gallagher, Gromley & Arendt, 2003). These breads are more prone to staling (Ahlborn, Pike, Hendrix, Hess & Huber, 2005) because gluten also plays a role in delaying the staling of bread through the formation of gas cell structure.

Gluten-free products are often also inferior in terms of nutritional quality when compared to their gluten-containing counterparts. Gluten-containing products are rich in micro-nutrients, whereas gluten-free alternatives are often low in B-group vitamins, calcium, vitamin D, iron, zinc, magnesium and fibre (Kupper, 2005; Arendt et al., 2008). This is due to gluten-free foods being made from refined flours and starches that contain low levels of nutrients. There are also very few gluten-free products being enriched or fortified to meet nutritional needs (Gallagher et al., 2004). Therefore, gluten-free products may be ineffective in providing the required nutrients needed by the body. This can worsen an already unbalanced diet consumed by coeliacs (Mariani, Viti, Montouri, La Vecchia, Cipolletta, Calvani & Bonamico, 1999). It is possible for coeliacs to use a number of nutrient-dense grains, seeds, legumes and nut flours, which offer increased variety, improved palatability and higher nutritional quality to the gluten-free diet. These grains and seeds include the

following: amaranth, buckwheat, flax, Indian rice grass, millet, teff, quinoa, and sorghum (Kupper, 2005). Some of these ingredients have been successfully used to improve the nutritional quality of gluten-free products especially in terms of fibre, protein and minerals (Arendt et al., 2008).

Currently, high quality gluten-free breads, with improved volumes, structure, mouth-feel, acceptability and shelf-life, have been achieved through the incorporation of functional ingredients (Lazaridou et al., 2007). Such ingredients include hydrocolloids, emulsifiers, and protein isolates which act as surface-active ingredients to stabilise the liquid films surrounding gas bubbles in fermenting batters (as reviewed by Taylor et al., 2006). It has also been hypothesized that the incorporation of proteins that are capable of forming cohesive networks will improve the structural quality of gluten-free breads (Cornish, Bekes, Eagles & Payne, 2006; Arendt et al., 2008; Schober, Bean, Boyle & Park, S. 2008). Proteins can improve structural quality by means of gelation, foaming, and increasing the elastic modulus of the dough through crosslinking reactions. Proteins are also used to enhance the sensory attributes of the bread through Maillard browning and flavour development.

2.5.1 Hydrocolloids

Hydrocolloids, gums and starches are used for their gelling and thickening ability in gluten-free goods. According to Gallagher et al. (2004), the baking industry widely utilizes starches and hydrocolloids to impart textural and appearance properties to cereal-based foods. Hydrocolloids often used in gluten-free products include xanthan gum, hydroxypropylmethylcellulose (HPMC), carboxymethylcellulose (CMC), guar gum and pectins (Lazaridou et al., 2007). The addition of hydrocolloids such as these has been shown to enhance gluten-free dough characteristics due to the formation of three-dimensional polymer networks in aqueous solutions (Arendt et al., 2008). This increases the solution's viscosity, leads to textural improvements and aids in the binding and retention of water (reviewed by Erickson et al., 2011). Schober (2009) reviewed the mechanism (Figure 2.3) by which hydrocolloids improve the texture of doughs and batters. These authors maintain that the increased viscosity of dough or batter following hydrocolloid addition, retains gases, which are incorporated during

mixing and fermentation, and minimizes their coalescence. The increased viscosity also prevents separation of the starch and other ingredients, thus improving dough homogeneity until gelatinization of the starch during baking.

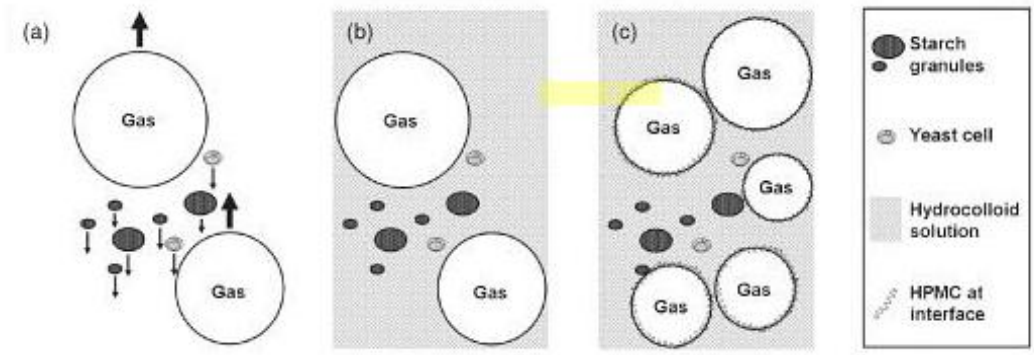

Figure 2.3: The effect of hydrocolloid addition on gas retention in doughs and batters: (a) The solution containing no hydrocolloids is unable to retain the gas bubbles produced through mixing and fermentation; settling of dough ingredients occurs. (b) The addition of a hydrocolloid such as xanthan gum increases the viscosity of the batter to such an extent that the ingredients and gas are suspended in the batter. (c) Surface active hydrocolloids, such as HPMC, add additional stability to the gas-liquid interface, preventing the coalescence of the bubbles. (from Schober, 2009)

Rotsch (1954) conducted studies on the role that starch plays in bread making, and found that bread dough without gluten can only retain gas if another gel is present as a gluten-like replacement. Ziobro, Korus, Witczak and Juszczak, (2012) investigated the replacement of maize starch with modified starch (acetylated distarch adipate [ADA], and hydroxyl-propyl distarch phosphate [HDP]) in gluten-free bread formulations. They found that the modified starches significantly increased the volume of the breads, and produced a higher number of cells with a smaller cell size and increased elasticity; a decrease in crumb hardness and chewiness was also observed. Lazaridou et al. (2007) explored the rheological behaviour of gluten-free dough formulations with added hydrocolloids. Farinography and rheometry conducted by these authors showed that the addition of xanthan gum produced dough with viscoelastic properties and strength that was on par with wheat flour dough.

However, the absence of a protein network increases moisture migration from the crumb to the crust, which leads to a dry crumbly texture and a finished product that was more prone to staling (as reviewed by Erickson et al., 2011). Andersson et al.

(2011) studied the extensional flow, viscoelasticity and baking performance of gluten-free zein-starch doughs supplemented with 2% HPMC and oat bran with a high concentration of β-glucan (28%). These authors found that hydrocolloid addition delayed staling. The zein-starch dough produced without hydrocolloids exhibited rapid age-related stiffening, which was believed to be due to crosslinks forming between peptide chains.

Schober et al. (2008) prepared gluten-free bread from commercial zein (20 g), maize starch (80 g), water (75 g), sugar (5 g), salt (5 g) and dry yeast (1 g) by mixing above zein's glass transition temperature at 40°C. Although zein bread without HPMC had a specific volume of 2.72 ± 0.10 ml/g and was able to retain some gas, it was not at a sufficient level to produce high quality bread. The addition of 2 g HPMC improved quality, and the resulting bread had a specific volume of 3.16 ± 0.08 ml/g, which was accompanied by a regular, fine crumb, a round top and good aeration, properties associated with wheat bread (Figure 2.4). An increase in viscosity was ruled out as a possible explanation for the volume increase following HPMC addition, because a zein dough with a 5% reduction in water level, that appeared to have similar dough properties to wheat dough, produced a baked loaf that had a specific volume of 2.54 ±0.23 ml/g. Instead, the additional HPMC was found to stabilize gas bubbles that were trapped by the protein network, at the gas – protein interface. Confocal laser scanning microscopy (CLSM) also revealed that the HPMC promoted the formation of a finer protein network, and dynamic oscillatory tests showed that HPMC made the zein above its T_g softer (it reduced the G').

Commercial gluten-free mixes usually contain only carbohydrates, which significantly limits the amount of protein (Ziobro, Witczak, Juszczak & Korus, 2013) and fibre in the diet (Torbica et al., 2010). Care must also be taken when using starches; should they be derived from wheat, they may contain trace amounts of prolamins, which can be toxic to coeliacs, even in low levels (Thompson, 2001; Gallagher et al., 2004).

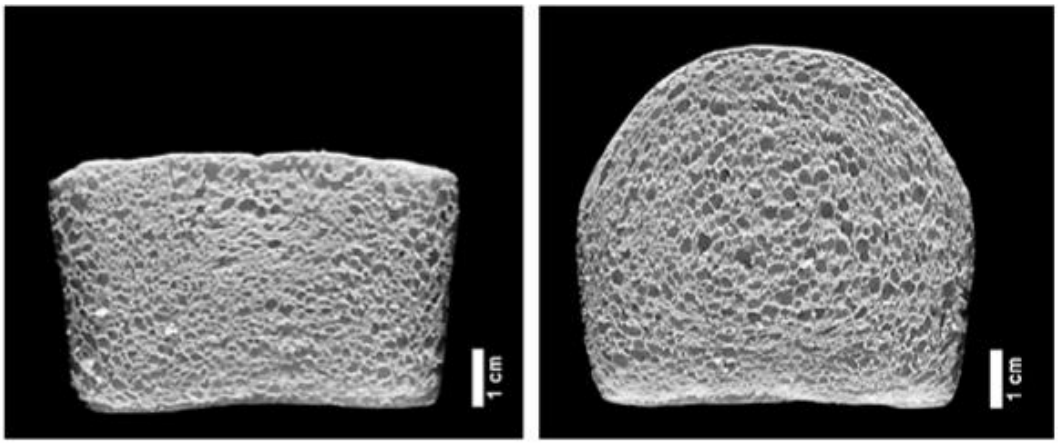

Figure 2.4: The effect of HPMC addition on the appearance of gluten free bread prepared from commercial zein (20 g), maize starch (80 g), water (75 g), sugar (5 g), salt (5 g) and dry yeast (1 g) at 40°C. Left: No HPMC added. Right: With 2 g HPMC added. (from Schober et al., 2008)

2.5.2 Non-cereal proteins

2.5.2.1 Dairy products and eggs

Dairy products are used in gluten-free bread formulations to provide a protein network (Arendt et al., 2008), increase water absorption and to enhance the handling properties of the batter (Gallagher, Gromley, & Arendt, 2003). They also contribute on a nutritional level (Gallagher et al., 2004), increasing the calcium content and the protein efficiency ratio (Arendt et al., 2008). However, allergenicity is a major factor that influences the use of milk and egg proteins in gluten-free products. Lactose intolerance in coeliac patients is a common pathology (Ojetti, Nucera, Migneco, Gabrielli, Lauritano, Danese, Zocco, Nista, Cammarota, Lorenzo, Gadbarrini & Gasbarrini, 2005), which affects approximately 50% of individuals (Murray, 1999). This is primarily due to a high level of intestinal villi damage and, therefore, an inability to produce the enzyme lactase (Ortolani & Pastorello, 1997; Murray, 1999; Moore, Schober, Dockery & Arendt, 2004; Gallagher et al., 2004). Thus, the incorporation of high lactose-containing dairy ingredients into gluten-free bread formulations is not advisable (Nunes, Ryan & Arendt, 2009; Arendt et al., 2008).

2.5.2.1.1 Dairy Products

Nunes et al. (2009) investigated the rheological characteristics of gluten-free bread formulations containing low lactose dairy ingredients. Sodium caseinate and milk protein isolate displayed a high water hydration capacity. This produced elastic batters, low bread volumes and a hard crumb. However, the addition of whey proteins had the opposite effect. Whey protein isolates and concentrates at a 90% water level significantly decreased the G' and G" of the dough. Gallagher et al. (2003) investigating the addition of seven different dairy powders (0, 3, 6 and 9% inclusion rates) to commercial wheat starch gluten-free flour obtained similar results. In general, the dairy powders that had high protein contents (skim milk powder, sodium caseinate and milk protein isolate) resulted in breads with low volumes and an increased crumb and crust hardness. Schober, Messerschmidt, Bean, Park, and Arendt, (2005) also found that the addition of skim milk powder had negative effects on sorghum bread quality. However, sensory testing by Gallagher et al. (2003) revealed that these breads had an appealing dark crust and a white crumb. When the water content was increased, various dairy powders including Molkin (a sweet whey), Kerrylac (fresh milk solids) and milk protein isolates at a 6% inclusion level, all increased bread volumes and softened the crumb and crust texture. Nunes et al. (2009) also found that crumb hardness seemed to be directly related to the amount of water that had been incorporated into the dough. Moore et al. (2004) conducted studies on different dairy and non-dairy gluten-free bread recipes with the objective of improving the nutritional quality of such breads. These authors found that commercial wheat flour and dairy containing gluten-free bread mixes, yielded significantly higher loaf volumes compared to their non-dairy and cereal-based gluten-free counterparts, though these gluten-free breads became brittle after two days of storage. CLSM showed that the crumb of these breads contained network-like structures, which resembled those of gluten (Figure 2.5). It was, therefore, concluded that the formation of a continuous protein phase is critical for the improvement in keeping quality of gluten-free bread.

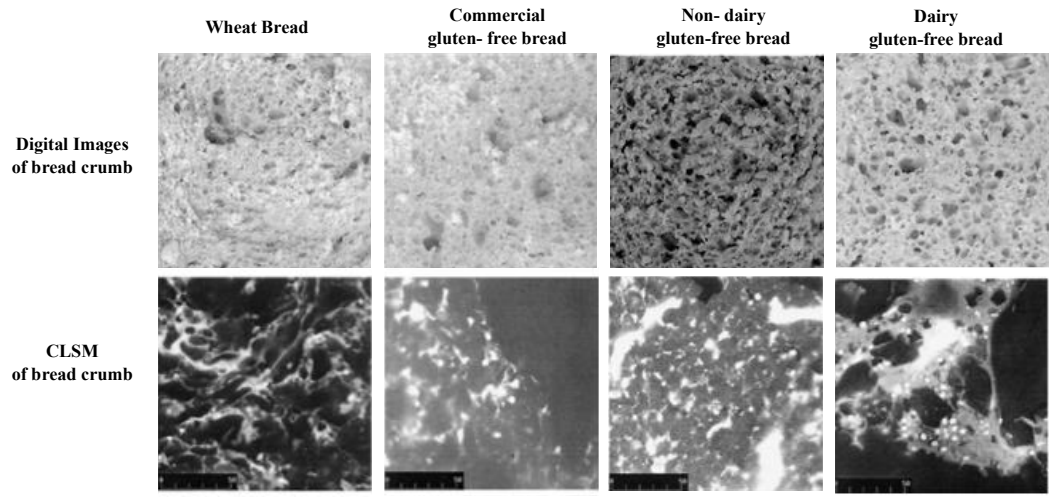

Figure 2.5: The crumb appearance and resulting microstructure, as viewed by CLSM, of different gluten-free breads. Wheat bread (wheat flour, salt, yeast, sugar and water), commercial gluten-free bread (wheat starch, milk solids, modified maize starch, soya flour, glucose, salt, stabilizer, HPMC, iron, thiamine, riboflavin, niacin, yeast, sugar and water), non-dairy gluten-free bread (brown rice flour, maize starch, buckwheat flour, soya flour, salt, yeast, sugar, sugar syrup, xanthan gum, water) and dairy gluten-free bread (brown rice flour, potato starch, maize starch, soya flour, skim milk powder, baking powder, salt, yeast, sugar, xanthan gum, konjac powder, water and egg). (from Moore et al., 2004.)

Van Riemsdijk, Pelgrom, Van Der Groot, Boom and Hamer (2011a) investigated whether a mesoscopically structured whey protein suspension could impart a gluten-like functionality in gluten-free doughs. These authors stated that sodium dodecyl sulphate-extraction of glutenin forms a gel layer (a glutenin macro-polymer, GMP, fraction), which contains protein particles that are 5-10 μm in size. The existence of these particles implies the presence of a dispersed protein phase within the gluten network and it is hypothesized that these protein particles account for the viscoelasticity, strain hardening and self-healing properties of gluten dough. Van Riemsdijk, Sprakel, Van der Goot and Hamer (2010) previously found through rheomicroscopy that whey protein particles (a 9% [w/w] stock solution of whey protein was prepared by stirring for 2 h at 25°C, followed by heating the solution at 68°C for 2.5 h) are able to form an elastic particle network in suspension due to a high degree of interactions within the particles. Van Riemsdijk, Pelgrom, Van Der Groot, Boom and Hamer (2011b) found through microscopy and strain and shear rate sweeps that the clustering of protein particles was favoured by the protein's ability to form sulphur bridges and, to a lesser extent, the small particle size. Van Riemsdijk et al. (2011a) investigated three types of mesoscopically-structured proteins: whey protein

aggregates, acid induced whey protein cold set gel, and whey protein particles. When placed in a protein-starch mixture, farinography indicated that the presence of the mesoscopically structured whey protein was essential for strain hardening and recovery properties of gluten-free bread. Without these particles, even with the addition of HPMC, a liquid-like mixture was present instead of a dough.

2.5.2.1.2 Eggs

Eggs can be added to gluten-free foods to increase nutritional value, improve colour and flavour, and to enhance the product's emulsifying, foaming, coagulation and gelation properties (Arendt et al., 2008). Crockett et al. (2011) investigated the effect of soy protein isolate and egg white solids on HPMC treated gluten-free dough systems. The purpose of the egg white was to provide additional structure to the dough, whilst soy protein isolate was used to increase disulphide linkages and improve the elasticity of baked goods. These authors found that at concentrations of 5 and 10%, soy protein isolate and egg white actually reduced the stability of the dough by decreasing the amount of available water, thus suppressing HPMC functionality by weakening its interactions with the starch matrix. Other authors, Kobylański, Pérez and Pilosof (2004), found that the addition of egg whites to maize cassava dough with added HPMC resulted in water binding by the egg whites, which in turn reduced the initial gelatinization temperature and improved loaf structure by hindering the gelatinization of starch during baking. When Crockett et al. (2011) investigated an increase in egg white solids (15%), they found that a primary protein scaffolding was formed, which resulted in an increased loaf volume. At levels above 15%, egg white solids in HPMC-treated cassava dough improve loaf volume and crumb regularity through the formation of an interconnected honeycomb matrix. Ziobro et al. (2013) investigated the enrichment of gluten-free dough with albumin, which lead to a significant increase in the specific volume of loaves; however, the viscoelastic properties of the dough were decreased. Moore et al. (2004) also found that egg proteins formed viscous solutions in gluten-free bread systems. However, CLSM revealed a film-like continuous protein structure, similar to that of wheat gluten. Ziobro et al. (2013) noted that the addition of albumin could adversely affect the smell acceptability of resulting breads.

2.5.2.2 Enzymes

One of the most important mechanisms for engineering food structures with desirable mechanical properties is through the crosslinking and aggregation of the protein molecules (reviewed by Storck, Da Rosa Zavareze, Gularte, Elias, Rosell & Guerra Dias, 2013). Sciarini, Ribotta, León and Pérez (2012) reported that enzymes are currently being added to gluten-free systems as means of modifying protein functionality through crosslinking, so that a continuous protein network can be created, enhancing the performance of gluten-free flours during bread making.

An enzyme that has received extensive attention for its ability to crosslink proteins is transglutaminase (Storck et al., 2013; Marco & Rosell, 2008b). Transglutaminase induces covalent cross-linking between the γ-carboxyamide group of glutamine residues and the ε-amino group of lysine residues (Renzetti, Dal Bello & Arendt, 2008), which is reported to improve firmness, elasticity, water-holding capacity, and heat stability in food systems (Kuraishi, Yamazaki & Susa, 2001). Crosslinking in soya bean enriched rice dough, as a result of transglutaminase, has been shown to produce a breadcrumb that has an increased hardness and a more continuous structure (Marco & Rosell, 2008b). Crosslinks within gluten-free systems containing skim milk powder or egg powder, at an appropriate protein substrate and enzyme ratio, have also been seen to improve dough volume (Moore, Heinbockel, Dockery, Ulmer & Arendt, 2006). According to Renzetti et al. (2008), transglutaminase improves the bread-making potential of gluten-free doughs by promoting network formation. These authors found that although transglutaminase improves the overall quality of gluten-free breads, the level of improvement differed. Buckwheat and brown rice flour showed significant improvements in the textural and structural characteristics in the resulting breads. In contrast, oat, sorghum and teff flour were only slightly affected by the addition of the enzyme, and maize flour showed that transglutaminase negatively affected the pseudo plastic behaviour of the batter. Protein source is a key element determining the impact of the enzyme, as cereals containing low levels of lysine, such as maize, limit the efficiency of transglutaminase (Erickson et al., 2011).

In cases where transglutaminase is ineffective, other enzymes such as glucose oxidase can be utilized. Glucose oxidase has been used as a polymerizing agent with varying

results, depending on the raw material employed (Sciarini et al., 2012). This enzyme has an oxidizing effect, due to the hydrogen peroxide that is released from its catalytic reaction. Bonet, Rosell, Caballero, Gómez, Pérez-Munuera and Lluch (2006) studied the macroscopic effect of increased glucose oxidase concentrations on the rheology of wheat dough, the characteristics of baked bread, and shelf-life. These authors found that the addition of glucose oxidase strengthened the wheat dough and improved the bread quality by the modification of the gluten proteins through the formation of disulphide and non-disulphide crosslinks. However, if excessive enzyme levels were added, then inverse effects were obtained, due to excessive crosslinking in the gluten network.

Advantages of using enzymes in gluten-free bread formulations is that they require milder processing conditions, have high specificity, are only required in catalytic quantities, and are less likely to produce toxic products (Singh, 1991). However, one of the disadvantages is that they are costly, and many consumers are averse to consuming foods produced with enzymes.

2.5.2.3 Legumes and soy

Legume proteins have functional properties that play an important role in food formulation and processing (Miñarro, Albanell, Aguilar, Gaumis & Capellas, 2012). Their role in gluten-free formulations is to provide a structural support for the starch and hydrocolloids (Ziobro et al., 2013). The addition of legume proteins to cereal proteins will also improve the amino acid profile and biological value of gluten-free bread. This is because these proteins contain relatively high amounts of complementary essential amino acids. Lysine from soy helps to make good the deficiency of lysine in maize, whilst methionine from maize helps to make up for the deficiency of methionine in soy (Marco & Rosell, 2008b; Miñarro et al., 2012).

However, the high allergenicity of soya, as well as associated digestive problems, has lead to further research on the use of alternative proteins as functional ingredients in gluten-free foods (Miñarro et al., 2012). These authors investigated the possibility of replacing soy protein with other legume proteins. The characteristics of gluten-free

bread formulations prepared with chickpea flour, pea protein isolate, carob germ flour or soya flour were evaluated. What these authors found was that the specific volume of chickpea flour bread was the highest, and that of the carob germ flour bread was the lowest (Figure 2.6). These authors stated that, because of its specific amino acid content, chickpea protein has higher foam expansion and stability values compared to pea and soya.

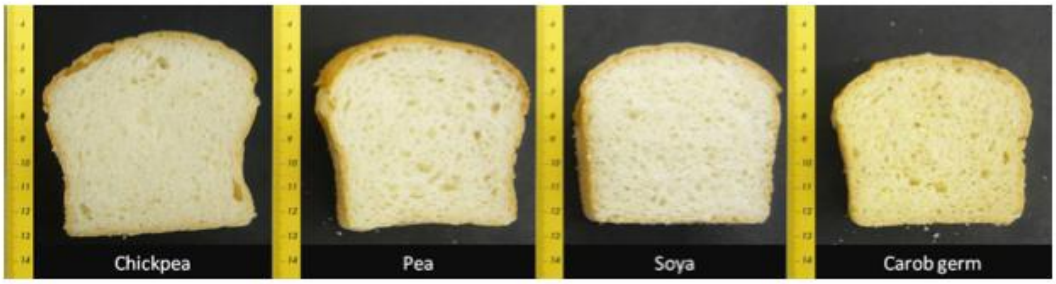

Figure 2.6: Digital images of legume flour gluten-free breads prepared with various proteins: chickpea flour, pea protein isolate, soya flour and carob germ flour (from Miñarro et al., 2012).

The structure-forming abilities of various proteins depend on their swelling ability and emulsification properties (Ziobro et al., 2013). According to Fukushima (2011), soy proteins are able to form heat-set gels that display similar properties to gluten. The application of heat causes the polypeptide chains to unfold, resulting in the exposure of amino acid residues and sulphhydryl groups on the protein's surfaces. These then crosslink by means of disulphide interchange reactions or hydrophobic bonding. The resulting gel, in which the starch may become embedded, has a three-dimensional network, which is capable of retaining water and gas. The good emulsifying ability of chickpea proteins means that its proteins, acting as emulsifiers, form a film or skin around oil droplets, preventing structural changes such as coalescence or creaming. This could have lead to an improved bread volume and finer crumb (Miñarro et al., 2012). Emulsifiers allow for the interaction between two chemically different phases and are used in baking to increase the stability of thermodynamically unstable systems (Sciarini et al., 2012). Their incorporation into gluten-free dough formulations has lead to softer crumb structures, as well as a reinforced dough structure. Miñarro et al. (2012) reported that carob germ, when hydrated, is capable of forming a network similar to, but weaker than, that of gluten. The low carob germ bread volume was attributed to a lack of prolamins present in the

carob germ flour, as well as an excess of hydrocolloid content, due to residual gums present in the flour. Miñarro, Normahomed, Guamis and Capellas, (2010) have also successfully formulated soy based, gluten-free bread with good baking and sensory characteristics. These authors found that breads made with legume flours showed good physico-chemical characteristics and an adequate sensory profile. Chickpea flour and pea protein isolate breads produced good results for all parameters studied; however, it was noted that during consumer preference testing the chickpea breads scored lower because of their strong chickpea taste. This indicates the importance of neutral-tasting ingredients in gluten-free formulations.

Another protein with a high functionality is marama bean protein. Amonsou, Taylor, Emmambux, Duodu and Minnaar (2012) compared the dough-forming properties of marama bean protein to that of soya and gluten. The extensibility of the marama bean dough was over twice that of gluten and soya, and it increased threefold when the moisture content of the dough was increased from 38% to 45%. The addition of the enzyme peroxidase helped in the formation of protein networks with dityrosine crosslinks, which resulted in the storage modulus of the dough increasing with time. The hydrophobicity and β-sheet content in marama protein was found to be higher than that of soya. This led these authors to believe the highly viscous and extensible rheological behaviour of marama protein to be related to its high β-sheet content, hydrophobic interactions and tyrosine crosslinks.

2.5.3 Modification of non-wheat cereal proteins

Much research is being conducted on the replacement of gluten with ingredients that are able to mimic its functional properties (García, Zaritzky & Califano, 2005; Sciarini et al., 2012.) It is recommended that grains from the Panicoideae tribes, such as maize, sorghum and millets, be utilized in gluten-free foods. These grains have proteins that do not elicit the same immunological response as those belonging to the Triticeae Tribe. However, flours from grains such as sorghum and maize do not normally form viscoelastic doughs. Modification of these non-wheat cereal grains is focused on functionalizing non-wheat cereal proteins to mimic gluten's viscoelastic nature, improving dough functionality as well as increasing gluten-free product

development (Erickson et al., 2011). Gluten-free bread dough of higher quality would eliminate the use of hydrocolloids, stabilizers and pregelatinized starch, which is usually used to provide gas occlusion and stabilizing mechanisms within the dough (Schober et al., 2007). Current research into the modification of these non-wheat cereal proteins involves investigations into temperature, defatting and acidification.

2.5.3.1 Temperature

The glass transition temperature (T_g) of the various proteins is very important in the creation of a manageable dough system. When a material is raised above its T_g it undergoes a physical state change from a brittle 'glass' to a molten 'rubber', which displays viscoelastic properties (Panchapakesan, Sozer, Dogan, Haung & Kokini, 2012). Zein, unlike gluten, is not able to form visco-elastic fibrils at room temperature, though it can be made functional in this way at higher temperatures (Mejia, Mauer & Hamaker, 2007). Schober, Bean, Tilley, Smith & Loerger (2011) state that zein aggregates in water above its T_g into an extensible, viscoelastic, gluten-like substance. Lawton (1992) investigated the relationship between dough properties and protein T_g in a zein-starch dough system. Mixing in a farinograph at 25, 30 and 35°C, produced a dough with viscoelastic properties and a gluten-like fibre network that was visible under a scanning electron microscope. It is believed that this network is responsible for the viscoelastic properties of the dough. Andersson et al. (2011) visualized this protein network in zein-starch doughs using CLSM (Figure 2.7).

Mejia et al. (2007) compared the secondary structure of a dough-like zein polymer to the structure present in a wheat viscoelastic system using Fourier Transform Infrared (FTIR) spectroscopy. These authors found that when the zein was in a hydrated viscoelastic state above its T_g (35°C), the native structure of the protein, which comprises approximately 68% α-helical conformation, is lost. The new structural arrangement is one that favours β-sheet conformations. This overall rearrangement is very similar to the structural changes observed in gluten viscoelastic polymers.

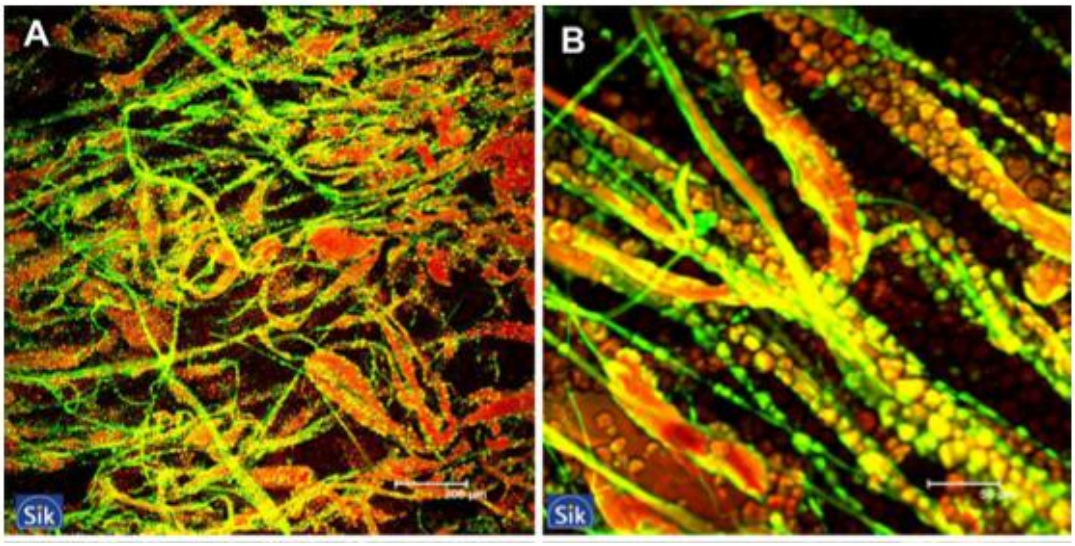

Figure 2.7: CLSM images showing the protein network of zein-starch doughs with 65% water. In the images, the zein is shown in green and the starch in red. **A**: 200 µm and **B**: 50 µm (from Andersson et al., 2011).

Lawton (1992) found that, although zein-starch dough, above its T_g, was not as strong as wheat dough, it was very extensible. Other authors have also found such dough to be weaker and more extensible than typical wheat dough (Schober et al., 2008; Schober et al., 2010.) Mejia et al. (2007) speculated that the addition of a protein that is capable of causing extensive and stable β-sheet formations within the zein-starch dough, could lead to an increased stability and relaxation time and, possibly, produce a viscoelastic dough with similar functionality to a wheat viscoelastic system.

Bugusu, Campanella and Hamaker (2001) conducted trials using zein to determine the effects of protein-body free prolamins on dough rheology and baking quality of wheat sorghum composite flours. In their trials the addition of zein above its T_g to sorghum-wheat composite doughs improved rheological and leavening properties of the doughs. When zein was added to the dough at temperatures above 35°C and at levels greater than 10%, mixing time was decreased and there was an increase in the loaf volume of the baked breads. This improvement in the functionality of the doughs shows that successful viscoelastic networks, capable of gas retention, were produced. These authors postulated that if the kafirin was freed from the protein bodies it would be capable of producing similar effects to the zein in dough systems. The addition of sorghum flour to wheat flour has negative effects on the rheological properties of the dough and loaf volume. This is probably as a result of the encapsulation of the

kafirins within the protein bodies (Bugusu et al., 2001). However, Schober et al. (2011) believe that it was the extraction and isolation techniques that prevented the viscoelastic functionality of kafirin from developing when heat was applied. Kafirin is extracted from dry milled sorghum using 70% (w/w) aqueous alchohol containing 0.35% sodium hydroxide and 0.5% sodium metabisulphide at 70°C (Emmambux and Taylor, 2003). Schober et al. (2011) extracted kafirin from dry milled sorghum, using a hydrophobic solvent (83% aqueous isopropanol). The resulting kafirin was able to aggregate in warm water, with the aid of a reducing agent, forming a substance that displayed viscoelastic properties. However, it quickly stiffened.

