Quality protein maize (QPM) zein:

Characterisation and functionality

By

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DECLARATION

I, Julia Thandikile Baloyi declare that the dissertation I hereby submit for the degree MSc Food Science at the University of Pretoria is my work and has not been previously submitted at any other higher institution. Reference material contained in this dissertation has been duly acknowledged.

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ABSTRACT

Quality protein maize (QPM) zein: Characterisation and functionality

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Quality protein maize (QPM) is a high-lysine maize type that was developed through breeding, to improve its grain hardness and agronomic performance. Despite QPM's nutritional advantage, this maize type has not been widely adopted mainly due to lack of a commercial market and probably because the functionality of its prolamin protein (zein) are largely unknown. In this study, zein extracted from white QPM was characterised. Its functional properties concerning the formation of bioplastic films, viscoelastic masses "doughs" and gels were investigated and compared to "total" zein from a related regular white Ethiopian maize, total zein from regular white South African maize and commercial zein. Amino acid analysis and SDS-PAGE confirmed that QPM had high lysine, cysteine and γ-zein contents. Bioplastic films were prepared from the zeins by casting from aqueous ethanol and from glacial acetic acid (GAA). QPM zein films were more opaque compared to other zein films cast from the same solvent. The opaqueness was attributed to the partial solubility of QPM zein when dissolved in the casting solvents. During preparation of viscoelastic masses, zein preparations were either mixed with a solvent (water or acetic acid) or coacervated from solution in GAA (simple coacervation). Neither QPM zein nor total zein preparations from regular maize formed viscoelastic masses. Possibly, the presence of β- or γ-subclasses hindered interaction of α-zein with the solvent, unlike with commercial zein (predominantly α-zein) which formed viscoelastic masses with water and 5.4% acetic acid treatments.

Interestingly, QPM zein formed a viscous mass when the prolamin was dissolved in GAA during coacervation. Hence, the firmness of the zeins was further investigated. It was thought that different levels of β- and γ-prolamins would contribute to gelation. Therefore, total

kafirin, kafirin without β-kafirin and kafirin without γ-kafirin preparations were used as controls. Compression testing showed that only total kafirin formed a true gel because its maximum compression force was the highest (9.61 N), compared to other zein and kafirin preparations (<1.57 N) which formed either runny liquids, solutions or colloids. Formation of a kafirin gel is attributable to disulphide crosslinking between γ-kafirin. To investigate the effect of disulphide bonding on prolamin gelation, reducing agent (5% 2-mercaptoethanol (BME)) was added to GAA. After inclusion of BME, QPM zein and kafirin without γ-kafirin formed gels, probably due to protein unfolding. Due to the abundant γ-zein, QPM zein is a potential raw material for producing bioplastic films with better barrier properties.

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1. INTRODUCTION

Cereal grains such as sorghum, millet and maize are the primary staple food sources consumed across Africa. These grains, however, are limiting in the essential (indispensable) amino acids: lysine and tryptophan, which are only obtainable from the diet (Delcour and Hoseney, 2010). Consuming cereal grains that are deficient in lysine and tryptophan may result in malnutrition (undernutrition); a global burden that is persisting in some parts of the world, particularly Africa (World Health Organization, 2017). In attempt to improve the nutritional intake and prevent protein-deficiency health problems, maize has been bio fortified with lysine through genetic engineering (Gunaratna et al., 2010; Wong et al., 2015).

Biofortified maize types with nearly double the lysine and tryptophan levels compared to regular maize have been developed through conventional crossbreeding practices (Shewry, 2007; Holding, 2014). These maize types are called quality protein maize (QPM). In African countries where maize is a staple cereal crop, consumption of QPM regularly has great potential to improve the nutritional status of people by reducing the prevalent protein-deficiency gap (Akalu et al., 2010; Nuss and Tanumihardjo, 2011). In developed countries such as the USA, approximately 40% of the maize produced in the country is used as a starch source for bioethanol production (Ranum et al., 2014). When substituted with regular maize, QPM has potential to lower the operational costs of bioethanol production (Lopez-Pereira, 1993). The solid co-product of bioethanol production; distillers dried grains solubles (DDGS) has a high protein content, and it is a major animal feed source (Swiatkiewicsz and Koreleski, 2008). Apart from using DDGS in animal feed, DDGS can be a possible starting raw material for the production of prolamin-based bioplastic films (Muhiwa et al., 2017).

In most countries, QPM has not been fully adopted as a food source because no incentives are being offered to encourage farmers to start producing this maize type on a commercial level. Also, the cost of the QPM seed is higher compared to regular maize (Vasal, 2002). Furthermore, practical applications where QPM can be used in food and non-food industries are still limited. This research will determine the characteristics and functional properties of isolated QPM zein, with respect to the formation of bioplastic films, viscoelastic masses "doughs" and gelation.

2. LITERATURE REVIEW

The literature review will provide an introduction on how QPM maize types were developed, followed by reviewing the characteristics and chemical composition of QPM. Thereafter, the literature review will focus on the characterisation and functional properties of zein and kafirin. The principles of forming prolamin bioplastic films, viscoelastic materials "doughs" and gels will also be discussed.

2.1 Development and characteristics of quality protein maize

Maize with improved protein quality (nearly double the lysine and tryptophan content) was first identified in the early 1920s (Vasal, 2002). This maize, called *opaque-2* maize, had undesirable soft kernels and poor agronomic characteristics (low pest resistance). In addition, the *opaque-2* maize grain yields were lower compared to that of regular maize (Gibbon and Larkins, 2005). These characteristics made it difficult to mill, process and store the *opaque-2* maize types. Hence, the need to improve the grain hardness and the poor agronomic properties of *opaque-2* maize, while maintaining its protein quality (high lysine and tryptophan content) led to further breeding programmes and the development of QPM (Vasal et al., 1980; Geevers and Lake, 1992).

QPM has higher levels of lysine, tryptophan, valine, methionine, cysteine, arginine, leucine and threonine compared to regular maize (Table 2.1). Also, QPM has lower levels of isoleucine, tyrosine, phenylalanine, glycine, glutamic acid, aspartic acid and threonine compared to regular maize (Abiose and Ikujenlola, 2014). The higher levels of lysine and tryptophan in QPM could be attributed to the presence of *opaque-2* genes that were introduced in the background of QPM during conventional breeding (Vasal et al., 1980; Hunter et al., 2002). *Opaque-2* genes code for proteins that have lysine and tryptophan in their amino acid sequence (Gaziola et al., 1999; Gibbon and Larkins, 2005). The mechanism that explains how *opaque-2* genes enhance the production of lysine and tryptophan (essential amino acids), specifically in QPM is, however, not well understood.

Table 2.1: Amino acid profile of quality protein maize and regular maize, g/100 g maize flour (dry basis)

Amino acid	Maize type									
	RM[1]	QPM[1]	RM[2]	QPM[2]	RM[3]	QPM[3]	RM[4]	QPM[4]	RM[5]	QPM[5]
Threonine	0.22	0.27	0.29	0.36	0.38	0.45	0.24	0.34	0.28	0.30
Tryptophan	0.05	0.07	0.06	0.08	0.06	0.10	0.06	0.10	0.08	0.12
Leucine	1.04	0.82	1.06	0.87	1.34	0.96	0.77	0.88	1.00	0.87
Valine	0.40	0.43	0.37	0.51	0.50	0.57	0.33	0.48	0.35	0.38
Methionine	0.17	0.16	0.17	0.18	0.22	0.21	0.17	0.15	0.18	0.19
Isoleucine	0.30	0.27	0.28	0.30	0.36	0.36	0.23	0.30	No data	No data
Cysteine	0.17	0.22	0.18	0.27	No data	No data	0.19	0.26	0.24	0.28
Lysine	0.21	0.28	0.25	0.36	0.27	0.43	0.23	0.36	0.27	0.41
Arginine	0.35	0.44	0.39	0.63	0.42	0.75	0.35	0.56	No data	No data

RM = regular maize, QPM = quality protein maize. [1]Osei et al. (1999); [2]Panda et al. (2011); [3]Ortega et al. (2008); [4]Osei et al. (1998) and [5]Zhai and Zhang (2007).

When consumed daily, QPM has potential to increase the growth of children and alleviate malnutrition in sub-Saharan Africa (Ortega et al., 2008; Akalu et al., 2010; Gunaratna et al., 2010; Nyakurwa et al., 2017). In terms of functionality, the QPM flour has higher pasting and increased viscosity properties (Cisse et al., 2013; Abiose and Ikujenlola, 2014) compared to that of regular maize. Furthermore, the protein digestibility of QPM is higher than that of regular maize (Zhai and Zhang, 2007). Thus, producing infant porridge using QPM instead of regular maize may be advantageous because more amino acids that are essential would be obtained from the diet (Akalu et al., 2010; Kaur et al., 2019).

The biological value of QPM ($\approx 80\%$) is similar to that of casein protein ($\approx 90\%$) and much higher compared to that of regular maize (between 40-50%) (Food and Agriculture Organization, 1992; Zhai and Zhang, 2007). Biological value refers to the amount of protein that is absorbed by the body for various metabolic functional uses (Prasanna et al., 2001). It has been reported that people who consumed QPM had high nitrogen retention compared to those who consumed regular maize (Bressani, 1991). Additionally, human studies have shown that lower quantities of QPM in the diet are required to meet the daily requirements for lysine recommended by the World Health Organization, compared to regular maize (Nuss and Tanumihardjo, 2011). Apart from human studies, the nutritional superiority and bioavailability of QPM has been demonstrated using pigs (Sullivan et al., 1989) and broiler chickens (Panda et al., 2013).

The quantity of prolamin protein (zein) present in QPM is nearly 50% less compared to that of regular maize types (Wallace et al., 1990; Holding, 2014). The lower zein content in QPM may be attributed to the delayed zein synthesis due to presence of *opaque-2* genes (Larkins et al., 1984) that lower the synthesis of 19 kDa and 22 kDa α-zein (Segal et al., 2003; Huang et al., 2004). In regular maize, zein first accumulates to form discrete protein bodies in the endosperm where they mature and develop (Shewry and Halford, 2002). In QPM, however, zein only starts to accumulate after several days and protein bodies then form at a slower rate (Holding and Larkins, 2006).

Of the prolamins (zein) present in QPM, the 27 kDa γ-zein is more abundant compared to other zein subclasses (α-, β- and δ-zeins) (Wallace et al., 1990). The increased synthesis of γ-zein is possibly a consequence of gene duplication at the 27 kDa locus in chromosome 7, where the γ-zein quantitative trait locus (QTL) (qγ27) and *opaque-2* genes are located (Lui et al., 2016). The authors proposed that *opaque-2* genes are responsible for duplicating the 15.26 kDa in the 27 kDa γ-zein locus. Hence, this results in overexpression of γ-zein. Apart from the study by Lui et al. (2016) which attempts to explain why γ-zein production increases in QPM, the mechanism of how *opaque-2* genes affect the synthesis of all QPM zeins (α-, β-, γ-, δ-) is still largely unknown.

QPM maize types have vitreous endosperms whereas regular maize types have opaque endosperms (Holding, 2014). The modification of the vitreous endosperms in QPM may be a pleiotropic effect of the *opaque-2* genes that were introduced in the background of the QPM during the breeding process (Torrent et al., 1997). In addition, it is likely that modification of

the maize endosperm (vitreous/opaque) may be attributable to a complex interplay of other factors such as γ-zein, arrangement of protein bodies within the starch matrix, structure of starch granules and the starch synthesis pathways (Geetha et al., 1991; Gibbon and Larkins., 2005; Wu et al., 2010; Wu et al., 2015). Gamma-zeins initiate the formation of protein bodies (Holding and Larkins, 2006). When γ-zein is abundant, numerous protein bodies are likely to be formed whereas the absence or suppression of the 27 kDa γ-zein synthesis may result in the reduction of the number of protein bodies formed (Yuan et al., 2014). When numerous protein bodies form, they surround the starch granules and this may result in the formation of crosslinked disulphide bonds on the periphery of the protein bodies (Lopes and Lopes, 1991), which consequently forms/develops vitreous endosperms. Since QPM zein has abundant 27 kDa γ-zein, it is probable that the interaction between the γ-zein and starch granules contributes to the formation of vitreous endosperm.

2.2 Structure, characterisation and functionality of zein and kafirin

2.2.1 Structure of zein and kafirin storage proteins

Zein and kafirin are the primary storage proteins of maize and sorghum. These proteins are entirely soluble in aqueous-alcoholic solutions, only in the presence of reducing agents. Zeins and kafirins are insoluble in water because they contain high levels of hydrophobic glutamine and proline residues (Larkins et al., 1984; Lasztity, 1996; Shewry and Halford, 2002; Delcour and Hoseney, 2010). In terms of amino acid composition, solubility and molecular weight, the zein and kafirin proteins are homologous to each other (De Rose et al., 1989; Shull et al., 1991). Therefore, they will be discussed together.

2.2.1.1 Zein and kafirin subclasses

Zein and kafirin comprise of three main prolamin subclasses, namely α-, β-, and γ- (Argos et al., 1982; Shewry and Tatham, 1990; Belton et al., 2006). The fourth prolamin subclass: δ-subclass is found in minute quantities.

Of the total prolamin fraction present in the vitreous endosperm, the α-prolamins of total zein account for approximately 70-85% (Lawton, 2002), whereas those of total kafirin consist of 80-85% (Watterson et al., 1993). Alpha-zein consists of two main classes, with apparent molecular weights of approximately 19 kDa and 22 kDa (Shewry and Tatham, 1990). Similarly, the α-kafirin is comprised of α1 or α2-kafirins, with apparent molecular weights of 24 - 29 kDa and 22 kDa respectively (Shull et al., 1991; Mazhar et al., 1993).

Argos et al. (1982) proposed a structural model for α-zein using results from circular dichroism spectroscopy. The model, as shown systematically in Figure 2.1A below, shows that α-zein consists of nine homologous antiparallel helices arranged within a cylinder. Each α-helix comprises 20 amino acids, most are hydrophobic, and a few are polar. The cylinders are proposed to join through hydrogen bonding between the glutamine residues at the end of each cylinder. Following on from the model of Argos et al. (1982), Matsushima et al. (1997) proposed that when in solution, α-zein has an extended structure. The authors proposed another model of α-zein suggesting that the antiparallel helices of α-zein would arrange themselves linearly along the helical axis. The glutamine-rich residues at the end of each

helix would join the cylinders to form hoops and loops (Figure 2.1B). Bugs et al. (2004) later suggested a model for α-zein consisting of a helical hairpin that coils to form a super helical structure (Figure 2.1C). So far, no structural model for α-kafirin has been proposed. However, it is probable that the α-kafirin has α-helical structures, similar to that of α-zein.
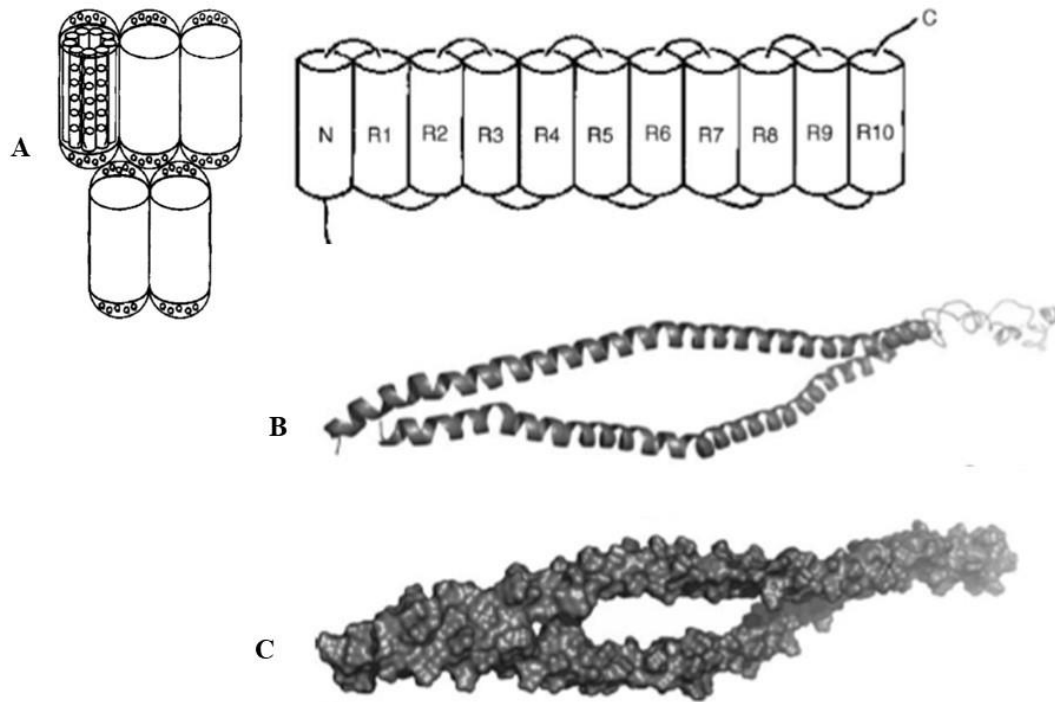
Figure 2.1 Structural models proposed for α-zeins. A - the α-helices arranged antiparallel to form a distorted cylinder as proposed by Argos et al. (1982). B- the α-helices arranged in antiparallel to form an extended structure as proposed by Matsushima et al. (1997). C- a super helix comprised of hairpin elements with α-helix, β- turns and β-sheet, as proposed by Bugs et al. (2004).