Oom, Pettersson, Taylor and Stading (2008) successfully produced a viscoelastic dough system using kafirin. These authors prepared kafirin and zein resins by dissolving the defatted proteins in aqueous ethanol at 75°C and adding oleic acid (Figure 2.8). Distilled water was used to precipitate the protein resins out of solution before kneading. The resulting kafirin and zein resins had similar extensional and rheological properties to those of gluten doughs, but the kafirin rapidly became stiff, with the G' of the resin increasing six times after 2 hrs. These authors used dynamic time scans (DMA) to observe the stiffening of the zein and kafirin-resins over a period of time. At the beginning of the run, the kafirin resin had a phase angle of 45°, which indicates an almost equal contribution of elastic and viscous properties. However, within 2000 s this angle had decreased to below 35°, which indicates an increase in the elastic contribution of the resin. The initial phase angle of the zein-resin was higher than the kafirins. It remained more or less the same for the first 1000 s and then, thereafter, the phase angle decreased slightly; thus it can be said that the zein resin maintained essentially equal viscous and elastic components. It was considered that the stiffening was related to crosslinking of the cysteine rich γ-kafirin monomers. Work by Schober et al. (2011) also points to the possibly of disulphide bonds reforming.

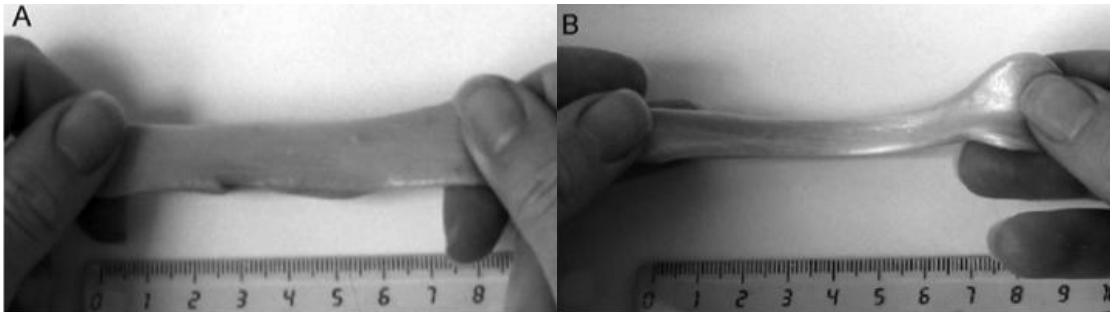

Figure 2.8: The appearance of viscoelastic kafirin (A) and zein (B) doughs. (from Oom et al., 2008)

A reduction in temperature leads to a change in polymer structure. Mejia et al. (2007) found that the viscoelastic properties of zein polymer was lost if the temperature was reduced below 25°C. This finding is similar to that of Lawton (1992), who showed that a dough could not be formed if the materials were mixed below 25°C. In addition, Lawton (1992) also found that the viscoelasticity of dough prepared above this temperature was lost upon cooling. Schober et al. (2008) found that zein fibres were not destroyed upon cooling to below the T_g, but that mechanical impact caused the brittle network to shatter. Reheating did not promote the formation of new fibres, which decreased the dough's resistance in uniaxial extension tests.

2.5.3.2 Defatting

Defatting has been shown to improve the aggregative and cohesive properties of zein, and to promote the formation of viscoelastic dough (Schober et al., 2010). These authors found that zein-starch dough with added HPMC increased in specific volume from 3.3 ml/g to 4.5 ml/g when the zein was bench scale defatted using chloroform at room temperature. Oom et al. (2008) also introduced a defatting stage during the preparation of their dough-like zein and kafirin resins. Schober et al. (2010) believe that the removal of the proteins' surface lipids improves their functionality by facilitating protein-protein interactions and improving water absorption. Although such doughs were capable of producing leavened bread, their high extensibility limited leavening.

Grosskreutz (1961) suggested that lipoproteins might contribute to the softness and plasticity of gluten through the formation of slip planes within the protein. Marco and Rosell (2008a) found that presence of hydrogenated vegetable fat in buckwheat and rice flours gave gluten-free bread good sensory attributes. However, other authors believe that lipids present in natural quantities do not contribute significantly to the rheological properties of dough. Instead their role is to act as surface–active compounds in stabilizing the gas-liquid interphase (Gan, Ellis, Schofield, 1995). The presence of HPMC provides a similar stabilizing action in zein-starch dough (Schober et al., 2008; Schober et al., 2010).

It has been hypothesised (Schober et al., 2010) that defatting in a solvent such as hexane or chloroform could also lead to denaturation, which would influence the protein's size, distribution and hydrophobicity due to the refolding and exposure of different side chains. Such physical changes would promote protein-interactions and improve the aggregation of zein. This could also result in improved aggregation as seen via the aggregation experiments and CLSM of zein-starch dough. However, size-exclusion high performance liquid chromatography (SE-HPLC) and reversed-phase high performance liquid chromatography (RP-HPLC) by these authors was inconclusive, and further studies were recommended using a benign method that does not mask small structural differences.

2.5.3.3 Plasticization and acidification

The principal role of a plasticizer is to improve the flexibility of a polymer by lowering the T_g. Plasticizers achieve this task by occupying the intermolecular spaces between polymer chains, thus reducing the secondary forces among them and changing the three-dimensional molecular organization of the polymers (Vieira, Da Silva, Dos Santos & Beppu, 2011). This, in turn, reduces the energy required for molecular motion and the formation of hydrogen bonds between chains. Plasticizers can include water, for hydration, and acids, for acidification (Oom et al., 2008). Bugusu et al. (2001) stated that gluten protein gains sufficient mobility due to thermal and water plasticization above T_g to form a thermoset network by disulphide crosslinking, which contributes to its rheological properties. Lawton (1992) showed

that the T_g of zein drops with increasing moisture content, but levels out at >16% moisture and does not fall much below room temperature. According to Santosa and Padua (2000), oleic acid is an effective, amphiphilic plasticizing agent. Oom et al. (2008) measured the viscoelastic properties of kafirin and zein resins, which had been plasticised with oleic acid. The T_g of the resins were in the range of -4 to -3°C, which was much lower than ≈20°C, the T_g of zein that had been plasticized with only water. Lawton (1992) found that the addition of dibutyl tartrate (Figure 2.9) as a plasticizer in concentrations up to 20% decreased the T_g of zein; however, it did not reduce it to below 50°C.

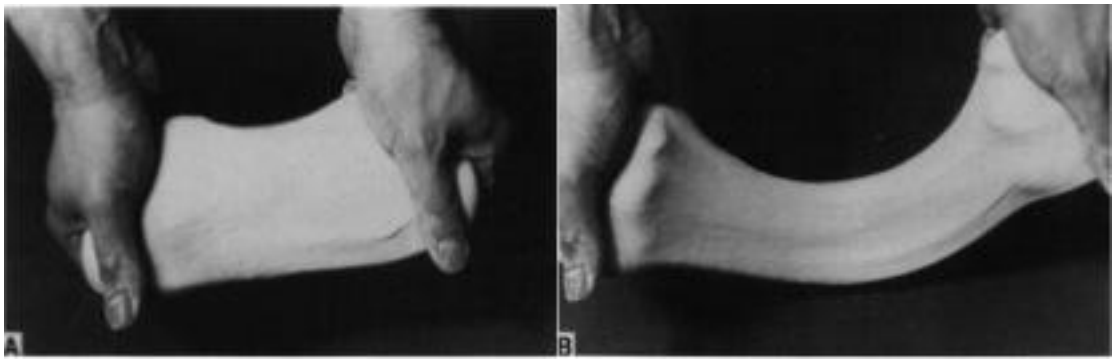

Figure 2.9: The appearance of zein-starch dough prepared with dibutyl tartrate; (A) Relaxed, (B) Extended (from Lawton, 1992).

Blanco, Ronda, Pérez & Pando (2011) conducted experiments aimed at improving the quality of gluten-free bread through the addition of various acids. These included acetic acid, lactic acid, citric acid and monosodium phosphate (an inorganic salt, which is expected to have an acidic behaviour in the dough). These authors noted that vinegar has been traditionally added to dough to improve the bread quality. The bread volume reached after baking was not significantly ($p<0.05$) affected by the type or concentration of the organic acid added to the dough. However, the addition of acetic acid did produce interesting results. At a low concentration of 0.2% the dough volume increased by 10% compared to the non-acidified bread dough (control). At a higher concentration, 0.4%, no change in volume was seen and at the highest concentration of 0.6% there was a decrease in dough volume. These researchers attributed this strange behaviour to the preservative properties of the acetic acid, giving it an ability to inhibit the functionality of yeast.

2.6 Sourdough processes

The use of sourdough shows much promise in increasing the quality of gluten-free breads, presenting an attractive alternative to conventional means of dough improvement through the incorporation of functional ingredients. Sourdough addition has a well-established role in improving the quality of gluten-free bread (Arendt et al., 2008). The acidification of flour by sourdough fermentation enhances the swelling properties of polysaccharides, thereby improving gas-holding abilities. Positive effects of this process include increased bread volume, improved crumb structure, aroma, flavour and an extended shelf-life (Clarke, Schober & Arendt, 2002; Katina, Heiniö, Autio & Poutanen, 2005). The nutritional properties of the bread are also improved (Moroni, Bello & Arendt, 2009).

Moore, Juga, Schober & Arendt (2007) investigated the effect of sourdough addition on the quality of gluten-free bread (the batter mix consisted of brown rice flour, buckwheat flour, maize starch, soya flour, xanthan gum, sugar syrup, salt, sugar and instant yeast). Three different strains of lactic acid bacteria (*Lactobacillus plantarum* 2115KW, *L. plantarum* FST 1.11, and *L. sanfranciscensis* TMW 1.52) were investigated and compared to non-acidified and chemically acidified gluten-free bread formulations. During fermentation, extrusion measurements revealed that the sourdoughs became softer over time due to the production of acids. Schober et al. (2007) state that acetic and lactic acids are common products of sourdough fermentations using *L. plantarum*. However, sourdough bread batters were noticeably firmer than both the non-acidified and chemically acidified dough. Examination by CLSM revealed that the protein particles (mainly soy and buckwheat protein) within the sourdough also degraded over time. These authors assumed that the lactic acid bacteria, in conjunction with the naturally present enzymes, partially digest the protein rich particles making the protein more accessible, so that it can bind water in the bread batters. The newly accessible proteins could also assist in sticking the remaining proteins together, creating larger aggregates and a more stable microstructure. Katina et al. (2005) found that the incorporation of a sourdough process into a wheat bread system causes significant increases in loaf volume. An increased volume was not evident in any of the sourdough breads prepared by Moore et al. (2007) due to the absence of a gluten-like protein network.

Schober et al. (2007) improved the quality of sorghum bread through the addition of sourdough fermentation. Originally 2% HPMC on a 105% water basis was used to improve the quality of 70% sorghum and 30% potato starch bread. However, the resulting bread had a flat top and holes in the crumb. Chemical acidification (batter pH 5.18) using 2.4% lactic acid led to acceptable bread with a small volume, and a slightly collapsed top. The addition of sourdough fermentation (batter pH 5.18) eliminated these problems (Figure 2.10). The specific volume and height of the sorghum breads with added HPMC, chemical acidification and sourdough fermentation were 2.60 cm^3/g and 10.7cm; 2.25 cm^3/g and 9.3 cm; and 2.68 cm^3/g and 12.2 cm respectively. Sourdough fermentation also increased the batter's resistance to deformation after gelatinization. This improvement through sourdough fermentation was seen during CLSM. Initially, aggregated protein, which could lead to interference in the starch gel, was present in the breadcrumb of the bread. After sourdough fermentation, only small isolated patches of protein bodies embedded in a matrix protein remained.

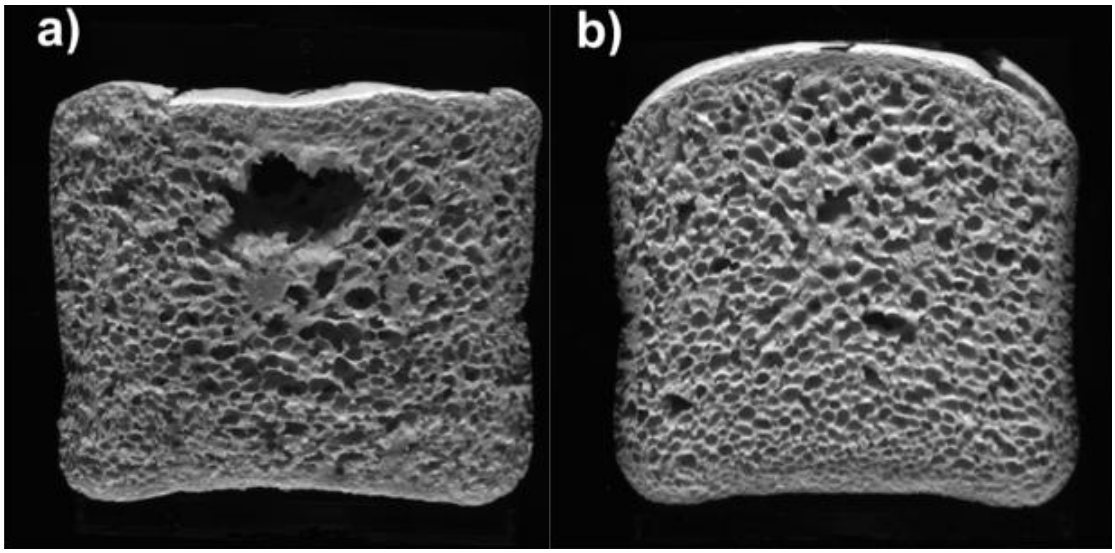

Figure 2.10: The effect of sourdough fermentation on the appearance of 70% sorghum breads with 2% added HPMC (a) no sourdough fermentation, (b) with sourdough fermentation (from Schober et al., 2007).

2.7 Conclusions

Producing a nutritiously sound gluten replacement that can provide structural scaffolding in gluten-free bread formulations, and which results in a high quality leavened bread that is acceptable to consumers, is a major technological challenge for food scientists. There has been some success with the use of proteins in gluten-free bread formulations, as they aid in improving structural stability, appearance and the nutritional value of foods. However, at present they need to be utilized in combination with other ingredients such as hydrocolloids and enzymes in order to gain increased functionality in a dough system. Concern over the allergenicity of proteins from dairy or legume sources should thus focus gluten-free work on the use of non-wheat cereal proteins. Two non-wheat cereal proteins that show much promise in this area are the prolamins, zein and kafirin, and they have been shown to exhibit viscoelastic properties similar to gluten. The use of sourdough fermentations (acidification) to modify the properties of such non-wheat cereal proteins naturally, in order to improve functionality in gluten-free bread formulations, warrants investigation.

3. HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Hypothesis 1:

By raising the temperature of a non-wheat cereal protein such as zein, kafirin and rice protein to above its glass transition temperature ($T_{g)}$ the aggregative and cohesive properties of the non-wheat prolamin protein can be improved to the extent where it exhibits viscoelastic properties.

The glass transition temperature is a critical temperature where the mechanical properties of amorphous polymers undergo a change (Panchapakesan et al., 2012). When the temperature of a solution is raised above the glass transition temperature, the amorphous matrix undergoes a physical state change from a brittle 'glass' to a molten 'rubber'. Upon cooling this change in state is reversed. Cereal proteins exist in an amorphous, metastable state that is sensitive to changes in moisture and temperature (Kokini, Cocero, Madeka & De Gaaf, 1994). Schober et al. (2010), Schober et al. (2008) and Lawton (1992) found that by raising zein, the maize prolamin, above its glass transition temperature of 40°C, the protein could be changed from a glassy state to a viscoelastic rubbery state. These authors showed that zein and zein in a model dough system with maize starch could form viscoelastic dough above its glass transition temperature. This leads to the ability to produce gluten-free bread from true dough systems rather than batters (Schober et al., 2011). Bugusu et al. (2001) added zein above its T_g to sorghum-wheat composite flour, which improved its rheological properties and the bread-making quality of the dough. Many similarities exist between the non-wheat cereal proteins zein and kafirin. The subclasses of these prolamins have similar molecular weights, composition, solubility and structure (Belton, Delgadillo, Halford & Shewry, 2006), which suggest that they could have similar functional properties when heated (Schober et al., 2011; Belton et al., 2006).

Hypothesis 2:

The addition of dilute organic acids, such as lactic acid and acetic acid (which are produced during sourdough fermentations), to non-wheat cereal proteins (zein, kafirin and rice protein) that have been heated above their glass transition temperature (T_g) will improve the rheological properties (strength, viscosity, extensibility and visco-elasticity) of the relevant non-wheat prolamin doughs.

Oom et al. (2008) maintained that acids could be used as plasticizers. The uptake of such plasticizers promotes the glass transition in cereal proteins (Bugusu et al., 2001), which promotes the formation of a viscoelastic rubbery state. Lawton (1992) found that dibutyl tartrate, an acidic organic salt, improves dough extensibility and produces dough that is similar in appearance to dough made from wheat flour. The acidic environment provided by the organic acids could also increase protein solubility (Maher Galal, Varriano-Marston & Johnson, 1978), which would promote an elongated protein structure that could be capable of forming a cohesive protein network.

Hypothesis 3:

Non-wheat cereal protein dough (zein, kafirin and rice protein) improved by the addition of dilute organic acids will be capable of retaining the gases produced during the bread-making process.

According to Katina et al. (2005) the incorporation of sourdough into a wheat bread system causes a significant increase in loaf volume. However, in a study by Moore et al. (2007) on using lactic acid bacteria to improve the properties of gluten-free breads, the sourdough did not increase the volume, owing to the lack of a gluten network. Lawton (1992), Schober et al. (2008) and Andersson et al. (2011) described gluten-like zein strands forming a network in the zein-starch dough. This network contributes to the physical stability of the dough on a macroscopic level (Schober et al., 2008) and its ability to retain gas bubbles. Sourdough acidification can positively impact on the structure-forming components of dough such as proteins (Arendt et al., 2007).

This could lead to an improved ability of the non-wheat prolamins' protein network to retain gas and produce leavened gluten-free bread.

3.2 Objectives

Objective 1:

To determine the effect of wet heat (raising the proteins above their glass transition temperatures, T_g) on the aggregation and cohesiveness of non-wheat cereal proteins such as zein, kafirin and rice proteins.

Objective 2:

To determine the effect of dilute organic acid addition, in various concentrations, on the aggregation and cohesiveness, and tensile properties of non-wheat cereal protein-based doughs made from zein, kafirin and rie protein.

Objective 3:

To determine the properties of non-wheat cereal protein-containing (zein, kafirin and rice protein) doughs made with dilute organic acids for use in leavened breads.

4. RESEARCH

The following research is written in the style of the scientific journal Chereal Chemistry. Figure 4.1 is a flow diagram of the experimental design.

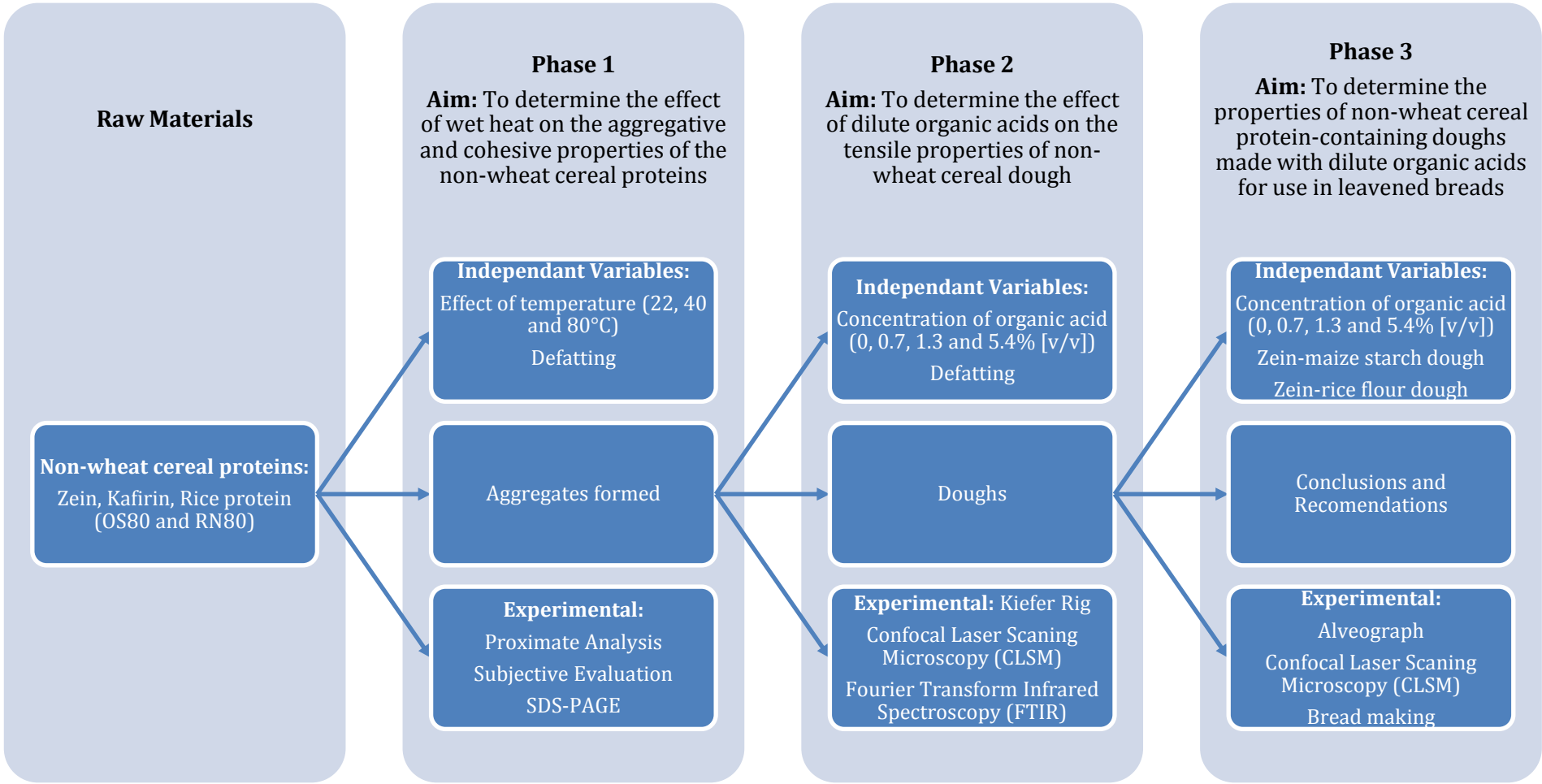

Figure 4.1: Experimental design employed to determine the effect of organic acids on the rheological properties of non-wheat cereal proteins and their use in gluten-free dough formulation.

IMPROVEMENT OF ZEIN DOUGH CHARACTERISTICS USING DILUTE ORGANIC ACIDS

ABSTRACT

The replacement of gluten in bread poses a major a technological challenge for food scientists. Breads lacking gluten have low volumes, poor crumb structure and lack consumer appeal. This investigation showed that the addition of organic acids (acetic acid and lactic acid) in increasing concentrations (0, 0.7, 1.3 and 5.4% [v/v]) dramatically increased the extensibility of zein dough above its glass transition temperature (40°C), and gave a structural element to starch and rice-flour dough formulations. CLSM revealed a protein fibril network, which became finer and more homogenous as the concentration of acid was increased. Alveography showed that low concentrations (0.7%) of organic acids improved the gas retention of zein-based dough. However, there was a limit to this improved dough functionality. High concentrations of organic acids (5.4%) created significantly softer dough with a decreased stability and gas-holding ability. The effect of this improved functionality was not evident in the baked breads due to the lack of an acid-tolerant leavening agent, which meant that the breads did not rise. SDS-PAGE showed that no oligomerization had taken place in the protein structure as a result of the acid addition. However, FTIR analysis indicated that organic acids, which promoted the formation of α-helices, decreased the β-sheet content of the protein. Partial solubilisation of protein by organic acids is believed to contribute to improved functionality of the zein dough; the positive charge created by an acidic environment is likely to have promoted an unfolding of the zein's secondary structure into an elongated form. Organic acids could also have a plasticizing effect, promoting the fluidity of the zein polymer. Thus, zein doughs prepared with low levels of organic acids show much potential for use as a gluten alternative in gluten-free bread formulations.

4.1 INTRODUCTION

The development of high quality gluten-free breads poses a major technological challenge to food scientists (Gallagher et al., 2004). The manufacture of such breads is challenging in that gluten, the main structure-forming protein in wheat dough, is responsible for the elastic and extensible properties characteristic of the dough (Moore et al., 2004). Without gluten as a structural component, quality leavened bread is difficult to produce.

Viscoelasticity is the critical attribute of gluten that allows the capture of carbon dioxide produced during dough fermentation (Erickson et al., 2011). Zein can display viscoelastic functionality that is similar to gluten when it is heated above its glass transition temperature (T_g) (Lawton 1992; Schober et al., 2008; Schober et al., 2010). In a hydrated dough system this temperature is approximately 35°C (Lawton 1992), however, Schober et al. (2008) used a temperature of 40°C in their experiments to obtain viscoelasticity. This difference in temperature could be due to the relative amount of water present in the system, which is known to act as a plasticizer and reduce T_g (Lawton, 1992). Although achievement of such viscoelastic functionality has been explored exclusively within zein systems (Schober et al., 2008; Andersson et al., 2011), there is minimal evidence as to whether this phenomenon could take place in other non-wheat cereal protein systems. However, similarities in the composition of the prolamins zein and kafirin suggest that it could (Belton et al., 2006; Oom et al., 2008; Schober et al., 2011).

The incorporation of sourdough into a wheat bread system causes a significant increase in loaf volume (Katina et al., 2005). However, in gluten-free breads, sourdough fermentation does not improve the volume of the baked breads, probably due to the lack of a protein network (Moore et al., 2007). The absence of this network gives gluten-free formulations a batter-like consistency with little structure (Erickson et al., 2011). However, a fibrous protein network is characteristic of viscoelastic zein dough (Lawton 1992; Schober et al., 2008; Andersson et al., 2011). This network displays similar characteristics to that of gluten dough, but it has a limited ability to retain gas (Schober et al., 2008) and is significantly more extensible (Schober et al., 2010). In order to create high quality zein-based bread, hydrocolloids such as

hydroxypropyl methylcellulose (HPMC) are required to stabilize the fibrous zein structure in the dough (Schober et al., 2008; Schober et al., 2010; Andersson et al., 2011).

Thus far, no systematic studies have been reported on the effect of acidic substances on the rheological properties of dough and the quality of the final fresh, gluten-free bread (Blanco et al., 2011). A limited modification of zein by chemical treatment, such that the protein molecules are partially unfolded, is likely to improve its functional properties (Zhang, Luo & Wang, 2011). Thus, the addition of organic acids to gluten-free dough containing a fibrous protein network, such as seen in zein dough, could improve the rheological properties of the dough. However, uncontrolled modification could lead to reduced functionality (Chiue, Iwami, Kusano & Ibuki, 1994; Chiue, Kusano & Iwami, 1997) due to fragmentation and shortening of the zein backbone (Zhu, Chen, Tang & Xiong, 2008).

Studying changes in zein dough rheology and structure under acidic conditions created by dilute organic acids should provide fundamental knowledge for predicting its properties and facilitating its application development for use in novel, non-wheat cereal, gluten-free dough systems.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The following protein samples were used in this investigation. Commercial zein from maize was obtained from Sigma-Aldrich, Johannesburg, South Africa (Sigma product code Z3625), unless stated all zein used was non-defatted. Kafirin was extracted from decorticated white tan-plant, non-tannin sorghum grain. The extraction method followed was that of Emmambux and Taylor (2003). Kafirin was extracted with 70% (w/w) absolute ethanol, 0.5% (w/w) sodium metabisulphite and 0.35% sodium hydroxide at 70°C for 1 hr. Two rice protein samples, Oryzatein Silk 80 (OS80) from Axiom Foods Inc. Los Angeles CA, and Remypro N80+ (RN80) from Beneo-Remy NY, Belgium were kindly donated by Daisy Health Foods, Johannesburg, South Africa. Kafirin microparticles and the residual kafirin (kafirin without γ-kafirin) were prepared as described by Taylor, Taylor, Belton and Minnaar (2009a) and Taylor, Bean, Ioerger and Taylor (2007), respectively.

Commercial maize starch (MAIZENA) and commercial wheat flour (SnowFlake - Strong Bread flour) were obtained from the outlet store Pick ‘n Pay, Hatfield, South Africa. Commercial rice flour (Entice 100% Pure Rice Flour, Daisy Health Foods) was kindly donated by Daisy Health Foods.

4.2.2 Methods

4.2.2.1 Proximate Analysis

Moisture, ash, fat and crude protein contents of the proteins were determined essentially according to the Approved Methods: 44-15A, 08-17, 30-25 and 46-19, respectively, of the American Association of Cereal Chemists (AACC, 2000). Moisture was determined by the loss in weight of the samples after drying for 3 h at 103°C. Ash was determined after ashing in a muffle oven at 550°C. Total fat was determined by the Soxhlet method, using petroleum ether (boiling point 40–60°C) to extract the fat. Crude protein (N × 6.25) was determined according to a Dumas

procedure. For wheat flour a conversion factor of N x 5.7 was used.

4.2.2.2 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

The different protein preparations used were characterized by SDS-PAGE under reducing and non-reducing conditions using the method described by Byaruhanga, Emmambux, Belton, Wellner, Ng and Taylor (2006). A vertical electrophoresis system (XCell SureLockTM Mini-Cell, Version H, Invitrogen, Carlsbad, CA) with a 4 to 12% polyacrylamide gradient gel (NuPAGE® Novex Gels, Invitrogen) measuring 8 cm x 8 cm and 1.0 mm thick with 15 wells was used. Samples, calculated to contain 5 mg protein, were placed in 3 ml plastic microfuge tubes together with a glass bead (to facilitate mixing). After adding 1.5 ml buffer (either reducing or non-reducing), the tubes were capped and the contents mixed on a vortex mixer. The sample tubes were then placed in a boiling water bath for 15 min, vortexing every 5 min. After removal of the glass beads, sample tubes centrifuged at 7 200 g for 5 min. Samples were then further diluted with buffer to give a final concentration of 1 µg protein/µl. Loading on to the gels was 10 µl for protein samples and 5 µl for the molecular weight standard. The electrophoresis was carried out at a constant voltage of 200 V, 80 mA and at 10 Watts/per gel (20 W / 2 gels) for 1 hr. The protein gels were stained with Coomassie Brilliant Blue R-250 overnight. After de-staining they were scanned on a flatbed scanner.

4.2.2.3 Aggregation and cohesiveness of the non-wheat cereal proteins

Analyses of aggregation and cohesiveness of the doughs was based on the methods of Schober et al., (2010). The tendency of zein, kafirin, and rice proteins to aggregate and form a dough-like substance in an aqueous environment was tested by short intense mixing with water at a range of temperatures (22°C, 40ºC, 80ºC respectively). To do this, 0.5 g protein preparation was warmed to the desired temperature and 1.875 g pre-warmed distilled water added. The suspension was vortexed at high speed for 5

s. Any doughs formed were removed from the excess water and were hand kneaded for 30 s, after which they were stretched and photographed. The same procedure was repeated using samples that had been defatted. They were defatted three times for one-hour intervals with n-hexane, one part protein: three parts n-hexane ratio, at ambient temperatures, and were then dried overnight under a fume hood at ambient temperature.

The protein samples that exhibited dough-like properties were then used to characterize the effects of dilute acetic and lactic acids (0.7%, 1.3% and 5.4% [v/v]) on the development of those dough-like properties. Acid concentrations were based on a 5.4% acetic acid solution in which the kafirin microparticles had been storred. The aggregates were prepared as before, except instead of using 1.875 g pre-warmed distilled water, solutions of organic acids acetic acid and lactic acid, in varying concentrations, were used. The doughs formed were removed from the excess acids and were hand kneaded for 30 s, after which they were stretched and photographed.

4.2.2.4. Tensile Properties

4.2.2.4.1 Extensibility and rheological properties

The tensile properties of the zein aggregates before and after treatment with organic acids were evaluated using a Kieffer rig (Abang Zaidel, Chin, Abdul Rahman & Karim, 2008), mounted on a TA-XT2 texture analyzer (Stable Micro Systems, Godalming, UK).

Zein was subjected to short intense mixing with water at 40°C as described above. The aggregates formed were removed from the excess water or acid solution, kneaded by hand for 30 s, and pressed into a cylindrical, longitudinally split rubber tube mould (70 mm long and 8 mm in diameter) to obtain a uniform size and shape. The moulded samples were placed over the vertical struts (30 mm apart) of the Kieffer rig and clamped in place at both ends. Samples were extended by means of a hook centred over the sample at a constant rate of 3.3 mm/s over a distance of 150 mm. This was the maximum hook displacement possible on the instrument. The test was performed

at ambient temperature (22ºC) and within 2 min, to prevent samples from cooling below their glass transition temperature. Samples were extended until they were no longer able to retain their original shape and broke, or until the maximum hook displacement was reached. The force over distance was recorded, as well as the peak force (N), extensibility until rupture (mm) and area under the curve (N × mm). From this information extensibility and rheological parameters were determined using formulae according to Abang Zaidel et al. (2008) and are stated in section 4.2.2.4.2. The above procedures were repeated using lactic acid and acetic acid solutions (0, 0.7, 1.3 and 5.4% [v/v]) in place of distilled water.

A similar approach was used to evaluate defatted zein as well as a zein-starch mixture (1:4) (w/w) that had been pre-warmed to 40ºC. To allow for better kneading of the zein-starch mixture, the quantities of ingredients used were increased; 1 g protein was prepared with 4 g maize starch and 4.4 g liquid. A larger cylindrical, longitudinally split rubber tube mould (70 mm long and 15 mm in diameter) was used to obtain a uniform size and shape. After an initial kneading for 5 min the samples were allowed to rest in an incubator at 40ºC for 2 min, after which a second kneading for 1 min was performed before the tensile tests were conducted. It was found that a second kneading step allowed for the proper hydration of the mixture and resulted in a better dough formation.

4.2.2.4.2 Derivation of extensibility and rheological parameters

Extensibility parameters were calculated using the following formulae adapted from Abang Zaidel et al. (2008). *Equation 1* was used to determine the original length of the dough (l_o) before being extended from its original position (y_o). In this case d, the distance between the vertical struts, was 30 mm. The length of the dough (l_t) and the hook displacement (y_t) at the point of fracture were calculated using *Equation 2*. The measured force (F_m) on the hook placed in the centre of the dough cylinder was divided equally over the dough lengths stretched on either side of the hook. The actual force (F_a) that acted upon the stretched dough was determined using *Equation 3*, while *Equation 4* shows the expression of the angle of deformation (α) in terms of the measured and actual force that acted upon the dough.

Equation 1: $l_o = 2\sqrt{[(d/2)^2 + (y_o)^2]}$

Equation 2: $l_t = 2\sqrt{[(d/2)^2 + (y_o + y_t)^2]}$

Equation 3: $Fa = F_m.l_t/4(y_t + y_o)$

Equation 4: $\sin \alpha = (F_m/2)/F_a = (y_t + y_o)/ (l_t/2)$

The extensibility parameters were used to determine the rheological parameters of strain, strain rate and stress. The Hencky strain (ε_H) acting on the dough was calculated using *Equation 5*. In *Equation 6*, the strain rate was calculated by differentiation of the Hencky strain with time, where v is the hook speed. The final cross-sectional area of dough (A_t) was calculated by assuming the volume of dough was constant throughout the test (*Equation 7*). The stress (σ) acting on the dough was calculated by dividing the actual force (F_a) by the final cross-sectional area of the dough cylinder (A_t), *Equation 8*. Extensional viscosity was calculated by dividing the stress experienced by the strain rate, *Equation 9*.

Equation 5: $\varepsilon_H = \ln (l_t/l_o) = [2\sqrt{[(d/2)^2 + (y_o + y_t)^2]}]/ [2\sqrt{[(d/2)^2 + (y_o)^2]}]$

Equation 6: $\dot{\varepsilon} = 4v(y_t + y_o)/ l_t^2$

Equation 7: $A_t = A_o l_o/ l_t$

Equation 8: $\sigma = F_a/A_t$

Equation 9: $\eta_E = \dot{\varepsilon} / \sigma$

By using these formulae the following parameters could be determined: peak force (N) at fracture (or at maximum force applied if the sample did not fracture), sample extension (mm), peak stress (kPa) at fracture (or at maximum force applied if the sample did not fracture), percentage strain and Hencky strain at fracture (or at maximum force applied if the sample did not fracture), extensional viscosity (kPa.s), Young's Modulus (kPa) and area under the curve (N.mm).

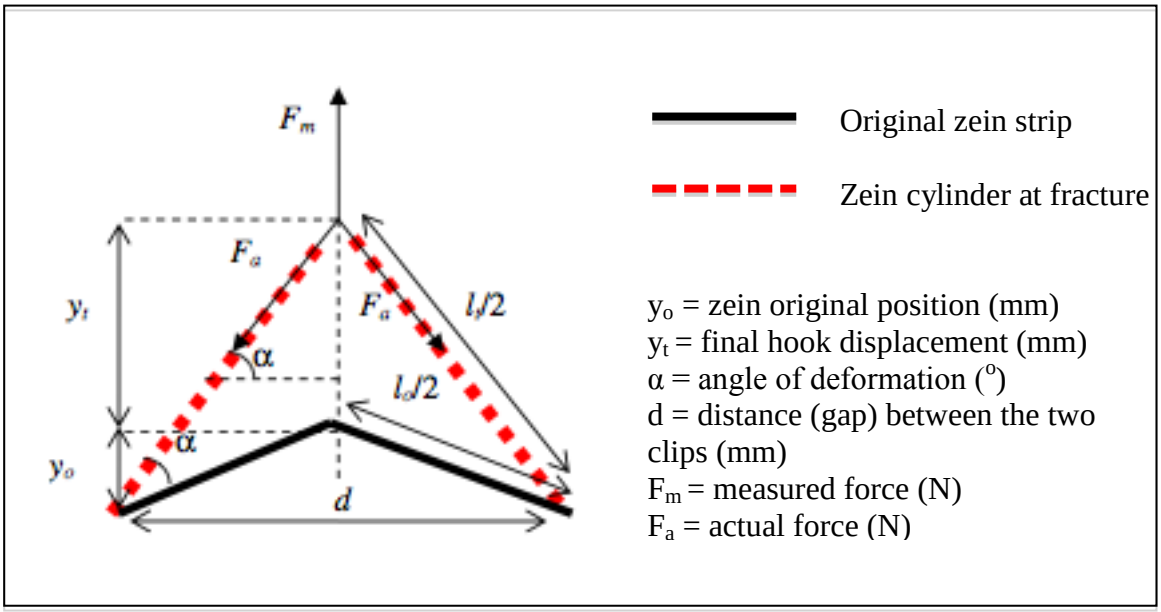

Figure 4.2: Schematic diagram of forces acting on zein dough cylinders and changes in length of zein dough cylinders during tensile tests using a Kieffer rig (adapted from Abang Zaidel et al., 2008).

4.2.2.5 Hydration properties of zein dough

Zein was subjected to short intense mixing with 5.6 g pre-warmed distilled water at 40°C, as described in 4.2.2.3. The protein aggregates were then gently patted dry with paper towel, to remove excess moisture, and subjected to a moisture analysis as previously described in 4.2.2.1. This procedure was repeated with zein prepared using 0.7, 1.3 and 5.4% (v/v) acetic acid and lactic acid.

4.2.2.6 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy was performed as described by Taylor, Taylor, Belton and Minnaar (2009b) to detect similarities and differences in the secondary structure of zein doughs prepared with varying concentrations of dilute organic acids at 40°C. The analysis was performed on freshly prepared zein dough. Samples were scanned in a Vertex 70v FTIR spectrophotometer (Bruker Optik, Ettlingen, Germany), using 64 scans, an 8 cm^{-1} band, and an interval of 1 cm^{-1} in the attenuated total reflectance (ATR) mode at a wave number 400-4000 cm^{-1}. At least four replicates were

performed for each treatment. The FTIR spectra were Fourier-de-convoluted with a resolution enhancement factor of 2 and a bandwidth of 12 cm^{-1}. There was a maximum 30 s delay from the moment the samples were taken out of the mixer to the beginning of the FTIR scan; the latter was considered the zero time point. The proportions of the α-helical conformations (≈ 1650 cm^{-1}) and the β-sheet structures (≈1620 cm^{-1}) were calculated as described by Anyango, (2013), where the heights of the peaks assigned to these secondary structures on the FTIR spectra were measured. The relative proportion of α-helical conformations was calculated using the following formula:

$$\% \text{ of } \alpha\text{-helical conformation} = \frac{\text{Abs } \alpha\text{-helix peak}}{\text{Abs } \alpha\text{-helix peak} + \text{Abs } \beta\text{-sheet peak}} \times 100$$

Where:

Abs α-helix peak = Absorbance at ≈ 1650 cm^{-1} after baseline correction

Abs β-sheet peak = Absorbance at ≈ 1620 cm^{-1} after baseline correction

4.2.2.7 Confocal Laser Scanning Microscopy (CLSM)

Protein autofluorescence was analysed by Confocal Laser Scanning Microscopy (CLSM, Zeiss 510 META, Jena, Germany) using a 488 nm excitation and the Plan-Neofluar 10x0.3 objective. The natural autofluorescence of the zein is strong enough to examine the microstructure of the protein without addition of fluorochromes. Dough samples were prepared as previously described. Both protein and protein-rice flour mixtures (1:4 ratio) were created. Once prepared, the doughs were stretched out as thinly as possible (15 mm x 5 mm x 1 mm) over a glass slide. For the samples that could not be stretched and the unstretched dough samples a very small piece of dough (5 mm x 5 mm x 2 mm) was cut and placed onto the slides. Each slide was prepared within 2 min using freshly made dough.

4.2.2.8 Alveography

An Alveograph (Chopin NG Consistograph, Paris, France) was used to analyse the bread-making quality of doughs made from zein-starch (1:4) and zein-rice flour mixtures (1:4) that contained different concentration of organic acids (0, 0.7, 1.3 and 5.4% v/v lactic acid and acetic acid). The tests were performed using the ICC method 121 (ICC, 1992), in conjunction with the Alveograph NG Consistograph instructional manual (Chopin, 2010). To make the flour mixtures, 50 g zein was added to 200 g maize starch or rice flour and the combination thoroughly mixed using an electric mixer for 5 min. The flour was then placed in a glass beaker, covered with parafilm, and warmed in a water bath to 50ºC for 1 h. The higher (compared to previous experiments) temperature of 50ºC was used to counter for any temperature losses that might occur during processing. Pre-warmed distilled water (200 g) was slowly added to the flour until a smooth homogenous dough was formed. The preformed dough was then kneaded for 8 min, formed into round patties and allowed to rest for 20 min before inflation took place. Dough kneading and resting was conducted at a temperature of 35ºC (the highest instrument setting). The pressure variation inside the bubbles was recorded on an Alveogram showing the dough resistance to extension (P, mm H_2O) and extensibility (L, mm). The curve configuration Ratio (P/L) was also determined as well as the deformation energy (W, J x 10^{-4}); this is the energy required to rupture the test piece.

4.2.2.9 Bread Making

Bread was made using two different leavening agents, yeast and baking powder, and followed a basic bread-making procedure adapted from Schober et al. (2008). The first step involved pre-mixing the dry ingredients (10 g protein, 40 g maize starch or rice flour, 2.5 g sugar, 1 g table salt and 1 g baking powder or instant dried yeast). The dry ingredients were then pre-warmed to 40°C for 1 h in a water bath. Liquid (distilled water or acid solution), 45 g at 40°C, was added to the dry ingredients. This was followed by manual mixing at 40°C (in a beaker sitting in a water bath) with the help of a spatula (that was warmed to 40°C). The dough was then manually kneaded on a baking tray (warmed to 40°C) until visibly smooth and homogenous. Doughs

containing the yeast were allowed to prove for 30 min before a second kneading and proving stage. The dough was then placed in a greased steel bread pan (top 8.5 × 5 cm, bottom 7.5 × 4 cm; depth 3 cm) and baked at 160°C for 20 min in a convection oven. After baking, the breads were allowed to cool for 1 hr. A subjective evaluation by observation was performed on the bread volume, crumb structure and texture. The crumb grain of both sides of the three most central 1 cm slices was recorded using a flatbed scanner.

4.2.2.10 Statistical Analysis

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) was performed. Means were compared at $p = 0.05$ using Fischer's Least Significant Difference Test (LSD).

4.3 RESULTS AND DISCUSSION

4.3.1 Proximate Composition

The moisture contents of both the rice proteins, significantly ($p<0.05$) higher than that of the zein and kafirin. The crude protein content of the different cereal protein preparations varied as shown in Table 4.1. Commercial zein and rice protein RN80 had the highest crude protein (87.8% and 87.0%, respectively) and kafirin had the lowest (68.9%). The low protein content of kafirin can probably be attributed to the presence of other materials such as carbohydrates, non-starch polysaccharides, fat and ash. Da Silva and Taylor (2004) and Pretorius (2008) found similar compositions for kafirin. The fat contents of the two commercial grades of rice protein concentrates did differ significantly but both values were nevertheless low.

Table 4.1: Proximate composition[1] of different non-wheat cereal proteins

Sample	Moisture (g /100 g)	Crude Protein (g / 100 g)	Ash (g /100 g)	Fat (g /100 g)
Zein	$4.91^{a} \pm 0.03$	$83.5^{d} \pm 0.2$ ($87.8^{f} \pm 0.2$)	$2.1^{a} \pm 0.0$ ($2.2^{a} \pm 0.0$)	$2.3^{c} \pm 0.2$ ($2.4^{c} \pm 0.2$)
Kafirin	$5.23^{a} \pm 0.31$	$65.3^{a} \pm 0.8$ ($68.9^{b} \pm 0.8$)	$17.6^{b} \pm 3.4$ ($18.6^{b} \pm 3.6$)	$3.6^{d} \pm 0.2$ ($3.8^{d} \pm 0.2$)
Rice Protein (OS80)	$7.91^{c} \pm 0.05$	$77.9^{c} \pm 0.0$ ($84.6^{e} \pm 0.0$)	$2.1^{a} \pm 0.0$ ($2.2^{a} \pm 0.0$)	$1.4^{b} \pm 0.4$ ($1.5^{b} \pm 0.4$)
Rice Protein (RN80)	$7.34^{b} \pm 0.12$	$80.6^{d} \pm 0.0$ ($87.0^{f} \pm 0.0$)	$1.3^{a} \pm 0.0$ ($1.4^{a} \pm 0.0$)	$0.4^{a} \pm 0.1$ ($0.4^{a} \pm 0.1$)

[1] Mean ± Standard Deviation of three replicates

( ) Values within brackets represent Dry Matter Basis

^{abc} numbers in columns with different superscript letters differ significantly ($p< 0.05$)

Rice flour is one of the most popular flours used in gluten-free baking (Marco & Rosell, 2008b). It does not impart negative effects on product quality and it has good attributes such as bland taste, white colour, ease of digestion and it possesses hypoallergenic properties (Blanco et al., 2011). The protein content of the rice flour

used in this study was significantly lower than that of wheat flour (Table 4.2). The protein content of maize starch was extremely low; this makes it an ideal medium to reflect the development and effect of protein networks in protein-starch doughs. The fat content of the flours was very low and did not differ significantly ($p \geq 0.05$).

Table 4.2: Proximate Composition[1] of rice and wheat flour samples, and maize starch

Sample	Moisture (g /100 g)	Crude protein (g / 100 g)	Ash (g /100 g)	Fat (g /100 g)
Rice Flour	$14.6^{b} \pm 0.1$	$7.2^{b} \pm 0.3$ ($8.4^{c} \pm 0.3$)	$0.44^{b} \pm 0.00$ ($0.52^{c} \pm 0.00$)	$1.03^{a} \pm 0.43$ ($1.20^{a} \pm 0.50$)
Wheat Flour*	$12.3^{a} \pm 0.1$	$12.5^{d} \pm 0.2$ ($14.3^{e} \pm 0.2$)	$0.51^{c} \pm 0.01$ ($0.59^{d} \pm 0.01$)	$0.98^{a} \pm 0.90$ ($1.11^{a} \pm 1.03$)
Maize starch	$11.7^{a} \pm 0.0$	$0.3^{a} \pm 0.2$ ($0.3^{a} \pm 0.2$)	$0.06^{a} \pm 0.01$ ($0.07^{a} \pm 0.01$)	$0.40^{a} \pm 0.20$ ($0.45^{a} \pm 0.23$)

[1] Mean ± Standard Deviation of three replicates

( ) Values within brackets represent values on a Dry Matter Basis

^{abc} numbers in columns with different superscript letters differ significantly ($p < 0.05$)
* a correction factor of N x 5.7 was used to calculate the crude protein content of wheat flour.

4.3.2 SDS-PAGE of the non-wheat cereal proteins

The different proteins exhibited different banding patterns (Figure 4.3). The monomer and dimer bands of the zein and kafirin (Oom et al., 2008) are visible in lanes B and lane C, respectively. Under reducing conditions the intensity of the bands increased. This is due to the breakage of disulphide bonds, which results in a lesser degree of polymerization (Emmambux & Taylor, 2009). A high level of polymerization could prevent hydration and, instead of forming an aggregate with soft dough-like properties, a hard, non-pliable, solid lump would be formed. Bonet et al. (2006) found that excessive crosslinking by glucose oxidase lead to decreased dough quality due to excessive strengthening of the protein network in wheat dough. A high level of polymerization is evident in the rice protein, OS80 as the large polymers of this protein were too big to filter through the polyacrylamide gel under non-reducing conditions.

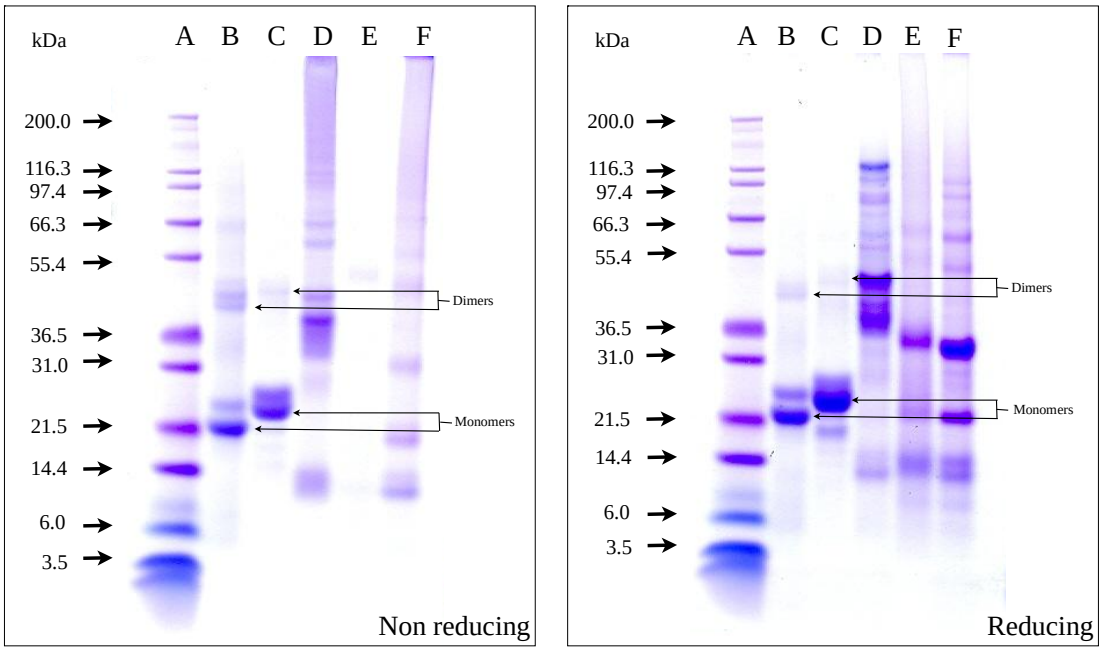

Figure 4.3: SDS-PAGE of the non-wheat cereal protein preparations. **A**: Molecular weight standards, **B**: Zein, **C**: Kafirin, **D**: Gluten, **E**: Rice protein (OS80), **F**: Rice protein (RN80).

4.3.3 EFFECT OF WET HEAT ON THE AGGREGATIVE AND COHESIVE PROPERTIES OF CEREAL PROTEINS

At ambient temperature (22ºC) there was no change in the physical properties of any of the proteins (zein, kafirin, rice proteins, kafirin microparticles, kafirin microparticles washed in distilled water and acetic acid, and residual kafirin) and none of the proteins formed aggregates upon agitation by vortex mixer (Figure 4.4, Table 4.3). Out of all the proteins tested, only the zein exhibited any change in its physical properties when there was an increase in the water temperature to 40°C and to 80°C. At these higher temperatures zein changed from a grainy dry material to a substance with a rubbery, dough-like consistency. At the higher temperature of 80°C a softer aggregate was formed. With all other proteins, only a slurry was evident after the wet heat treatments.

The change in the physical properties of zein can be explained by the protein having being raised above its glass transition temperature (T_g). As stated, for amorphous polymers, or amorphous regions within semi-crystalline materials, the glass transition

temperature is a critical temperature at which the material's mechanical properties undergo a dramatic change (Bugusu et al., 2001; Panchapakesan et al., 2012). This reversible transition is characterized by a change in state from hard and relatively brittle to molten or rubbery. This glass transition in cereal prolamins is promoted by the addition of heat as well as the uptake of plasticizers (Bugusu et al., 2001). Even at a relatively high moisture content, the T_g of zein is above room temperature (Lawton, 1992). The T_g of zein lies within the temperature range of 30 to 40°C (Schober et al., 2011). This explains the presence of elastic, rubbery dough-like properties when there was an increase in temperature from 25ºC to 40ºC and to 80ºC. Other authors including Lawton (1992), Schober et al. (2010) and Kokini et al. (1994) have seen this type of behaviour in zein at 40°C.

Defatted zein seemed to produce a much softer and smoother aggregate compared to its non-defatted counterpart (Table 4.3). Schober et al. (2010) hypothesized that non-defatted zein particles have difficulty absorbing water and that a thin layer of surface lipids might hamper the protein-protein interactions between the zein particles. Thus, through the removal of the lipids the aggregation and cohesiveness of zein is improved, which would lead to better dough-forming properties.

When the zein aggregates were cooled to below 22ºC they became brittle and stiff and no longer exhibited any elastic behaviour (Table 4.3). This is due to the reversibility of the T_g from a rubbery to a glassy state. Bugusu et al. (2001) state that below the T_g polymers have a low molecular mobility and diffusivity and thus the material is in a rigid glassy state. Panchapakesan et al. (2012) state that when zein is below its T_g, the polymer chain is incapable of diffusing within a random matrix due to intermolecular interactions fixing the molecules in their positions. Thus, below the T_g polymer chains are less flexible and have a rigid structure.

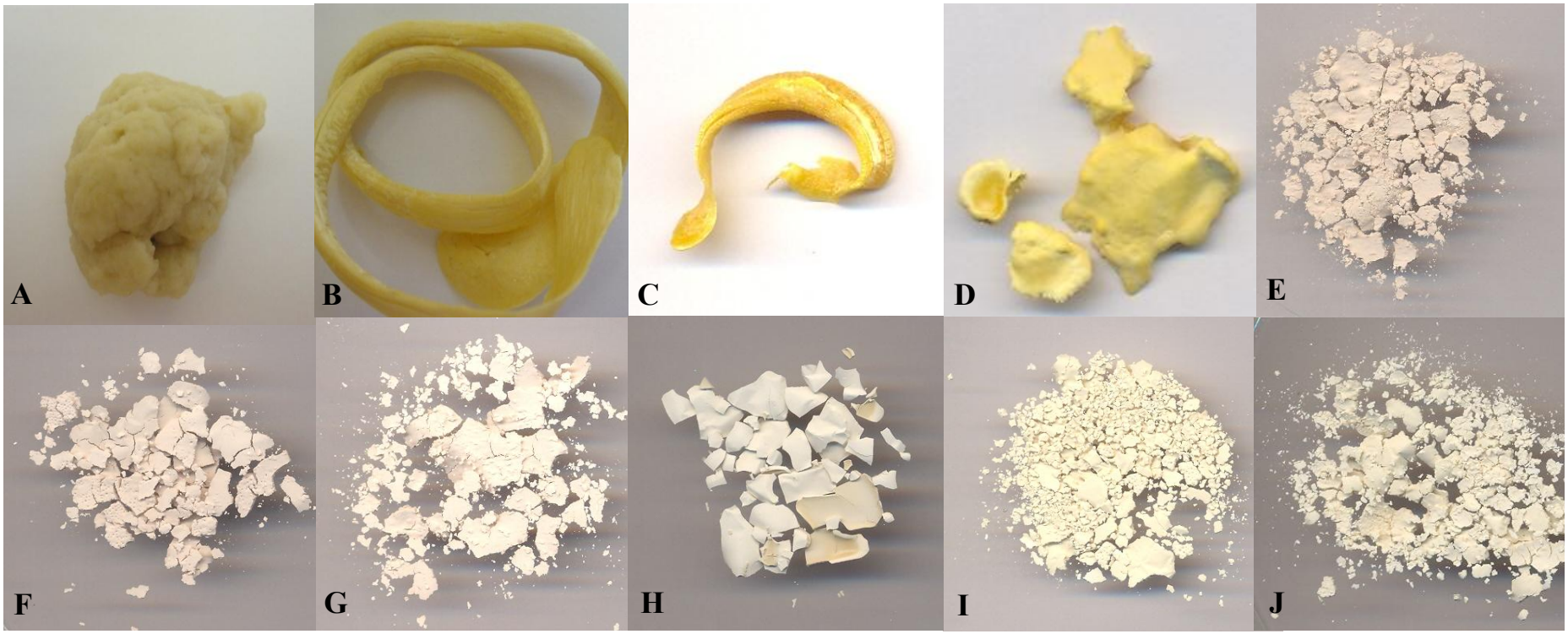

Figure 4.4: The general appearance of cereal proteins after water treatment. **A**: Gluten dough. **B**: Freshly prepared zein dough at 40°C. **C**: Dried zein dough after preparation at 40°C. **D**: Dried zein slurry after preparation at 22°C. **E**: Dried kafirin slurry (22, 40 and 80°C) **F**: Dried kafirin microparticles washed in distilled water (22, 40 and 80°C). **G**: Dried kafirin microparticles in acetic acid (22, 40 and 80°C). **H**: Dried residual kafirin (22, 40 and 80°C). **I**: Dried rice protein OS80 (22, 40 and 80°C). **J**: Dried rice protein RN80 (22, 40 and 80°C).

Kafirin did not aggregate to form dough even at 80°C. Neither did the defatting process lead to any aggregative properties. Due to the many similarities between kafirin and zein (Belton et al., 2006) it had been postulated that they would behave in similar ways when heated above their T_g, and thus demonstrate similar functional properties. As reviewed by Bugusu et al. (2001), several studies on zein and kafirin have shown that they are analogous in a number of ways, including electrophoretic banding patterns, extractability, immune-specificity, and amino acid composition. Their subclasses of α-, β-, and γ- prolamins are also similar with regard to molecular weight, composition, solubility and structure (Belton et al., 2006).

A reason for the kafirin failing to aggregate could be the presence of the γ-kafirin subclass. Gamma-kafirin is rich in cysteine residues, and these can form disulphide crosslinks (Duodu, Taylor, Belton & Hamaker, 2003). These links could prevent binding of the proteins, which in turn would inhibit dough development (Oom et al., 2008). Highly polymerized proteins could also prevent adequate hydration, and although they would improve dough strength they would have minimal dough-like properties. SDS-PAGE conducted by Oom et al. (2008) showed that kafirin, similar to that used in this present work, contained both α-kafirin and γ-kafirin fractions, and that commercial zein contained mainly α-zein. The absence of the γ-fraction could explain why the zein aggregate was formed. However, in this present work, when the experiment was repeated using the residual kafirin, kafirin that had at least most of its γ-kafirin fraction extracted, still no aggregates formed. Oom et al. (2008) investigated whether viscoelastic functionality of kafirin could be achieved by mixing the prolamin above its T_g. These authors were able to produce resin films of kafirin with rheological properties similar to those of gluten by means of plasticization with oleic acid. However, viscoelastic dough could not be formed upon hydration and mixing with starch above the kafirin's T_g. These authors speculated that kafirin's inability to form viscoelastic dough when raised above its T_g is due to its high hydrophobicity, which resulted in inadequate hydration and plasticization by water. It is probably due to this high hydrophobicity that even the residual kafirin did not form an aggregate.

Kafirin microparticles were investigated because Van Riemsdijk et al. (2011a)

hypothesized that several gluten properties originate from a particle structure present in the gluten network. These authors produced elastic dough using a structured whey protein particle suspension. To ensure that the proteins were adequately hydrated, a liquid/colloidal kafirin microparticle suspension in 5.4% acetic acid solution, as well as microparticles washed in distilled water, were used. It was speculated that the larger surface area provided by the voids present in the microparticles (Anyango et al., 2012) could also improve the aggregation and cohesiveness of the kafirin. Variations in the protein structure may also have a direct impact on the T_g itself; which could also indirectly affect the affinity of the biopolymer for a plasticizing agent such as water (Noel, Parker, Ring & Tatham, 1995), which affects the T_g of an amorphous protein (Kokini et al., 1994). However, aggregates did not form when the kafirin microparticles were subjected to wet heat.

One of the underlying problems that may influence the aggregative and cohesive properties of kafirin could be the method by which the kafirin was extracted. The kafirin used during these experiments was extracted in 70% (w/w) absolute ethanol, 0.5% (w/w) sodium metabisulphite and 0.35% sodium hydroxide at 70°C. Schober et al. (2011) found that crude kafirin, isolated from dry milled sorghum flour using similar extraction techniques did not aggregate in water into a viscoelastic dough over a wide range of temperatures (10, 50, 60, 70 and 85°C). They found that in order to achieve the aggregation of kafirin in warm water the protein needed to be isolated from dry milled sorghum with a more hydrophobic extractant such as 83% isopropanol. However, even when this was done the aggregate that formed became firm and lost its extensibility very quickly. From this work they suggested that hydrophobic interactions rather than disulphide bonds were key to the gluten-like functionality of zein and kafirin.

No viscoelastic dough could be formed when the rice proteins were kneaded with water. As reviewed by Gujral and Rosell (2004), this could be due to rice protein containing very little prolamin (2.5-3.5%). In rice, the major storage proteins are the glutelins (65–85%), while prolamins are the minor fraction (Marco & Rosell, 2008b). These authors suggested that the different ratio of storage proteins in rice compared with that of wheat may be related to the prevention of the development of a viscoelastic network.

Table 4.3: Subjective evaluation of the aggregative and cohesive properties of zein and kafirin at elevated temperatures and after defatting

Sample	22°C	40° C	80° C
Zein	• Protein does not hydrate • A wet powder is formed, not a dough • Breaks/crumbles upon kneading	• Forms small balls immediately once placed onto the vortex mixer • After kneading it resembles chewing gum • Has slight elastic properties • Is extensible and can be stretched. • Becomes brittle upon cooling • Is shiny in appearance • Fibrous strands are visible	• Forms small balls immediately once placed onto the vortex mixer • After kneading it resembles chewing gum • Is very soft, seems as though the hotter the samples the softer they are • Has slight elastic properties • Is extensible and can be stretched. • Gets brittle upon cooling • Fibrous strands are visible
Defatted Zein	• A slurry (just a wet powder) • Finer powder compared to non-defatted zein	• Uniform, smooth texture • Dough appears to be softer after defatting • Has elastic properties • Dough is easily stretched • Becomes brittle upon cooling • Fibrous strands are visible	• Appears to be softer then non-defatted protein. • Is elastic and very extensible • Seems as though the hotter the samples the softer they are • Fibrous strands are visible
Kafirin	• A slurry • Some wet powder in the bottom of the container • Not a dough	• A slurry with some wet powder is formed • Not a dough	• A slurry is formed
Defatted Kafirin	• A slurry is formed • Appears no different to the non-defatted kafirin	• A slurry is formed	• A slurry is formed

4.3.4 EFFECT OF ORGANIC ACIDS ON ZEIN DOUGH

4.3.4.1 Tensile properties of zein dough at 40ºC

When heated above their glass transition temperatures (T_g) all the zein doughs that were formed, with and without added dilute lactic acid or acetic acid, reached the maximum possible extension (270 mm) on the Kieffer rig without breaking (Table 4.4). However, the effect of the organic acids on the dough extensibility could be seen clearly when the doughs were manually stretched (Figure 4.5); the higher the concentration of acid added, the more extensible the doughs became.