The β-prolamins account for about 10-15% of the total zein fraction (Shewry and Halford, 2002). Beta-zein has a molecular weight of approximately 14-16 kDa (Lawton, 2002), while that of β-kafirin is about 19 kDa (Mazhar et al., 1993). In general, β-prolamins are rich in methionine and cysteine residues (Shukla and Cheryan, 2001). The latter can polymerise through disulphide bonding (Duodu et al., 2003). For this reason, more β-prolamins from zein and kafirin are extracted in the presence of reducing agents (Pomes, 1971; El Nour et al., 1998). In kafirin, the β-kafirin seems to exist in monomers (El Nour et al., 1998), thus it is unlikely that it will form high molecular weight polymers. To date, no model has been published to illustrate the structure of β-zein or β-kafirin. However, it has been proposed that β-zein consists of β-sheets and random coils, with the few α-helices present (Shewry and

Tatham, 1990). Similar to the proposed structure of β-zein, it is probable that the β-kafirin also consists of a mixture of β-sheets and random coils.

The γ-prolamins of zein and kafirin are hydrophobic and rich in cysteine residues that can form disulphide bonds (Duodu et al., 2003). These bonds can only be cleaved by reducing agents (Shewry and Tatham, 1990). Both γ-zein and γ-kafirin are extracted in the presence of a reducing agent (El Nour et al., 1998; Lawton, 2002). Of the two prolamins, the γ-kafirin is considered more hydrophobic because the free energy of hydration for γ-kafirin (-124.5 kcal/mol) is more negative compared to γ-zein (-113.6 kcal/mol) (Duodu et al., 2003). Recently, Bicudo et al. (2018) suggested that γ-zein has a helical structure. However, the molecular structure of γ-kafirin has not yet been published. Since kafirin and zein are extensively homologous to each other (De Rose et al., 1989), it may be assumed that γ-kafirin has a helical structure, similar to that of γ-zein.

2.2.2 Characterisation of zein and kafirin proteins

Studies that characterise zein and kafirin have shown that both prolamins have complex protein fractions. The subclasses (α-, β-, δ-, γ-) of zein and kafirin can be characterised quantitatively by high-performance liquid chromatography (HPLC) (Sastry et al., 1986; Wilson, 1991), capillary electrophoresis (Rodriguez-Nogales et al., 2006) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Shull et al., 1991; El Nour et al., 1998). The HPLC and capillary electrophoresis techniques are accurate and efficient. However, the sample preparation is usually complex. In comparison to HPLC, the SDS-PAGE is relatively inexpensive and straightforward to use.

2.2.2.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis is a technique that can be used to separate proteins under the influence of an electric field. When an electric field is applied, ions move through the medium towards the anode or cathode (electrodes that attract or repel ions), with a characteristic velocity that is dependent on electrical parameters (current, voltage) and the medium (ionic strength, pH) in which the ions are moving (Fritsch and Krause, 2003). The higher the current, the faster the migration. Current is influenced by voltage, thus, when the difference in potential between

them is large, the rate of migration also increases. Dense or more viscous mediums retard the movement of ions during electrophoresis (Yada et al., 1996). SDS-PAGE separates proteins based on apparent molecular weight only (Friedman et al., 2009). The mobility of proteins is influenced by the protein's net charge, shape and size (Fritsch and Krause, 2003). During SDS-PAGE, proteins are first treated with sodium dodecyl sulphate (SDS), an anionic detergent that wraps and binds to the protein polypeptides, conferring an overall uniform negative charge (Garfin, 1990). This eliminates the effect of the proteins' net charge during separation. Additionally, SDS denatures proteins by altering their secondary and tertiary structure, resulting in uncoiled and linear polypeptides (Yada et al., 1996). Thus, the effect of the shape of the protein is also eliminated. The polyacrylamide gel is composed of pores with different sizes. Therefore, it acts as a molecular sieve to restrain the movement of proteins. Upon application of an electric current, the negatively charged proteins migrate from the negatively charged electrode (cathode) towards the positively charged electrode (anode) (Yada et al., 1996). Proteins with the least molecular mass pass most rapidly through the polyacrylamide gel pores and therefore they migrate faster and more freely towards the anode compared to proteins with greater mass. After protein separation, the gel is then stained with a dye (normally Coomassie Brilliant Blue R-250) to visualise the proteins, which appear as discrete bands (Roy and Kumar, 2014).

SDS-PAGE has shown that total zein (from regular maize) and total kafirin prolamins are heterogeneous, both containing different levels of α-, β-, δ- and γ-prolamin subclasses (Mazhar et al., 1993; El Nour et al., 1998; Nunes et al., 2005; Anderson and Lamsal, 2011). Wallace et al. (1990) found that QPM zein has abundant γ-zein when compared to total zein extracted from regular maize. The high γ-zein content is a characteristic of QPM (Holding, 2014). Furthermore, the QPM zein had significantly lower 19 kDa, 22 kDa α-zein and β-zein subclasses compared to total zein from regular maize (Wallace et al., 1990). Most SDS-PAGE work has been done on commercial zein (predominantly α-zein) and total zein (from regular maize). Apart from the study of Wallace et al. (1990), no recent work has been published where SDS-PAGE was used to characterise the composition of QPM zein.

2.2.2.2 Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2D-PAGE is a more powerful electrophoretic technique than SDS-PAGE for separating different proteins. 2D-PAGE separates proteins using two different techniques in sequence, which are performed in two different dimensions at right angles to each other. The first separation is by isoelectric focusing (IEF), which separates proteins on the basis of their isoelectric point (pI) in a linear pH gradient that increases from the low pH anodic end to the high pH cathodic end (Herbert et al., 1997; Fritsch and Krause, 2003). The second separation is by SDS-PAGE which separates proteins based on their molecular weights (Friedman et al., 2009), as described above. During IEF, an immobilized pH gradient (IPG) strip is first rehydrated with a solution of the protein. The IPG strip contains amphoteric molecules (called ampholytes) that migrate through the linear pH gradient over a range of up to pH 2-11 (Fritsch and Krause, 2003). Proteins are amphoteric molecules, having either a net positive or a negative charge. If the pH of the buffer solution is below a particular protein's pI, the protein will have a positive net charge and conversely a protein becomes negatively charged when the pH of the solution is above its pI. When an electric current is passed, voltage is created and the charged proteins move towards electrodes of the opposite charge (i.e. positively charged proteins migrate towards the cathode and negatively charged proteins migrate towards the anode, respectively) (Friedman et al., 2009). When the ampholytes reach a relative pH that is equal to the protein's pI (net charge of protein becomes zero), migration stops and at this point, the protein remains stationary and becomes sharply focused (Fritsch and Krause, 2003). After IEF, the IPG strip is placed horizontally across the top of a polyacrylamide gel and SDS-PAGE is performed to further separate proteins based on their molecular mass (Friedman et al., 2009).

Elhassan et al. (2018) has used 2D-PAGE to separate different kafirin types. The authors reported that kafirins from genetically modified high protein digestibility sorghum lines have missing γ-kafirin bands, whereas those from waxy-high protein digestibility sorghum lines have missing β-kafirin. Little work has been published where QPM zein has been characterised using either SDS-PAGE or 2D-PAGE. In the current study, the composition of QPM zein was characterised using both techniques.

2.2.3 Functionality of prolamin protein

The functionality of prolamins can be affected by various factors including the composition of the prolamin (presence of α-, β-, γ- and δ- subclasses), hydrophobicity, temperature, pH and prolamin concentration (Lawton, 1992; Duodu et al., 2003; Taylor et al., 2005; Wang and Padua, 2010). Prolamin proteins can form different structures such as viscoelastic materials, bioplastic films, scaffolds and microparticles (Taylor et al., 2013). These structures can be interchangeable. For example, total kafirin films can be cast from protein microparticles prepared using glacial acetic acid (Anyango et al., 2011). Also, total zein viscoelastic materials (doughs) can be formed by casting the prolamin into films first, followed by sonicating in water to produce fibrils that can be worked into a viscoelastic mass (King et al., 2016).

2.2.3.1 Microparticles and bioplastic films

2.2.3.1.1 Principles of forming microparticles

Protein microparticles can be formed using various methods, the most common ones being: liquid-liquid dispersion processes (Zhong and Jin, 2009), evaporation-induced self-assembly (EISA) techniques (Wang and Padua, 2010) and coacervation processes (Taylor et al., 2009; Anyango et al., 2011). Such protein microparticles have potential practical use in the pharmaceutical industries where they could be utilised in drug delivery systems, tissue or gene engineering systems (Paliwa and Palakurthi, 2014; Taylor et al., 2014). In addition, microparticles can be used in food industries as coating materials or stabilising agents for colloidal solutions (Gao et al., 2014).

Zein and kafirin prolamins can self-assemble into nanostructures that can form films, rods, spheres or microparticles (Taylor et al., 2013). During the formation of microparticles, the amphiphilicity of the prolamin is the major driving force of self-assembly (Wang and Padua, 2012). Zein is amphiphilic, and this may be attributed to its amino acid sequence (Shukla and Cheryan, 2001). Kim and Xu (2008) reported that when the concentration of aqueous ethanol is lower than 90%, zein aggregates into micelle-like structures, with its hydrophilic sides exposed to the solvent. As the concentration of aqueous ethanol increases to greater than 90%, the hydrophilic sides of the zein orient towards the aqueous ethanol. Similar to zein, it

may be assumed that total kafirin has amphiphilic properties. Apart from the work of Kim and Xu (2008), Wang et al. (2004) have previously studied the amphiphilic behaviour of zein.

At the nanoscale level, Wang and Padua (2012) proposed a model to explain the mechanism of self-assembly in zein proteins (Figure 2.2). In this model, the authors suggested that zein proteins unfold to form β-sheets structures. The β-sheets align side by side, and they interact by forming hydrogen bonds through the glutamine residues that are on the bottom of the α-zein. The β-sheets structures then pack in opposite sides, enabling them to form a ribbon that coils into discs and eventually spheres. When solvent evaporation takes place, the β-sheets structures may shrink, consequently resulting in self-assembly of proteins.
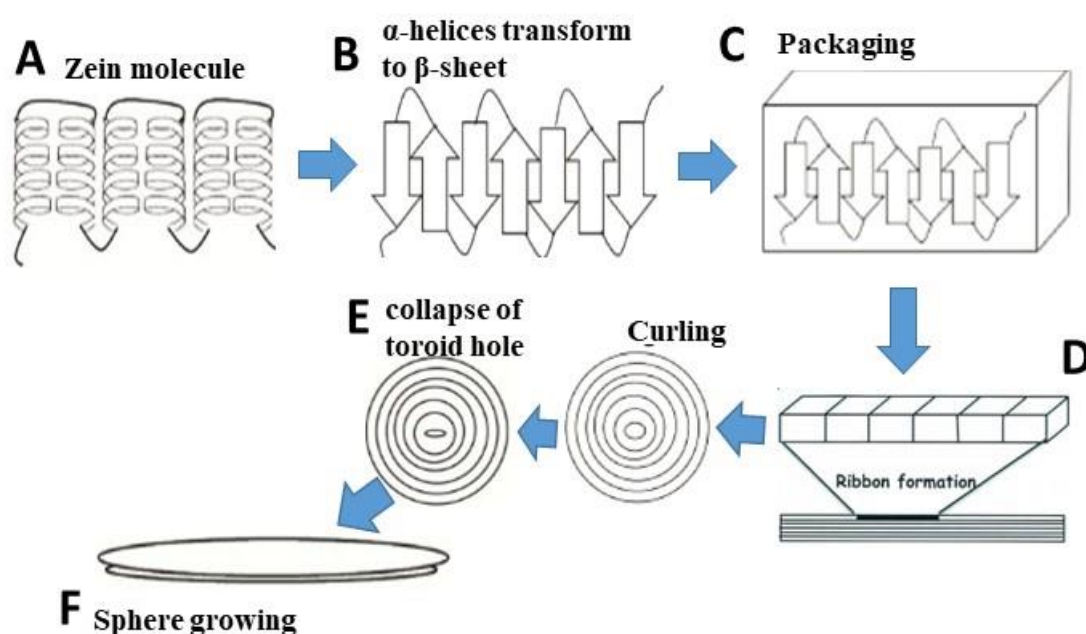
Figure 2.2 Possible mechanism for zein self-assembly from single molecules to nanospheres according to Wang and Padua (2012) (redrawn by Taylor et al., 2013).

Taylor (2008) proposed a model for the self-assembly and aggregation of kafirin into microparticles. In this model, kafirin is first dissolved in glacial acetic acid, followed by addition of water to precipitate the protein out of the solvent (coacervation process). Addition of water reduces the concentration of the acetic acid, enabling the prolamin to become insoluble in the solution and eventually precipitate out of solution as spheres (Taylor et al., 2013). Instead of using organic solvents, kafirin microparticles can be produced by coacervation process using aqueous ethanol as a solvent (Taylor et al., 2009).

The models proposed by Taylor (2008) and Wang and Padua (2012) are common in that the prolamin protein first undergoes structural changes to enhance protein aggregation. The model proposed by Taylor (2008) may apply to the formation of total zein microparticles, considering both total zein and total kafirin proteins contain all subclasses (α-, β-, γ-, δ-). On the other hand, the model that was proposed by Wang and Padua (2012) may apply to prolamin proteins with high α-helical content that can be transformed into β-sheet structures.

During the formation of microparticles, it may be crucial to consider the effect of various solvents on the secondary structure of the prolamin protein. Gamma-zein has an α-helical structure (Bicudo et al., 2018), thus it may be possible that γ-enriched zein prolamins will self-assemble into microparticles by a similar mechanism as proposed by Wang and Padua (2012). Anyango et al. (2013) was able to form microparticles from total kafirin enriched with 15% γ-kafirin subclass. These microparticles were smaller and non-vacuolated compared to the control total kafirin microparticles that had vacuoles. Even though most work on the formation of prolamin microparticles has been done using either total kafirin or total zein preparations, no one has reported on the formation of prolamin microparticles using QPM zein.

2.2.3.1.2 Principles of forming bioplastic prolamin films

Zein and kafirin are hydrophobic prolamins with excellent film-forming properties and higher water permeability compared to some synthetic plastic films (Wittaya, 2012). To date, research on the formation of bioplastic films using prolamin proteins such as zein, kafirin and gluten has gained recognition, and this is mainly fuelled by environmental safety concerns that arise when synthetic plastics are disposed of (Avio et al., 2017). However, the development of plant-based bioplastics on a commercial scale is an expensive process (Hernandez-Munoz et al., 2003; Zhang and Mittal, 2010; Taylor et al., 2013; Corradini et al., 2014).

Prolamin bioplastic films can be produced using various methods and techniques. Most freestanding bioplastic films are produced by using the casting method, which involves dissolving prolamin in a solvent followed by heating and drying the protein solution to form a film (Zhang and Mittal, 2010; Corradini et al., 2014). The prolamin protein type and choice of casting solvent are important factors that may influence the properties of free-standing

films (Taylor et al., 2005; Xiao et al., 2015). Oxidising agents, crosslinking agents, plasticizers, addition of nanoparticles and physical technologies/treatments have shown to improve the functionality of prolamin films (Paramawati et al., 2001; Kim et al., 2004; Lawton, 2004; Byaruhanga et al., 2006; Xu et al., 2012; Anyango et al., 2013; Pankaj et al., 2014; Taylor et al., 2016a; Dong et al., 2018; Huo et al., 2018; Kaur et al., 2013; Wei et al., 2018. Of the methods that have been investigated, plasticisers and crosslinking agents have great potential to enhance mechanical properties and film flexibility.

2.2.3.1.2.1 Research into the modification of zein and kafirin bioplastic films

Plasticizers such as polyethene glycol (PEG), glutaraldehyde and glycerol have been used to improve the functional properties of zein and kafirin films (Lawton, 2004; Xu et al., 2012; Huo et al., 2018; Shi et al., 2012). Plasticizers can be defined as small molecular weight molecules that are incorporated during film formation, with the aim of improving film functionality (Krotcha, 2002). Although plasticizers have the potential to improve various film properties such as mobility (Athamneh et al., 2008), elongation (Huo et al., 2018), barrier, mechanical and tensile strenght (Paramawati et al., 2001; Anyango et al., 2011; Masamba et al., 2015), they tend to leach out of the film during film formation (Taylor et al., 2013). Leaching out of plasticizers is problematic because it may consequently reduce the mechanical strength or increase in water vapour permeability of prolamin films (Taylor et al., 2013).

Thus, the use of chemical crosslinking agents may be a preferred alternative to improve zein and kafirin film functionality (Parris and Coffin, 1997; Anyango et al., 2011; Khalil et al., 2015; Masamba et al., 2015). Crosslinking agents enhance the formation of intermolecular disulphide bond crosslinkages between protein polypeptides (Kim and Xu, 2008). This brings the protein polypeptides together during film formation, thereby improving film functionality (Anyango et al., 2011).

Apart from using crosslinking agents, prolamin film functionality may be influenced by the presence of γ- prolamins that can form disulphide crosslinkages (Duodu et al., 2003; Anyango et al., 2013). Studies have shown that total kafirin films have better film functionality (barrier properties, water vapour and tensile strength) compared to zein films (Buffo et al., 1997; Byaruhanga et al., 2005), possibly due to the presence of abundant

hydrophobic γ-kafirin, which crosslink through disulphide bonding (Duodu et al., 2003). Even though most literature on prolamin functionality has been done on commercial zein, total zein (from regular maize) and total kafirin, no work has been done to investigate if films can be cast from QPM zein.

2.2.3.2 Formation of viscoelastic doughs and viscoelastic materials

2.2.3.2.1 Improving the functionality of gluten-free doughs systems using flour

Development of gluten-free baked products has been challenging, mainly due to the absence of a protein substitute that gives the same functionality as wheat gluten (Taylor et al., 2016b). Gluten-free flours obtained from cereals such as maize, rice or sorghum do not form gluten when worked into a dough. Gluten is a wheat prolamin protein complex that confers viscoelasticity in wheat doughs (Shewry and Halford, 2002; Goesaert et al., 2005). Thus various additives, technologies and processes have been used to mimic the structural gas-holding properties of wheat gluten using gluten-free flours (Matos and Rosell, 2014; Wang et al., 2017). For example, the inclusion of pre-gelatinised starch, dough sheeting and natural fermentation processes have been applied to non-gluten dough systems (Satin, 1988; Edema et al., 2013; Ngemakwe et al., 2014; Khuzwayo, 2016; Padalino et al., 2016). Pre-gelatinised starch acts as a binder while dough sheeting distributes proteins within the dough (Khuzwayo, 2016). In gluten-free dough systems, the application of fermentation can break down the protein peptides into amino acids that may improve the texture, quality, aroma and flavour of the baked product (Thiele et al., 2002; Arendt et al., 2007). Research has shown that hydrocolloids such as xanthan gum, hydroxypropylmethylcellulose (HPMC), carboxymethyl carbon (CMC), gelatine, alginate and guar gum improve the water binding, viscosity and gas holding capacity of gluten-free batters (Antony and Artfield, 2008; Sciarini et al., 2010; Ngemakwe et al., 2014). Pulse flours from lentils, chickpeas and dry beans increase the binding capacity of batters (Tosh and Yada, 2010), thus improving the dough handling properties. Also, oxidising enzymes such as glucose oxidase enhance the formation of elastic-like batters, and this may consequently improve the quality of sorghum and maize bread quality by preventing collapsing at the top and increasing bread volume (Renzetti and Arendt, 2009).

2.2.3.2.2 Research into formation of viscoelastic materials using zein and kafirin proteins

Understanding the role and functionality of zein and kafirin is crucial for advances to be made towards improving the gluten-free dough systems (Taylor et al., 2016b).

The four factors that affect the functionality of prolamins, particularly from non-wheat cereals during formation of doughs (also referred to viscoelastic materials) have been identified (Taylor et al., 2018). These are glass transition temperature (T_g), prolamin's secondary structure, hydrophobicity and prolamin composition (level of α-, β-, δ- and γ-subclasses). Commercial zein (predominantly α-zein) alone, or commercial zein plus starch can form viscoelastic doughs when mixed with water or acetic acid above the glass transition temperature (T_g) of zein (Lawton, 1992; Sly et al., 2014). The zein protein polymers occur in a glassy state when prepared with solvents below the prolamin's T_g. As temperature increases and exceeds T_g, the reactivity of zein increases and the polymers change into a rubbery state, which enhances zein aggregation (Erickson et al., 2012).

Erickson et al. (2012) suggested that β-sheet conformation affects the functionality of zein during formation of viscoelastic materials. Using total zein (containing all subclasses), King et al. (2016) found out that the β-sheet content of the total zein in distilled water was higher compared to that when the preparation is dry. Despite the high β-sheet content, the author could not form viscoelastic materials from total zein even after preparing with distilled water above the T_g of zein. Thus, it is unlikely that β-sheet content would have an effect on the functionality of total zein during formation of viscoelastic materials (King et al., 2016). Recent work has shown that viscoelastic materials from total zein can be produced by casting the protein into films with glacial acetic acid, followed by sonication in water (King et al., 2016). Alternatively, the prolamin can be dissolved in glacial acetic acid, followed be rapid addition of water (simple coacervation process) to precipitate the protein from solution (Taylor et al., 2018). In addition to total zein, stable total kafirin viscoelastic materials can be produced through the simple coacervation process (Elhassan et al., 2018; Taylor et al., 2018). The methods of King et al. (2016) and Elhassan et al. (2018) are similar in that prolamins are first dissolved in glacial acetic acid. This increases the α-helical content of both zein and kafirin. Hence, the level of α-prolamin may be a possible factor to consider when attempting to form viscoelastic materials using total zein or total kafirin.

Following on the work of Elhassan et al. (2018) and Taylor et al. (2018), Oguntoyinbo et al. (2018) found that the functionality of total zein and total kafirin viscoelastic materials was influenced by the conditions of their formation by coacervation; final acetic acid concentration and initial concentration of protein. When the final acid concentrations were 0.5-0.1% and 5-10% for zein and kafirin respectively, interconnected fibrils were formed and these could be worked into cohesive dough-like viscoelastic materials that resemble the gluten viscoelastic material. To enable formation of fibrils, a minimum protein concentration of 5-10% is required (Oguntoyinbo et al., 2018). At low acid concentrations (5% and 0.1%), zein exhibited viscous flow characteristics and the viscoelastic materials formed were softer than kafirin. In contrast, kafirin viscoelastic materials displayed high elasticity during storage and this may be attributed to the additional polymerisation that took place during storage. Thus, it is probable that prolamin composition (subclasses present) and solvent used (glacial acetic acid) influences functionality of prolamins during formation of viscoelastic materials.

Acetic acid has been used to modify the secondary structure of zein doughs. Using commercial zein, Sly et al. (2014) showed that increasing the concentration of acetic acid led to the formation of softer viscoelastic materials and this may have been due to the increase in the α-helical conformation that was observed. However, King et al. (2016) could not form viscoelastic materials when total zein was prepared in the acetic acid treatment. The presence of other subclasses (β-, δ- and γ-) may have hindered with the formation fibrils, unlike commercial zein that is predominantly α-zein. Since the work of Lawton (1992) and Sly et al. (2014) demonstrated that only commercial zein forms viscoelastic materials when prepared in distilled water or acetic acids above T_g of zein, it may be suggested that the functionality of zein during formation of viscoelastic materials is affected by the prolamin composition, in terms of subclasses present.

Kafirin is considered more hydrophobic than zein due to the presence of γ-prolamins that are abundant compared to total zein (Duodu et al., 2003). Similar to the work of Lawton (1992), Oom et al. (2008) attempted to make a kafirin-starch viscoelastic mass using lactic acid as a plasticiser. The authors could not form a viscoelastic material. Instead, they formed a kafirin resin, which was extensible and similar to commercial zein resin. Over time, however, the kafirin resin became stiff, making it difficult to stretch compared to the commercial zein-starch resin (Figure 2.3c-d). The authors proposed that the extensive disulphide bonding and

crosslinking between cysteine-rich kafirin monomers were responsible for the stiffening of kafirin viscoelastic materials over time.
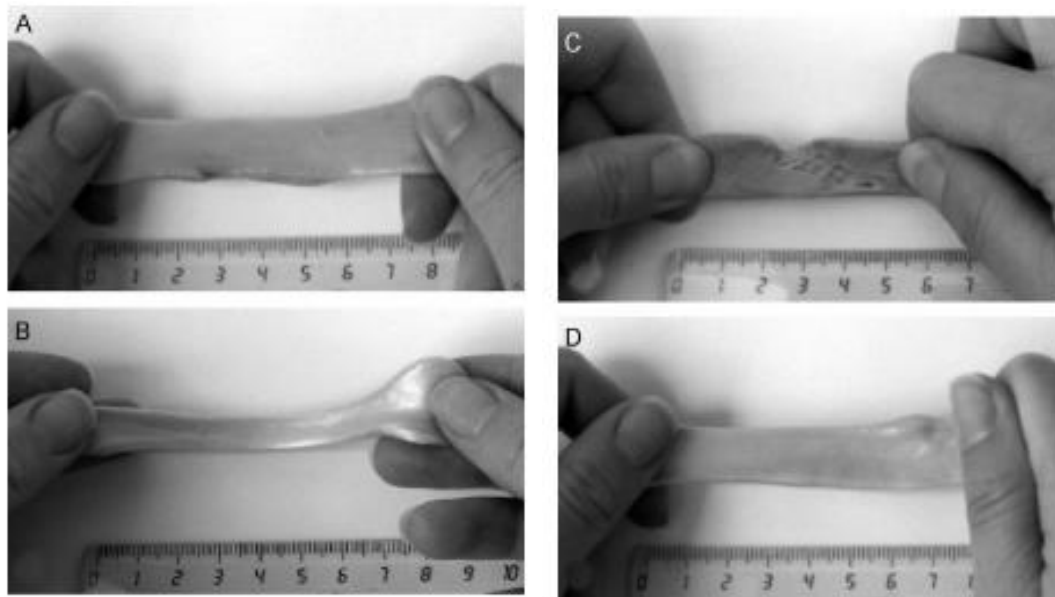
Figure 2.3: Zein and kafirin resins; kafirin (A) and zein (B) stretched immediately after making kafirin (C) and zein (D) stretched after 2.5 hours (Oom et al., 2008)**.**

Schober et al. (2011) suggested that disulphide bonding is not desirable for the formation of total zein viscoelastic materials. Taylor et al. (2018) suggested that disulphide bonds formed between the cysteine-rich γ-kafirin could be responsible for retaining the softness and elastic behaviour of the viscoelastic materials. Findings of Schober et al. (2011) and Taylor et al. (2018) may suggest that either disulphide bonding can be a limiting factor or it may contribute towards the formation of viscoelastic materials using γ-enriched prolamins.

Essentially, all studies on the formation of viscoelastic materials from zein have been done using either commercial zein or total zein extracted from regular maize. However, no attempts have been made to produce a viscoelastic material using QPM zein. As it has been demonstrated that total kafirin (Elhassan et al., 2018) and total zein (King et al., 2016; Taylor et al., 2018) can form viscoelastic materials, it is likely that QPM zein can also form viscoelastic materials, despite it being rich in γ-zein.

2.2.3.3 Formation of prolamin gels

Protein prolamin gels can be formed through aggregation, crosslinking or interaction of polymer chains at different sites (Clark and Ross-Murphy, 1987; Clark and Farrer, 1995). Formation of gels through protein aggregation or chain interaction may involve non-covalent interactions while crosslinking usually involves disulphide bonding (Clark and Ross-Murphy, 1987; Clark and Farrer, 1995). During gel formation, protein polymers partially unfold and this alters their secondary structure to enable more interactions between polymers (Kim and Xu 2008). As a result, a three-dimensional protein network is formed which in most cases is stabilised by non-covalent interactions such as hydrogen bonding and Van de Waals interactions (Zayas, 1997; Totosaus et al., 2002).

One study showed that kafirin more readily forms gels compared to zein (Johns and Brewster, 1917), and this may be due to disulphide bonding between γ-prolamins. Similarly, using zein, Nonthanum et al. (2012) reported that zein samples with added γ-zein formed gels when compared to commercial zein (predominantly α-zein) which had less γ-zein content. Low temperatures induce reversible changes to the protein, while high temperatures thermally damage the protein resulting in the denaturation of polypeptides (Selling et al., 2007). High pH promotes the formation of disulphide bonds in γ-enriched zein (Nonthanum et al., 2013). Furthermore, Fu (2000) suggested that when the level of zein in the aqueous ethanol is higher than 20%, zein gelation is likely to occur. Thus, it may be possible to assume that zein and kafirin gelation may be attributed to a complex interplay of factors such as prolamin type, pH, temperature, and concentration of prolamin in the solvent.

Even though the literature on total zein and total kafirin gelation is still limiting, it would be worth investigating the functionality of QPM zein gelation because this prolamin has abundant γ-prolamin, similar to total kafirin.

2.3 Conclusions

This literature review shows that QPM has a unique amino acid composition, with higher lysine and tryptophan contents compared to regular maize. Furthermore, the QPM has unique zein composition, consisting of less α-zein and abundant γ-zein. There is evidence that factors such as prolamin type, prolamin composition (subclasses present), prolamin hydrophobicity and concentration of prolamin in the solvent influence the formation of viscoelastic materials using prolamin proteins alone (zein and kafirin). Disulphide bond formed between γ-prolamins play an essential role in improving the functional properties barrier of zein and kafirin films. The functionality of QPM zein may be compared to that of kafirin because of its abundant γ-prolamin that form disulphide crosslinkages. Despite the composition of QPM zein, this prolamin has potential to form viscoelastic materials and bioplastic films with better barrier properties of films, similar to total kafirin.

Furthermore, QPM has potential to form gels and this may be attributable to the high levels of γ-zein, which can form disulphide crosslinkages. Even though QPM has nutritional advantages, there is limited research on prolamin functionality with respect to gelation. Knowledge of QPM zein functionality would open doors to prospective applications where QPM can be used, in both food and non-food industries.

3. HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Hypothesis 1

QPM zein film functionality (buffer uptake) will differ from that of total zein (from regular maize) or commercial zein films because of the high proportions of the cysteine-rich hydrophobic γ-prolamins (Holding, 2014). Gamma zein has a helical structure (Bicudo et al., 2018). When QPM zein is dissolved in either aqueous ethanol or glacial acetic acid (casting solvents), it is likely that the γ-zein polypeptides will unfold (Li et al., 2012). This will increase the polypeptide surface area for solvation and polymer-polymer interactions. Disulphide crosslinkages will be formed between γ-zein polypeptides (Duodu et al., 2003). This will bring the zein polypeptides closer together, resulting in formation of a compact protein network that will be stabilised by hydrogen bonding, ionic bonding, Van der Waals forces and disulphide bonding (Nowick, 2008), while creating a barrier that limits the movement of liquids. As a result, QPM zein films will absorb less buffer when compared to either total zein films or commercial zein films cast from the same solvent.

Hypothesis 2

QPM zein will form viscoelastic materials when prepared in a solvent (distilled water or acetic acid) or coacervated out of glacial acetic acid (GAA). Above its transition temperature, hydrated zein exists in a rubbery state. This enables the formation of fibrils that can be worked into a viscoelastic material (Lawton, 1992). Total zein and total kafirin can form stable viscoelastic materials when they are first dissolved in GAA followed by adding water (simple coacervation process) (Elhassan et al., 2018; Taylor et al., 2018). Since total kafirin is comparable to QPM zein due to the high γ-prolamin content, QPM zein will be expected to form a viscoelastic material using the coacervation process.

Hypothesis 3

QPM zein will form a gel when the prolamin is prepared in GAA. When mixed with GAA, zein polypeptides will unfold in the coil region (Li et al., 2012). Protein unfolding will enhance the mobility of the zein polypeptides, consequently increasing the surface area for polymer-polymer interactions to take place. As storage time increases, more intermolecular disulphide crosslinkages will be formed between the γ-zein polypeptides, resulting in a compact three-dimensional structure that is stabilised by hydrogen bonds, Van der Waals forces (Nowick, 2008). Compared to zein, kafirin was found to readily form a gel (Johns and Brewster, 1917), and this was attributable to polymerisation of γ-kafirin through disulphide crosslinking. Similarly, zein samples enriched with abundant γ-prolamins compared to zein with less γ-prolamins. Since QPM zein has abundant γ-zein, it will form a gel more readily when compared to commercial zein and total zein from regular maize.

3.2 Objectives

Objective 1

To determine the characteristics of two quality protein maize (QPM) types based on protein content, zein yield and composition of zein protein. The aim is to verify that samples obtained were, in fact, true QPM maize types.

Objective 2

To determine if QPM zein can be cast into films that have better film functional properties compared to commercial zein films or total zein (from regular maize) films. The aim is to determine the effect of different prolamin preparations (QPM zein, commercial zein, total zein from regular maize) on the physical appearance and buffer uptake of zein films.

Objective 3

To determine the effect of different treatments (distilled water, acetic acid) and techniques (coacervation process) on the functionality of QPM zein, with the aim of forming a QPM zein viscoelastic mass.

Objective 4

To determine the effect of glacial acetic acid and storage time on the functional properties of QPM zein. The aim is to form a QPM zein gel.

4. EXPERIMENTAL DESIGN
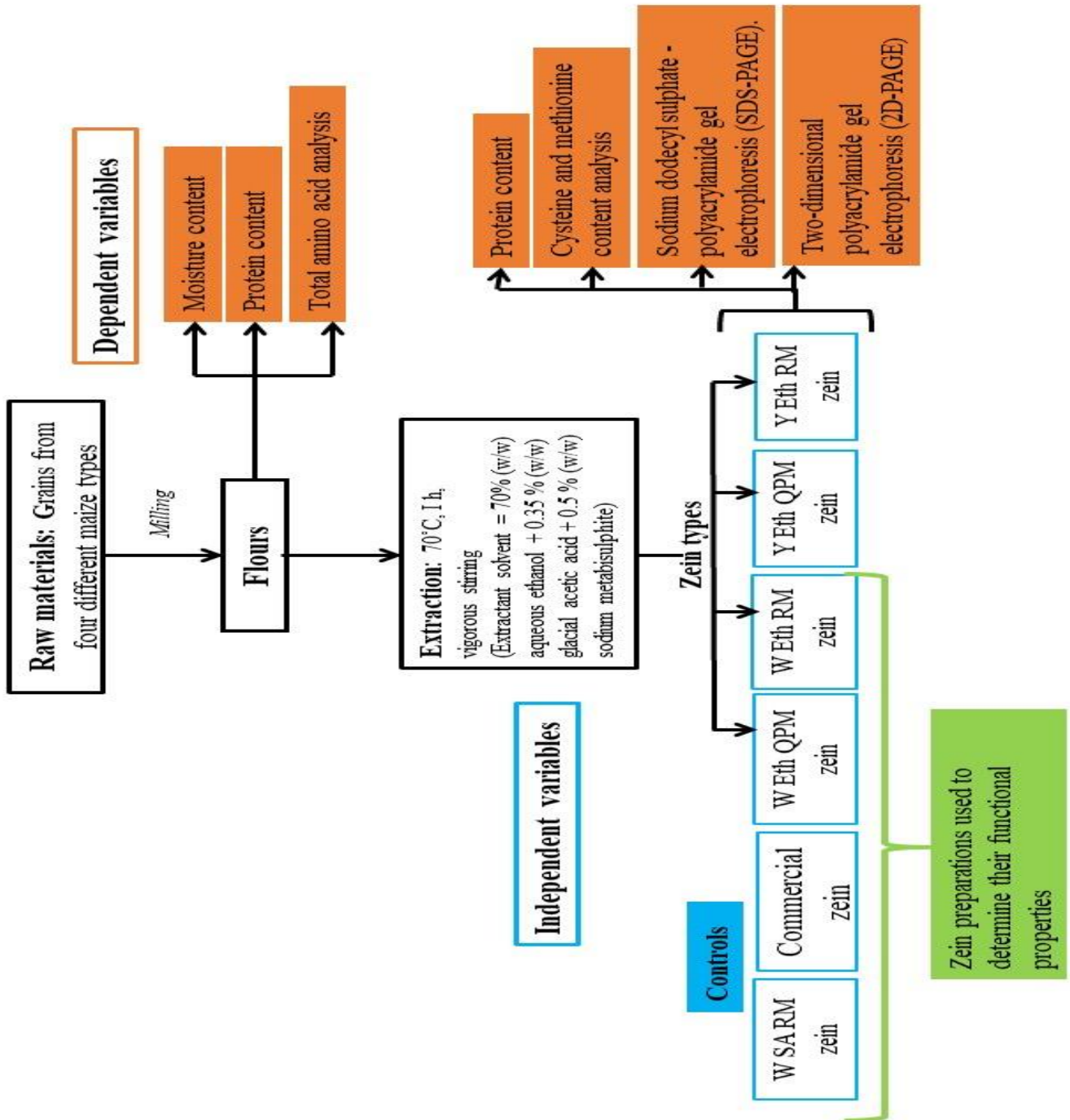
Figure 4A: Experimental design to characterise the composition of four different maize flours and zein extracted from four different maize types. W SA RM = total zein from regular white South African maize; W Eth QPM zein = zein from white QPM; W Eth RM = zein from regular white Ethiopian maize, Y Eth QPM = zein from yellow QPM; Y Eth RM = zein from yellow Ethiopian regular maize.

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Continuation of Experimental design:
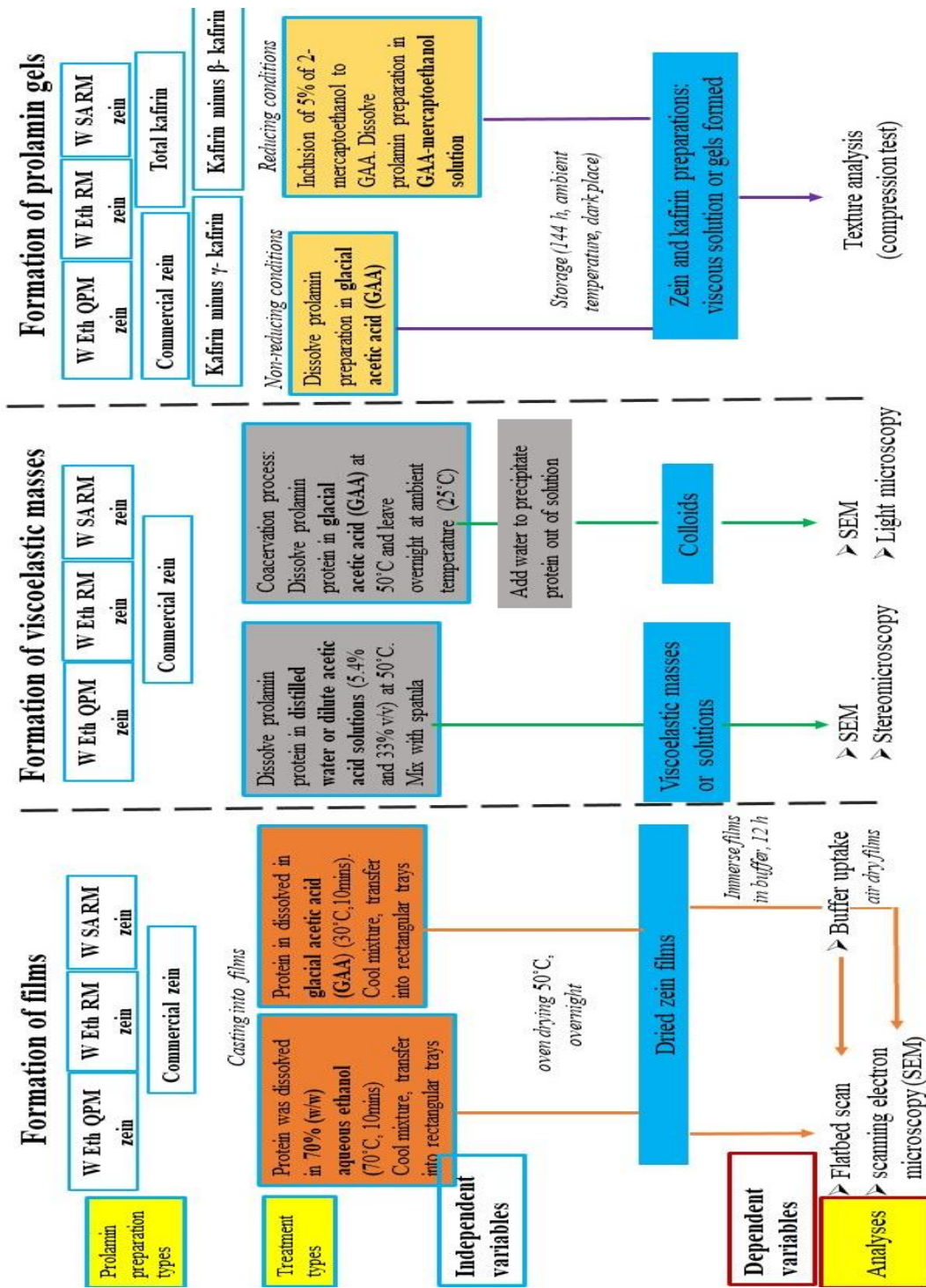
Figure 4B: Experimental design to determine the effect of prolamin preparation types on the functionality of the protein, with respect to formation of bioplastic films, viscoelastic materials and gels. Abbreviations: W Eth QPM zein = zein from white QPM; W Eth RM = zein from white Ethiopian regular maize, W SA RM = total zein from white South African regular maize.

5. RESEARCH CHAPTER

5.1 Abstract

Quality protein maize (QPM) zein has an unusual zein composition, with less α-zein and abundant γ-zein compared to zein from regular maize. In the current study, analyses showed that yellow and white QPM varieties obtained from Ethiopia had higher lysine contents and they gave lower zein yields compared to regular maize. Moreover, the QPM zeins had higher levels of cysteine compared to their regular counterparts, confirming that they were true QPM. QPM zein could be cast into films. Furthermore, QPM zein films cast from glacial acetic acid (GAA) were extremely flexible without the inclusion of plasticisers. When zein films were immersed in buffer (pH 6.8), QPM zein films cast from GAA absorbed the least amount, probably due to disulphide crosslinking between γ-zein molecules. When zein preparations were dissolved in GAA followed by addition of water (coacervation process), all zein preparations formed colloids. Interestingly, QPM zein formed a more viscous solution when dissolved in GAA. After inclusion of 5% 2-mercaptoethanol (BME) in GAA, QPM zein and kafirin without β-kafirin formed gels, and this was attributed to protein unfolding. In conclusion, the functionality of QPM zein differs from that of regular maize zein and this is attributable to the abundant γ-zein.

5.2 Introduction

Zein from quality protein maize (QPM) has less α-zein and abundant γ-zein (which is rich in cysteine) compared to zein from regular maize (Holding, 2014). The presence of abundant γ-zein in QPM zein is likely to affect its functionality. To date, research on zein functionality has involved either commercial zein (predominantly α-zein) or total zein (containing α-, β-, γ- and δ- subclasses) extracted from regular maize. However, studies that compare the functional properties of zeins from QPM and those from regular maize types are lacking.

Zein can be used to make bioplastics that have the potential to replace synthetic plastics (Arcan and Yemenicioglu, 2011). Commercialization of zein and kafirin bioplastics has however been slow (Taylor et al., 2013), possibly because prolamin-based films are brittle and have poor functional properties or because the production of prolamin films on a large scale is an expensive process. Hence, there is a need to improve prolamin film functionality in order to increase its applications within the food and non-food industries. Modification of zein films can be achieved by incorporating plasticisers (Lawton, 2004), using oxidising agents (Taylor et al., 2016a) and enzymes (Kim et al., 2004; Anyango et al., 2011; Turasan et al., 2016). A potential alternative to improve the barrier properties of zein films would be the use of QPM zein as a raw material because it has abundant γ-zein that forms disulphide cross-linkages that could improve film barrier properties and other aspects of film functionality.

Hydrated commercial zein can form viscoelastic materials (also referred to as gluten-like doughs) when prepared in distilled water or dilute acetic acid above the glass transition temperature (T_g) of zein (Lawton, 1992; Taylor et al., 2018). Total zein can be manipulated into viscoelastic materials by dissolving it in glacial acetic acid (GAA) then casting into a film (King et al., 2016). Similar to the method used by King et al. (2016), viscoelastic materials have also been formed from total zein using a coacervation process (Oguntoyinbo et al., 2018). The functionality of zein or kafirin, with respect to formation of viscoelastic materials can be influenced by factors such as prolamin subclasses (in terms α-, β- and γ-zein levels), prolamin secondary structure (i.e. β-sheet content), T_g, prolamin hydrophobicity and the level of protein in solvent (Oguntoyinbo et al., 2018; Taylor et al., 2018). However, no work has been done to determine if QPM zein can form viscoelastic materials.

Formation of zein gels has been described as a "troublesome" characteristic of zein (Shukla and Cheryan, 2001). One study has shown that total kafirin readily form gels in aqueous ethanol when compared to total zein (Johns and Brewster, 1917). Formation of kafirin gels can be attributable to disulphide crosslinking between γ-kafirin. Furthermore, two studies on zein gelation showed that zein samples containing γ-zein could form gels compared to zein without γ-subclass (Nonthanum et al., 2012; Nonthanum et al., 2013). Apart from the work of Nonthanum et al. (2012) and Nonthanum et al. (2013), recent literature on zein gelation is still limiting.

Hence, the functional properties of QPM zein, with respect to the formation of bioplastic films, viscoelastic materials "dough" or zein gelation will be determined in the current study.

5.3 Materials and Methods

5.3.1 Materials

Zein preparations were extracted from four different maize types. Whole grain white QPM from Ethiopian variety (MHQ 138), regular white maize from Ethiopian variety (MH 140) and yellow QPM Ethiopian maize variety (BHPQY 545) (coded W Eth QPM, W Eth RM and Y Eth QPM, respectively). Dr K. Abegaz of Hawassa University, Ethiopia very kindly provided these. Yellow regular maize from South African variety (coded Y SA RM) was purchased from a local store in Pretoria, South Africa in the form of crushed maize. Commercial zein, total zein from white South African maize (coded W SA RM), total kafirin, kafirin without β-kafirin, kafirin without γ-kafirin preparations were also used as controls. Commercial zein was purchased from Sigma-Aldrich, Johannesburg, South Africa (Product code: Z3625). Total zein from W SA RM and the three kafirin preparations types (total kafirin, kafirin without β-kafirin, kafirin without γ-kafirin) were prepared as described by Njila (2017) and Elhassan et al. (2018), respectively.