The tensile tests revealed that the addition of the dilute organic acids substantially altered the physical properties of the zein doughs. In all cases the addition of the organic acid had a softening effect on the dough; the greater the concentration of acid, the greater the effect. This softening was seen in the maximum force (peak force) that was required to extend the dough. This force decreased as the concentration of the dilute organic acid increased. This was seen for both lactic acid and acetic acid and indicates that the dilute organic acids decreased the doughs' resistance to extension. The resistance to extension (peak force) of the zein doughs prepared with both acids at concentrations of 1.3 and 5.4%, were significantly lower ($p < 0.05$) than that of the control zein dough without acid and at a low acid level of 0.7%.

When the concentration of lactic acid and acetic acid was increased from 0.7 to 5.4%, the peak stress experienced by the doughs during extension was significantly ($p< 0.05$) decreased from 115 to 18 kPa with the lactic acid, and from 112 to 9 kPa with the acetic acid. This same trend was seen for the area under the force displacement curve. When the concentration of acid was increased from 0.7 to 5.4%, the area under the curve decreased from 612 to 118 N.mm and from 547 to 48 N.mm for the lactic acid and acetic acid, respectively. The same was found for the modulus of elasticity (Young's modulus), which decreased from 31.1 to 10.3 kPa and from 30.7 to 15.8 kPa for lactic acid and acetic acid, respectively. This indicates that the elasticity (the ability of the dough to resume its normal shape after being stretched or compressed) and stiffness (rigidity) of the zein dough decreased with organic acid addition.

The addition of the organic acids also greatly influenced the extensional viscosity of the different doughs. The extensional viscosity of zein dough without dilute organic acids was much higher than that of the doughs prepared with dilute organic acids. At a concentration of 0.7% neither the doughs prepared with lactic acid nor the doughs prepared with acetic acid differed significantly ($p \geq 0.05$) from the dough prepared with distilled water. However, dough prepared with no acid had an extensional viscosity of almost 1.5 and 6.7 times higher than the 1.3% and 5.4% lactic acid-containing zein doughs, respectively, and had an extensional viscosity of 1.7 and 13.9 times higher than that of the 1.3% and 5.4% acetic acid-containing doughs, respectively. Thus, without the organic acids the dough was much more firm. This firmness relates to the strength of the dough.

The Hencky strain (ε_H) takes into consideration the strain path of the dough (Abang Zaidel et al., 2008). Dough that has been set up on a Kieffer rig experiences strain in three directions and not just one. The Hencky strain value provides a more accurate measurement of the final strain present when deformation of the dough occurs. However, the percentage strain and Hencky strain (ε_H) of the different samples at maximum hook displacement generally did not differ. Only the 5.4% acetic acid decreased the strain, indicating that very soft dough was formed.

Table 4.4: Tensile properties[1] of zein dough prepared with dilute organic acids, lactic acid and acetic acid

Treatment	Concentration (%)	Peak Force (N)	Extension (mm)	Peak stress (kPa)	Strain at maximum hook displacement (150 mm) (%)	Strain at maximum hook displacement (150 mm) (ε_H)	Extensional Viscosity (η_E, kPa.s)	Young's Modulus (*E*, kPa)	Area under the curve (N.mm)
Distilled Water	**0**	$3.47^{a} \pm 0.43$	$270^{a} \pm 0.0$	$122.8^{a} \pm 10.8$	$1002^{a} \pm 4$	$2.31^{a} \pm 0.00$	$5625^{a} \pm 510$	$29.8^{a} \pm 0.4$	$612^{a} \pm 51$
Lactic acid	**0.7**	$3.25^{a} \pm 0.67$	$270^{a} \pm 0.0$	$114.9^{a} \pm 23.5$	$1000^{a} \pm 6$	$2.30^{a} \pm 0.01$	$5251^{a} \pm 756$	$31.1^{a} \pm 5.4$	$612^{a} \pm 135$
	1.3	$2.40^{b} \pm 0.44$	$270^{a} \pm 0.0$	$84.8^{b} \pm 15.5$	$1003^{a} \pm 2$	$2.31^{a} \pm 0.00$	$3888^{b} \pm 314$	$19.8^{b} \pm 4.0$	$440^{b} \pm 69$
	5.4	$0.52^{c} \pm 0.10$	$270^{a} \pm 0.0$	$18.2^{c} \pm 3.6$	$1002^{a} \pm 2$	$2.31^{a} \pm 0.00$	$834^{c} \pm 165$	$10.3^{c} \pm 7.6$	$118^{c} \pm 18$
Acetic acid	**0.7**	$3.15^{a} \pm 0.40$	$270^{a} \pm 0.0$	$111.6^{a} \pm 14.2$	$1001^{a} \pm 4$	$2.30^{a} \pm 0.00$	$5104^{a} \pm 645$	$30.7^{a} \pm 2.6$	$547^{a} \pm 91$
	1.3	$2.00^{b} \pm 0.14$	$270^{a} \pm 0.0$	$70.9^{b} \pm 4.9$	$996^{a} \pm 6$	$2.30^{a} \pm 0.01$	$3227^{b} \pm 204$	$20.9^{b} \pm 0.9$	$316^{b} \pm 21$
	5.4	$0.25^{c} \pm 0.10$	$270^{a} \pm 0.0$	$9.0^{c} \pm 3.7$	$984^{b} \pm 12$	$2.29^{b} \pm 0.01$	$404^{c} \pm 169$	$15.8^{c} \pm 2.5$	$48^{c} \pm 83$

[1] Mean ± Standard Deviation of three replicates
ε_H = Hencky strain/true strain
^{abc} = numbers in columns with different superscript letters differ significantly ($p < 0.05$)

As stated, none of the zein doughs broke upon extension with the Kieffer rig. The fact that the zein doughs were very extensible (Figure 4.5) confirmed findings by Lawton (1992) who was able to extend zein starch doughs, mixed at 30°C and 35°C, the entire length of an Extensigraph without them breaking. The peak stress usually occurs when the materials break or fracture. However, in this case none of the zein doughs did. Fracture of dough membranes during fermentation and baking restricts the bread volume that can be obtained (Dunnewind, Sliwinski, Grolle & Van Vliet, 2004). Thus, the failure of the zein dough to fracture at high extensions shows promise for its use to improve dough volumes in gluten-free systems.

Oom et al. (2008) found that oleic acid plasticized zein resins. Plasticized melts of zein showed both high extensional viscosity and strain hardening during contractional flow measurements. In this present work, the zein dough prepared with low concentrations of dilute organic acids (0.7%) had high extensional viscosities. As the concentration of dilute organic acid increased (1.3 to 5.4%), the extensional viscosity of the dough decreased. This is not ideal in terms of the bread-making quality of the dough. Oom et al. (2008) maintained that extensional rheology measurements have shown that good bread-making wheat flours have greater extensional viscosity and strain hardening than poor bread-making wheat flours. These authors also found that zein resins had a higher extensional viscosity than gluten resin. Andersson et al. (2011) used hyperbolic contraction flow at 40°C to measure the extensional viscosities of zein-starch doughs containing hydrocolloids. They found that these doughs gave high extensional viscosities at magnitudes just above wheat dough.

Oom et al. (2008) measured the extensional rheological properties of zein-resin and obtained a Henchy strain of 5.3. This is more than twice that of the values obtained in this present work. These high values could be a result of the method in which the zein-resins were prepared, because they were dissolved in ethanol, plasticized with oleic acid and then precipitated out before kneading to form a resin.

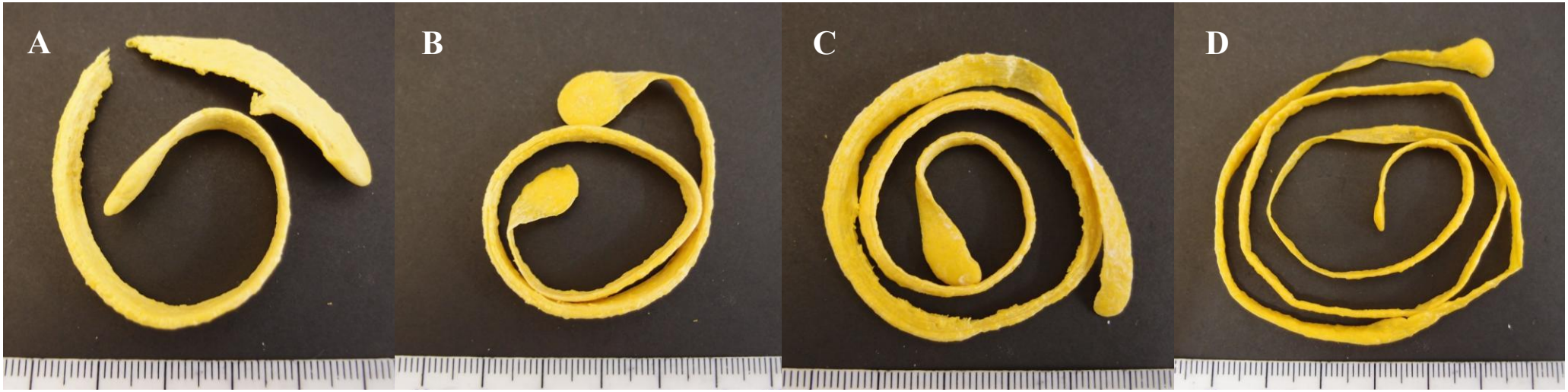

Figure 4.5: Photographs illustrating the extensibility of 1 g zein dough prepared with dilute organic acids at 40°C after manual stretching for 10 seconds. **A**: Distilled water, **B**: 0.7% acetic acid, **C**: 1.3% acetic acid, **D**: 5.4% acetic acid. The same trend of increasing extensibility as the concentration of acid increased was followed with dough prepared with lactic acid.

4.3.4.2 Effects of defatting and dilute organic acids on zein dough tensile properties

The defatted zein dough mixed with distilled water at 40°C was much softer and required less force to extend the samples when compared to zein that had not been defatted (Table 4.5). The addition of organic acids produced the same trends in the tensile properties as was seen in the non-defatted zein dough (Table 4.4). Although defatting resulted in a marked decrease in the amount of force required to extend the samples and improved the properties of the doughs, that is, it became softer and more extensible, this difference was masked by the addition of the organic acids. Thus, the addition of organic acid had a greater effect on the zein dough than defatting.

Other authors have not noted the softening effect of defatting on zein dough, for example Schober et al. (2010). However, what was perceived in this present work as a softening effect could also be perceived as an improvement in overall dough quality through the formation of fine protein fibrils. Schober et al. (2010) found that HPMC containing zein-starch dough was improved through defatting, which facilitated protein fibril aggregation and promoted the formation of gluten-like strands and thus led to stronger dough. Erickson et al. (2011) concluded that the removal of certain polar compounds such as carotenoids and lutein by chloroform extraction caused the zein to aggregate more readily. By removing the fat, which was present in and around the protein bodies of the zein, the protein-protein interactions within the zein dough were improved. Studies conducted by Schober et al. (2010) on the surface lipid removal from zein, found that baking performance of defatted zein dough containing the hydrocolloid HPMC was enhanced through larger volumes and lower width to height ratios. As explained, these authors hypothesized that the zein particles would have difficulty absorbing water and that a thin layer of surface lipids might hamper the protein-interactions between the zein particles.

From the work conducted, one can see that it is possible to produce non-defatted zein dough, which has similar properties to its defatted counterpart, through the addition of dilute organic acids. This would remove the need for the expensive and potentially toxic process of defatting in order to enhance the baking performance of zein dough.

Table 4.5: The tensile properties[1] of defatted zein dough prepared with dilute organic acids.

Treatment	Concentration (%)	Peak Force (N)	Extension (mm)	Peak stress (kPa)	Strain at max hook displacement (150 mm) (%)	Strain at max hook displacement (150 mm) (ε_H)^2	Extensional Viscosity (η_E, kPa.s)	Young's Modulus (E, kPa)	Area Under the curve (N.mm)
Distilled Water	**0**	2.80^{b} ± 0.59	270^{a} ± 0.0	73.1^{b} ± 15.4	1000^{a} ± 5	1.30^{a} ± 0.01	3336^{b} ± 705	23.6^{a} ± 3.7	418^{c} ± 23
Lactic acid	**0.7**	2.71^{b} ± 0.65	270^{a} ± 0.0	70.5^{b} ± 16.8	994^{a} ± 14	2.29^{a} ± 0.01	3206^{b} ± 787	17.5^{ab} ± 6.7	398^{c} ± 13
	1.3	2.15^{ab} ± 0.72	270^{a} ± 0.0	56.1^{ab} ± 18.6	995^{a} ± 9	2.30^{a} ± 0.00	2567^{ab} ± 868	13.4^{b} ± 1.3	324^{b} ± 25
Acetic acid	**0.7**	2.37^{b} ± 0.36	270^{a} ± 0.0	61.8^{b} ± 9.4	1000^{a} ± 6	2.29^{a} ± 0.02	2809^{ab} ± 434	20.1^{ab} ± 3.5	337^{b} ± 28
	1.3	1.41^{a} ± 0.06	270^{a} ± 0.0	36.6^{a} ± 1.6	1000^{a} ± 5	2.30^{a} ± 0.01	1669^{a} ± 86	14.2^{b} ± 2.1	278^{a} ± 16

[1] Mean ± Standard Deviation of three replicates
ε_H = Hencky strain/true strain
^{abc} = numbers in columns with different superscript letters differ significantly ($p < 0.05$)
Samples prepared with 5.4% dilute organic acids were too soft to be registered by the Kieffer rig

4.3.4.3 CLSM of zein dough prepared with dilute organic acids

The 3D images from the CLSM clearly show the green autoflorescence of zein, as well as a dramatic change in appearance in the zein dough when it was subjected to wet heat at 40°C (Figure 4.6). As the protein was heated above its T_g, it changed from a chunky granular appearance to that of a fine fibrous network (Figure 4.6a). This fibrous network was also visible in the zein doughs with added organic acids. The networks appeared to become smoother in consistency as organic acids were added. Examination of a mid-section Z-slice (Figure 4.6b) of the 3D image showed that the fibrils in the zein doughs prepared with the organic acids became finer as the concentration of acid increased. When the zein dough was stretched the protein fibrils aligned (Figure 4.7). The stretched dough prepared with only distilled water appeared to have imperfections on its surface. There were a few imperfections on the surfaces of the zein dough prepared with 1.3% organic acids. However, the number of imperfections reduced greatly as the level of organic acid was raised to 5.4%.

Erickson et al. (2011) reviewed that when exceeding its T_g, zein's reactivity is increased and thus its propensity for aggregation and crosslinking is enhanced, which allows for the development of extensive fibrous protein networks similar to those seen in wheat doughs. These fibres range in size from μm to mm and are present in all zein starch doughs that have been heated to temperatures above the T_g of zein (Lawton, 1992; Schober et al., 2008; Andersson et al., 2011). Lawton (1992) considered that these fibres, which are similar in appearance to wheat gluten fibres, were responsible for the viscoelastic properties of zein-starch dough. In this present work these fibrous networks could be seen during the manual kneading and stretching of the zein protein, as well as by CLSM. The alignment of these fibrils, seen during the stretching of the dough (Figure 4.7), could explain the high extensibility of the zein doughs on the Kieffer rig (Tables 4.4 and 4.5). Andersson et al. (2011) stated that fibre formation could be promoted through the increased input of mechanical energy during mixing.

As stated, the addition of organic acids seemed to create a finer protein network. A finer protein network was also found for zein-starch dough containing hydrocolloids (Andersson et al., 2011; Schober et al., 2008). Andersson et al. (2011) also observed by CLSM that by decreasing the water content of zein-starch doughs from 75 to 65%,

the fibrous structures present changed from chunky, coarse strands to uniform, fibrous strands. These authors hypothesized that the finer protein network in zein-dough with added HPMC allows for fewer interactions between zein peptides and thus decreases the effect of cross-linking. Schober et al. (2008) hypothesized that in the case of zein with added hydrocolloid HPMC, the weaker interactions between protein chains might contribute to the tendency of zein to form very extendable strands. Meijia et al. (2007) found that when zein was mixed at 35°C, changes in its secondary structure suggested that the protein loses its predominantly α-helical-based native structure and undergoes a structural rearrangement that favours β-sheet structures. Thus, a viscoelastic system is formed. Maher Galal et al. (1978) hypothesized that, in an acidic environment, the sizable positive net charge present increases the solubility of the proteins. The imperfections visible on the surface of the films when the acid was either not present or present only in low concentrations (1.3%) could represent proteins that have been partially or incompletely solubilized. These areas of insoluble proteins could be weak areas in the dough's protein network, and could reduce the extensibility of the dough through the formation of small pinholes, which could lead to crack intitation and propagation. This could help explain the fracture dynamics seen during the tensile tests and alveography.

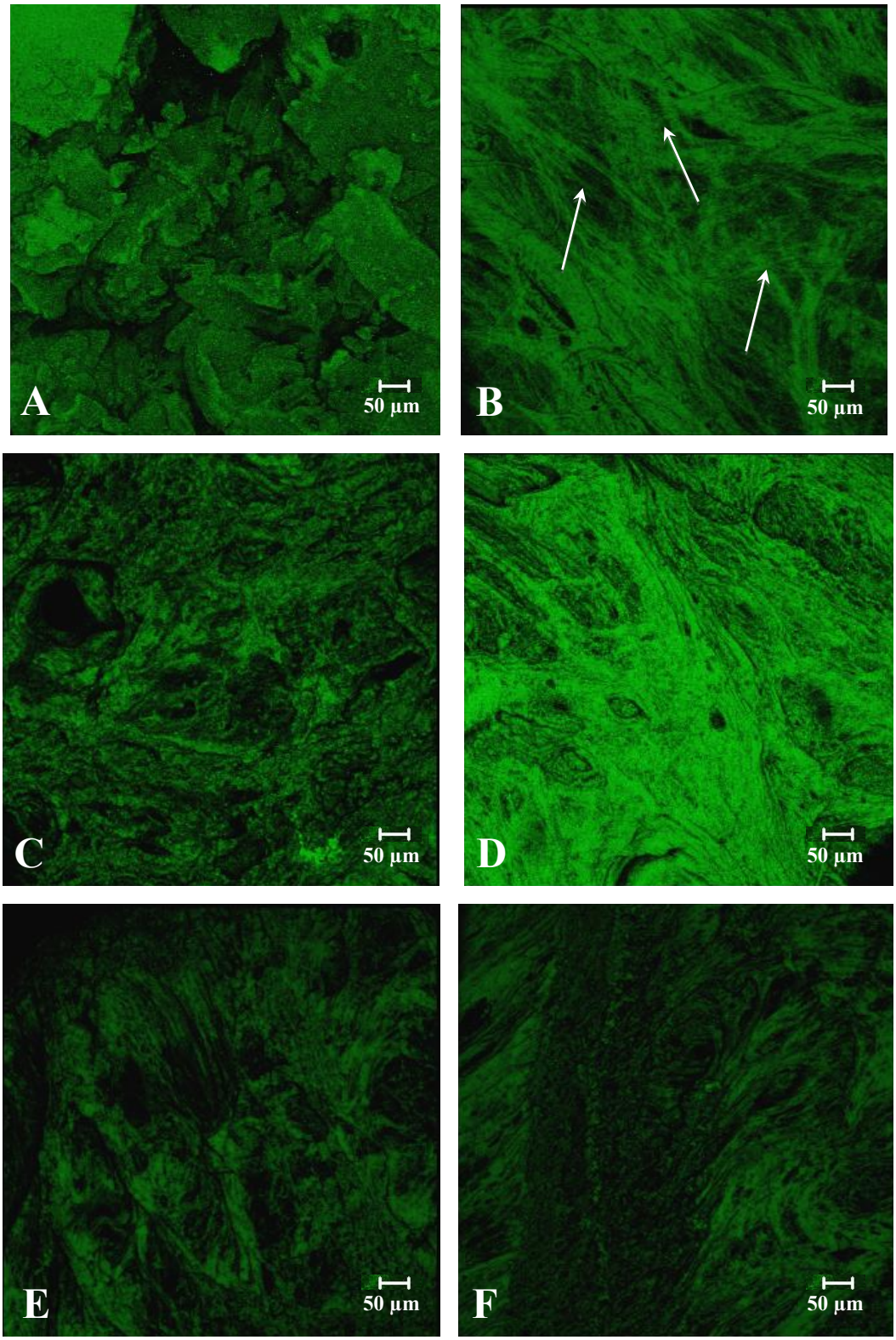

Figure 4.6a: CLSM of zein dough prepared with various concentrations of dilute organic acids. 3D images viewed from the top, **A**: Zein in distilled water at ambient temperature, **B**: Zein in distilled water at 40°C, **C**: Zein in 1.3% lactic acid at 40°C, **D**: Zein in 5.4% Lactic acid at 40°C, **E**: Zein in 1.3% acetic acid at 40°C, **F**: Zein in 5.4% acetic acid at 40°C. Fibrils are indicated by arrows.

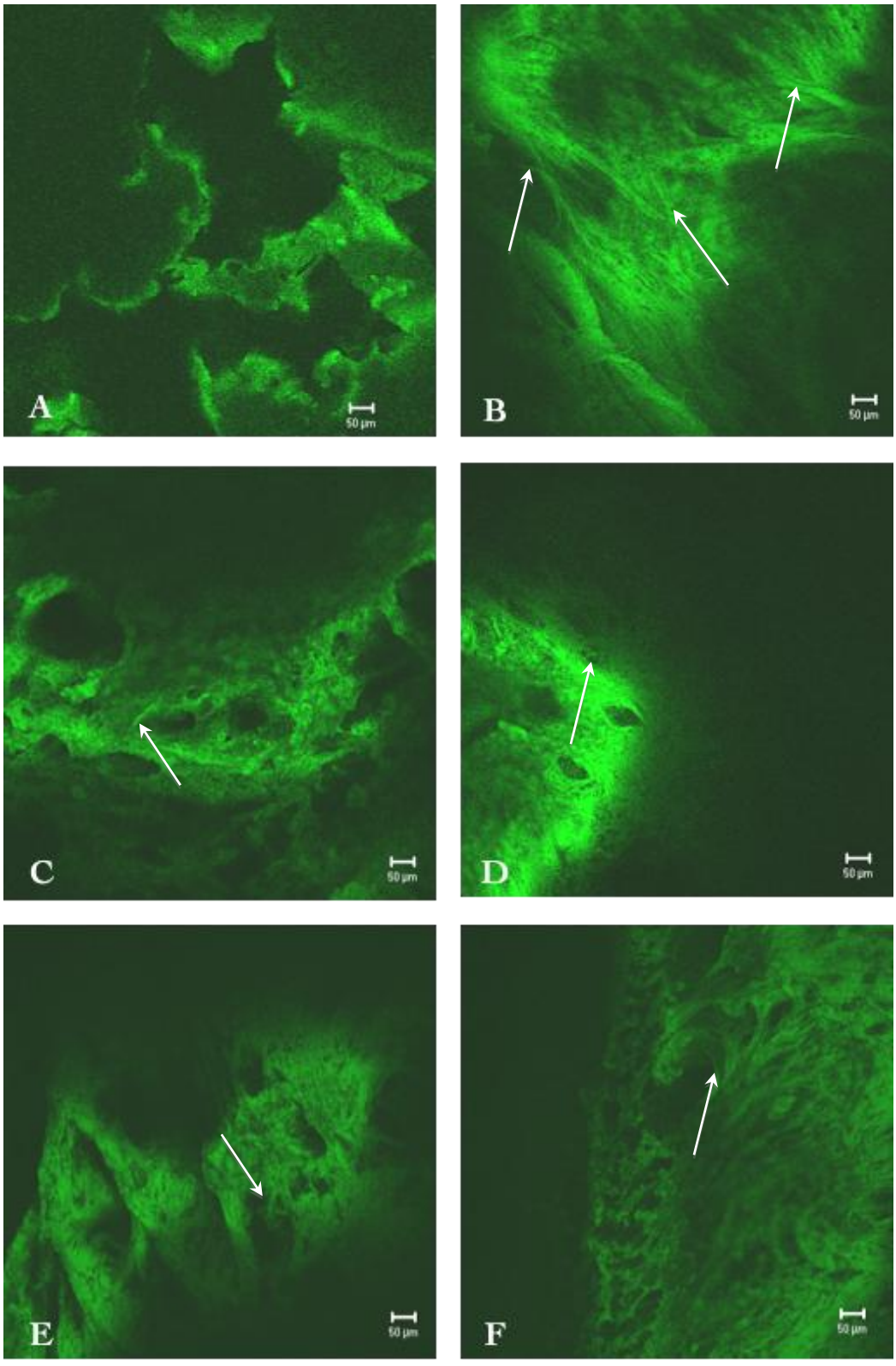

Figure 4.6b: CLSM of zein dough prepared with various concentrations of dilute organic acids. Micrographs of a mid Z-slice from a 3D CSLM image **A**: Zein in distilled water at ambient temperature, **B**: Zein in distilled water at 40°C, **C**: Zein in 1.3% lactic acid at 40°C, **D**: Zein in 5.4% Lactic acid at 40°C, **E**: Zein in 1.3% acetic acid at 40°C, **F**: Zein in 5.4% acetic acid at 40°C. Fibrils are indicated by arrows.

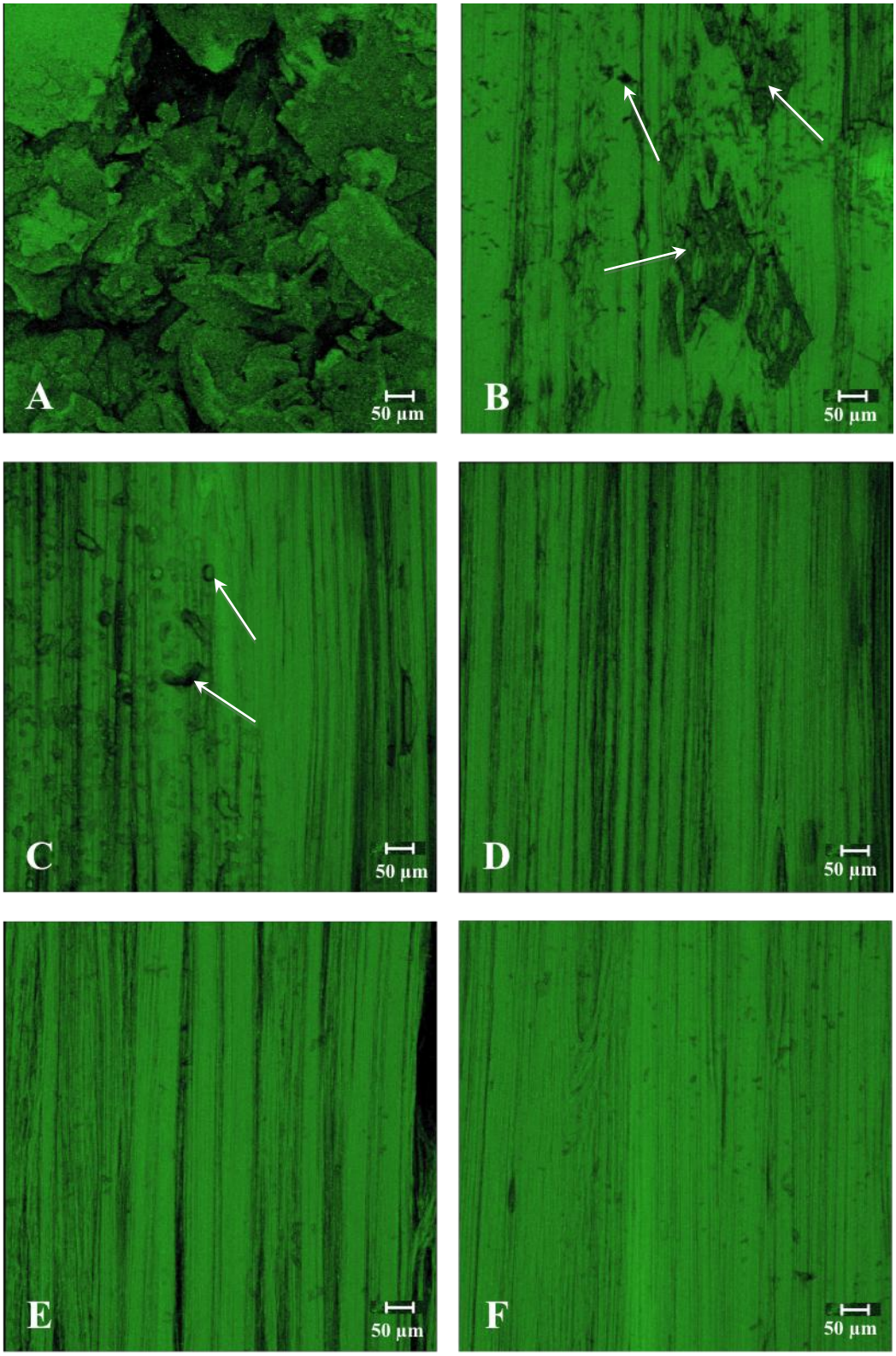

Figure 4.7: CLSM of stretched zein dough prepared with various concentrations of dilute organic acids: 3D images viewed from the top. **A**: Zein in distilled water at ambient temperature, **B**: Zein in distilled water at 40°C, **C**: Zein in 1.3% lactic acid at 40°C, **D**: Zein in 5.4% Lactic acid at 40°C, **E**: Zein in 1.3% acetic acid at 40°C, **F**: Zein in 5.4% acetic acid at 40°C. Impurities are indicated by arrows.

4.3.4.4 Effect of organic acids on the hydration of zein doughs

The addition of organic acids, at a concentration of 0.7 and 1.3%, to zein dough did not change the hydration or water holding capacity of the dough significantly. However, the 5.4% concentrations appeared to decrease the water holding capacity of the dough slightly (Table 4.6).

Zein dough prepared with organic acids has a high extensibility. It was expected that organic acids would act as plasticizers, aiding in the uptake of water into the dough system. Plasticizers make the proteins more extensible by forming a lubricating layer between protein molecules, which also tends to decrease the tensile strength of the protein (Byaruhanga, Erasmus, Emmambux & Taylor, 2007). Amonsou et al. (2012) found that the extensibility of marama protein increased considerably (3-fold) when the moisture content was increased from 38 to 40% due to the plasticization effect of water. Studies of wheat dough using a farinograph have shown that water uptake or consistency was increased by adding organic acids in the absence of salt (Tanaka, Furukawa & Matsumoto, 1967; Maher Galal et al., 1978). Beck et al. (1996) as cited in Lawton (2004) showed that compression moulded zein samples containing different plasticizers absorbed water to different degrees depending on the type of plasticizer in the sample. These authors further concluded that water is a very good plasticizer for zein because it is absorbed by both the zein and its plasticizers and ultimately affects the tensile strength of the zein films.

It is possible that organic acids could influence the hydrophobic interactions within the protein through denaturation of the protein, thus promoting the formation of a highly functional aggregated protein. Schober et al. (2011) suggested that hydrophobic interactions are key to zein's gluten-like functionality. The slight decrease in moisture content when dough was prepared with 5.4% organic acids could be explained if the higher concentrations of acids altered protein hydrophobicity.