5.3.2 Methods

5.3.2.1 Maize milling and extraction of zein

The maize samples were separately milled into flour using a laboratory hammer mill (Falling Number 3100, Huddinge, Sweden) with a screen particle size of 0.5 mm, to obtain a powder with maximum 500 μm particle size. The milled flour was packed in airtight, polyethylene ziplock-type bags and stored at 9-10°C for further analysis.

Zein was extracted from the milled maize flour using 70% (w/w) aqueous ethanol containing 0.35% (w/w) glacial acetic acid and 1.0% (w/w) sodium metabisulphite. Milled maize flour (500 g) was weighed into a 5 L plastic bucket with a tight fitting lid and a central hole for a stirring paddle. The aqueous ethanol-based solution was then added to the flour and extraction was carried at 70°C for 1 h with vigorous stirring. After extraction, the protein solution was centrifuged at 1000 x g for 5 min at 25°C to obtain a clear supernatant containing zein. The supernatant was decanted into stainless steel trays, and the ethanol was allowed to evaporate in a fume hood at 25°C for 3 d. The resulting precipitate was washed

with minimal amounts of cold distilled water (8°C) and filtered under vacuum using a Buchner funnel and two Whatman No 4 filter papers (150 mm diameter). The precipitate was dried overnight in a fume hood at 25°C. The dried zein was re-milled into a powder using an air-cooled knife type laboratory mill (IKA A11 Basic mill (Staufen, Germany)). It was then weighed to determine the zein yield.

The zein was defatted with n-hexane at a ratio of one part zein to three parts hexane (w/w), with mixing for 45 min at 25°C. The suspension was allowed to stand for 15 min, and the hexane was decanted. After repeating the process three times, the suspension was filtered under vacuum and air dried in a fume hood. Zein protein recovered after defatting was weighed, packed in zip-lock type bags and stored at 9-10°C for further analysis.

5.3.3 Analyses

5.3.3.1 Moisture and protein content of raw materials and zein protein

The moisture contents of the maize flours were determined by the AACC Approved Method 44-15A Air oven method (AACC, 2000).

The total protein contents of maize flours and zein preparations were determined by Dumas total combustion method, following the AACC Approved method 46-30 (AACC, 2000) using a conversion factor of N x 6.25.

5.3.3.2 Total amino acids, cysteine and methionine

5.3.3.2.1 Total amino acids

The total amino acid compositions of the four maize flours (W Eth QPM, W Eth RM, Y Eth QPM and Y SA RM) were determined by the Southern Africa Grain Laboratory (SAGL) (Pretoria, South Africa), using an AccQ-tag method, a Waters Acuity H-Class with Empower software (Waters, Millipore Corp, Milford, MA). Maize flour (400 mg) was first hydrolysed with 6 M HCl (24 h) and then derivatised with 6-aminoquinolyl-N-hydroxysuccinimidyl carbonate. The stable derivatives obtained were then analysed by reverse phase UPLC, using an Acc-Q Tag Ultra 2.1 x 100 mm, 17 μm particle size C-18 column, with a PDA detector at

an absorbance of 260 nm, at flow rate of 0.7 ml/min. The mobile phase consisted of standards AccQ-Tag Ultra eluent A & B with gradient separation. The following standards: (A) Amino Acid Standard H from Pierce (Prod no: 20088; Thermo-Fischer Scientific, Johannesburg, South Africa) and (B) L-Novaline (N-7627, Sigma-Aldrich, Johannesburg, South Africa) were used. The amino acids analysed were aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, isoleucine, leucine, phenylalanine and lysine.

5.3.3.2.2 Cysteine and methionine

The cysteine and methionine contents of the six zein preparations (W Eth QPM, W Eth RM, Y Eth QPM, Y SA RM, W SA RM and commercial zein) were determined by SAGL, using a modified Pico Tag method with Empower Software (Waters Millipore). Zein was first oxidised with performic acid to convert cysteine and methionine quantitatively to cysteic acid and methionine sulfone, respectively. Hydrogen bromide was then added to stop the reaction and the zein samples were dried. After drying, zein (400 mg) was hydrolysed with 6 M HCl for 24 h, then derivatised with phenylisothiocyanate (PITC) to produce phenyltiocarbamyl (PTC) amino acids, which were analysed using reversed phase HPLC. The HPLC system consisted of a UV detector (wavelength 254 mm), a binary pump, an autosampler and a column. A Pico Tag 3.9 mm x 150 mm C-18 column (Waters Millipore) was used with a flow rate of 101.5 ml/min. The mobile phase consisted of (A) sodium acetate buffer and (B) acetonitrile-water (60/40), with gradient separation. The following standards were used; cysteic acid (C-7630, Sigma-Aldrich) and methionine sulfone (M-0876, Sigma-Aldrich).

5.3.3.3 Electrophoresis

5.3.3.3.1 SDS –PAGE

SDS-PAGE was performed on the zein and kafirin preparations, under both reducing and non-reducing conditions using a procedure described by Anyango et al. (2013). A 4-12% polyacrylamide gradient gel (NuPAGE® Novex gel, Invitrogen (Carlsbad, CA)) with 15 wells was used, together with a vertical electrophoresis system (XCellSureLock TM Mini-Cell, VersionTM. Protein solutions were prepared by dissolving in either reducing

(containing 2-mercaptoethanol) or non-reducing conditions to give a final concentration of 1µg protein/µl. The protein samples were loaded into the gel wells using a 10 µm syringe. Electrophoreses was carried out at a constant voltage of 200 V, 80 mA and 10 W for 1 h. The gels were stained with Coomassie Brilliant Blue R-250 overnight, then rinsed with the destaining solution for 15 min. After destaining, the gels were recorded using a flat-bed scanner.

5.3.3.3.2 2D-PAGE

2D-PAGE was further used to separate zein and kafirin fractions based on subclasses, isoelectric point as well as by apparent molecular weight. For isoelectric focusing, a ZOOM® IPG System (Waltham, MS) was used according to the manufacturer's instruction manual (Invitrogen, 2012). Prolamin preparations were solubilised in buffer solution (containing 60 µl Ampholite and 3 ml DeStreak Rehydration Solution). The resulting protein solution was diluted with DeStreak Rehydration Solution to obtain a final protein concentration of 2 µg protein/µl. A ZOOM® strip (7 cm) was rehydrated with 140 µl protein solution (with concentration two µg/µl), and the strip was placed in a ZOOM® IPG RunnerTM Cassette to equilibrate for 1 h. The strip was placed in a ZOOM® IPG Runner TMMini Chamber, with conditions set at 200 V for 20 min, 450 V for 15 m, 750 V for 15 min and 2000 V for 45 min, to perform the isoelectric focusing separation. After separation, the strips were placed in ZOOM Equilibration Trays. NuPAGE® LDS Sample buffer (9 ml) and sample reducing buffer (1 ml) were added to the equilibrium trays to equilibrate the strip. After 15 min, the equilibration solution was discarded, and an alkylation solution (232 mg iodoacetamide in 10 ml NuPAGE® LDS sample buffer) was prepared. The alkylation solution was added to the equilibrium tray (with the strip). After 15 min, the strip was placed in the NuPAGE Novex 4-12% Bis-Tris ZOOM® Gel well and 0.5% agarose solution was added. The running buffer was prepared and electrophoresis was carried out in the second dimension, at a constant voltage of 200 V, 80 mA and 10 W for 1 h. The gels were then stained with Coomassie Brilliant Blue R-250 overnight then rinsed with the destaining solution for 15 min. After destaining, the gels were recorded using a flat-bed scanner.

5.3.3.4 Zein film formation

Zein films were prepared without addition of plasticizers, using 70% (w/w) aqueous ethanol or glacial acetic acid (GAA) as casting solvents, based on a method described by Anyango et al. (2011), with some modifications. Zein preparation (1.2 g, protein equivalent) was weighed into a 100 ml Erlenmeyer flask. The casting solvent [70% (w/w) aqueous ethanol or GAA] (8.8 g) and a magnetic stirrer were added to the flask. The weight of the flask, solvent and stirrer were recorded, and the flask was then covered with aluminium foil before heating (70°C for aqueous ethanol and 30°C for GAA) using a heated magnetic stirrer plate. The magnetic stirrer was used to facilitate complete solubilisation of prolamin protein in casting solvent. After heating for 10 min, the solution was cooled for 2 min to ambient temperature (25°C). The casting solvent (aqueous ethanol or GAA) was added to replace the solvent lost by evaporation. The prolamin solutions (3 g) were transferred into rectangular silicone baking trays (28 mm x 69 mm), and the trays were gently swirled for 30 s to distribute the contents evenly over the bottom of the trays. The trays were placed on a level surface (confirmed by a spirit level) in an oven (not forced draft), then dried overnight for 50°C. After drying, the zein films were placed in zip-lock bags for storage at 9-10°C.

5.3.3.4.1 Zein film buffer uptake

The dry zein films (28 mm x 69 mm) were weighed and placed in cylindrical transparent plastic containers (diameter 105 mm x height 38 mm). Sodium phosphate buffer 0.2 M (pH 6.8) was added to the containers until the films were completely immersed. The containers were covered with lids, then placed in a shaking water bath (30 resolutions/min) for 12 h at 39°C. After this period, the films were removed from the container. The buffer solution was removed from the surface of films by gentle blotting using white paper towels. The films were then weighed and photographed. The percentage of buffer uptake by films was calculated using the following formula:

%buffer uptake = [(mass of films after immersing in buffer (mg) – initial mass of dry film (mg)) / initial mass of dry films (mg)] * 100

5.3.3.5 Preparation of zein viscoelastic materials

Formation of viscoelastic materials using distilled water or acetic acids was performed according to a method described by King et al. (2016).

5.3.3.5.1 Formation of viscoelastic materials using distilled water

Zein preparation (0.2 g, protein equivalent) and 0.4 g distilled water were weighed separately into plastic centrifuge tubes (8 mm diameter x 96 mm height). The centrifuge tubes were pre-warmed to 50°C in a water bath. When the temperature was reached, distilled water was added to the zein powder to give a final concentration of 33% (w/w) protein. The protein suspension was immediately vortexed at high speed for 20 s. Then, it was mixed with a spatula to ensure that the zein was hydrated with the solvent. If a viscoelastic mass was formed, it was kneaded with the fingers for 30 s then manually stretched for 5 s before being photographed. Where viscoelastic materials were not formed, the slurry or suspensions were decanted into a beaker and photographed.

5.3.3.5.2 Formation of viscoelastic materials using acetic acid

Viscoelastic materials were prepared using the same procedure as above (see 5.3.3.5.1). Instead of distilled water, 5.4% (v/v) and 33% (v/v) acetic acids were used as solvents.

5.3.3.5.3 Coacervation of zein in GAA with water

Preparation of viscoelastic materials using the coacervation process was done according to the method described by Elhassan et al. (2018). For coacervation, 0.5 g (protein equivalent) of the zein preparation and 4.0 g of GAA were weighed into separate 50 ml Oak Ridge-type plastic centrifuge tubes (8 mm diameter x 96 mm height) with sealing caps. The centrifuge tubes were pre-warmed to 50°C. Upon reaching the temperature, the zein was added to the GAA to give a final protein concentration of 11% protein in solution. The zein solution was mixed using a spatula to ensure complete solubilisation. The zein that did not dissolve in the solvent was further vortexed at high speed for 1 min, followed by warming to 50°C. The zein

solutions were left to stand overnight at ambient temperature (25°C) in the dark with the caps tightly sealed to prevent evaporation of GAA. Distilled water (8.1 ml) was rapidly added to the zein solution, followed by stirring using a spatula. Zein fibrils that were formed were kneaded and moulded with fingers for 30 s. Where fibrils were not formed, the slurry/colloidal suspensions were decanted into a beaker then photographed.

5.3.3.6 Firmness of zein and kafirin preparations during gelation

The firmness of zein and kafirin preparations was determined under both reducing and non-reducing conditions, using a method described by Adeboye and Emmambux (2017), with modifications. Under non-reducing conditions, zein or kafirin preparations (1.4 g, protein equivalent) and 2.8 g GAA were separately weighed and mixed at 25°C to give a final concentration of 33% (w/w) protein. The protein preparations were poured into small cylindrical shaped plastic containers (13.2 mm height; 12.4 mm diameter) and placed in air-tight zip-lock-type bags at 25°C. After 2 h, the firmness of the materials was determined using an EZ-L SHIMADZU Texture Analyser (Tokyo, Japan) fitted with a 10 mm diameter cylindrical probe. The materials were compressed 5 mm using a pre-load force of 0.02 N and the test speed of 10 mm/min. The same materials were further stored in ziplock-type bags at 25°C, and their firmness was determined after 24, 48, 96 and 144 h respectively. For gelation analysis under reducing conditions, 5% 2-mercaptoethanol (BME) (reducing agent) was dissolved in GAA to form a GAA-BME solution. Kafirin and zein preparations were prepared with the GAA-BME mix, as described above.

5.3.3.7 Microscopy

5.3.3.7.1 Scanning electron microscopy (SEM)

The surface morphology of zein films (before and after immersing in 0.2 M sodium phosphate buffer) was studied using a Zeiss Ultra PLUS Field Emission Gun SEM (Oberkochen, Germany). Films were dried in the silica gel desiccator for 48 h. Pieces of the dried films (5 mm x 5 mm) were cut using a razor blade then mounted on an aluminium stab with double-sided tape. The films were coated with carbon before viewing.

SEM was also used to study the structure of the viscoelastic materials or suspensions that were formed when zein preparations were dissolved in distilled water or acetic acid. Viscoelastic materials (approx. 5 mm x 5 mm) were stretched and mounted on double-sided tape. Where viscoelastic materials were not formed, one drop of the prolamin suspension was also mounted on double-sided tape. The samples were air dried in desiccators for 3 d, then coated with carbon before viewing.

5.3.3.7.2 Stereomicroscopy

A stereo light microscope (Nikon SMZ 800 Stereomicroscope, Tokyo, Japan) was used to study the surface structure of the viscoelastic materials and the protein suspensions formed when zein was prepared under distilled water and acetic acid treatments. Viscoelastic materials (5 mm x 5 mm) or two drops of the protein suspensions were placed on glass slides, then photographed.

5.3.3.7.3 Light microscopy

The structure of zein colloids formed after the coacervation process were viewed using a Nikon Optiphot Transmitted Light Microscope (Tokyo, Japan) with phase contrast. One drop of the colloid was placed on a glass slide then viewed.

5.3.3.8 Statistical analyses

All experiments were performed at least twice. One-way and two-way ANOVA were performed using STATISTICA 8 (StartSoft, Tulsa, US. The means were compared using Fisher's Least Significant Difference Test (LSD) at a 95% level of confidence.

5.4 Results and discussion

5.4.1 Extraction and characterisation of zein from quality protein maize (QPM)

5.4.1.1 Moisture, protein content of maize flour and zein extraction

Table 5.1 shows the moisture and protein contents of maize flours, yield and purity of zein extracted from four different maize types.

Table 5.1: Moisture content, protein content and yield of zein preparations from four types of maize

| Maize type | Maize | | | Zein | | |
	Moisture content (g/100 g) (as is basis)	Protein content (N x 6.25) (g/100 g maize) (db)	Zein preparation extracted (g/100 g maize) (db)	Zein preparation yield (g/100 g protein)	Zein preparation purity (g protein/100 g preparation) (db)
W Eth QPM	$5.89^a \pm 0.28$	$9.72^b \pm 0.01$	2.90	29.8	$72.9^b \pm 0.97$
W Eth RM	$5.83^a \pm 0.18$	$10.0^c \pm 0.21$	5.26	52.4	$81.5^{c} \pm 0.18$
Y Eth QPM	$5.92^a \pm 0.64$	$8.94^a \pm 0.13$	2.22	24.6	$57.9^a \pm 0.39$
Y SA RM	$6.24^a \pm 0.09$	$10.6^c \pm 0.28$	3.22	30.5	$78.3^{bc} \pm 0.07$

W Eth QPM = White Ethiopian Quality Protein Maize; W Eth RM = White Ethiopian Regular maize; Y Eth QPM = Yellow Ethiopian Quality Protein Maize; Y SA RM = Yellow South African regular maize. Mean ± standard deviation of two duplicate analyses, db = dry basis. Mean values in the same column with a different superscript are significantly different ($p \leq 0.05$). n = 2

There was no significant difference ($p > 0.05$) in the moisture contents of the four maize types (Table 5.1). These were within the range of most cereal grains (Delcour and Hoseney, 2010). Moisture content influences microbial activity, which affects the shelf life and quality

of cereal grains. The higher the moisture content, the greater the microbial activity (Weinberg et al., 2008). The moisture contents of the QPM and regular maize flours obtained from the current study are similar to what was reported by Prasanna et al. (2001).

The protein contents of white and yellow QPM maize flours (9.72 and 8.94 g/100g protein respectively) were significantly lower (p ≤ 0.05) compared to their regular maize counterparts (10.0 g/100g and 10.6 g/100 g protein, respectively) (Table 5.1). Abiose and Ikujenlola (2014) found that there was no significant difference in the total crude protein content of QPM (9.72 g/100 g protein) and that of regular maize (9.80 g/100g protein). On the other hand, Nuss and Tanumihardjo (2011) reported that the crude protein content of QPM was slightly higher (9.8 g/100 g) compared to regular maize (9.4 g/100 g protein). The result obtained from the present study, that QPM had a lower protein content than regular maize was not expected. Thus, it can be proposed that QPM has better protein quality due to the high lysine and tryptophan content (Holding, 2014).

The amount of zein extracted from W Eth QPM was nearly 50% less than that extracted from W Eth RM (Table 5.1). This result was anticipated and may be attributed to the delayed synthesis of zein in QPM maize (Larkins et al., 1984). The synthesis of zein in QPM is possibly influenced by the presence of the *opaque-2* genes that are responsible for the formation of high-lysine QPM maize types (Hunter et al., 2002). The amount of zein extracted from W Eth QPM was slightly higher than the zein extracted from Y Eth QPM, but similar to that of Y SA RM zein. The similarities in zein yield (on a grain basis) between W Eth QPM and Y SA RM were not anticipated. However, this may suggest that Y SA RM was a mixture of QPM and regular maize.

The purities of the W Eth QPM zein and Y Eth QPM zein preparations were lower by approximately 11% and 35%, compared to their respective regular maize controls (p ≤ 0.05) (Table 5.1). This may be attributed to the fact that less zein was extracted from these maize varieties and this increased the chances of the extracted protein being impure. The zein preparation yield results indicated that W Eth QPM had a lower zein content compared to W Eth RM. In contrast, the yield of zein extracted from Y Eth QPM and Y SA RM were similar. Total amino acid analysis, with particular attention to lysine content, was then conducted to confirm the identity of the four maize types.

5.4.1.2 Amino acid analyses of maize flour and extracted zein

Table 5.2: Amino acid composition (g /100 g protein) of the four types of maize

Amino acid	W Eth QPM	W Eth RM	Y Eth QPM	Y SA RM
Lysine	4.17^b	2.16^a	3.42^{ab}	3.97^{ab}
Histidine	3.65^b	2.17^a	3.47^b	2.74^a
Serine	4.68^a	4.94^a	4.76^a	4.92^a
Arginine	5.67^c	4.01^a	5.43^{bc}	5.39^b
Aspartic acid	5.94^a	6.20^a	7.39^a	7.24^a
Glutamic acid	16.8^a	20.0^b	15.51^a	17.2^b
Proline	9.52^{bc}	9.05^b	9.74^c	7.28^a
Threonine	3.76^c	3.36^a	3.70^{ab}	3.69^{ab}
Alanine	5.86^a	7.32^{bc}	5.94^{ab}	6.67^c
Glycine	4.68^b	3.40^a	4.54^b	4.30^b
Tyrosine	2.61^a	2.75^a	2.51^a	2.84^a
Valine	5.30^a	4.66^a	5.10^a	4.73^a
Isoleucine	2.88^a	3.08^a	3.14^a	3.07^a
Leucine	8.80^a	12.9^b	9.63^b	10.1^c
Phenylalanine	4.01^a	4.90^{ab}	4.48^{ab}	4.40^b

Mean of two duplicate analyses. Mean of values in the same row with a different superscript are significantly different ($p \leq 0.05$)

Amino acid analysis showed that W Eth QPM, Y Eth QPM and Y SA RM were high-lysine maize types because their lysine contents were higher (>3.42 g/100 g protein) (Table 5.2) compared to that of regular maize (approx. 2.86 g/100 g protein) (Henley et al., 2010). The high lysine contents of W Eth QPM and Y Eth QPM were expected, and this may be attributed to the presence of *opaque-2* genes that are responsible for the production of lysine-

rich proteins (Huang et al., 2004). However, the mechanism of how *opaque-2* genes affect the protein pathways through down-regulating the synthesis of 19-21 kDa and 22-24 kDa α-zeins while simultaneously increasing the production of lysine in QPM is not well understood (Segal et al., 2003). Prasanna et al. (2001) reported similar findings on the lysine contents of QPM and regular maize. Surprisingly, the lysine contents of Y Eth QPM and Y SA RM were similar (3.42 g/100 g and 3.97 g/100 g respectively), possibly suggesting that the former maize type was possibly a mixture of QPM and regular maize. In contrast, the lysine content of W Eth RM was lower compared to that reported by Henley et al. (2010) and Tandzi et al. (2017), confirming that W Eth RM was a regular maize variety.

In the current study, the tryptophan content was not measured because a separate technique would have been required to quantify the individual amino acid (Kambhampati et al., 2019). Various methods can be used to determine the tryptophan content of food protein. However, most of these methods require the protein to first undergo hydrolysis (acid, alkaline or enzymatic) in vacuum sealed tubes at high temperatures, followed by neutralising and dilution before quantifying directly using ion-exchange, HPLC or gas chromatography (Adebiyi et al., 2005). Acid hydrolysis methods involve heating the protein in 6 M hydrochloric acid solution in vacuum sealed tubes at a high temperature (above 100˚C) (Friedman and Finley, 1971). Unlike other amino acids, tryptophan is prone to oxidative deamination, thus this amino acid is likely to be destroyed before quantifying (Kambhampati et al., 2019). Wu and Tanoue (2001) reported that enzyme hydrolysis methods partially hydrolyse the tryptophan especially when the protein in heated, hence, this method is not widely used. Alkaline hydrolysis can be performed using barium hydroxide or sodium hydroxide at 110˚C for 16 h in an autoclave, followed by addition of a standard (usually α-methyl-tryptophan) before analysing using HPLC (Bech-Andersen, 1991; Delhaye and Laundry, 1993). Among the hydrolysis methods, alkaline hydrolysis is most preferred when determining tryptophan content in food.

The amino acid data indicated that W Eth QPM, Y Eth QPM and Y SA RM were high-lysine maize types, whereas W Eth RM was a regular maize variety. After extraction, the cysteine and methionine contents of the six zein preparations were then determined to confirm their identity.

Table 5.3: Cysteine and methionine contents (g/100 g protein) of zein preparations

Amino acid	W Eth QPM	W Eth RM	Y Eth QPM	Y SA RM	W SA RM	Commercial zein
Cysteine	$3.80^d \pm 0.32$	$1.83^b \pm 0.11$	$3.03^c \pm 0.12$	$2.73^c \pm 0.02$	$2.69^c \pm 0.04$	$0.98^a \pm 0.02$
Methionine	$0.84^a \pm 0.06$	$1.50^b \pm 0.03$	$0.62^a \pm 0.02$	$2.65^d \pm 0.12$	$3.09^e \pm 0.07$	$2.02^c \pm 0.03$

Mean ± standard deviation of two duplicate analyses. Mean of values in the same row with a different superscript are significantly different (p ≤ 0.05)

The cysteine content of W Eth QPM zein was significantly higher (3.80 g/100 g protein) (p ≤ 0.05) compared to the other zein preparations (<3.03 g/100 g protein) (Table 5.3). The higher cysteine content observed in the white QPM zein preparation was expected, and may be attributed to the pleiotropic effect of *opaque-2* modifier genes that reduce the α-zein synthesis while subsequently increasing the synthesis of cysteine-rich γ-zein (Geetha et al. 1991; Lopes et al., 1995; Hunter et al. 2002). Also, *opaque-2* modifier genes which are located near the 27 kDa γ-zein locus in chromosome 7 enhance the overexpression of the 27 kDa γ-zein in QPM (Lui et al., 2016).

On the other hand, the cysteine contents of W Eth RM zein and commercial zein were significantly lower (<1.83 g/100 g protein) (p ≤ 0.05) compared to those of the Y Eth QPM, Y SA RM and W SA RM zein preparations, which were the same (>2.69 g/100 g protein). This was unexpected as it would be unlikely for Y Eth QPM zein to have the same cysteine content as the Y SA RM and W SA RM zeins extracted from regular maize types. This result may be attributed to the different maize genotypes since W SA RM and Y SA RM zein were extracted from regular South African maize types. The methionine contents of yellow and white QPM zeins were significantly lower (<0.84 g/100 g protein) (p ≤ 0.05) compared to the regular maize varieties (>1.50 g/100 g protein) (Table 5.3). Similar to the cysteine content, the low methionine content in QPM may be a consequence of the *opaque-2* genes, which reduces the expression and accumulation of methionine-rich δ- 10 kDa zein in QPM (Kirihara et al., 1988; Hunter et al., 2002).

The high cysteine content of W Eth QPM zein confirmed that this zein preparation was from a true QPM maize type. Thus, SDS-PAGE was performed to further confirm the identity of the QPM zein preparations, by comparing their zein subclasses composition with those of regular maize.

5.4.1.3 Electrophoresis

5.4.1.3.1 SDS-PAGE of different zein and preparations

SDS-PAGE, under reducing conditions, showed that all the zein preparations [W SA RM (the standard), W Eth RM, W Eth QPM, Y SA RM and Y Eth QPM] had two major monomer bands and a few dimer bands (Figure 5.1 B). The monomer bands were identified as α-zein, of apparent molecular weights between 20 and 25 kDa (Shull et al., 1991). Of interest were the W Eth QPM and Y Eth QPM zein monomer bands (approx. 25kDa) (Figure 5.1B, tracks 4 and 6), which were more intense compared to their respective regular maize controls (tracks 2, 3 and 5). This result indicates that the white and yellow QPM zein preparations had higher levels of γ-zein, confirming what had been observed with the cysteine content of W Eth QPM zein (Table 5.3).

In addition to the darkly stained monomer bands (approx. 25 kDa), both W Eth QPM and Y Eth QPM zein had higher molecular weight bands (indicated by solid arrows, tracks 4 & 6, Figure 5.1B). These bands are similar to the 49 kDa band that was discovered in sorghum prolamin protein (kafirin) by Evans et al. (1987) and later identified as γ-kafirin by El Nour et al. (1998). Since kafirin is homologous to zein (De Rose et al., 1989), it is likely that the high molecular weight bands observed in W Eth QPM and Y Eth QPM zein preparations in the present study were γ-zein.
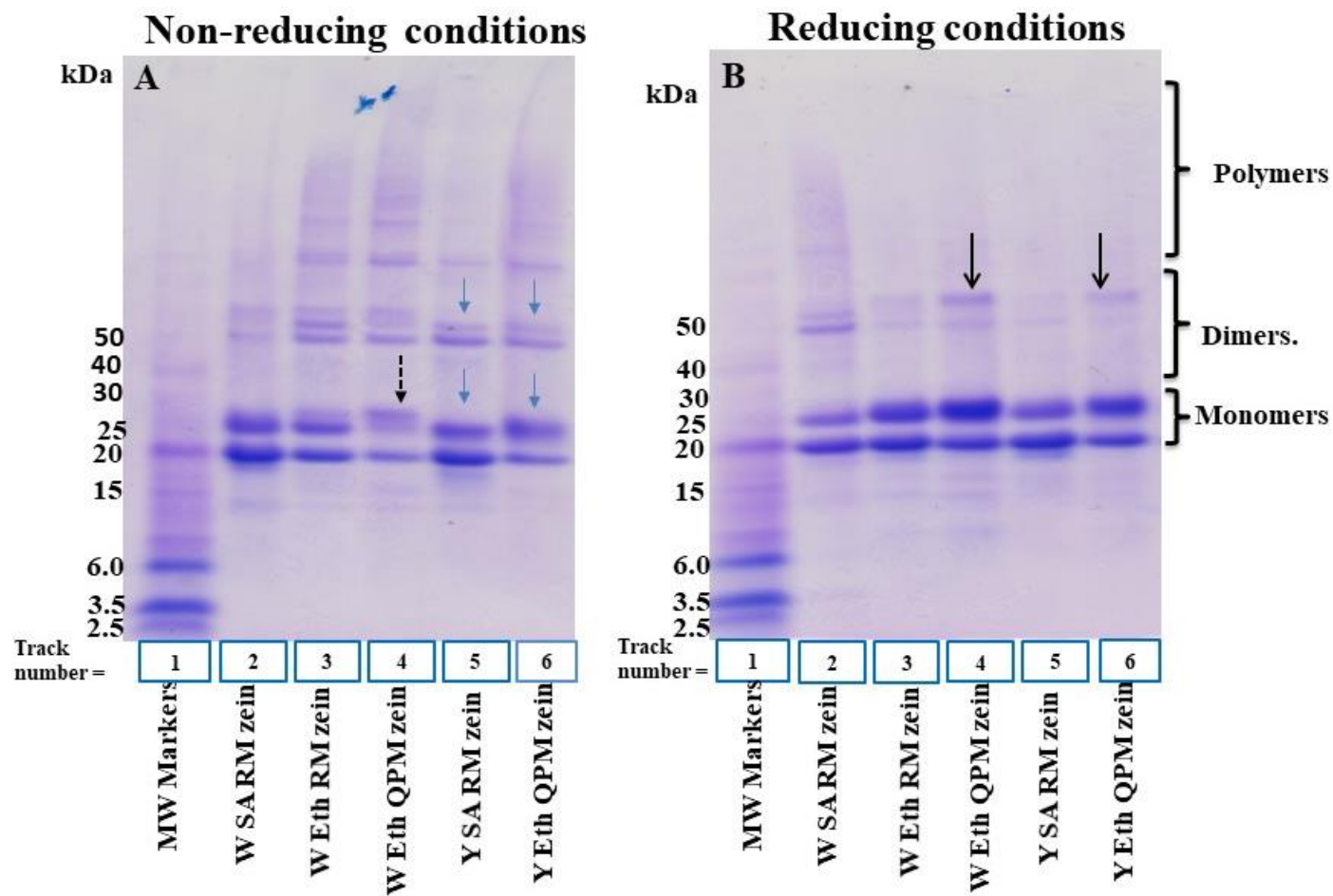
Figure 5.1: SDS-PAGE of five zein preparations, under non-reducing (A) and reducing (B) conditions. Track 1 = Molecular Weight Standard, track 2 = zein from white South Africa regular maize (standard), track 3 =zein from white Ethiopian regular maize, track 4 = zein from white Ethiopian QPM, track 5 = zein from yellow South African regular maize and track 6 = zein from yellow Ethiopian QPM.