Table 4.6: The moisture content[1] of freshly made zein dough prepared with different concentrations of organic acids

Type of Acid	Concentration of acid			
	0*	**0.7**	**1.3**	**5.4**
Lactic Acid	66.4^{ab} ± 0.3	66.8 ^{ab} ± 1.5	66.8 ^{ab} ± 2.4	63.7 ^{a} ± 1.5
Acetic Acid		67.2 ^{b} ± 0.4	65.6 ^{ab} ± 2.2	63.6 ^{a} ± 0.5

* Distilled water
[1] Mean ± Standard Deviation of three replicates
^{abc} = numbers in columns with different superscript letters differ significantly ($p<0.05$)

4.3.4.5 SDS-PAGE of zein dough prepared with different concentrations of dilute organic acids

All the zein samples that were heated above 40°C with added organic acids, lactic acid or acetic acid, at concentrations of 0.7, 1.3 and 5.4% (v/v) gave the same band pattern as the zein prepared in distilled water (Figure 4.8). Distinct zein monomer, dimer and trimer bands were observed in all the samples. From the results obtained there was no clear effect of the acids on the degree of polymerization taking place. Under non-reducing conditions the zein appeared to be more polymerized, as shown by the darker dimer and trimer bands and the lighter monomer bands. Under reducing conditions the disulphide bonds are broken (Emmambux and Taylor, 2009), which increased the number of monomers present and resulted in the darker monomer banding. It was hypothesized that organic acids might have been able to break down the crosslinks between polymers, leading to a softer dough, and resulting in more monomers being present as the concentration of acid was increased. However, this is not supported by evidence from SDS-PAGE. Crosslinking as a result of acid addition was not evident either. Zhang et al. (2011), also found that under a variety of pH levels no obvious fragmentation or oligomerization was detected in zein using SDS-PAGE. This indicates that changes in the protein structure brought about by the organic acids could be taking place in the secondary structure of the zein.

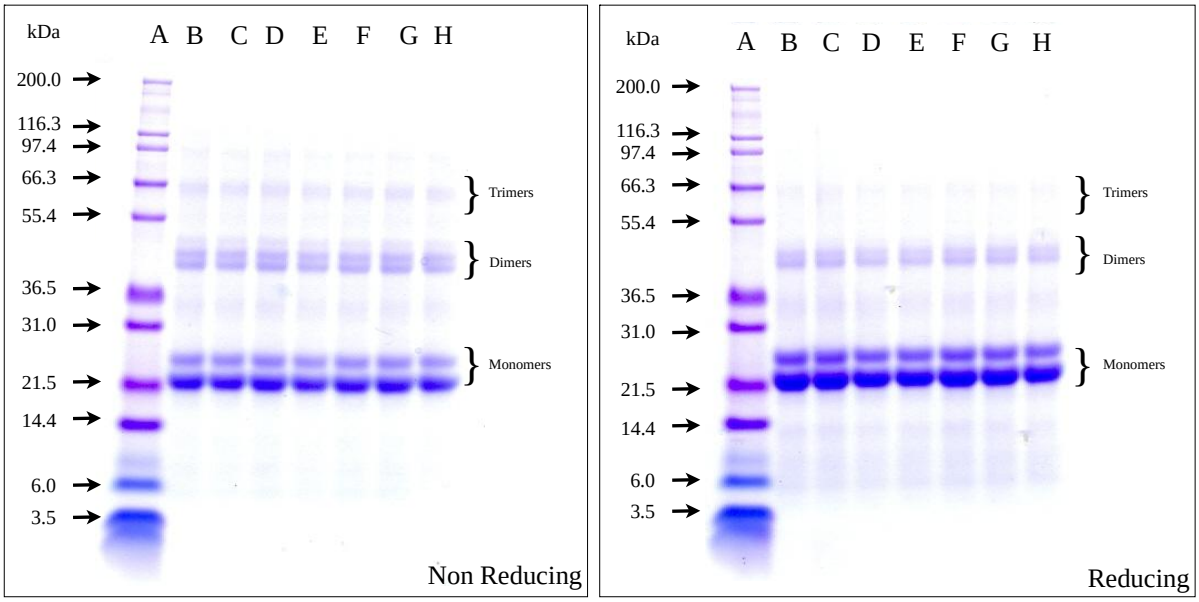

Figure 4.8: SDS-PAGE of zein dough prepared with dilute organic acids at 40°C. **A**: Molecular standard **B**: Zein in distilled water **C**: Zein in 0.7% lactic acid **D**: Zein in 1.3% lactic acid **E**: Zein in 5.4% lactic acid **F**: Zein in 0.7% acetic acid **G**: Zein in 1.3% acetic acid **H**: Zein in 5.4% acetic acid

4.3.4.6 FTIR analysis of zein dough prepared with different concentrations of dilute organic acids

FTIR was used to gain insight into the secondary structure of zein dough that was prepared with different concentrations of organic acids. In the amide I region, with unprocessed dry zein powder the α- helical conformation was favoured (Table 4.7, Figure 4.9). However, with the addition of distilled water or low levels of organic acids (0.7 and 1.3%), the relative level of the α- helical conformation was diminished. Interestingly, the higher concentration of organic acid (5.4%) slightly increased the proportion of α-helical conformation present in the doughs. In the amide I region, the addition of distilled water decreased wavenumber associated with α- helical conformation, while the concentration of 5.4% organic acid (both acetic acid and lactic acid) increased it slightly. In the amide II region the addition of organic acids increased the level of α- helical conformation.

Table 4.7: FTIR of freshly made zein dough at 40°C prepared with different concentrations of organic acid

Treatment	Concentration (%)	α-helix Wavenumber (cm^{-1})	β-sheet Wavenumber (cm^{-1})	Ratio (α:β)	Relative % α-helical conformation
			Amide I		
Zein Powder	**N/A**	1645.04 ^{e} ± 0.57	1618.55 ^{a} ± 0.95	1.52 ^{d} ± 0.09	60.32 ^{d} ± 1.47
Distilled Water	**100**	1643.30 ^{ab} ± 0.95	1626.34 ^{bc} ± 4.77	1.05 ^{a} ± 0.03	51.24 ^{a} ± 0.73
Lactic Acid	**0.7**	1642.81 ^{a} ± 0.95	1627.95 ^{c} ± 0.50	1.08 ^{ab} ± 0.05	51.82 ^{ab} ± 1.19
	1.3	1643.06 ^{a} ± 0.57	1628.07 ^{c} ± 1.23	1.08 ^{ab} ± 0.04	51.84 ^{ab} ± 1.00
	5.4	1644.29 ^{cde} ± 0.50	1624.24 ^{b} ± 0.81	1.25 ^{c} ± 0.03	55.55 ^{c} ± 0.54
Acetic Acid	**0.7**	1643.55 ^{abc} ± 0.00	1626.34 ^{bc} ± 1.02	1.10 ^{ab} ± 0.04	52.31 ^{ab} ± 0.91
	1.3	1644.05 ^{bcd} ± 0.57	1628.07 ^{c} ± 0.74	1.13 ^{b} ± 0.03	53.05 ^{b} ± 0.71
	5.4	1644.54 ^{de} ± 0.00	1625.73 ^{bc} ± 0.57	1.22 ^{c} ± 0.03	55.01 ^{c} ± 0.56
			Amide II		
Zein Powder	**N/A**	1537.61 ^{ab} ± 0.57	1510.12 ^{a} ± 0.50	0.94 ^{a} ± 0.05	48.18 ^{a} ± 1.28
Distilled Water	**100**	1541.07 ^{ab} ± 0.00	1516.56 ^{bc} ± 0.51	1.45 ^{bc} ± 0.06	59.20 ^{b} ± 1.02
Lactic Acid	**0.7**	1541.69 ^{ab} ± 0.47	1516.56 ^{bc} ± 0.50	1.45 ^{bc} ± 0.09	59.13 ^{b} ± 1.51
	1.3	1542.06 ^{b} ± 0.81	1516.07 ^{b} ± 0.50	1.42 ^{b} ± 0.06	58.62 ^{b} ± 1.01
	5.4	1542.06 ^{b} ± 0.01	1516.81 ^{bc} ± 0.01	1.73 ^{d} ± 0.30	63.08 ^{d} ±3.70
Acetic Acid	**0.7**	1541.57 ^{ab} ± 0.57	1516.81 ^{c} ± 0.00	1.49 ^{bc} ± 0.04	59.78 ^{bc} ± 0.62
	1.3	1541.57 ^{ab} ± 0.57	1516.56 ^{bc} ± 0.50	1.56 ^{bc} ± 0.05	60.88 ^{bcd} ± 0.77
	5.4	1535.50 ^{a} ± 12.47	1516.56 ^{bc} ± 0.50	1.62 ^{cd} ± 0.04	61.75 ^{cd} ± 0.64

*Distilled water, [1] Mean ± Standard Deviation of three replicates, ^{abc} = numbers in columns with different superscript letters differ significantly ($p<0.05$)

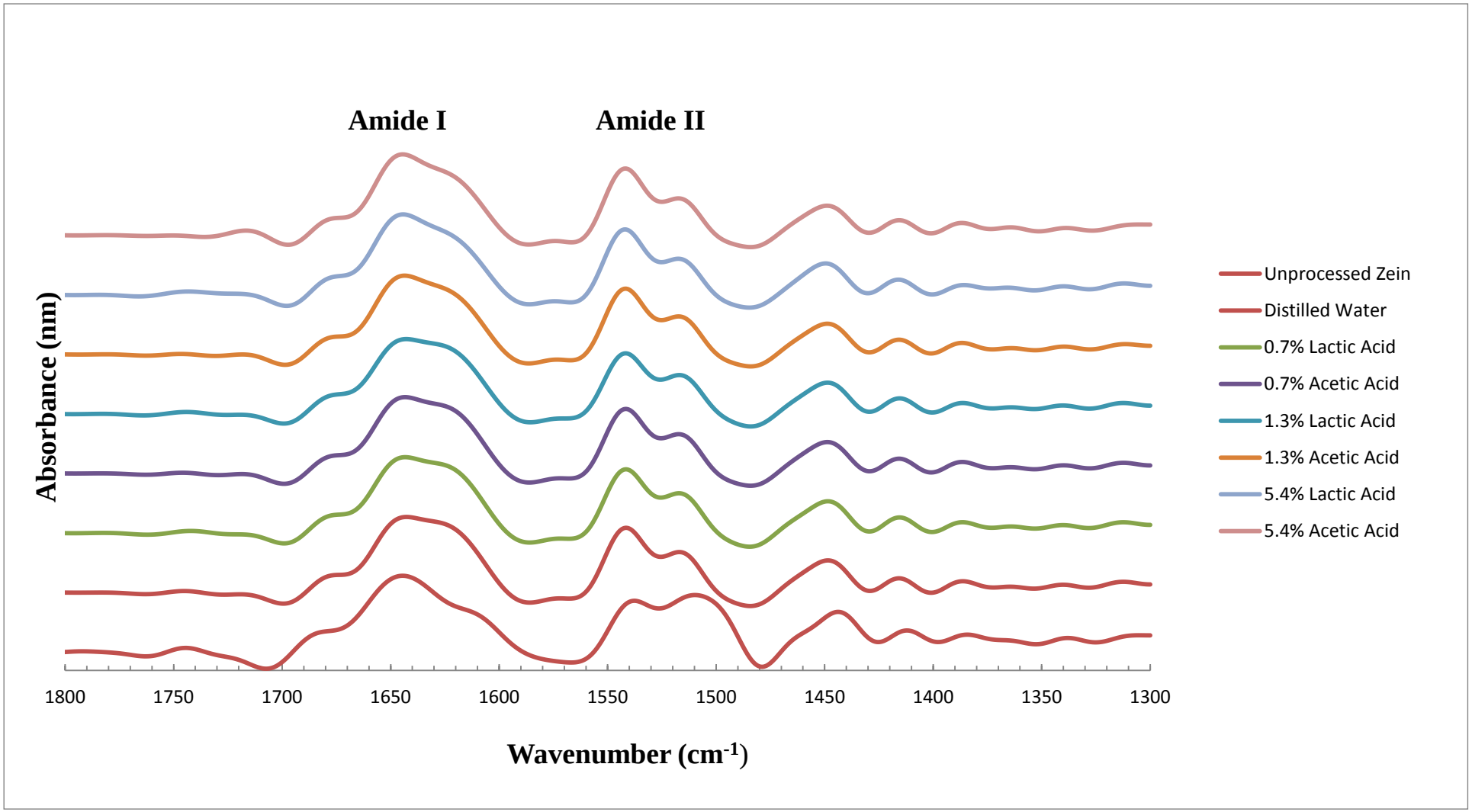

Figure 4.9: FTIR of freshly made zein dough at 40°C prepared with different concentrations of organic acid. Abs α-helix peak = Absorbance at ≈ 1650 cm^{-1} and 1540 cm^{-1} after baseline correction, and Abs β-sheet peak = Absorbance at ≈ 1620 cm^{-1} and 1515 cm^{-1} after baseline correction.

According to Shewry and Tatham (1990) there are between 40 and 60% α-helical conformation present in the secondary structure of α-zein, and only a small amount of β-sheet conformation is present when the protein is dissolved in aqueous ethanol. Recent literature attributes the high extensibility demonstrated by zein doughs above their T_g being most probably due to the high amount of β-sheet conformation present in the protein under these conditions (Erickson et al., 2011). Amonsou et al. (2012) found that the highly viscous and extensible rheological behaviour of marama protein was probably related to its high β-sheet conformation, hydrophobic interactions and tyrosine crosslinks. Mejia et al. (2007) had also found that the amount of β-sheet conformations present in zein increased from 30 to 48% when the protein was raised above its T_g. These observations are consistent with findings in this current work. However, Mejia et al. (2007) further found that the β-sheet content decreased significantly back to 30% when the temperature of the zein polymer was decreased from 35 to 25°C, coinciding with a loss of viscoelasticity of the zein polymer. All these observations could imply that a predominance of β-sheet interactions in the network is critical to the viscoelastic properties of the zein polymer, as has been suggested for gluten dough systems (Popineau, Bonefant, Cornec & Pezolet, 1994). Belton (1999) reviewed that gluten has a high level of β-sheets and β-turns in its structure.

It appears that the addition of dilute organic acids, acetic acid and lactic acid, increased the solubility of the zein, and increasing concentration of organic acid promoted increasing levels of α-helices in the dough. Such α-helices are held together by a combination of hydrogen, ionic, hydrophobic and limited covalent (disulphide) bonds (Bourtoom, 2008). Belton et al. (2006) reviewed that various factors influence the wide range of α-helical conformation present in such protein. These include differences in protein fraction used, as well as the different solvents and methods used for deconvolution.

Differences seen between the Amide I and Amide II bands could be explained by the different sensitivities of these regions, with the Amide II band being the less sensitive of the two spectral regions (Singh, 2000). However, it should also be noted that the samples used were freshly prepared dough, thus the liquid water present in the dough could have led to –OH vibrations in this region.

4.3.5 EFFECT OF DILUTE ORGANIC ACIDS ON ZEIN-MAIZE STARCH AND ZEIN-RICE FLOUR DOUGH SYSTEMS

4.3.5.1 Effect of dilute organic acids on zein- maize starch dough tensile properties

The addition of maize starch to the zein in a 4:1 ratio produced an elastic dough that was much softer and noticeably more fibrous (Figure 4.10) than dough made of pure zein. However, when zein-maize starch dough was allowed to cool down from 40°C to ambient temperatures, it quickly became very brittle.

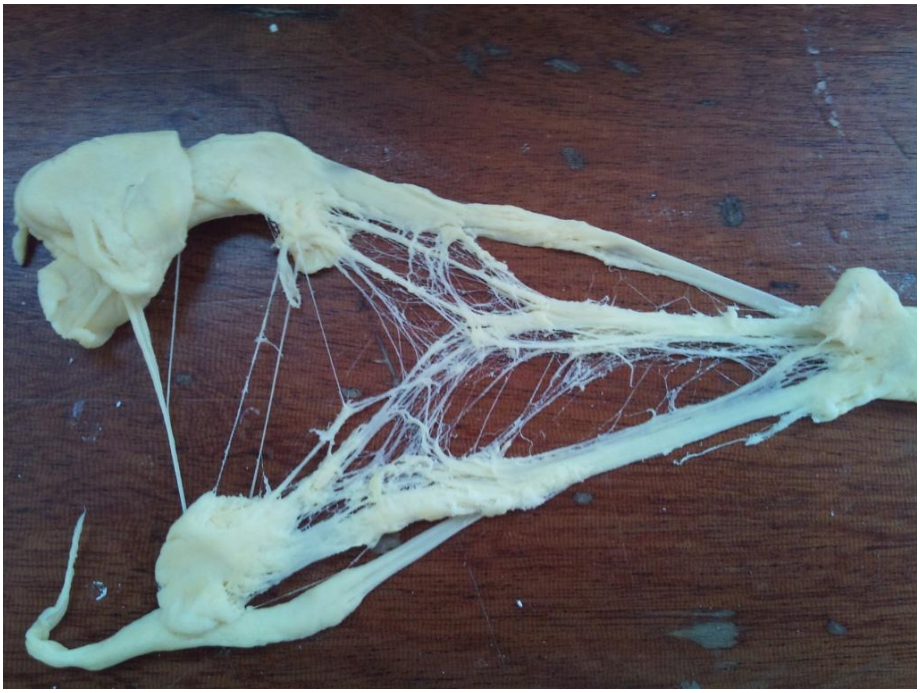

Figure 4.10: The fibrous appearance of zein- maize starch dough prepared with a 5.4% acetic acid solution

Data obtained from the tensile tests (Table 4.8) showed that zein-maize starch dough prepared without added organic acids fractured upon extension with the Kieffer rig at 90 mm. However, all the samples that were prepared with the organic acids at levels of 0.7 and 1.3% reached the maximum extension of 270 mm. Preparation with dilute organic acids had a softening effect on the dough. This is evident in that the dilute organic acids decreased the amount of force (N) required to extend the dough from 2.33 N when the dough prepared with no organic acid, to 1.33 and 1.15 N for dough prepared with 1.3% lactic acid and acetic acid respectively. The doughs prepared with 5.4% dilute organic acids were too soft for the Kieffer rig to register.

Table 4.8: The tensile properties[1] of a zein-maize starch mixture (1:4) prepared with lactic acid and acetic acid

Treatment	Concentration (%)	Peak Force (N)	Extension (mm)	Peak stress (kPa)	Strain at max hook displacement (150mm) (%)	Strain at max hook displacement (150mm) (ε_H)[2]	Extensional Viscosity (η_E, kPa.s)	Young's Modulus (E, kPa)	Area Under the curve (N.mm)
Distilled Water	**0**	2.33 ^{a} ± 0.38	90^{a} ± 16.0	60.8 ^{a} ± 9.8	413 ^{a} ± 52	1.42 ^{a} ± 0.13	1171^{a} ± 171	63.4^{a} ± 1.5	178 ^{a} ± 51
Lactic acid	**0.7**	1.95 ^{b} ± 0.08	270 ^{b} ± 0.0	51.0 ^{b} ± 2.1	1003 ^{b} ± 1	2.31 ^{b} ± 0.00	2324 ^{c} ± 99	32.3 ^{b} ± 3.6	407 ^{c} ± 39
	1.3	1.33 ^{c} ± 0.12	270 ^{b} ± 0.0	34.6 ^{c} ± 3.1	1001 ^{b} ± 1	2.30 ^{b} ± 0.00	1584 ^{b} ± 141	33.6 ^{b} ± 7.3	271^{b} ± 31
Acetic acid	**0.7**	1.28 ^{c} ± 0.11	270 ^{b} ± 0.0	33.3 ^{c} ± 2.8	997 ^{b} ± 2	2.21 ^{b} ± 0.08	1404 ^{ab} ± 218	24.6 ^{b} ± 7.4	331 ^{bc} ± 63
	1.3	1.15 ^{c} ± 0.22	270 ^{b} ± 0.0	30.1 ^{c} ± 5.7	999 ^{b} ± 6	2.30 ^{b} ± 0.01	1374 ^{ab} ± 252	11.4 ^{c} ± 6.1	187 ^{a} ± 11

[1] Mean ± Standard Deviation of three replicates
ε_H = Hencky strain/true strain
^{abc} = numbers in columns with different superscript letters differ significantly ($p < 0.05$)
Samples prepared with 5.4% dilute organic acids were too soft to be registered by the Kieffer rig

Acetic acid tended to soften the dough more than lactic acid. Acetic acid at both 0.7 and 1.33% concentrations reduced the force required to extend the samples by a similar ($p \geq 0.05$) and significant ($p < 0.05$) extent, respectively. In the case of the dough prepared with lactic acid there was a significant ($p < 0.05$) reduction in the force required to extend dough prepared with 0.7% addition, but the use of 1.33% was required to achieve a further significant reduction in force that matched figures seen at both addition levels of acetic acid. A similar trend was observed with measurements of peak stress. Dough without organic acids had a peak stress 10 to 30 kPa higher than that of doughs prepared with organic acids. The percentage strain and Hencky strain of dough prepared without organic acids were both significantly lower ($p <0.05$) than when doughs were made with organic acids. The extensional viscosity of the dough tended to increase when organic acids were added but this change was only significant in the case of lactic acid, where the magnitude of the increase observed at 0.7% addition was significantly reduced at a concentration of 1.3%. The modulus of elasticity (Young's modulus) of the zein-maize starch doughs was higher than that of the doughs made from pure zein (Table 4.4 and 4.5). Preparations of zein-maize starch doughs with dilute organic acids reduced the modulus of elasticity by almost half. The area under the curve of the samples initially increased when either acetic or lactic acids were used at 0.7% and then decreased at the higher concentration of organic acid. The low area under the curve of dough prepared without organic acids was due to the dough fracturing before maximum extension was reached. All these observations point to a softening effect of the organic acids on the dough.

As stated, Lawton (1992) showed that zein and zein in a model dough system with maize starch could form viscoelastic doughs when above its T_g temperature. The addition of the starch seems to interfere with the protein-protein interactions, thus creating a much softer and more fibrous product (Figure 4.10). Schober et al. (2008) theorized that protein-starch interactions, rather than protein-protein interactions, influenced the rheological behaviour of zein-maize starch doughs. In the current work, the zein-maize starch dough, although cohesive, was not strong enough to withstand great extension using the Kieffer rig. It appears that preparation with the organic acid facilitates the uptake of water within the dough system, improving the starch-protein interactions and increasing dough extensibility. Marco and Rosell

(2008b) maintained that the plasticising effect of water is crucial to the bread-making properties of gluten-free rice flour and in low protein-starch breads. Mixograms conducted by these authors showed that the addition of water at increasing levels of 65, 70 and 95% reduced the maximum consistency or torque of gluten-free breads to 1.45, 107 and 0.81 Nm, respectively.

Studies of wheat dough using a farinograph showed that water uptake or consistency was increased by adding organic acids in the absence of salt (Tanaka et al., 1967). Maher Galal et al. (1978) reported that the addition of a combination of organic acids to dough increased water absorption, while dough development time, stability and tolerance to mixing were decreased. The softening effect of the added organic acids confirms previous research by Moore et al. (2007), who found that chemical acidification caused the gluten-free batter to become more fluid-like. Blanco et al. (2011) made similar observations when they investigated the effect of various organic acids (acetic acid, lactic acid and citric acid) on the properties of gluten-free batter. These authors showed that when the acids were initially added to the batter, the viscoelastic moduli of the dough were altered. The G' increased slightly from 2105 Pa in the non-acidified dough to 2490 Pa and 2270 Pa when prepared with acetic acid and lactic acid were added, respectively. As the dosage (based on % flour) of these acids was increased so the G' and the G" of the batter decreased to 2040 Pa and 1910 Pa. The reverse was found when citric acid was added. However, these authors reported that type or concentration of acid was not seen to have any significant effect on the viscoelastic moduli of the batter. In apparent contradiction, Moore et al. (2007) found that biologically acidified gluten-free dough (obtained through the incorporation of sourdough into the formulation) significantly increased batter firmness.

4.3.5.2 Alveography

Zein-maize starch doughs and zein-rice flour doughs exhibited similar patterns of behaviour in the alveograph (Table 4.9, Table 4.10). In both cases, when the dough was mixed with only distilled water, a soft, lumpy mixture was formed which, although it was fibrous and cohesive, did not exhibit any dough-like properties and was easily pulled apart. If left for a short resting period, there was visible phase separation between the protein and starch in the dough (Figure 4.11, A). This phase separation took the form of a starchy liquid surrounding the dough. The extent to which starch leached out of the dough increased if extra water was introduced. Thus, it was not possible to process these doughs using the alveograph.

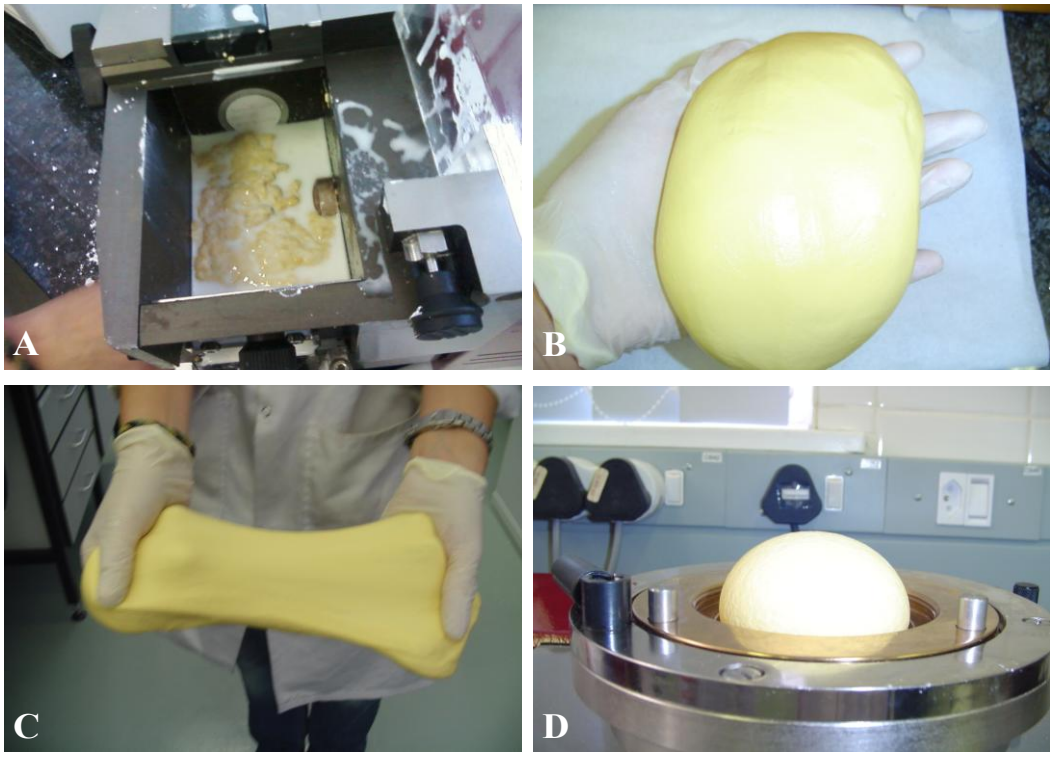

Figure 4.11: Alveography of zein-maize starch dough with added organic acid (lactic acid, 1.3%). **A**: Phase separation when no organic acid was added, **B**: Appearance of dough with added acid after kneading, **C**: Malleability of dough with added acid after kneading, **D**: Alveography of the dough. Similar results were observed with acetic acid

Dough prepared with dilute organic acids improved the quality of the dough to an extent where it could be processed and assessed using the alveograph (Figure 4.11, D; Table 4.10). The magnitude of this influence was dependent on the concentration of acid added, but and in all cases small tears or holes in the dough prevented the formation of bubbles similar in size

to those obtained with wheat flour dough. Stability values (P) similar to those observed with strong wheat flour (around 103 mm H_2O) where achieved in the zein-maize starch dough (Table 4.10) when using 1.3% lactic acid or 0.7 and 1.3% acetic acid. However, when using an acid concentration of 5.4%, the stability of the dough decreased by almost half. Similar observations were made in the zein-rice flour doughs. Organic acids at 0.7 and 1.3% resulted in P values similar to (and greater than) those of strong wheat flour, with additions of 5.4% reducing stability. Stability reduction at the highest acid concentration, however, only reached statistical significance with acetic acid. The zein-rice flour combination tended to produce stronger doughs than the zein-maize starch combination.

A similar pattern was seen with the distensibility (L) measurements of the doughs (Table 4.10). In the zein-maize starch dough, L values approximately twice those seen with strong wheat flour where obtained with the use of 1.3% lactic acid and 0.7 and 1.3% acetic acid. Although the distensibility was significantly reduced when 5.4% concentrations of organic acids were used, the figures obtained still matched those seen with strong wheat flour. In the zein-rice flour dough 0.7 and 1.3% organic acids resulted in L values around twice that of strong wheat flour, with the highest acid concentration of 5.4% again significantly reducing the values, which nevertheless matched those found with wheat. Unfortunately, the strength of these 5.4% acid doughs was very low. This weakness, or low stability (P), could have resulted from the dough bubble tearing before a proper indication of the true extensibility of the dough could be obtained.

In this work, distensibility (L) refers to the biaxial extensibility of the dough. Biaxial extensibility is used as an indicator of the handling characteristics of the dough (Rosell, Rojas & Benedito de Barber, 2001) because it gives valuable information about the performance of dough during fermentation and baking. Whilst the use of organic acids at 0.7 and 1.3% elicited high distensibility figures, these values were seen to fall at the highest level of 5.4% lactic and acetic acids. This fall may not truly reflect actual changes in the extensibility of the dough, because the dough stability (P) at this concentration of acid was quite low. The decreased dough stability could have led to tears in the dough surface during inflation before maximum extensibility could be reached (Table 4.9). Furthermore, the tensile data obtained with the Kieffer rig (Table 4.4 and 4.5) shows that the greater the concentration of acid, the more extensible the dough becomes.

Table 4.9: Subjective evaluation of doughs prepared with different concentrations of organic acids using an alveograph

Organic Acid	Concentration of Acid (%)			
	0*	**0.7**	**1.3**	**5.4**
		Zein: Maize starch (1:4)		
Lactic Acid	• A fibrous mixture formed • Samples were too soft • Not cohesive or adhesive enough to measure using the alveograph	• A small bubble formed • The dough cooled quickly	• The dough split at the sides where it touched the machine • A good bubble formed • Dough cracks	• Holes appeared on the surface of the dough • A small bubble formed • Very soft dough
Acetic Acid	• The starch leached out of the mixture when standing	• A small bubble formed • Dough was yellow in colour	• Samples crack at sides upon inflation • Small bubble	• Very small bubble, samples get holes in the surface.
		Zein: Rice Flour (1:4)		
Lactic Acid	• Very soft dough • Quite fibrous • Not cohesive or adhesive enough to measure using the alveograph	• Lighter in colour than the samples containing maize starch • Forms a better dough patty than the maize starch samples • No cracks at the side • A small bubble formed	• Quite a large bubble formed • No holes • Continued to inflate for a while	• A soft, large bubble formed
Acetic Acid		• A small bubble formed • Same as with the lactic acid	• A good bubble formed • No holes	• A soft bubble formed • Medium in size

* Distilled Water

Table 4.10: Viscoelastic behaviour of a zein-maize starch dough (1:4) and zein-rice flour dough (1:4) prepared with lactic acid and acetic acid solutions[1] as measured using an alveograph.