SDS PAGE under non-reducing conditions showed that all the zein preparations comprised of monomers, dimers and oligomers (Figure 5.1A). This indicates polymerisation through disulphide bonding between cysteine-rich γ-zein molecules. Of interest, W Eth QPM and Y Eth QPM zeins had more dimers and polymers compared to the other preparations, indicating that these preparations had more γ-zein compared to other zein preparations (Figure 5.1A - track 4 and 6). Both W Eth RM and W Eth QPM zein preparations exhibited two separate bands, with molecular weights between 25 kDa and 30 kDa (dotted arrow, Figure 5.1A - track 3 and 4). Presumably, the two bands were 25 kDa α-zein and 28 kDa γ-zein respectively (Shull et al., 1991). The second band was more visible in W Eth QPM, indicating the presence of more γ-zein (black dotted arrow, track 4, Figure 5.1A) compared to Y Eth QPM (track 6). This finding suggests that Y Eth QPM was a mixture of QPM and regular maize, as previously indicated by the same lysine and cysteine contents of the Y SA RM and Y Eth QPM (Tables 5.2 and 5.3).

In overall, the data obtained from the zein yield, lysine content and cysteine content showed that Y Eth QPM was similar to Y Eth RM. SDS-PAGE revealed that under reducing conditions, W Eth QPM and Y Eth QPM zein preparations were rich in γ-zein. Non-reducing conditions showed that Y Eth QPM zein had similar characteristics with Y SA RM zein. For this reason, zeins from Y SA RM and Y Eth QPM were not used for further analyses. Additionally, 2D-PAGE was conducted to confirm the presence of abundant γ-zein in W Eth QPM. The 2D-PAGE electrophoregram of W Eth QPM zein was compared with the different zein preparations (W Eth RM, W SA RM, commercial) and three kafirin preparations (total kafirin, kafirin without β-kafirin and kafirin without γ-kafirin).

5.4.1.3.2 2D-PAGE of different zein and kafirin preparations

2D-PAGE showed that W Eth QPM zein had less α-zein (particularly 24 kDa), abundant γ-zein (28 kDa) and fewer higher molecular weight γ-zein spots (approx. 55 kDa) (Shull et al., 1991) compared to W Eth RM zein (Figure 5.2A). On the other hand, W Eth RM had low levels of γ-zein and more α_1- and α_2-zein monomers compared to W Eth QPM zein (Figure 5.2A). The difference in zein composition between the two preparations was expected.
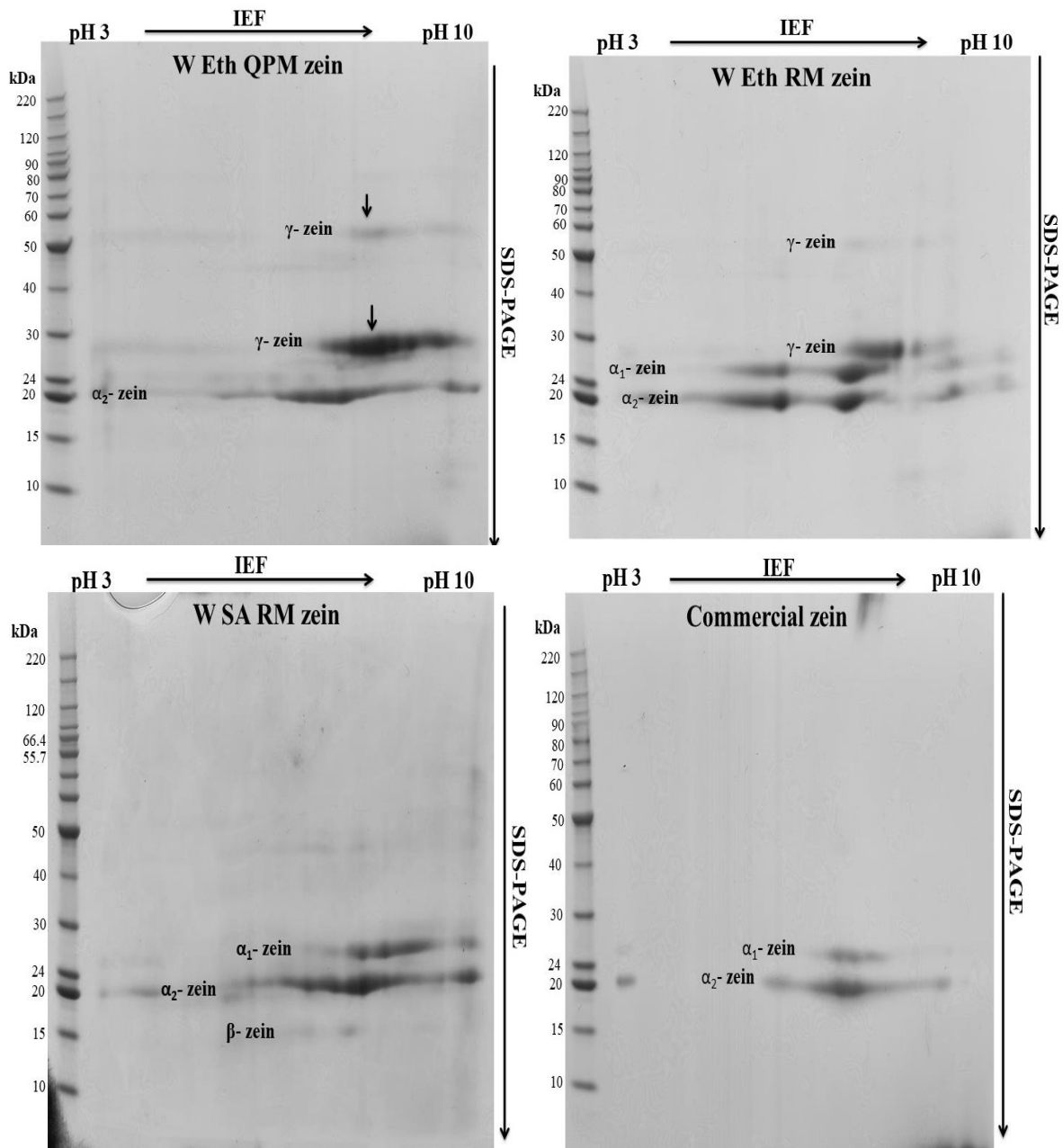
Figure 5.2: 2D-PAGE of W Eth QPM zein, W Eth RM zein, W SA RM zein and commercial zein preparations.

The W SA RM zein preparation had two major α-zein bands and a faint β-zein band with an apparent molecular weight of 15 kDa (Figure 5.2A). The absence of γ-zein in the W SA RM zein was not expected, and this contradicts the cysteine content data (Table 5.3), which showed that the cysteine content of the W SA RM zein was similar to that of Y Eth QPM and Y SA RM zein preparations. Cysteine and methionine residues can form complexes that are difficult to separate during SDS-PAGE (Esen et al., 1988). Since W SA RM zein has high levels of methionine compared to the other zein preparations (Table 5.3), it is possible that

the formation of cysteine-methionine complexes affected the mobility of cysteine rich γ-zein and methionine-rich β-zein by SDS-PAGE. In contrast to the total zein preparations, only α-zein was present in commercial zein. This result was anticipated. The composition of commercial zein, in terms of subclasses present, is mainly attributed to the extraction process, because reducing agents are not used during the extraction process (Anderson and Lamsal, 2011).
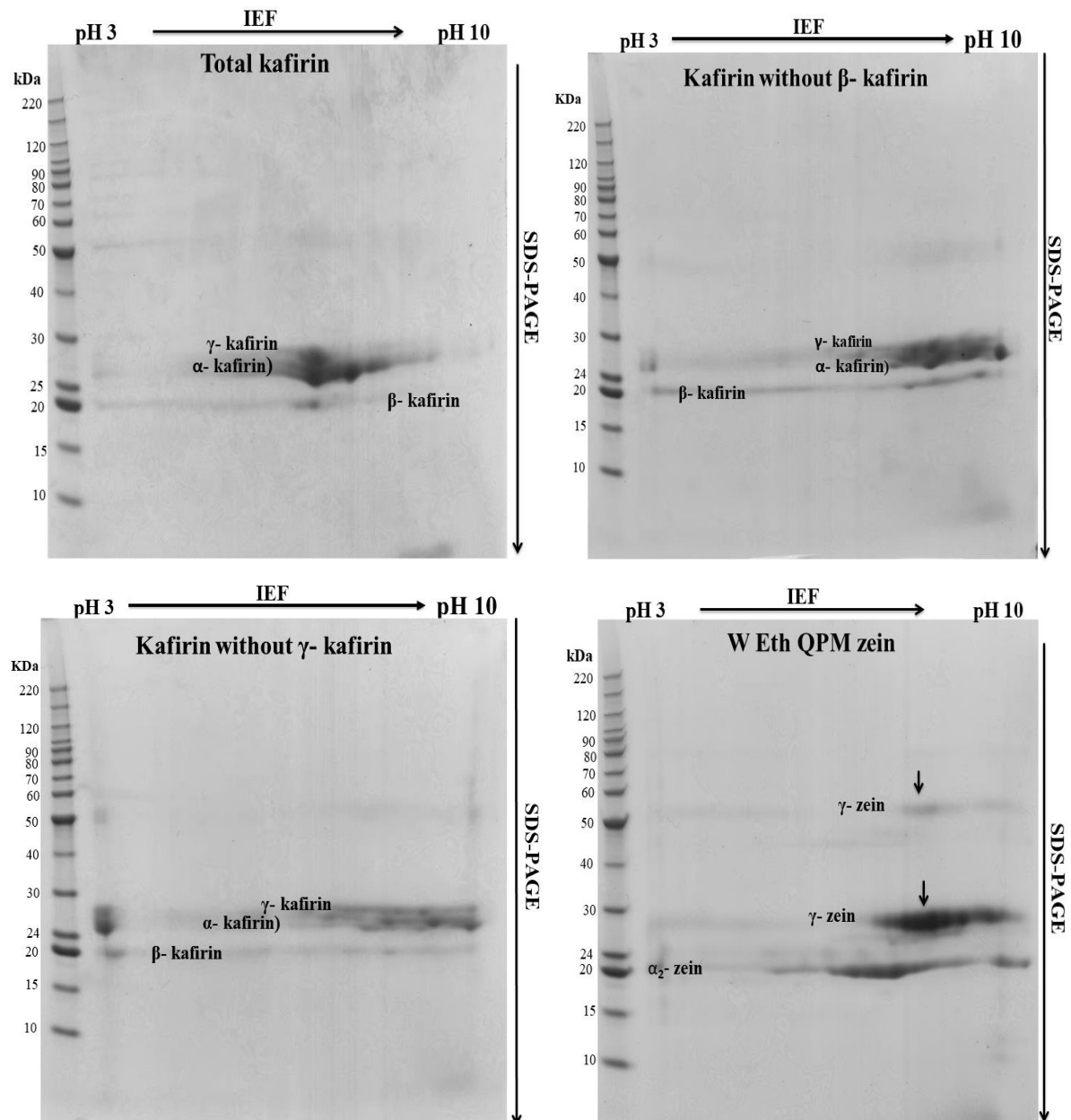
Figure 5.2B: 2D-PAGE of total kafirin, kafirin without β-kafirin, kafirin without γ-kafirin and W Eth QPM zein preparations.

2D-PAGE of total kafirin revealed four major spots (Figure 5.2B), which, with reference to Shull et al. (1991), were identified as α_1-, α_2-, β- and γ-kafirin subclasses, respectively. Among these, the γ-kafirin subclass was more abundant compared to other kafirin subclasses. The γ-kafirin spot of total kafirin was presumably just on top of the α-kafirin band, and this is similar to the γ-zein spot of W Eth QPM zein preparation (Figure 5.2B).

Similar to total kafirin, both kafirin without γ-kafirin and kafirin without β-kafirin preparations had four spots (identified as $\alpha1$, $\alpha2$, β- and γ-kafirin respectively). However, the four kafirin spots observed in the latter two kafirin preparations differed in intensity compared to the total kafirin. It could be possible that the absence of other subclasses in the kafirin without γ-kafirin preparation and the kafirin without β-kafirin preparation affected the mobility of the kafirin polypeptides during electrophoresis.

2D-PAGE also revealed that the W Eth QPM zein and total kafirin preparations had abundant γ-prolamins in comparison to the other zein and kafirin preparations (Figure 5.2B). To determine the functional properties of zein with respect to the formation of bioplastic films, viscoelastic materials and gels, four zein preparations (W Eth QPM, W Eth RM, W SA RM and commercial zein) were studied.

5.4.2 Functionality of zein

5.4.2.1 Film formation

5.4.2.1.1 Effects of zein type and casting solvent on the physical appearance of zein films

All the zein preparations, including the W Eth QPM zein, could be cast into films from 70% (w/w) aqueous ethanol and GAA respectively. Generally, all zein films cast from aqueous ethanol were brittle, as shown by the cracks on their surface after casting (Figure 5.3A - top row films A-D). Brittle films are difficult to work with and this limits their potential applications. Therefore, plasticisers are usually added during film formation to reduce film brittleness (Lai and Padua, 1997). In the current study, however, plasticisers were omitted in order to compare the effect of the prolamin preparations only on the properties of the films, with particular attention to the films from W Eth QPM zein.

The W Eth QPM zein films cast from aqueous ethanol were more opaque and yellow-brown compared to the W Eth RM zein films (control) cast from the same solvent, which were transparent (Figure 5.3A, top row films). The opaqueness of W Eth QPM zein films cast from aqueous ethanol was possibly due to the partial solubility of the prolamin in aqueous ethanol during film formation. SEM did show that W Eth QPM zein films had few undissolved proteins on the surface after casting (Figure 5.4A). Muhiwa (2016) observed similar lumps of undissolved protein on the surfaces of total kafirin films.

In addition to opaqueness, the W Eth QPM zein films cast from aqueous ethanol were stiff, with a few cracks on the surface when compared to other zein prolamin films cast from the same solvent (Figure 5.3A - top row films). Using commercial zein, Kim et al. (2004) reported that cross-linked films were stiff, with rough surfaces compared to non-cross-linked films. The authors proposed that the stiffness of the films was a result of crosslinking. Since the W Eth QPM zein preparation had a high cysteine content compared to other zein preparations (Table 5.3), disulphide bond crosslinking between γ-zein residues of W Eth QPM zein may be the possible explanation why these films were stiff.

After casting, the W SA RM zein films cast from aqueous ethanol had gel-type aggregates on the surface (shown by arrows, Figure 5.3A, film C). Similar gel-aggregates were also

observed on the surfaces of W SA RM zein films cast from GAA (Figure 5.3B, film K). Formation of gel-type aggregates on the surfaces of W SA RM zein films was possibly contributed by the methionine content of this zein preparation, which was the highest (3.09 g/100 g protein) compared to other zein preparations (< 2.69 g/100 g protein) (Table 5.3). Methionine-rich proteins are unstable and prone to gelation (Pomes, 1971). In addition to the high methionine content, it could be possible that the casting solvent (aqueous ethanol or GAA) may have contributed because W SA RM zein films cast from aqueous ethanol had fewer gel aggregates compared to the W SA RM zein films cast from GAA (Figure 5.3 A & B, films C and K).

Commercial zein films cast from aqueous ethanol were yellow. These films were neither transparent nor opaque because the yellow film colour made it difficult to see through. Muhiwa (2016) and Njila (2017) have reported similar physical characteristics of commercial zein films. SEM showed that all the zein films cast from aqueous ethanol had smooth surfaces, with white specks or fragments on the surface of the films (marked SS or F, Figure 5.4A). The white fragments could be carbon deposited on the surfaces during carbon coating before viewing using SEM.