Treatment	Concentration (%)	Stability (P, mm H_2O)	Distensibility (L, mm)	Curve configuration Ratio (P/L)	Deformation Energy (W, J x10^{-4})
Strong Wheat Flour	0	103.2 ^{de} ±10.3	79.6 ^{a} ± 16.9	1.36 ^{d} ± 0.40	301.2 ^{c} ± 28.9
Zein-Maize starch dough (1:4)					
Distilled Water	0	Samples were too soft and were not cohesive or adhesive enough to measure			
Lactic acid	0.7	75.4 ^{bc} ± 9.2	81.6 ^{a} ± 30.4	0.84 ^{bc} ± 0.18	166.0 ^{b} ± 35.6
	1.3	92.2 ^{cde} ± 8.5	176.5 ^{c} ± 45.9	0.44 ^{a} ± 0.23	302.6 ^{c} ± 47.9
	5.4	41.4 ^{a} ± 10.8	83.0 ^{a} ± 25.2	0.51 ^{ab} ± 0.12	44.0 ^{a} ± 0.2
Acetic acid	0.7	90.0 ^{cd} ± 8.3	140.2 ^{bc} ± 38.2	0.82 ^{abc} ± 0.39	328.0 ^{cd} ± 79.3
	1.3	106.0 ^{def} ± 10.7	179.8 ^{c} ± 27.6	0.65 ^{abc} ± 0.20	310.4 ^{c} ± 46.2
	5.4	52.2 ^{a} ± 7.5	67.3 ^{a} ± 20.9	0.84 ^{abc} ± 0.30	49.0 ^{a} ± 7.1
Zein-Rice Flour dough (1:4)					
Distilled Water	0	Samples were too soft and were not cohesive or adhesive enough to measure			
Lactic acid	0.7	123.8 ^{f} ± 5.6	164.0 ^{c} ± 39.4	0.84 ^{bc} ± 0.22	420.4 ^{e} ± 87.2
	1.3	106.0 ^{def} ± 17.5	185.5 ^{c} ± 26.8	0.66 ^{abc} ± 0.22	335.3 ^{cde} ± 77.8
	5.4	86.8 ^{cd} ± 8.3	91.2 ^{ab} ± 27.0	1.00 ^{cd} ± 0.23	158.8 ^{b} ± 31.7
Acetic acid	0.7	113.0 ^{ef} ± 18.1	153.4 ^{c} ± 38.3	0.85 ^{bc} ± 0.38	412 ^{de} ± 65.0
	1.3	97.7 ^{de} ± 16.6	141.7 ^{bc} ± 44.5	0.87 ^{abc} ± 0.48	369.0 ^{cde} ± 28.2
	5.4	59.6 ^{ab} ± 16.4	90.6 ^{ab} ± 30.3	0.85 ^{bc} ± 0.45	117.0 ^{ab} ± 59.1

[1] Mean ± Standard Deviation of three replicates, ^{abc} = numbers in columns with different superscript letters differ significantly ($p < 0.05$)

Dough resistance to deformation or stability (P) is a predictor of the ability of the dough to retain gas (Rosell et al., 2001). When zein-containing doughs were produced without the addition of organic acids, they were incapable of holding gas. When organic acids were added the ability of the dough to retain gas increased. This is most likely due to the formation of a cohesive network of fibrils. As the concentration of acid is increased further to 5.4% the dough become so soft that its stability was reduced. Softer dough cannot withstand as much stress without tearing when compared to firmer, stronger dough. Maher Galal et al. (1978) hypothesized that softening of dough in an acidic environment is due to the sizeable positive net charge of an acidic environment. This positive net charge increases the protein solubility through an increase in the intramolecular electrostatic repulsion. The repulsion leads to an unfolding of the proteins and an increased exposure of the hydrophobic groups, and also prevents the formation of new bonds. The net result of this is a weakening of the structure, which has a softening effect. Studies by Clarke et al. (2002) on wheat dough using a farinograph showed that the addition of sourdough or acid significantly increased the degree of softening after 10 and 20 min. Hoseney (1994) also commented on this softening effect and the weakening of wheat dough structure as a result of the decreased pH.

The curve configuration ratio (P/L) provides information on the elastic resistance and extensibility balance of flour (Rosell et al., 2001), and gives an indication as to the overall bread-making quality of the flour through a combination of dough strength and distensibility (Chopin, 2010). Commercial strong bread flour dough had a significantly higher P/L ratio than any of the zein doughs produced (Table 4.10). This indicates that, when compared to strong wheat flour, zein based doughs will produce poor quality leavened breads. However, when compared to rice flour dough that has no added zein, or organic acids, zein based doughs prepared with organic acids produce superior dough.

The deformation energy (W) figures for zein-maize starch doughs made with 0.7% and 1.3% lactic acid, as well as those for zein-rice flour made with acetic or lactic acids at 0.7 and 1.3%, were similar to if not higher than those for strong wheat flour dough (301 W). When the concentration of acid was increased to 5.4% the deformation energy dramatically decreased, indicating significantly softer dough. At

this level, the deformation energy of the strong wheat flour was some six times greater than that of the samples containing maize starch, but only two to two and a half times that of samples containing rice flour. This suggests that doughs made with rice flour tend to be stronger than doughs made with maize starch. This could be due to better zein-rice flour component interactions than zein-maize starch interactions. The higher protein and fat content in the rice flour (Table 4.2) might have also contributed to these findings by improving dough structural stability.

Compared to zein, which has non-polar side groups (Andersson et al., 2011), starch is more hydrophilic in nature and thus can interact more strongly with water (Corradini, Souto de Mederiros, Carvalho, Curvelo & Mattose, 2006). This could account for the phase separation that was seen in the zein-maize starch "dough" prepared with water. By competing with the protein for water, the starch could prevent adequate hydration of the zein, which could in turn reduce dough strength, as seen during the tensile tests when the dough prepared with distilled water fractured (Table 4.8). Andersson et al. (2011) found that zein doughs containing no hydrocolloid, HPMC, experienced a similar phase separation, which resulted in starchy liquid surrounding the dough after mixing. The use of HPMC was found to add structural stability to zein-starch doughs, but it also increased the water-binding capacity of the dough. An increase in water-binding capacity is unlikely the effect of organic acids in this study (Table 4.6). It is also worth mentioning that the acid could potentially cause partial starch hydrolysis (BeMiller and Whistler, 1996).

4.3.5.3 Appearance of distended zein-rice flour doughs

The influence of the different concentrations of organic acid could be seen clearly when 5 g of the zein-rice flour dough was distended over a petri dish (Figure 4.12). Dough prepared with distilled water could not be distended. As the concentration of acid was increased, the stability of the dough clearly decreased. At low concentrations (0.7%) the surface of the dough was smooth and uniform with very few small holes (Figure 4.12a and b: A, B). When the concentration of acid was increased, so the surface of the dough became more uneven, with the fibrous strands appearing to clump together. The holes present in the dough became larger and more numerous. At

the highest level of organic acid (5.4%) it became evident that the stability of the dough was very low (Figure 4.12a and b: C, D). The cohesive and uniform appearance had completely collapsed and large fibrous strands were visible.

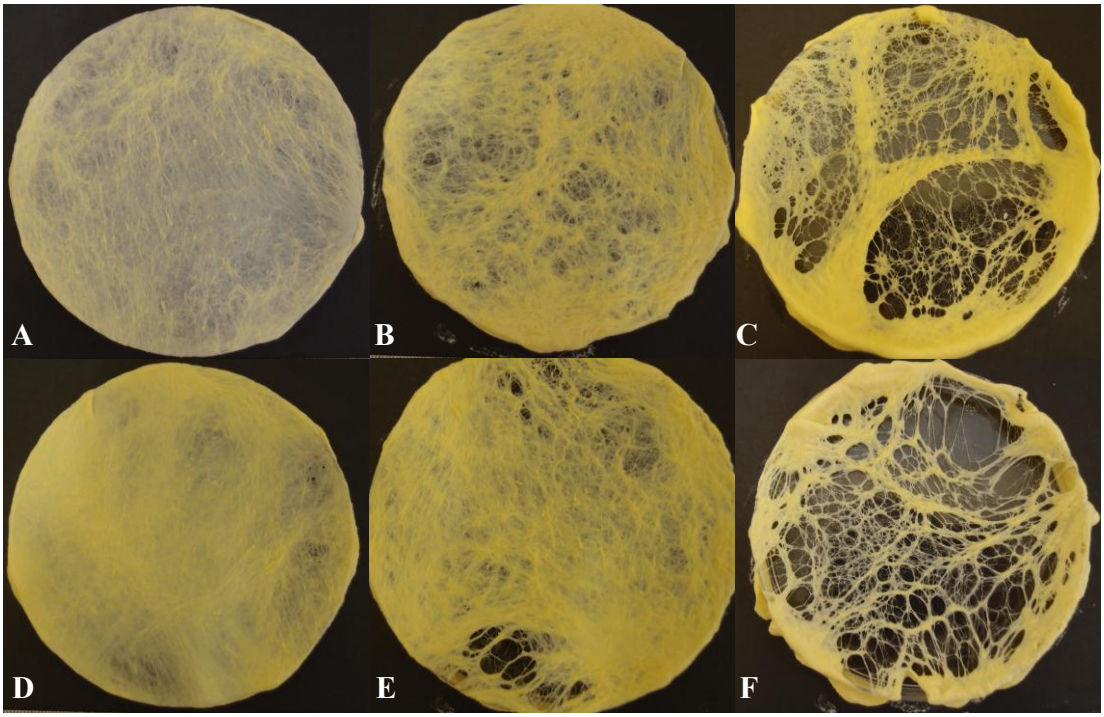

Figure 4.12a: The appearance of zein-rice flour dough that has been distended over petri dishes. **A**: 0.7% acetic acid **B**: 1.3% acetic acid **C**: 5.4% acetic acid **D**: 0.7% lactic acid **E**: 1.3% lactic acid **F**: 5.4% lactic acid.

A review by Rochet and Lansbury (2000) gives insight into the fibrilization pathway of zein prepared with organic acids. These authors describe the evolution of oligomeric intermediates of amyloid fibrils, which are highly ordered, insoluble aggregates of proteins or polypeptides (Chiti & Dobson, 2006) that can be induced under acidic conditions. According to Rochet and Lansbury (2000), protein spheres undergo self-association to form protofibrils. Two protofibrils may then intertwine to produce a wound structure that may undergo further conformational rearrangements to form a mature fibril. These oligomeric intermediates of amyloid fibrils could describe the clumping of the zein protein fibrils that was seen when the concentration of organic acid was increased. Fibril formation *in vitro* is dependent on conformational stability of the protein. The solubility of a protein is a major factor in amyloid fibril formation because, when proteins are destabilized, the formation of amyloid fibrils is enhanced (Schmittschmitt & Scholtz, 2003).

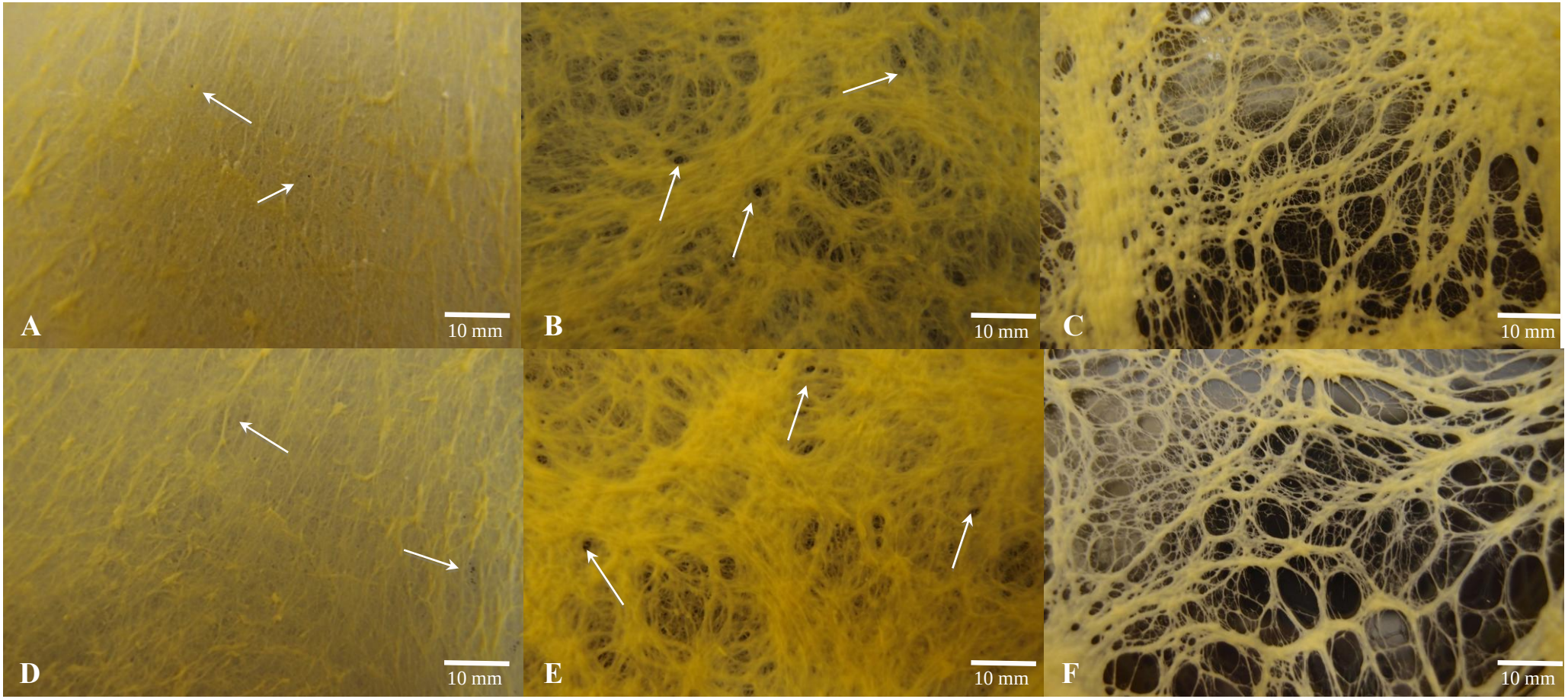

Figure 4.12b: The appearance of zein-rice flour dough that has been distended over petri dishes. Close up view. **A**: 0.7% acetic acid **B**: 1.3% acetic acid **C**: 5.4% acetic acid **D**: 0.7% lactic acid **E**: 1.3% lactic acid **F**: 5.4% lactic acid. Arrows indicate small holes.

4.3.5.4 CLSM of zein-rice flour dough

The microstructure of zein-rice flour dough (Figure 4.13 and Figure 4.14) was investigated by CLSM and compared to that of wheat dough (Figure 4.15). The autofluorescence of the zein or gluten in the dough was clearly seen. The dark areas on the images are areas of starch granules within the rice flour.

When zein-rice flour doughs were prepared at 40°C, there was evidence of protein fibril formation (Figure 4.13a). This change in structure could be seen in the rice dough as the zein changed from a granular to a fine fibrous form. Kneading the dough created a continuous multidirectional textured network of protein fibrils. As was previously seen with pure zein dough, dough prepared with organic acids produced a smoother, finer protein network. As the concentration of organic acid was increased, so it became more and more difficult to distinguish between the individual fibres because they appeared to make up a homogenous mass. The z-slices of the 3D images clearly show the individual fibrils that make up the different layers of the complex protein networks (Figure 4.13b). Upon stretching of the dough made without organic acids, the fibrous protein network that was visible in the unstretched dough appeared fractured and not as continuous (Figure 4.14a and 4.14b). The dark voids represented by the rice flour particles also appeared to become larger. In dough prepared with organic acids, the extended protein network seemed to retain its textured appearance. This shows promise for the commercial possibilities of zein-based doughs because during proofing and baking the protein network needs to retain its structural integrity in order to retain gases produced as a result of fermentation. However, the protein network in zein-rice flour dough was very different to that of strong wheat flour dough, which appeared more globular and less fibrous in structure (Figure 4.15).

The continuous and extensive microstructure of gluten has been described in the 'gluten sheet' model (reviewed by Kontogiorgos, 2011). In this model, the gluten network is considered to be formed by crosslinked protein sheets that are layered one over the other, forming a three-dimensional structure. Such a three-dimensional structure could be seen in the CLSM 3D images of zein dough (Figures 4.13a and 4.14a); the z-slices from the 3D images show the individual layers of fibres that make up the 3D image (Figure 4.13b and 4.14b). Kontogiorgos (2011) state that upon

extension of gluten sheets, perforation might occur, which might eventually lead to disruption of the network and possible appearance of fibre-like structures. The zein network, however, was always fibrous, which could indicate a lack of crosslinks and thus a weaker structure compared to gluten. This difference in structure could shed light on the fundamental difference between zein and gluten, which influences their performance in doughs.

CLSM studies by Bugusu et al. (2002) also showed this fine structure of zein fibres. These authors found that when zein was added to wheat dough it tended to form thinner and finer networks than were formed by gluten. Both protein networks were similar in that they formed a continuous and extensive matrix in which the flour and other dough ingredients were evenly distributed. Moore et al. (2004) observed similar networks with the CLSM of wheat dough, though these networks were absent in commercial gluten-free batter, non-dairy gluten-free batter and dairy gluten-free batter. Blanco et al. (2011) reviewed that a lack of a proper network that can hold carbon dioxide during proofing, similar to that of gluten, is the reason why rice flour is unable to produce a leavened product. Thus, zein fibrils could add a structural element to gluten-free doughs and batters (Schober et al., 2008).

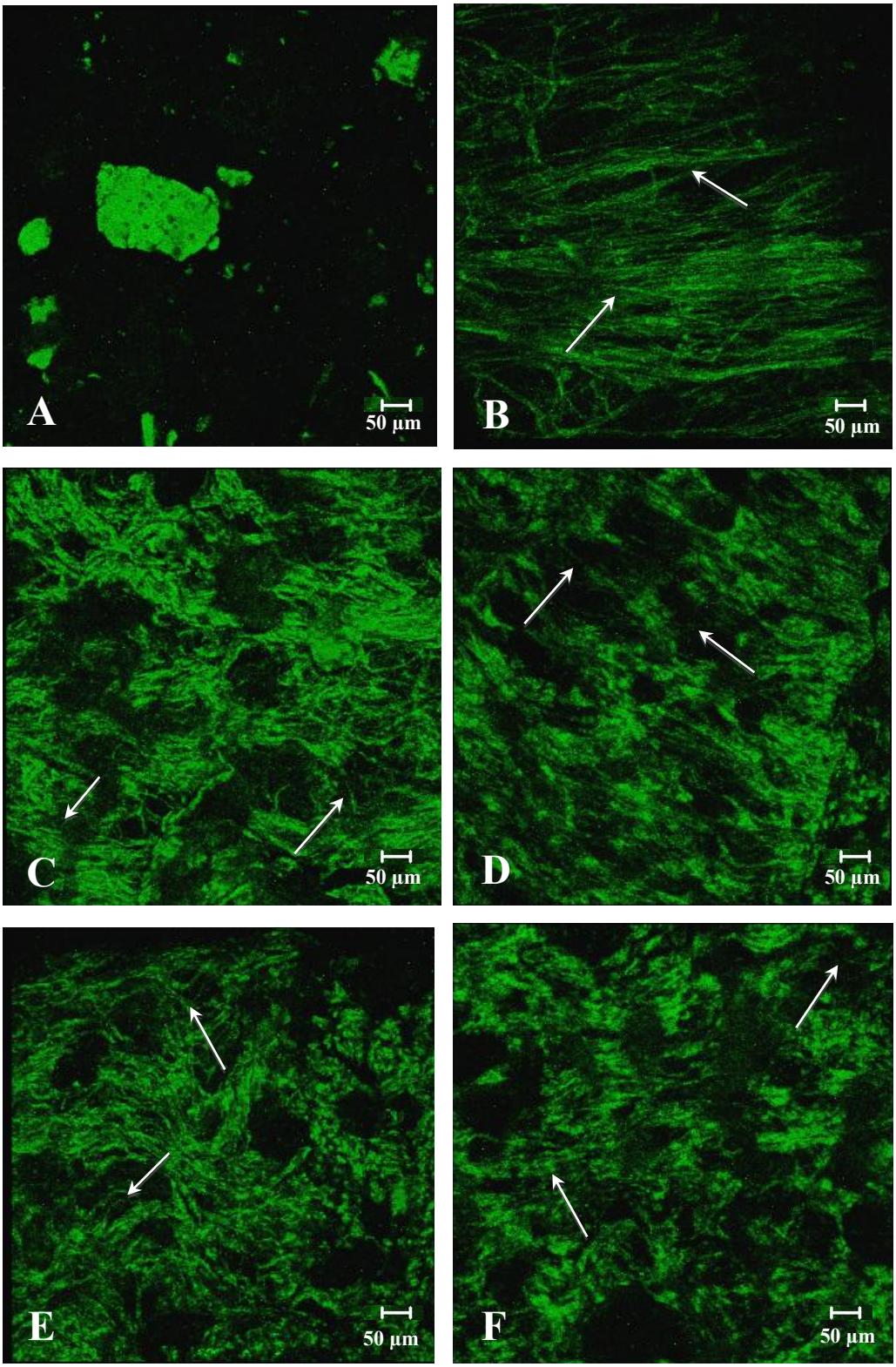

Figure 4.13a: CLSM of zein-rice flour dough prepared with various concentrations of dilute organic acids: 3D images viewed from the top. **A**: Zein in distilled water at room temperature **B**: Zein in distilled water at 40°C **C**: Zein in 1.3% lactic acid at 40°C **D**: Zein in 5.4% Lactic acid at 40°C **E**: Zein in 1.3% acetic acid at 40°C **F**: Zein in 5.4% acetic acid at 40°C. Fibrils are indicated by arrows.

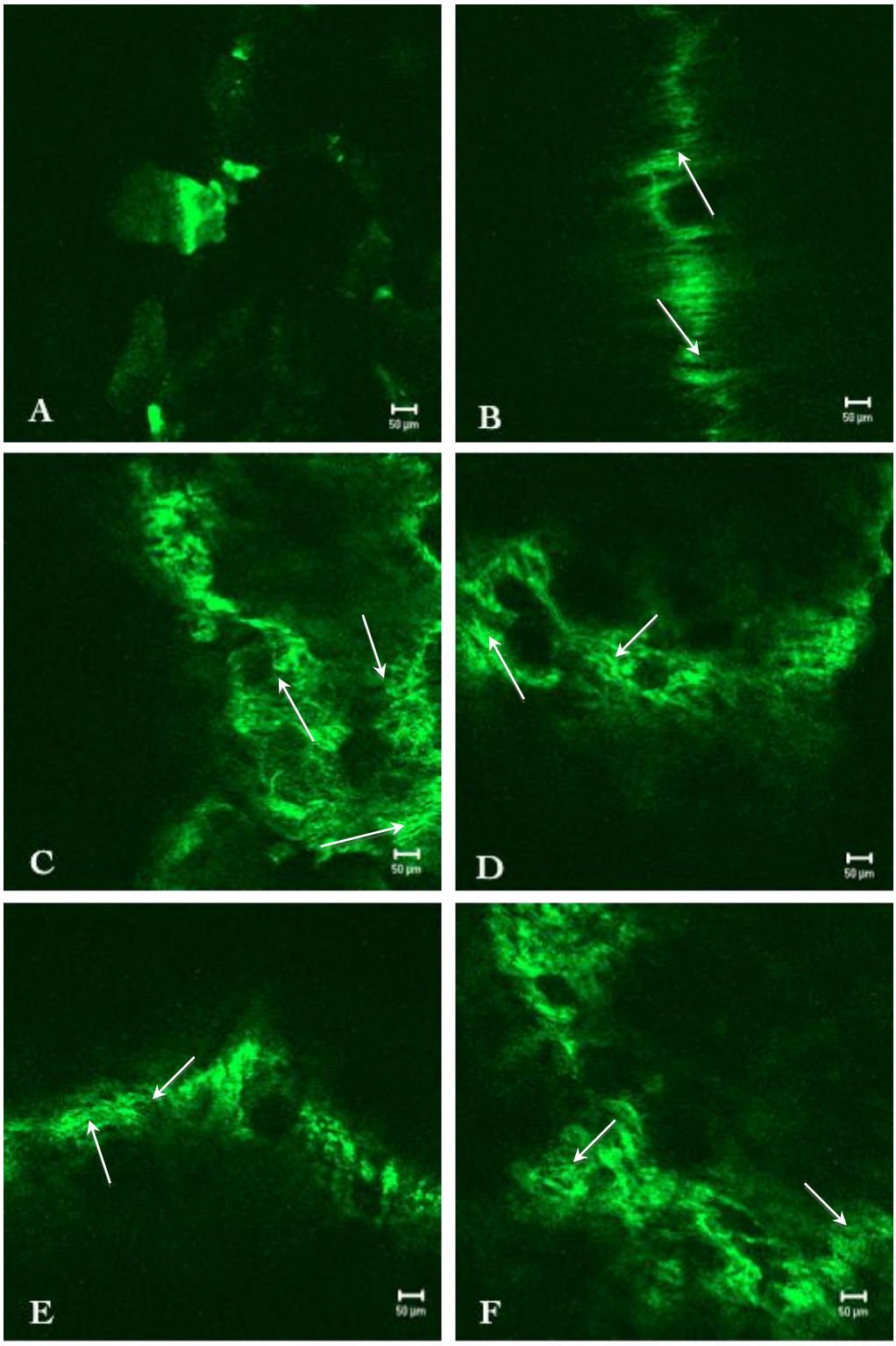

Figure 4.13b: CLSM of zein-rice flour dough prepared with various concentrations of dilute organic acids: Micrographs from a mid Z-slice of a 3D CSLM image. **A**: Zein in distilled water at room temperature **B**: Zein in distilled water at 40°C **C**: Zein in 1.3% lactic acid at 40°C **D**: Zein in 5.4% lactic acid at 40°C **E**: Zein in 1.3% acetic acid at 40°C **F**: Zein in 5.4% acetic acid at 40°C. Fibrils are indicated by arrows.

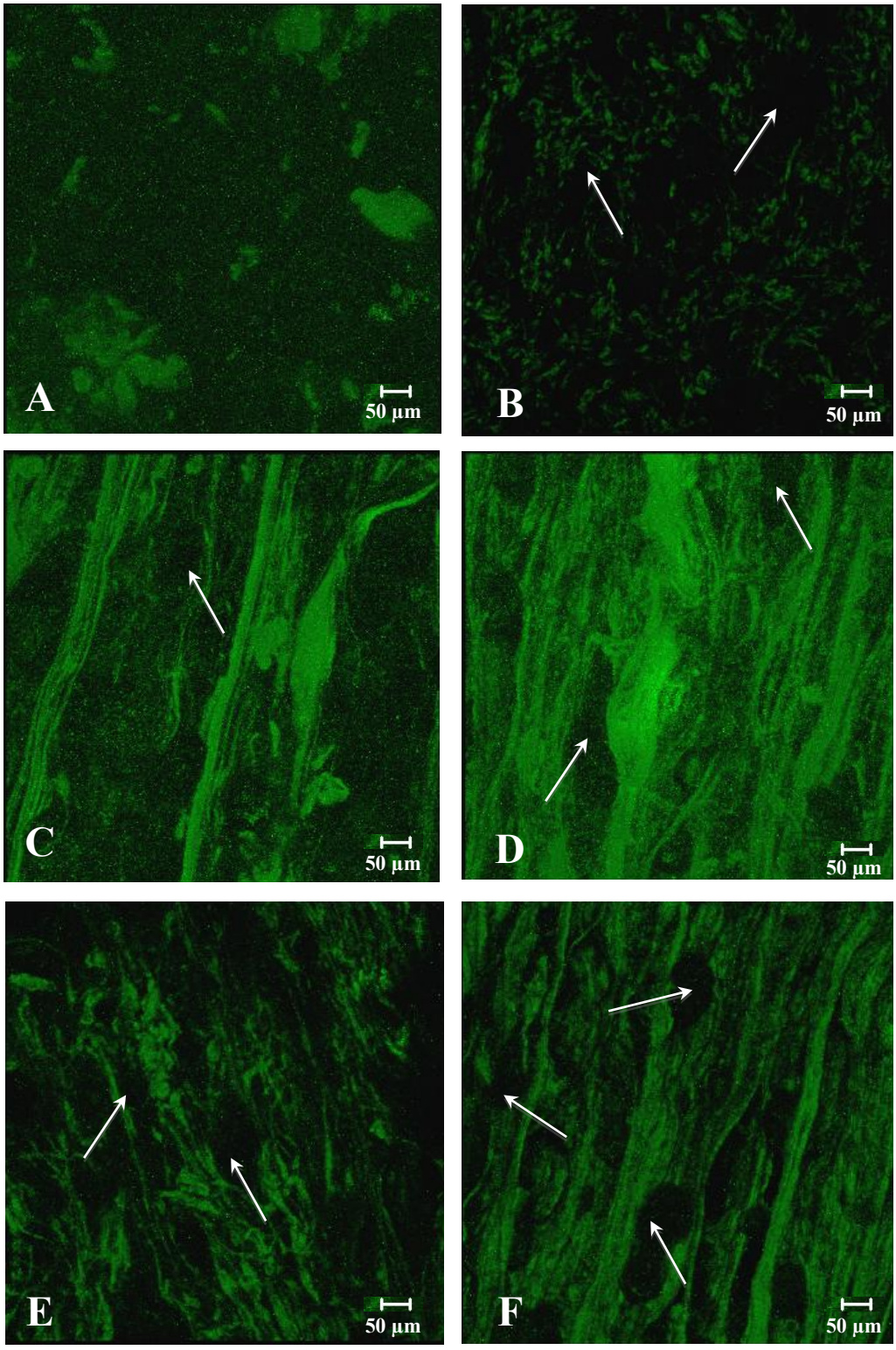

Figure 4.14a: CLSM of stretched zein-rice flour dough prepared with various concentrations of dilute organic acids: 3D images viewed from the top. **A**: Zein in distilled water at room temperature **B**: Zein in distilled water at 40°C **C**: Zein in 1.3% lactic acid at 40°C **D**: Zein in 5.4% lactic acid at 40°C **E**: Zein in 1.3% acetic acid at 40°C **F**: Zein in 5.4% acetic acid at 40°C. Dark regions of rice flour particles are indicated by arrows.

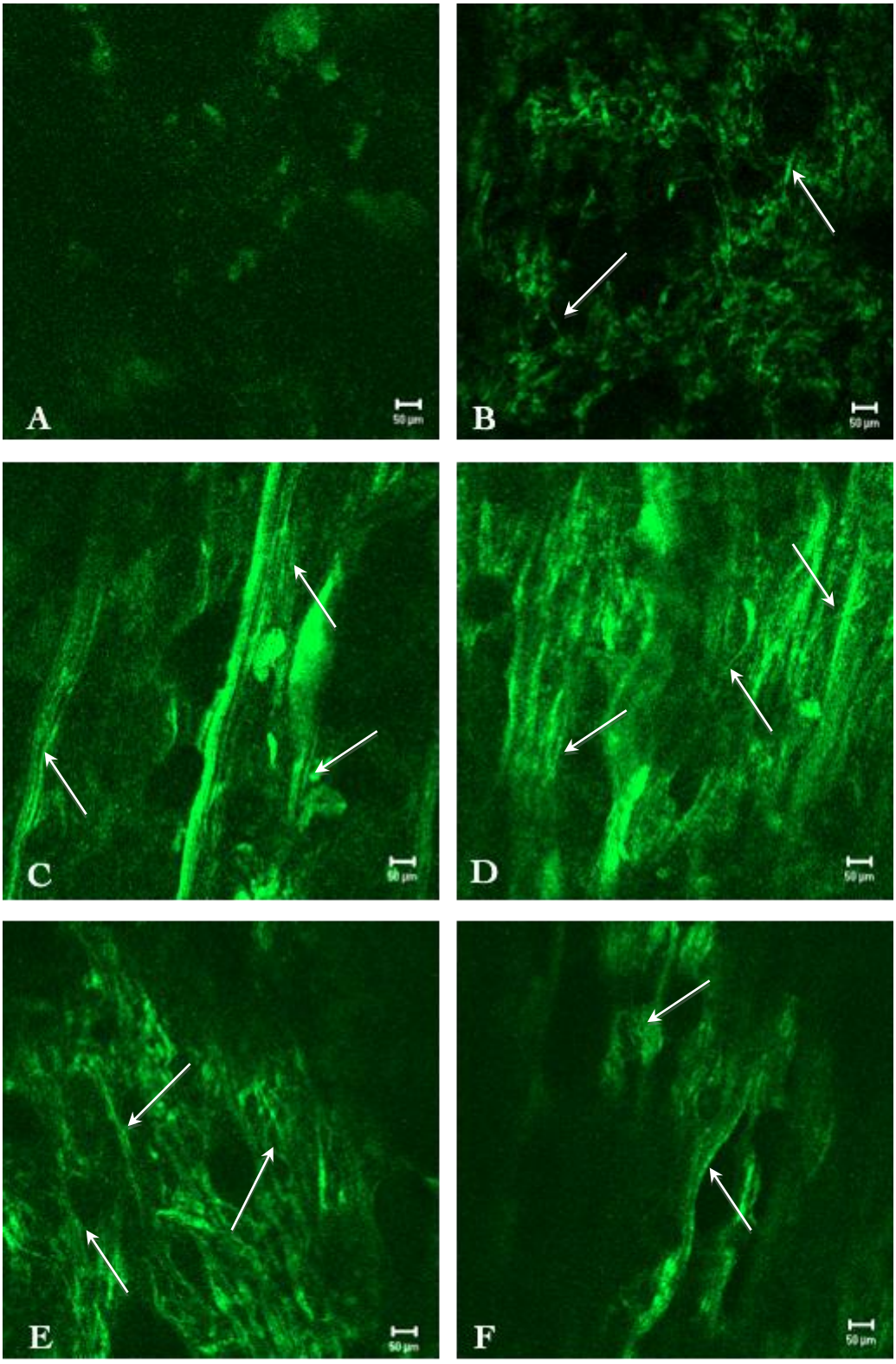

Figure 4.14b: CLSM of stretched zein-rice flour dough prepared with various concentrations of dilute organic acids: Micrographs from a mid Z-slice of a 3D CSLM image. **A**: Zein in distilled water at room temperature, **B**: Zein in distilled water at 40°C, **C**: Zein in 1.3% lactic acid at 40°C, **D**: Zein in 5.4% Lactic acid at 40°C, **E**: Zein in 1.3% acetic acid at 40°C, **F**: Zein in 5.4% acetic acid at 40°C. Fibrils are indicated by arrows.

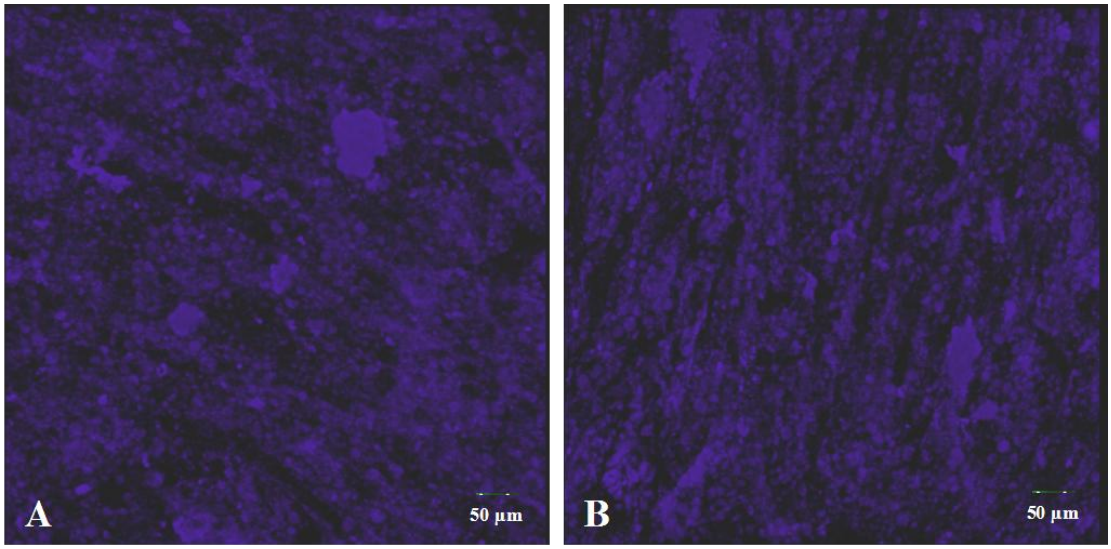

Figure 4.15: CLSM 3D images, viewed from the top, of strong wheat flour dough **A**: Unstretched and **B**: Stretched

4.3.5.5 Bread Making

Making bread from the zein-based dough proved to be quite challenging, and in all cases poor quality bread was produced (Figure 4.16). The addition of zein increased the loaf volume of rice-flour bread (A) and the breads made with zein-based dough prepared with distilled water produced the best crumb structure and volume (B). The zein-based doughs prepared with the organic acids at a 0.7% concentration followed this in terms of quality, as the breads produced had a slightly increased volume (C and F) compared to the pure rice-flour bread. As the concentration of organic acid (lactic acid and acetic acid) was increased from 1.3 to 5.4% so the visible crumb structure and volume decreased. A few large gas pockets appeared in the breads, which became increasingly dense and compact in appearance. The crusts of these breads were observed to be very uneven. At a level of 5.4% organic acid, large cavities were visible in the upper centre of the breads, below the crust. In general, a thick crust was visible whenever organic acids were used, and all the breads were yellow in colour and had pungent acidic maize-like aromas.

The results obtained from the baking support those obtained from the alveography of the zein doughs (Table 4.10). The low P/L ratios indicated that zein-based flours have poor bread-making potential when compared to strong wheat flour. However, the

zein-based flours did improve the baking potential of rice flour. The lack of adequate leavening could be due to the absence of a leavening agent that is able to produce the required amount of carbon dioxide for rising to take place when acidic conditions prevail. Zein-based doughs made with organic acids, even in low concentration (0.7, 1.3 & 5.4%) appeared to inactivate the yeast and cause the rapid release of carbon dioxide from the baking powder. The zein-starch dough produced very dry bread with a gum-like, starchy consistency. However, the zein-rice flour doughs displayed small gas bubbles during the proofing stages and produced better quality bread. Although the zein-rice flour bread was spongy to touch, the crumb structure of the breads was not uniform, soft, elastic or well aerated. All the breads, including the ones made without organic acids had a very dense texture.

Similar trends resulting from the addition of acetic acid and lactic acid were observed by Blanco et al. (2011). These authors found that the addition of 0.2% acetic acid to gluten-free rice flour dough increased the volume of the breads by 10% compared to the non-acidified control breads. While an increased concentration of 0.4% acetic acid did not change the volume of the bread, the highest level of 0.6% led to a bread volume that was smaller than that of the control. It was also found that as the level of acetic acid increased, so the crumb cell area decreased. The same trend in bread volume was seen with the addition of lactic acid. However, the addition of lactic acid initially decreased the crumb cell area, but at a level of 1.2% it increased it slightly. These authors postulated that the small volume of breads made with high concentration of acids was due to a “preservative effect” of the acid on the yeast. These authors suggested that the un-dissociated form of the acid could pass through the membrane of the yeast cell by simple diffusion. Following this, the acid would dissociate, liberating protons and lowering the internal pH of the cell, thus preventing cell growth and gas production, which affect dough development. Thus, the acid would either kill the yeast or inhibit gas production by the yeast cells. These authors also found that the addition of acidic food additives such as acetic acid, lactic acid, citric acid and monosodium phosphate did not alter the rice flour doughs rheological properties, such as viscoelasticity.

Schober et al. (2008) concluded that the fibrous network of zein itself holds only a small amount of gas. The excessive extensibility of zein led to flat appearing zein

"hearth-type" rolls (Schober et al., 2010). According to Schober et al. (2008) the frequent occurrence of large void spaces below the crust in zein bread suggests that a structural weakness exists in the dough. Structural weaknesses, created by the 5.4% organic acids, could have led to gas accumulation under the crust during the proofing and baking phases, which would have forced the dough apart and created voids (E and H). Erickson et al. (2011) reviewed that the fibrous microstructure of zein can be fractured due to mechanical treatments and cooling below the T_g. Such fractures cause structural limitations in the dough that are responsible for the formation of large voids during the cooling of the zein-based breads (Schober et al., 2008).

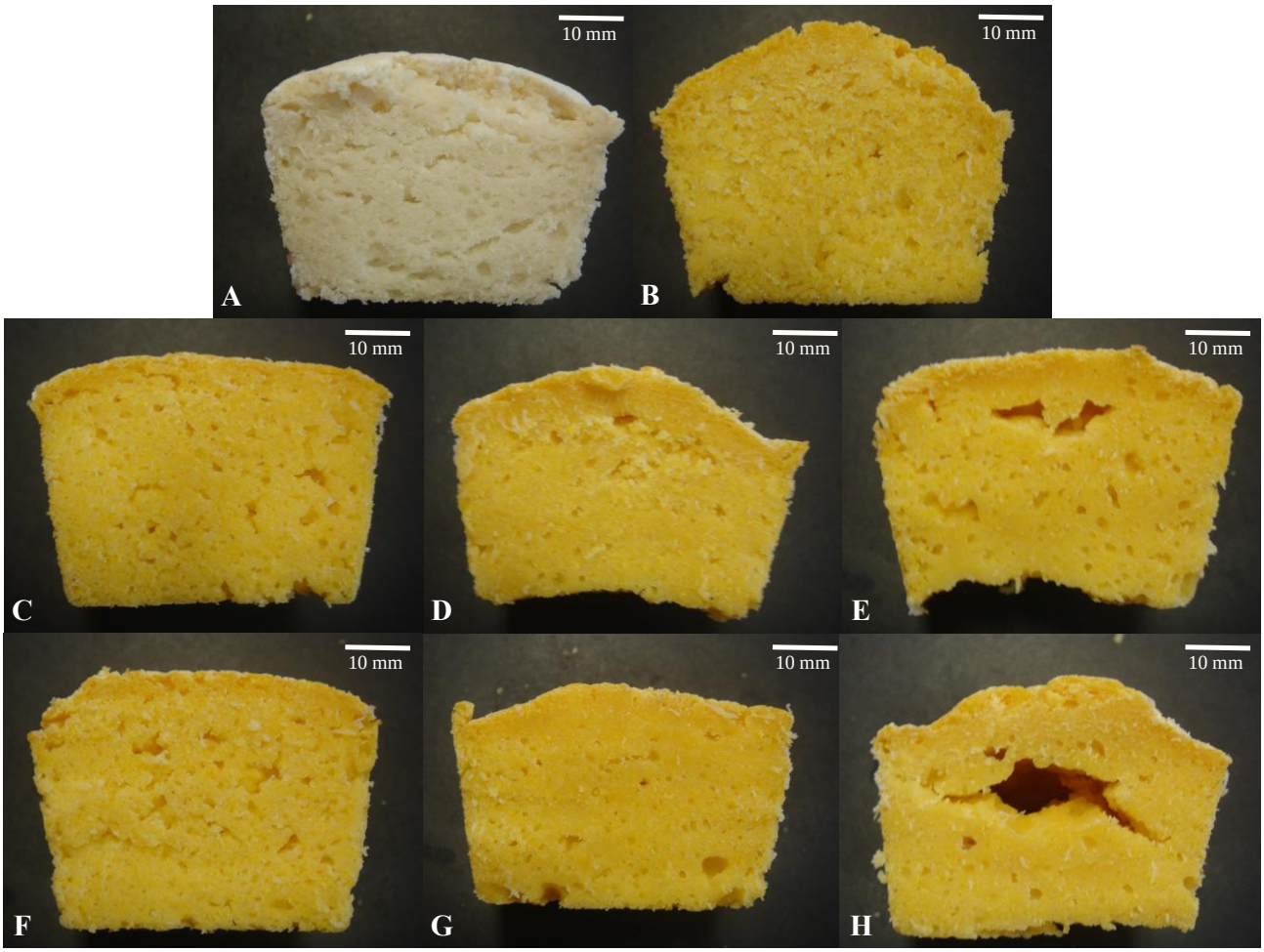

Figure 4.16: Cross sections of chemically leavened zein based (20%) rice flour breads prepared with different concentrations of organic acids. **A**: Control, rice flour bread containing no zein **B:** distilled water, **C**: 0.7% lactic acid, **D**: 1.3% lactic acid, **E**: 5.4% lactic acid, **F**: 0.7% acetic acid, **G**: 1.3% acetic acid, **H**: 5.4% acetic acid.

4.4 CONCLUSIONS

Zein forms a viscoelastic dough when heated above its T_g of 40°C. The rheological properties of this dough are altered by the addition of the organic acids, acetic acid and lactic acid. Addition of these organic acids in increasing concentrations caused a softening of the dough and a dramatic increase in extensibility, which was accompanied by a substantial decrease in dough stability when high levels (5.4%) of acid were used. SDS-PAGE did not indicate that any polymerization or crosslinking had taken place in dough as a result of the organic acids. However, FTIR revealed that the high β-sheet content observed in doughs above their T_g decreased when organic acids were added. CLSM demonstrated that the addition of organic acids to zein dough promotes the formation of fine protein fibrils, which are capable of aggregating together and, upon kneading, are able to form a cohesive network. At low concentrations of organic acid (0.7%) this network is capable of gas retention. However, as the concentration of acid was increased to 5.4%, the gas holding ability of the dough diminished due to a decrease in dough stability. Zein protein networks that do form are still significantly weaker than those formed by gluten, most probably due to the absence of crosslinks. The finer protein network is believed to increase protein-protein interactions that could lead to clumping of the protein fibrils if very high concentrations of acid are used. The effect of organic acids on the bread-making potential of the zein dough could not be determined due to the lack of an acid tolerant leavening agent.

In conclusion, zein dough prepared with dilute organic acids shows potential to improve the rheological properties of gluten-free doughs. The quality of bread made with this dough is likely to still be poor, and indicates that the presence of an extensive protein network alone does not guarantee satisfactory gas holding ability during baking. Harnessing the inherent structural potential of the protein network of zein prepared with organic acids in order to achieve improved viscoelastic functionality offers an alternative novel approach to gluten-free bread making.

5. GENERAL DISCUSSION

The maintenance of zein dough above its T_g temperature of 40°C proved to be a challenge during the practical component of this research. This discussion deals with the relevant principles behind the T_g temperature of zein as well as the strengths and limitations of the methodologies used during the experiments. The possible influences of organic acids on the conformational state of zein in dough are also discussed. Lastly, the implications of this research and its future application are considered.

5.1 Methodological Considerations

The greatest methodological challenge experienced when working with zein dough was maintaining it above the T_g temperature of the protein. The T_g of zein, approximately 40°C (Schober et al, 2008), is an important physical parameter with regard to zein functionality. Above this temperature, the protein's propensity for aggregation and cross-linking is increased (Erickson et al., 2011), the polymer is rendered more mobile (Sperling, 1992; Kokini et al., 1994), and there is an increase in adhesive forces present (Panchapakesan et al., 2012). In this present work, it was very evident that the favourable structural changes in zein brought about by exceeding its T_g temperature were unstable and were quickly reversed when the temperature dropped. This temperature challenge was evident in all aspects of the practical work, from analysis using the Kieffer rig, to the CLSM, FTIR, alveography and even during bread making. In all cases, the viscoelastic functionality of the zein dough, which is characteristic when the protein is above the T_g, was lost when the dough cooled.

The rapid cooling of zein during handling at room temperature proved problematic before and during the various stages of dough processing. The extension of zein dough during tensile tests conducted on the Kieffer rig created a larger surface area, which promoted a more rapid cooling and hardening of samples as they approached maximum extension. When zein dough was inflated using an alveograph such rapid cooling was once again seen. A drop in dough temperature, as the dough was inflated, could have led to the tearing and fracturing experienced before maximum distensibility was reached. The high extensibility of zein dough is dependent on the

presence of a viscoelastic rubbery state, without it the brittle protein network cannot expand as gases are produced during fermentation (Erickson et al., 2011). The rate of cooling is also dependant on the size of the samples being processed. Small sample sizes, such as those required for analysis by FTIR and CLSM, have a large surface area to volume ratio, and are therefore more prone to accelerated cooling.

Consistent instrumental readings, with low standard deviations, could only be obtained when the zein dough was kept above 40°C. This required a very rapid and organized processing technique. The use of a water bath, a heated working surface, as well as the pre-warming of any equipment, such as spatulas and baking tins used all helped to reduce the heat loss during the practical work. In an ideal situation, the various experimental equipment used would be housed inside a large-scale incubator, unfortunately such an incubator was not available for use during this study.

Conversely, elevated temperatures during baking stages can also prove problematic for zein dough. The current baking experiments were conducted at a lower temperature (160°C) than usual, because the normal baking temperature of 220°C softened the dough to such an extent that it became too weak and could not retain any gases that were produced. Tensile tests also showed that softer aggregates were formed when zein dough was incubated at 80°C. When temperatures are elevated in excess of the T_g, the reactivity and mobility of the polymer is also increased (Sperling, 1992). An increased reactivity could enhance the protein's tendency to aggregate and crosslink (Madeka & Kokini, 1996). However, increasing the mobility of the dough could also decrease its strength, and ultimately the gas holding ability. As mentioned, the alveography conducted in this work showed that zein doughs with a low structural stability were unable to retain gases during inflation.

One limitation of the equipment used in this practical work was that the Kieffer rig only had a maximum possible extension of 150 mm. This proved too short for the experiments as conducted here, because the high extensibility of the dough meant that none of the samples broke before the maximum possible extension was reached. The use of a smaller sample size, or a different set-up with a greater possible maximum extension would give insight into the true extensibility and fracture point of zein dough.

5.2 The potential influence of organic acids on the conformational state of zein

It was found that preparing zein dough with dilute organic acids influenced the functional properties of the resulting dough. The greater the concentration of acid used, the greater the effect. Zein has a stable structure and in order to produce large changes in its tertiary and secondary structures, high concentration of denaturants, changes in pH, or elevated temperatures are required (Selling, Hamaker & Sessa, 2007). The addition of acid has no single effect on the conformational state of proteins (Fink, Calciano, Goto, Kurotsu & Palleros, 1994). From this present work it appears that the organic acids affect the zein protein in various ways. Apart from partially solubilizing the protein (Taylor, Taylor, Dutton & De Kock, 2005a; Taylor, Taylor, Dutton & De Kock, 2005b), the acid could have physically altered the structure of the zein through denaturation and deamination and it could also have acted as a plasticizer aiding in the plasticization of the zein with water.

Theoretically, the acidic environment created by addition of organic acids could possibly increase the solubility of zein, promoting the formation of extended protein structures. From the experimental work conducted, it was found that addition of organic acids significantly influenced the extensibility of the zein dough when in a viscoelastic state. Solubilisation of protein would account for the increasing extensibility of the zein prepared with increasing concentrations of organic acids. Evidence for the existence of the thin extended structure of the zein when it is in solution is convincing. Tatham, Field, Morris, I'Anson, Cardle, Dufton and Shewry (1993) showed, through small-angle X-ray scattering (SAXS) and intrinsic viscosity measurements of zein solutions in 70% (v/v) ethanol, that α-zein in solution has an extended structure. The structural length varied between 10 and 24 nm depending on the type of analysis and interpretation of data by these authors. Li, Li, Xia, Zhang, Wang and Huang (2012) found through static light scattering that zein solubilized more easily in acetic acid than in aqueous ethanol due to the protonation of the protein by the acid.

Intramolecular charge repulsion is also a driving force for unfolding the protein structure during acidic denaturation (Fink et al., 1994), where irreversible structural

changes take place (Selling et al., 2007). Under many unfolding conditions, however, the denatured protein may retain substantial structure (Fink et al., 1994). Thus, Zhang et al. (2011) suggested that partial unfolding of the zein protein molecular structure by means of controlled chemical treatments is necessary to improve functional properties. However, uncontrolled modifications could lead to a reduced functionality due to fragmentation and truncation of the zein backbone.

During the present study, improved functionality was seen in zein dough prepared with organic acids at low concentrations. However, when the concentration of organic acid was increased, the resulting high extensibility weakened the dough and it lost its structural stability. According to Maher Galal et al. (1978), the formation of new bonds after a protein structure unfolds can be prevented by the presence of strong intermolecular electrostatic repulsive forces. This results in further weakening of the structure and thus a softening effect. Such a softening effect and a weakening of the protein's structural stability was seen when the zein dough was prepared with organic acids. The doughs became increasingly weaker and softer as the concentration of acid was increased.

Fink et al. (1994) investigated the acid-induced unfolding of several monomeric proteins as a function of ionic strength. They found that acids induce a wide range of effects. Proteins that these authors categorised as being type I (which included β-lactamase, cytochrome c and apomyoglobin) when titrated with HCl in the absence of salts, experienced initial unfolding in the vicinity of pH 3-4 and then refolded to a molten globule-like conformation. Type II proteins (α-lactabumin and carbonic anhydrase) did not completely unfold upon acid titration, however instead they transformed into a molten globule state at a pH of 3. These authors state that the exact behaviour of a protein at a given pH is a complex interplay between a variety of stabilizing and destabilizing forces, some of which are sensitive to the environment. In particular, protein conformation is sensitive to denaturants and temperature, which cause destabilization, as well as to the presence of salt and anions, which affect the proteins electrostatic interactions (Fink et al., 1994). The effect of an increase in ionic strength, resulting from the presence of an acid, could explain the differences seen here between doughs prepared with different concentrations of organic acid; the higher the levels of organic acid, the greater the resulting positive net charge and thus

the more pronounced the effect on the structure of the protein network in the zein dough systems. It is thus clear that alteration of pH away from zein's Pi and above its T_g has a major influence on zein's functionality.

During unfolding, reactive groups on the surface of the proteins polymer may become exposed. Maher Galal et al. (1978) noted that increased intramolecular electrostatic repulsion created by acidic environments leads to the unfolding of gluten proteins and an increased exposure of hydrophobic groups. Zein is a hydrophobic protein that contains high levels of non-polar hydrophobic amino acids such as leucine, alanine, proline and phenylalanine (Wall & Paulis, 1978). The exposure of zein's hydrophobic domains could facilitate protein-protein aggregation through hydrophobic reactions (Schober et al., 2010). These increased protein-protein interactions could explain the tendency of the zein fibrils to 'clump' together into larger, more pronounced protein fibers when high levels of organic acids were used. This clumping was clearly evident in the distended zein-rice flour doughs prepared with organic acids at 5.4%. It could also have resulted in the dough's poor ability to capture and hold gases during alveography, as well as the decreased stability of the dough.

An increase in surface hydrophobicity would also allow greater interactions with solvents (Cabra, Arreguin, Vazquez-Duhalt & Farres, 2006). Kim and Xu (2008) described how zein, which is amphiphilic (Schober et al., 2011), could aggregate when evaporated into films. Kim and Xu (2008) postulate that protein aggregates orientate their hydrophilic moieties towards the solvent medium when the solvent is more polar than 90% ethanol, but away from the solvent medium at polarities less than this.

Another explanation for the formation of fibrils in zein could be self-assembly, such as occurs with amyloid fibril formation. Amyloid fibrils are highly ordered, insoluble aggregates of proteins or polypeptides (Chiti & Dobson, 2006), which have fibrous qualities and are characterized by high levels of β-sheet conformations (Erickson et al., 2011). They are typically associated with neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Rochet & Lansbury, 2000). This model system of fibril self-assembly could lead to a mechanistic understanding of the structural transformations that are responsible for the development of zein's aggregation and

viscoelastic functionality (Erickson et al., 2011).

Fibril formation *in vitro* is dependent on the conformational stability of the protein. The solubility of a protein is a major factor in amyloid fibril formation because, when proteins are destabilized, the formation of amyloid fibrils is enhanced (Schmittschmitt & Scholtz, 2003). Rao (2008) reviewed that fibrils can be obtained by altering conditions such as pH, temperature, concentrations of salt or metal ions, and through the use of solvents and chaotropic agents. At low pH levels the protein's acidic side chains become protonated and the build-up of positive charges results in coulombic repulsion of like charges (Pedersen & Otzen, 2008). The outstanding strength of these structures is due to the backbone hydrogen atoms of the β-sheet providing an extensive hydrogen-bonding network (Chiti & Dobson, 2006). However, while amyloid fibrils, as viewed by electron microscopy, generally share this elongated fibrillar appearance, they often present as mixtures of different morphologies (Pedersen & Otzen, 2008). The substructures of these morphologies are intertwined bundles of protofilaments that are 2 to 3 nm in diameter (Figure 5.1) (as reviewed by Rao, 2008).

Figure 5.1: Evolution of oligomeric intermediates of the fibrilization pathway. Spheres undergo self-association (i) to form protofilbrils. Two protofibrils may intertwine (ii) to produce a wound structure that may undergo further conformational rearrangements (iii) to form a mature fibril. Two protofibrillar constituents of the mature fibril are shown in the cross-section. (from Rochet & Lansbury, 2000).

Such structures were seen during the distensibility tests; the finer fibrils created by the high levels (5.4%) of organic acids, as seen by CLSM, clumped together and the formation of larger fibrils was enhanced. According to Pedersen and Otzen (2008), the tendency of a protein to form amyloid fibrils is highly modulated by amino acid composition with sequences rich in hydrophobic aromatic amino acids and β-branched chains forming fibrils more easily.

Deamination of zein can also occur under acidic conditions such as those used in the present work. Cabra et al. (2006) found that different pH treatments (0.5-2.0 M hydrochloric acid or sodium hydroxide), with and without the application of heat, induced deamination of zein and improved the protein's emulsifying abilities. Zhang et al. (2011) observed that under acidic conditions the structure of zein changed dramatically. However, as in this work, these authors showed by means of SDS-PAGE that no obvious zein fragmentation or oligomerization had taken place. They also found, through FTIR analysis, that under acidic conditions the content of α-helical, β-sheet and β-turn conformations in zein decreased. These changes were attributed to deamination of glutamine to glutamic acid. However, such changes in the secondary structure of zein dough were not evident in the practical work conducted in this study. This absence of noticeable deamination could be explained by the high molarity of the acid treatments used by Cabra et al. (2006), which indicates that only extremes of pH induce deamination.

FTIR during this study revealed that by raising the temperature of the zein to above the T_g, the β-sheet content increased, and the addition of organic acids in increasing concentrations promoted the formation of α-helical conformation. The initial increase in β-sheet content when zein is raised above its T_g is thought to promote the viscoelastic properties of the polymer (Erickson et al., 2011; Mejia et al., 2007). Amonsou et al. (2012) observed that the highly viscous and extensible rheological behaviour of marama bean protein was probably related to the high proportion of β-sheets present, hydrophobic interactions and tyrosine crosslinks. Beta-sheets are believed to contribute substantially to the elastic behaviour of gluten in wheat dough (Belton, 1999), and β-sheet and disulphide bridge formation seems to contribute to the stabilization of gluten protein polymers (Mejia et al. 2007).

When organic acids were added to zein dough in increasing concentrations (0.7, 1.3 & 5.4%), so the β-sheet content decreased. This could explain the destabilization of the zein dough that was observed during the alveography. The lack of high molecular weight subunits in maize zein (Anderson & Lamsal, 2011) might also explain why the extended β-sheet alignments that occur upon formation of the viscoelastic zein polymer do not remain stable and quickly dissipate upon cooling. Zein also contains

half the amount of cysteine (0.8 g/100g protein) compared to gluten, which could affect the strength, stability and functionality of the dough system due to a lack of disulphide bond formation (Mejia et al., 2007). Initially it was thought that the addition of organic acids to zein could increase the amount of crosslinking that was taking place in the dough. However, crosslinking as a result of organic acid addition was not evident from the SDS-PAGE conducted in this experimental work. Schober et al. (2011) suggested that hydrophobic interactions play a central role in zein aggregation and that disulphide bonds are undesirable. Matsushima, Danno, Takezawa & Izumi (1997) suggested that α-zein in solution could consist of α-helices stacked in a linear fashion along the direction of the long axis. Thus, the increasing number of α-helices seen in the dough as the concentration of organic acids increased, could have influenced extensibility as the α-helices aligned in this linear fashion. Schober et al. (2011) suggests that α-zein in water aggregated into oligomeric structures by means of the hydrophobic surfaces on the α-helices. Thus, the higher the number of α-helices present in dough, the greater the protein-protein interactions present and thus the greater the tendency for aggregation to take place.

Because the addition of an organic acid into the zein dough system altered the tensile properties of the doughs to produce a less viscous, rubbery state, it is believed that the acid could have plasticized the zein protein and could have aided in the plasticization of the zein with water. A plasticizer is an additive that is used to affect the phase behaviour of polymers (Panchapakesan et al., 2012) by increasing plasticity or fluidity. By embedding themselves between polymer chains, small plasticizer molecules increase spacing and free volume. This allows polymers to move past one another more freely, even at lower temperatures, leading to a reduction in the T_g. According to Kester and Fennema (1986) and Krochta (2002) a plasticizer competes with the protein chains for hydrogen bonding and electrostatic interactions, causing a decrease in the intermolecular forces between adjacent protein chains. As noted earlier, the addition of an acid also decreases the intermolecular forces between adjacent protein chains by changing the charge on the amino acid side groups along the protein (Maher Galal et al., 1978). In gluten, gliadins form a matrix within the long glutenin polymer networks and contribute to resistance to extension by forming a viscous environment (Belton, 1999). However, upon increasing the gliadin content, the gluten's resistance to extension decreased and the extensibility increased

(Sliwinski, Kolster, Prins and Van Vliet, 2004). Thus, it has been said that gliadins act like a plasticizer, promoting viscous behaviour and extensibility of gluten (Abang Zaidel, Chin & Yusof, 2010; Kontogiorgos, 2011). This is similar to what was experienced with zein dough; increasing the amount of organic acid incorporated in the zein system, led to a reduction in resistance to extension and increase in extensibility.

The addition of an organic acid could also have aided in the uptake of water into the dough system. Studies of wheat dough using a farinograph showed that water uptake or dough consistency was increased by adding organic acids in the absence of salt (Tanaka et al., 1967; Maher Galal et al., 1978). Lawton (2004) describes findings by Beck et al (1996) that compression molded zein samples containing different plasticizers absorbed water to different degrees depending on the type of plasticizer in the sample. These authors stated that water is a very good plasticizer for zein because it is absorbed by both the zein and its plasticizers and ultimately affects the tensile strength of the zein films. As discussed by Panchapakesan et al. (2012) the mechanism by means of which water acts like a plasticizer can be explained in terms of a free volume effect where the free volume is inversely proportional to the number average molecular weight. In other words, a low molecular weight substance such as water leads to an increased free volume, which in turn allows for increased mobility of the polymer. The absorption of too much water can have a weakening affect. Lawton (2004) found that the addition of triethylene glycol, a hygroscopic plasticizer, caused zein films to absorb too much water, which led to a weakening of the film structure. However, in this present work, although organic acids caused zein dough in the current study to soften and weaken, hydration tests did not show any significant change in the moisture content of the dough.

5.3 Way Forward

This study has demonstrated that zein-based doughs prepared with organic acids have properties that can be developed and exploited in the production of gluten-free bread. Future work should address a number of issues that could include finding a suitable leavening agent that is acid tolerant, improving dough strength through crosslinking

of protein networks and improving the sensory attributes of the dough in order to increase consumer acceptability. An investigation should also be conducted into biological acidification through natural sourdough fermentation.

During bread making, the leavening of acidic zein-based dough with either yeast or a chemical leavening agent proved problematic. Acid-tolerant yeasts, such as those used during sourdough fermentation (Katina et al., 2005; Katina 2005), could be employed to help overcome this problem. The use of weaker, less volatile organic acids, such as citric acid, could also be investigated to improve the aroma and taste of the breads; acetic acid proved itself to be exceptionally pungent and unappealing.

Ultimately, the sensory properties of dough prepared with organic acids are going to influence consumer acceptability of the final baked product. The zein dough and bread had a pungent and unappealing acidic-maize smell and were bright yellow in colour. Thus, apart from improving the dough's internal structure through acidification, focus should also be directed towards improving its appearance, smell and taste. Schober et al. (2007) found, through informal taste panels, that U.S. consumers disliked excessive acidity in sourdough breads. The problem of an unappealing colour could be addressed through the use of zein extracted from white rather than yellow maize.

Although the addition of organic acids improved functionality, zein-based dough was still much softer and weaker than its gluten counterpart. Methods to improve dough strength and stability could include the use of the cysteine rich β- and γ-zeins (Schober et al., 2011). These zein fractions are more likely to form disulphide crosslinks, which if used in controlled quantities could improve the strength of the dough without compromising on viscoelastic functionality. Since the number and pattern of disulphide crosslinks of wheat glutenin polymers also affect dough strength (Shewry, Popineau, Lafiandra, Halford, Tatham, & Belton, 2003), the manipulation of the crosslinking pattern in the zein polymer, with and without other added proteins, should also be evaluated.

A natural sourdough fermentation of the zein based bread flour could also be investigated. Schober et al. (2007) examined the effect of sourdough fermentation on

the structure of sorghum based gluten-free bread and found an improvement in volume and crumb structure. Enzymes inherently present in sourdough fermentations could be examined for their potential to modify and strengthen zein protein structures (Moore et al., 2007).

6. CONCLUSIONS AND RECOMMENDATIONS

Zein displays viscoelastic functionality when it is warmed above its T_g temperature of 40°C. This viscoelastic functionality is highly temperature dependent, however, and is completely lost upon cooling to below the T_g point. Preparation of zein dough with organic acids, lactic acid and acetic acid, above its T_g temperature greatly improves its viscoelastic functionality, though this functionality is still dependent on temperature.

The addition of acid to the zein dough makes it easier to handle. There is a softening effect and dramatic increase in extensibility as the concentration of organic acid is increased. It is believed that these changes in zein dough functionality can be attributed to the solubilizing effect of the acids on the protein's secondary structure, promoting the formation of finer fibrils, which then clump together by means of protein-protein interactions. The acids may act as plasticizers in their own right, thereby improving the mobility of the protein polymer.

The higher the concentration of acid used, the greater the effect that was observed on dough functionality. Unfortunately, improved zein dough functionality does not correspond directly with the potential usefulness of the dough in a bread dough system. The use of low concentrations (0.7 & 1.3%) of organic acids improves the functionality of zein-rice flour and zein-maize starch doughs to the extent that the protein network within the dough is capable of retaining gases. Higher concentrations of organic acids (5.4%) produce dough with a further increase in functionality in terms of cohesiveness and extensibility, but the stability of the protein network is reduced. Thus, only zein doughs prepared with low levels of organic acids are likely to serve as a practical alternative to gluten in gluten-free dough systems for individuals suffering from coeliac disease.

It is recommended that improvements on this current work should focus on increasing dough strength and investigating the potential of biological acidification through means of natural sourdough fermentations.

7. LITERATURE CITED

AACC International. 2000. *Approved Methods of the American Association of Cereal Chemists*, 10th Ed. Approved Methods 08-17, 30-25, 44-15A and 46-19. American Association of Cereal Chemists. St. Paul, MN.

Abang Zaidel, D.N., Chin, N.L., Abdul Rahman, R. and Karim, R. 2008. Rheological characterization of gluten from extensibility measurement. *Journal of Food Engineering*. 86: 549-556.

Abang Zaidel, D.N., Chin, N.L., & Yusof, Y.A. (2010). A review on rheological properties and measurements of dough and gluten. *Journal of Applied Science*. 10: 2478-2490.

Achour, A., Thabet, Y., Sakly, W., Mankai, A., Sakly, N., Ayadi, A., Sfar, M.T., Amri, F., Harbi, A., Essoussi, A.S., Krifa, A., Ajmi, S. and Ghedira, I. 2010. IgA anti-actin antibodies in celiac disease. *Gastroenterologie Clinique et Biologique*. 34: 483-487

Ahlborn, G.J., Pike, O.A., Hendrix, S.B., Hess, W.M. and Huber, C.S. 2005. Sensory, mechanical, and microscopic evaluation of staling in low-protein and gluten-free breads. *Cereal Chemistry*. 82: 328-335.

Alaedini, A. and Green, P.H.R. 2005. Narrative Review: Celiac Disease: Understanding a complex autoimmune disorder. *Annals of Internal Medicine*. 142: 289-298.

Alm, V., Fang, C., Busso, C. S., Devos, K. M., Vollan, K., Grieg, Z. R. O. A. and Rognli, O. A. 2003. A linkage map of meadow fescue (Festuca pratensis Huds.) and comparative mapping with other Poaceae species. *Theoretical and Applied Genetics*. 108: 25-40.

Amonsou, E.O. 2010. Characterisation of marama bean protein. PhD thesis. University of Pretoria: Pretoria, South Africa.

Amonsou, E.O., Taylor, J.R.N., Emmambux, M.N., Duodu, K. G. and Minnaar, A. 2012. High viscous dough-forming properties of marama protein. *Food Chemistry*. 134:1519-1526.

Anderson, T.J. and Lamsal, B.P. 2011. Zein extraction from corn, corn products, and coproducts and modifications for various applications: a review. *Cereal Chemistry*. 88:159-173.

Andersson, H., Öhgren, C., Johansson, D., Kniola, M. and Stading, M. 2011. Extensional flow, viscoelasticity and baking performance of gluten-free zein-starch doughs supplemented with hydrocolloids. *Food Hydrocolloids.* 25: 1587-1595

Anyango, J.O. 2013. Physico-chemical modification of kafirin microstructures for application as biomaterials. PhD thesis. University of Pretoria: Pretoria, South Africa.

Anyango, J.O., Duneas, N., Taylor, J.R.N. and Taylor, J. 2012. Physicochemical modification of kafirin microparticles and their ability to bind bone morphogenic protein-2 (BMP-2), for application as biomaterial. *Journal for Agricultural and Food Chemistry*. 60: 8419-8426.

Arendt, E.K., Ryan, L.A.M. and Dal Bello, F. 2007. Impact of sourdough on the texture of bread. *Food Microbiology*. 24: 165-174.

Arendt, E.K., Morrissey, A., Moore, M.M., and Dal Bello, F., 2008. Gluten-free breads. Pages 289-302 in: Gluten-free Cereal Products and Beverages. E.K. Arendt and F. Dal Bello, eds. Elsevier: United Kingdom.

Beck, M.I., Tomka, I. and Waysek, E. 1996. Physio-chemical characterisation of zein as a film coating polymer. A direct comparison with ethyl cellulose. *International Journal of Pharmaceutics*. 141:137-150.

Belton, P.S. 1999. On the elasticity of wheat gluten. *Journal of Cereal Science* 29: 103-107.

Belton, P.S., Delgadillo, I., Halford, N.G., and Shewry, P.R. 2006. Kafirin structure and functionality. *Journal of Cereal Science.* 44: 272-286.

BeMiller, J.N. and Whistler, R.L. 1996. Charbohydrates. Pages 157-224 in: Food Chemistry. O.R. Fennema (Ed.) Third edition. Marcel Dekker, Inc. New York.

Blanco, C.A., Ronda, F., Pérez, B. and Pando, V. 2011. Improving gluten-free bread quality by enriching with acidic food additives. *Food Chemistry.* 127: 1204-1209.

Bonet, A., Rosell, C.M., Caballero, P.A., Gómez, M., Pérez-Munuera, I. and Lluch, M.A. 2006. Glucose oxidase effect on dough rheology and bread quality: A study from macroscopic to molecular level. *Food Chemistry*. 99: 408-415.

Bourtoom, T. 2008. Edible films and coatings: characteristics and properties. *International Food Research Journal.* 15:1-8.

Bugusu, B.A., Campanella, O. and Hamaker, B.R. 2001. Improvement of sorghum-wheat composite dough rheological properties and breadmaking quality through zein addition. *Cereal Chemistry.* 78:31-35.

Bugusu, B.A. Rajwa, B. and Hamaker, B.R. 2002. Interaction of maize zein with wheat gluten in composite dough and bread as determined by confocal laser scanning microscopy. *Scanning.* 24: 1-5.

Byaruhanga, Y.B., Erasmus, C., Emmambux, M.N., and Taylor, J.R.N., 2007. Effect of heating cast kafirin films on their functional properties. *Journal of the Science of Food and Agriculture* 87:167-175.

Byaruhanga, Y.B., Emmambux, M.N., Belton, P.S., Wellner, N., Ng, K.G. and Taylor, J.R.N. 2006. Alteration of kafirin and kafirin film structure by heating with microwave energy and tannin complexation. *Journal of Agriculture and Food Chemistry*. 54: 4198-4207.

Cabra, V., Arreguin, R., Vazquez-Duhalt, R. and Farres, A. 2006. Effect of

temperature and pH on the secondary structure and processes of oligomerization of 19 kDa alpha-zein. *Biochemica et Biophysica Acta.* 1764: 1110-1118.

Chiti, F. and Dobson, C.M. 2006. Protein misfolding, functional amyloid, and human disease. *Annual Review of Biochemistry*. 75: 333–366.

Chiue, H., Kusano, T. and Iwami, K. 1997. Deamidation-induced fragmentation of maize zein, and its linked reduction in fatty acid-binding capacity as well as antioxidative effect. *Food Chemistry*. 58, 111–117.

Chiue, H., Iwami, K., Kusano, T. and Ibuki, F. 1994. Decreased antioxidative activity of maize zein in response to deamidation rate. *Bioscience Biotechnology and Biochemistry*. 58: 198–199.

Chopin. 2010. Alveograph NG Consistograph Instructional manual. CHOPIN groupe Tripette et Renand. Villeneuve-la-Garenne, Paris, France.

Ciacci, C., Maiuri, L., Caporaso, N., Bucci, C., Giudice, L.D., Massardo, D.R., Pontieri, P., Fonzo, N.D., Bean, S.R., Loergerf, B. and Londei, M. 2007. Celiac disease: In vitro and in vivo safety and palatability of wheat-free sorghum food products. *Clinical Nutrition*. 26: 799-805.

Clarke, C.I., Schober, T.J. and Arendt, E.K. 2002. Effect of single strain and traditional mixed starter cultures on rheological properties of wheat dough and on bread quality. *Cereal Chemistry*. 79: 640-647.

Cornish, G.B., Békés, F., Eagles, H.A. and Payne, P.I. 2006. Prediction of dough properties for bread wheats. Pages 243-280 in: Gliadin and Glutenin, The unique Balance of Wheat Quality. C. Wrigley, F. Békés and W. Bushuk, eds. American Association of Cereal Chemists. St. Paul, MN.

Corradini, E., Souto de Mederiros, E., Carvalho, A.J.F., Curvelo, A.A.S. and Mattose, L.H.C. 2006. Mechanical and morphological characterization of starch/zein blends plasticized with glycerol. *Journal of Applied Polymer Science.* 101: 4133-4139.

Coultate, T. 2009. Food: The Chemistry of its Components. Fifth Edition. The Royal Society of Chemistry, Cambridge. Pages 198-208.

Crockett, R., Ie, P. and Vodovotz, Y. 2011. Effects of soy protein isolate and egg white solids on the physicochemical properties of gluten-free bread. *Food Chemistry*. 129: 84–91.

Da Silva, L.S. and Taylor, J.R.N. 2004. Sorghum bran as a potential source of kafirin. *Cereal Chemistry*. 81: 322-327.

Denery-Papini, S., Nicolas, Y. and Popineau, Y. 1999. Efficiency and limitations of immunochemical assays for the testing of gluten-free foods. *Journal of Cereal Science*. 30: 121-131.

Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E. and Schuppan, D. 1997. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nature Medicine*. 3:797–801.

Dunnewind, B., Sliwinski, E.L., Grolle, K. and Van Vliet, T. 2004. The Kieffer dough and gluten extensibility rig – An experimental evaluation. *Journal of Texture Studies.* 34: 537–560.

Duodu, K.G., Taylor, J.R.N., Belton, P.S. and Hamaker, B.R. 2003. Factors affecting sorghum protein digestibility. *Journal of Cereal Science*. 38: 117–131.

Duodu, K.G., Tang, H., Grant, A., Wellner, N., Belton, P.S. and Taylor, J.R.N. 2001. FTIR and solid state 13C NMR spectroscopy of proteins of wet cooked and popped sorghum and maize. *Journal of Cereal Science.* 33: 261-269.

Emmambux, M.N. and Taylor, J.R.N. 2003. Sorghum kafirin interaction with various phenolic compounds. *Journal of the Science of Food and Agriculture.* 863: 402-407.

Emmambux, M.N. and Taylor, J.R.N. 2009. Properties of heat-treated sorghum and maize meal and their prolamin proteins. *Journal of Agricultural and Food Chemistry*. 57:1045-1050.

Erickson, D.P., Campanella, O.H. and Hamaker, B.R. 2011. Functionalizing maize zein in viscoelastic dough systems through fibrous, β-sheet-rich protein networks: An alternative, physiochemical approach to gluten-free breadmaking. *Trends in Food Science and Technology.* 24:74-81.

Fasano, A. and Catassi, C. 2001. Current approached to diagnosis and treatment of celiac disease: An evolving spectrum. *Gastroenterology*. 120: 636-651.

Fasano, A., Berti, I., Gerarduzzi, T., Not, T., Colletti, R.B., Drago, S., Elitsur, Y., Green, P.H., Guandalini, S., Hill, I.D., Pietazak, M., Ventura, A., Thorpe, M., Kryszak, D., Fornaroli, F., Wasserman, S.S., Murray, J.A. and Horrath, K. 2003. Prevalence of coeliac disease in at-risk and not-at-risk groups in the United States: a large multi- center study. *Archives of Internal Medicine.* 163: 286–292.

Feighery, C. 1999. Clinical review: coeliac disease. *British Medical Journal.* 319: 236- 239.

Fink, A.L., Calciano, L.J., Goto, Y., Kurotsu, T. and Palleros, D.R. 1994. Classification of acid denaturation of proteins: intermediates and unfolded states. *Biochemistry.* 33: 12504-12511.

Fukushima, D., 2011. Soy proteins. Pages 210-232 in: Handbook of Food Proteins. G.O. Phillips and P.A. Williams, eds. Woodhead Publishing Limited: Cambridge.

Gallagher, E., Gromley, T.R. and Arendt, E.K. 2003. Crust and crumb characteristics of gluten free breads. *Journal of Food Engineering***.** 56: 153-161.

Gallagher, E., Gormley, T.R. and Arendt, E.K. 2004. Recent advances in the formulation of gluten-free cereal-based products. *Trends in Food Science and Technology*. 15: 143-152.

Gan, Z., Ellis, P.R. and Schofield, J.D., 1995. Gas cell stabilisation and gas retention in wheat bread dough. *Journal of Cereal Science.* 21: 215-230

García, E. M., Zaritzky, E. N., & Califano, N. A. 2005. Effect of composition on rheological properties of gluten-free dough disks for "empanadas". Page 399 in: Proceedings of the 2nd Mercosur Congress on Chemical Engineering, 4th mercosur congress on process systems engineering (14th-18th August). Rio de Janeiro: Argentina.

Gianfrani, C., Auricchio, S. and Troncone, R. 2005. Adaptive and innate immune responses in celiac disease. *Immunology Letters*. 199: 141-145.

Grosskreutz, J.C. 1961. A lipoprotein model of wheat gluten structure. *Cereal Chemistry*. 38: 336-349.

Gujral, H.S. and Rosell, C.M. 2004. Improvement of the breadmaking quality of rice flour by glucose oxidase. *Food Research International.* 37:75-81.

Hager, A. and Arendt, E.K. 2013. Influence of hydroxypropylmethylcellulose (HPMC), xanthan gum and their combination on loaf specific volume, crumb hardness and crumb grain characteristics of gluten-free breads based on rice, maize, teff and buckwheat. *Food Hydrocolloids.* 32: 195-203.

Hoseney, R. C. 1994. Principles of cereal science and technology. Second edition. American Association of Cereal Chemists. St Paul, MN. Page 378.

ICC-Standard. 1992. Method for using of the Chopin Alveogaph (Rheological Properties); No. 121. International Association for Cereal Science and Technology. Vienna, Austria.

Kasarda, D.D. 2001. Grains in relation to celiac disease. *Cereal Foods World*. 46: 209–210.

Katina, K. 2005. Sourdough: A tool for the improved flavour, texture and shelf-life of wheat bread. PhD dissertation, University of Helsinki: Helsinki, Finland.

Katina, K., Heiniö, L.R., Autio, K. and Poutanen, K. 2005. Optimization of sourdough process for improved sensory profile and texture of wheat bread. *LWT Food Science and Technology*. 39:1189-1202.

Kester, J.J., and Fennema, O.R. 1986. Edible films and coatings: A review. *Food Technology*. 40: 47-59.

Kim, S. and Xu, J. 2008. Aggregate formation of zein and its structural inversion in aqueous ethanol. *Journal of Cereal Science*. 47: 1-5.

Kobylański, J. R., Pérez, O. E. and Pilosof, A. M. R. 2004. Thermal transition of gluten-free doughs as affected by water, egg white, and hydroxypropylmethylcellulose. *Thermochimica Acta*. 411: 81–89.

Kokini, J.L., Cocero, A.M., Madeka, H. and De Gaaf, E. 1994. The development of state diagrams for cereal proteins. *Trends in Food Science and Technology*. 5: 281-288.

Kontogiorgos, V. 2011. Microstructure of hydrated gluten network. *Food Research International*. 44: 2582-2586.

Krochta, J.M. 2002. Proteins as raw materials for films and coatings: definitions, current status, and opportunities. Pages 1-41 in: Protein-based Films and Coatings. A. Gennadios, ed. CRC Press: Boca Raton, Florida.

Kupper, C. 2005. Dietary guidelines and implementation for celiac disease. *Gastroenterology*. 128: 121-127.

Kuraishi, C., Yamazaki, K. and Susa, Y. 2001. Transglutaminase: Its utilization in the food industry. *Food Reviews International*.17: 221-246.

Lawton, J.W. 1992. Viscoelasticity of zein-starch doughs. *Cereal Chem*istry. 69: 351-355.

Lawton, J.W. 2004. Plasticizers for zein: their effect on tensile properties and water absorption of zein films. *Cereal Chemistry.* 81: 1-5.

Lazaridou, A., Duta, D., Papageorgiou, M., Belc, N. and Biliaderis, C.G. 2007. Effects of hydrocolloids on dough rheology and bread quality parameters in gluten-free formulations. *Journal of Food Engineering.* 79: 1033–1047.

Li, Y., Li, J., Xia, Q., Zhang, B., Wang, Q. and Huang, Q. 2012. Understanding the dissolution of α-zein in aqueous ethanol and acetic acid solutions. *Journal of Physical Chemistry.* 116: 12057-12064.

Lindsay, M.P. and Skerritt, J.H. 1999. The glutenin macropolymer of wheat flour doughs: structure–function perspectives. *Trends in Food Science and Technology*. 10: 247–253.

Lohi, S., Mustalahti, K., Kaukinen, K., Laurila, K., Collin, P., Rissanen, H., Lohi, O., Bravi, E., Gasparin, M., Reunanen, A. and Mäki, M. 2007. Increasing prevalence of coeliac disease over time. *Alimentary Pharmacology and Therapeutics.* 26: 1217-1225.

Madeka, H. and Kokini, J. L. 1996. Effect of glass transition and cross-linking on rheological properties of zein: development of a preliminary state diagram. *Cereal Chemistry.* 73: 433-438.

Maher Galal, A., Varriano-Marston, E., and Johnson, J. A. 1978. Rheological dough properties as affected by organic acids and salt. *Cereal Chemistry*. 55:683-691.

Marco, C. and Rosell, C. M. 2008a. Functional and rheological properties of protein enriched gluten-free composite flours. *Journal of Food Engineering*. 88: 94-103.

Marco, C. and Rosell, C.M. 2008b. Breadmaking performance of protein enriched, gluten-free breads. *European Food Research and Technology*. 227: 1205-1213.

Mariani, P., Viti, M.G., Montouri, M., La Vecchia, A., Cipolletta, E., Calvani, L. and Bonamico, M. 1999. The gluten-free diet: a nutritional risk factor for adolescents with celiac disease? *Journal of Pediatric Gastroenterology and Nutrition.* 27: 519–523.

Matsushima, N., Danno, G., Takezawa, H. and Izumi, Y. 1997. Three-dimensional structure of maize α-zein proteins studied by small-angle X-ray scattering. *Biochemica et Biophysica Acta.* 1339:14-22.

Mejia, C.D., Mauer, L.J. and Hamaker, B.R. 2007. Similarities and differences in secondary structure of visco-elastic polymers of maize α-zein and wheat gluten proteins. *Journal of Cereal Science.* 45: 353–359.

Miñarro, B., Albanell, E., Aguilar, N., Gaumis, B. and Capellas, M. 2012. Effect of legume flours on baking characteristics of gluten-free bread. *Journal of Cereal Science.* 1-6.

Miñarro, B., Normahomed, I., Guamis, B., Capellas, M., 2010. Influence of unicellular protein on gluten-free bread characteristics. *European Food Research and Technology*. 231:171-179.

Moore, M.M., Juga, B., Schober, T.J. and Arendt, E.K. 2007. Effect of lactic acid bacteria on properties of gluten-free sourdoughs, batters, and quality and ultrastructure of gluten-free bread. *Cereal Chemistry.* 84: 357-364.

Moore, M.M., Schober, T.J., Dockery, P. and Arendt, E.K. 2004. Textural comparisons of gluten-free and wheat-based doughs, batters, and breads. *Cereal Chemistry*. 81: 567-574.

Moore, M.M., Heinbockel, M., Dockery, P., Ulmer, H.M. and Arendt, E.K. 2006. Network formation in gluten-free bread with application of transglutaminase. *Cereal Chemistry*. 83: 28-36.

Moroni, A.V., Bello, F.D. and Arendt, E.K. 2009. Sourdough in gluten-free bread-making: An ancient technology to solve a novel issue. *Food Microbiology*. 26: 276-284.

Murray, J. A. 1999. The widening spectrum of celiac disease. *American Journal of Clinical Nutrition.* 69: 354–365.

Noel, T.R., Parker, R., Ring, S.G. and Tatham, A.S. 1995. The glass-transition behaviour of wheat gluten proteins. *International Journal of Biological Macromolecules*. 17: 81-85.

Nunes, M.H.B., Ryan, L.A.M. and Arendt, E.K. 2009. Effect of low lactose dairy powder addition on the properties of gluten-free batters and bread quality. *European Food Research and Technology*. 229: 31-41.

Ojetti, V., Nucera, G., Migneco, A., Gabrielli, M., Lauritano, C., Danese, S., Zocco, M.A., Nista, E.C., Cammarota, G., Lorenzo, A.D., Gadbarrini, G. and Gasbarrini, A. 2005. High prevalence of celiac disease in patients with lactose intolerance. *Journal of Gastroenterology*. 71: 106-110.

Oom, A., Pettersson, A., Taylor, J.R.N. and Stading, M. 2008. Rheological properties of kafirin and zein prolamins. *Journal of Cereal Science*. 47: 109-116.

Ortolani, C. & Pastorello, E. A. 1997. Study of Nutritional Factors In Food Allergies and Food Intolerance. European Commission: Brussels, Luxembourg. Pages 26-45.

Panchapakesan, C., Sozer, N., Dogan, H., Haung, Q. and Kokini, J.L. 2012. Effect of different fractions of zein on the mechanical and phase properties of zein films at nano-scale. *Journal of Cereal Science*. 55: 174-182.

Pedersen, J.S. and Otzen, D.E. 2008. Amyloid- a state in many guises: Survival of the fittest fibril fold. *Journal of Protein Science.* 17: 2-10.

Popineau, Y., Bonefant, S., Cornec, M., and Pezolet, M. 1994. A study of infrared spectroscopy of the conformations of gluten proteins differing in their gliadin and glutenin compositions. *Journal of Cereal Science* 20: 15–22.

Pretorius, C. 2008. Kafirin and zein as coatings for the controlled release of amino acid supplements. MSc Dissertation. University of Pretoria: Pretoria, South Africa.

Rao, S.P. 2008. Amyloid Fibrils in Biomaterials. PhD thesis. University of Canterbury: Canterbury, New Zealand.

Renzetti, S., Dal Bello, F., and Arendt, E.K. 2008. Microstructure, fundamental rheology and baking characteristics of batters and breads from different gluten-free flours treated with a microbial transglutaminase. *Journal of Cereal Science*. 48: 33–45.

Rochet, J.C. and Lansbury, P.T. 2000. Amyloid fibrillogenesis: Themes and variations. *Current Opinion in Structural Biology*. 10:60-68

Rosell, C.M., Rojas, J.A. and Benedito de Barber, C. 2001. Influence of hydrocolloids on dough rheology and bread quality. *Food Hydrocolloids.* 15: 75-81.

Rotsch, A. 1954. Chemical and baking—technological investigation with artificial doughs. *Brot und Gebäck*. 8:129–130

Sanchez, H.D., Osella, C.A. and De La Torre, M.A. 2002. Optimization of gluten-free bread prepared from cornstarch, rice flour, and cassava starch. *Journal of Food Science.* 67: 416- 419.

Santosa, F.X.B. and Padua, G.W. 2000. Thermal behaviour of zein sheets plasticized with oleic acid. *Cereal Chemistry*. 77: 459–462

Schmittschmitt, J.P. and Scholtz, J.M. 2003. The role of protein stability, solubility, and net charge in amyloid fibril formation. *Journal of Protein Science.* 12:2374-2378.

Schober, T. J. 2009. Manufacture of gluten-free specialty breads and confectionery products. Pages 130-180 in: Gluten-Free Food Science and Technology. E. Gallagher, ed. Wiley-Blackwell, Hoboken, NJ.

Schober, T.J, Bean, S.R. and Boyle, D.L. 2007. Gluten-free sorghum bread improved by sourdough fermentation: biochemical, rheological, and microstructural background. *Journal of Agricultural and Food Chemistry*. 55: 5137-5146

Schober, T.J., Bean, S.R., Boyle, D.L. and Park, S. 2008. Improved viscoelastic zein-starch doughs for leavened gluten-free breads: Their rheology and microstructure. *Journal of Cereal Science.* 48: 755-767.

Schober, T.J., Moreau, R.A, Bean, S.R. and Boyle, D.L. 2010. Removal of surface lipids improves the functionality of commercial zein in viscoelastic zein-starch dough for gluten-free bread making. *Journal of Cereal Science.* 52: 417-425.

Schober, T.J., Bean, S.R., Tilley, M., Smith, B.M. and Loerger, B.P. 2011. Impact of different isolation procedures on the functionality of zein and kafirin. *Journal of Cereal Science.* 54: 241-249.

Schober, T.J., Messerschmidt, M., Bean, S.R., Park, S.H. and Arendt, E.K. 2005. Gluten-free bread from sorghum: quality differences among hybrids *Cereal Chemistry.* 82: 394–404.

Sciarini, L.S., Ribotta, P.D., León, A.E. and Pérez, G.T. 2012. Incorporation of several additives into gluten-free breads: Effect on dough properties and bread quality. *Journal of Food Engineering.* 111: 590-597.

Selling, G.W., Hamaker, S.A.H. and Sessa, D.J. 2007. Effect of solvent and temperature on secondary and tertiary structure of zein by circular dichroism. *Cereal Chemistry*. 84: 265-270.

Shewry, P.R. and Halford, N.G. 2002. Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany*. 53: 947-958.

Shewry, P.R. and Tatham, A.S. 1990. The prolamin storage proteins of cereal seeds: structure and evolution. *Biochemistry Journal*. 267: 1-12.

Shewry, P. R., Popineau, Y., Lafiandra, D., Halford, N. G., Tatham, A. S. and Belton, P. S. 2003. The high molecular weight subunits of wheat glutenin and their role in determining wheat processing properties. *Advances in Food and Nutrition Science*. 45: 219–302.

Singh, H. 1991. Modification of food proteins by covalent crosslinking. *Trends in Food Science and Technology*. 2: 196– 200.

Singh, B.R., 2000. Basic aspects of the technique and applications of infrared spectroscopy of peptides and proteins. In: Singh, B.R., (Ed.), Infrared Analysis of Peptides and Proteins, Principles and Application. American Chemical Society Symposium Series, American Chemical Society, Washington, DC, pp. 2-37.

Sliwinski, E.L., Kolster, P., Prins, A. and Van Vliet, T. 2004. On the relationship between gluten protein composition of wheat flours and large-deformation properties of their doughs. *Journal of Cereal Science*. 39: 247–264.

Smith, B.M. 2012. Functionality of corn and sorghum proteins in viscoelastic dough systems. PhD dissertation. Kansas State University: Manhattan, Kansas.

Sollid, L. M. and Khosla, C. 2005. Future therapeutic options for celiac disease. *Nature Clinical Practice-Gastroenterolgoy and Hepatology*. 2: 140-147.

Sperling, L.H., 1992. Introduction to Physical Polymer Science. Second Edition. Wiley-Interscience, New York. Pages 349-427.

Storck, C. R., da Rosa Zavareze, E., Gularte, M. A., Elias, M. C., Rosell, C. M. and Guerra Dias, A. R. 2013. Protein enrichment and its effects on gluten-free bread characteristics. *LWT-Food Science and Technology*. 53: 346-354.

Sturgess, R., Day, P., Ellis, J.H., Lundin, K.E.A., Gjertsen, H.A., Kontakou, M. and Ciclitria, P. J. 1994. Wheat peptide challenge in coeliac disease. *Lancet.* 343: 758-761.

Tanaka, K., Furukawa, K. and Matsumoto, H. 1967. The effect of acid and salt on the farinogram and extensigram of dough. *Cereal Chemistry*. 44: 675–680.

Tatham, A.S., Field, J.M., Morris, V.J., I'Anson, K.J., Cardle, L., Dufton, M.J. and Shewry, P.R. 1993. Solution conformational analysis of the α-zein proteins of maize. *Journal of Biological Chemistry.* 268: 26253-26259.

Taylor, J.R.N. 2004. Grain production and consumption: Africa. Pages 70-78 in: Encyclopedia of Grain Science. C. Wrigley, H. Corke and C.E. Walker, Eds. Elsevier: London.

Taylor, J., Bean, S.R., Ioerger, B.P. and Taylor, J.R.N. 2007. Preferential binding of sorghum tannins with gamma-kafirin and the influence of tannin binding on kafirin digestibility and biodegradation. *Journal of Cereal Science*. 46:22–31.

Taylor, J., Taylor, J.R.N., Belton, P.S. and Minnaar, A. 2009a. Formation of kafirin microparticles by phase separation from organic acid and their characterization. *Journal of Cereal Science.* 50: 99-105.

Taylor, J., Taylor, J.R.N., Belton, P.S. and Minnaar, A. 2009b. Preparation of free-standing films from kafirin protein microparticles: mechanism of formation and functional properties. *Journal of Agricultural and Food Chemistry.* 57: 6729-6735.

Taylor, J., Taylor, J.R.N., Dutton, M.F. and De Kock, S. 2005a. Identification of kafirin film casting solvents. *Food Chemistry*. 90: 401-408.

Taylor, J., Taylor, J.R.N., Dutton, M.F. and De Kock, S. 2005b. Glacial acetic acid- a novel food-compatible solvent for kafirin extraction. *Cereal Chemistry.* 82:485-487.

Taylor, J.R.N., Schober, T.J. and Bean, S.R. 2006. Novel food and non-food uses for sorghum and millets. *Journal of Cereal Science*. 44: 252-271.

Thompson, T. 2001. Wheat starch, gliadin, and the gluten-free diet. *Journal of the American Dietetic Association.* 101: 1-4

Torbica, A., Hadnadev, M. and Dapčević, T. 2010. Rheological, textural and sensory properties of gluten-free bread formulations based on rice flour and buckwheat flour. *Food Hydrocolloids.* 24:626-632.

Uthayakumaran, S., Gras, P.W., Stoddard, F.L. and Bekes, F. 1999. Effect of varying protein content and glutenin-to-gliadin ratio on the functional properties of wheat dough. *Cereal Chemistry*. 76: 389–394.

Van Riemsdijk, L.E., Pelgrom, P.J.M., Van Der Groot, A.J., Boom, R.M. and Hamer, R.J. 2011a. A novel method to prepare gluten-free dough using a meso-structured whey protein particle system. *Journal of Cereal Science*. 53:133-138.

Van Riemsdijk, L.E., Pelgrom, P.J.M., Van Der Groot, A.J., Boom, R.M. and Hamer, R.J. 2011b. New insights on the formation of colloidal whey protein particles. *Journal of Food Hydrocolloids*. 25: 333-339.

Van Riemsdijk, L., Sprakel, J., Van der Goot, A. and Hamer, R. 2010. Elastic networks of protein particles. *Food Biophysics.* 5: 41-48.

Vieira, M.G.A., Da Silva, M.A., Dos Santos, L.O., and Beppu, M.M. 2011. Natural-based plasticisers and biopolymer films: A review. *European Polymer Journal.* 47: 254–263.

Wall, J.S. and Paulis, J.W. 1978. Corn and sorghum grain proteins. Pages 135-219 in: Advances in Cereal Science and Technology. Vol II. Y. Pomeranz, ed. American

Association of Cereal Chemists: St. Paul, MN.

Wieser, H., 2007. Chemistry of gluten proteins. *Food Microbiology* 2: 115-119

Zhang, B., Luo, Y. and Wang, Q. 2011. Effect of acid and base treatments on structural, rheological and antioxidant properties of α-zein. *Food Chemistry.* 124: 210-220.

Zhu, L., Chen, J., Tang, X. and Xiong, Y. 2008. Reducing, radical scavenging, and chelation properties of in vitro digests of alcalase-treated zein hydrolysate. *Journal of Agricultural and Food Chemistry*. 56: 2714–2721.

Ziobro, R., Korus, J., Witczak, M. and Juszczak, L. 2012. Influence of modified starches on properties of gluten-free dough and bread. Part II: Quality and staling of gluten-free bread. *Journal of Food Hydrocolloids.* 29: 69-74.

Ziobro, R., Witczak, T., Juszczak, L. and Korus, J. 2013. Supplementation of gluten-free bread with non-gluten proteins. Effect on dough rheological properties and bread characteristic. *Food Hydrocolloids* (in press).