Similar to the aqueous ethanol treatment, the W Eth QPM zein films cast from GAA were visually more opaque and light brown compared to the W Eth RM zein, WSA RM zein and commercial zein films cast from the same solvent (GAA). The W Eth QPM zein films cast from GAA had bubbles within the film (indicated by yellow arrows - Figure 5.3B, film I). The bubbles were probably formed during film preparation because when the W Eth QPM zein preparation was dissolved in GAA, a considerable amount of foaming was observed. Foaming was probably an indication that the solution was very viscous. When the solution was poured into the silicone dish before oven drying, bubbles were still present in the solution, showing that the bubbles were still entrapped within the viscous solution. SEM, however showed that the W Eth QPM zein films had spherical protein particles on the surfaces, after casting (marked UP - Figure 5.4B). In contrast to W Eth QPM zein films which were visually opaque, the W Eth RM zein and commercial zein films cast from GAA were smooth and clear, with consistent surfaces (Figure 5.3B, top row film J & L). However, SEM revealed that the surfaces of these films had more spherical particles, compared to the ones that were observed on W Eth QPM zein films (marked UP - Figure 5.4B).
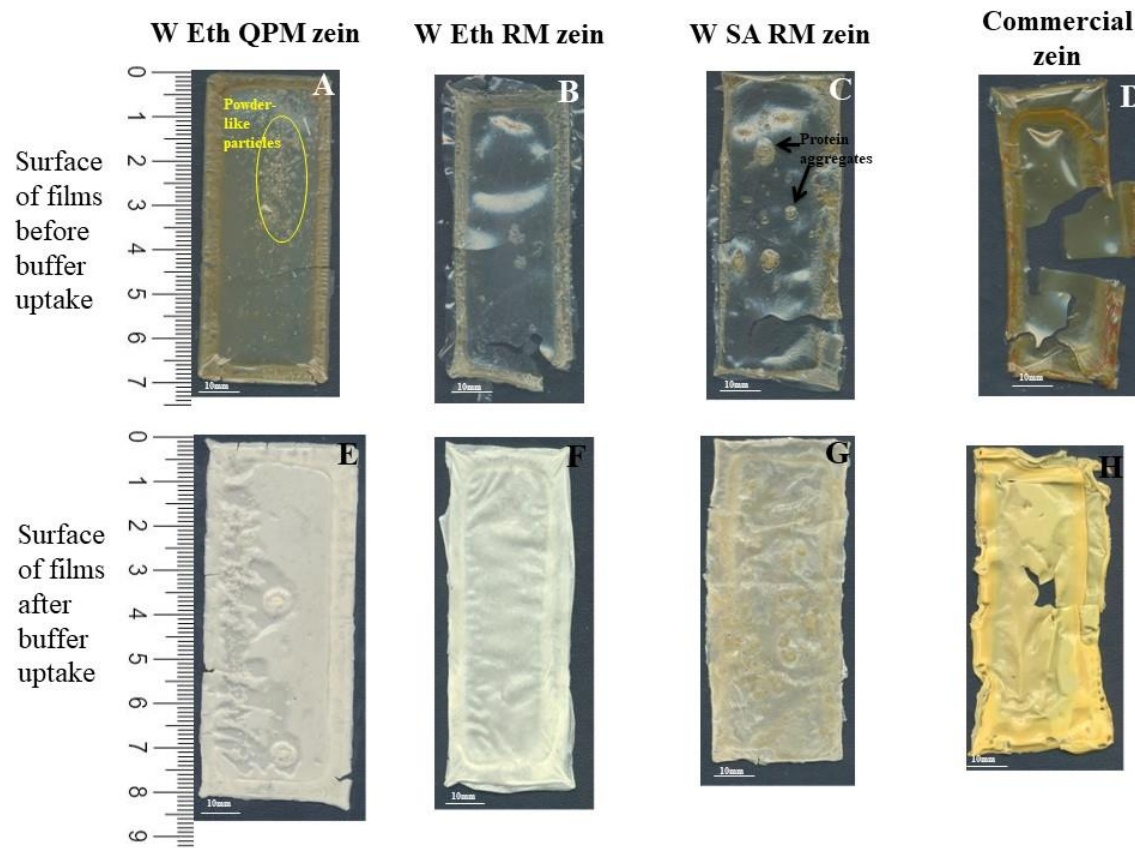
Figure 5.3A: Effect of zein preparations on the physical appearance, colour (top row films) and the buffer uptake of films (bottom row films) **cast from 70% (w/w) aqueous ethanol**. Column 1 = zein from white Ethiopian QPM; column 2 = total zein from white Ethiopian regular maize, column 3 = total zein from white South African regular maize, column 4 = commercial zein.

(**A – D**) appearance of zein films before buffer uptake (i.e. before immersing of films in 0.2 M sodium phosphate buffer (pH 6.8), with agitation for 12 hours. (**E – H**) appearance of zein films after buffer uptake.
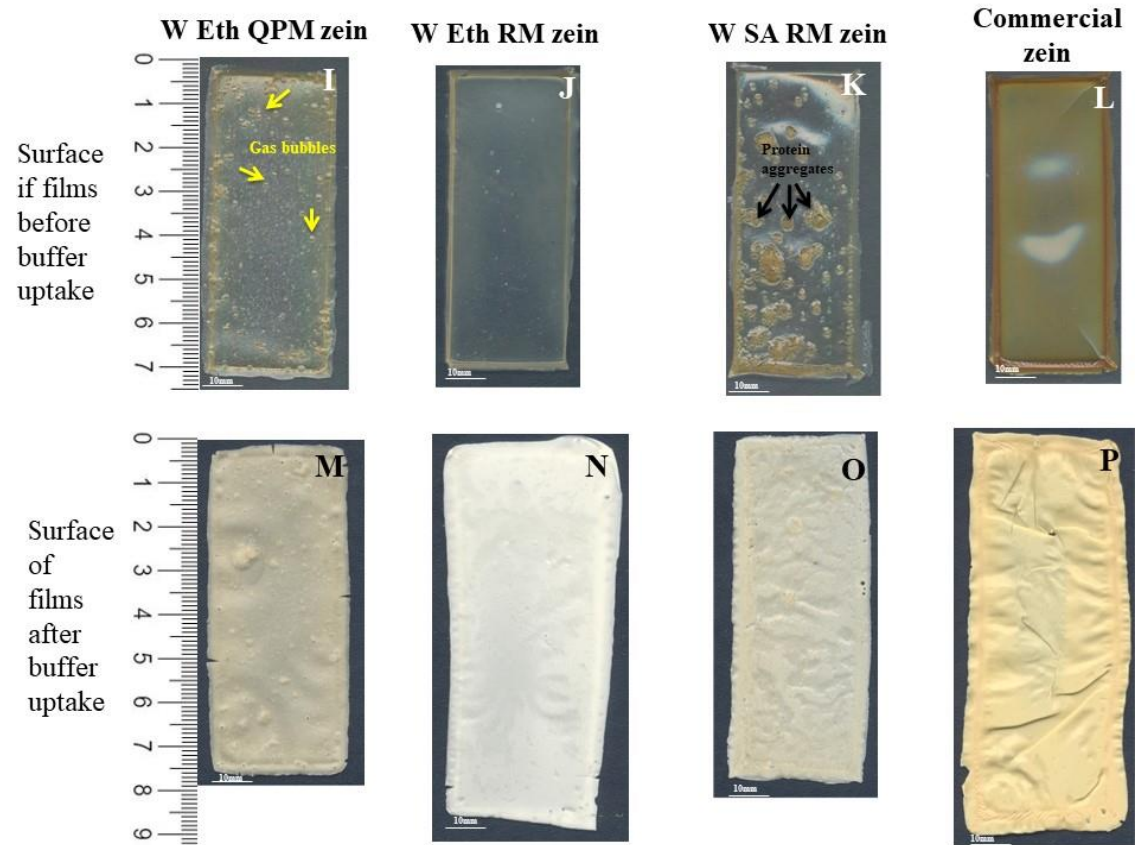
Figure 5.3B: Effect of zein preparations on the physical appearance, colour (top row films) and the buffer uptake (bottom row films) of **films cast from glacial acetic acid (GAA).** Column 1 = zein from white Ethiopian quality protein maize; column 2 = total zein from white Ethiopian regular maize, column 3 = total zein from white South African regular maize, column 4 = Commercial zein

(I – L) appearance of zein films before buffer uptake (i.e. before immersing of films in 0.2 M sodium phosphate buffer (pH 6.8), with agitation for 12 hours **(M – P)** appearance of zein films after buffer uptake.
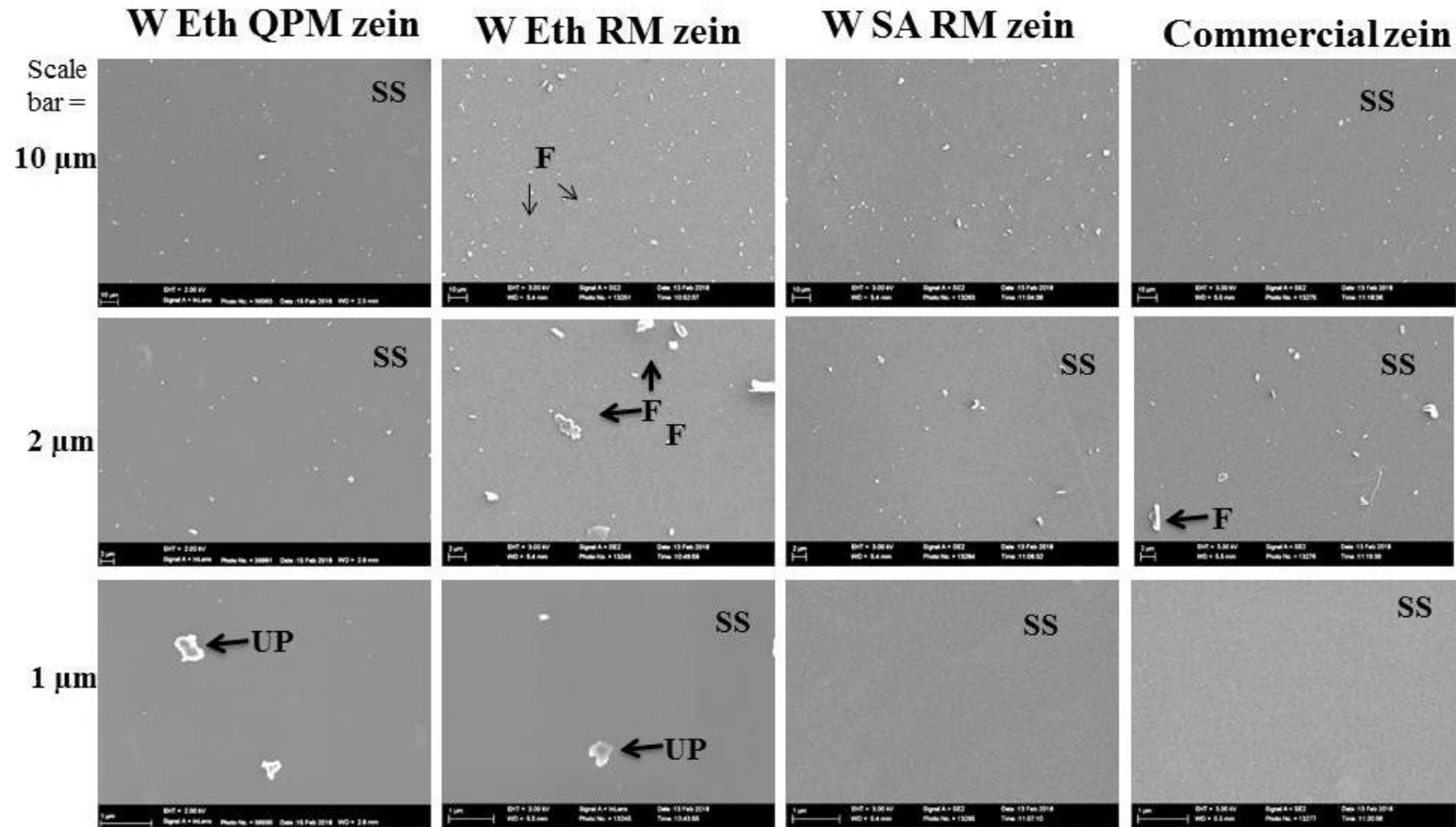
Figure 5.4A: SEM showing the surface morphology of different zein films (column 1 = zein from white QPM, column 2 = total zein from white Ethiopian regular maize, column 3 = total zein from white South African regular maize, column 4 = commercial zein) prepared using **70% w/w aqueous ethanol,** before immersing films in 0.2 M sodium phosphate buffer (pH 6.8). SS = smooth surface, F = fragments on film surface, UP = undissolved protein
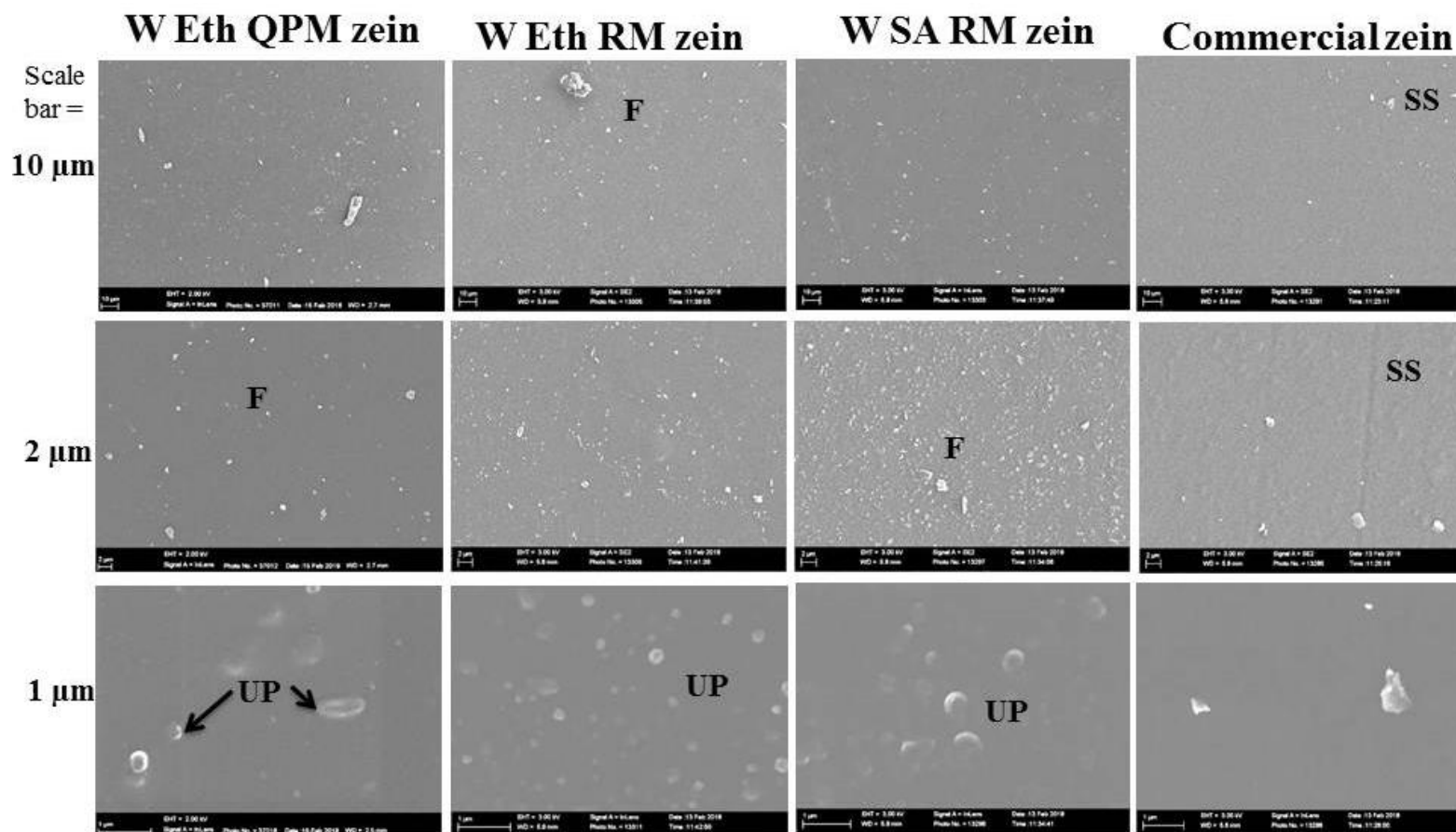
Figure 5.4B: SEM showing the surface morphology of different zein films (column 1 = zein from white QPM, column 2 = total zein from white Ethiopian regular maize, column 3 = total zein from white South African regular maize, column 4 = commercial zein) prepared using **glacial acetic acid,** before immersing films in 0.2 M sodium phosphate buffer (pH 6.8). SS = smooth surface, F = fragments on film surfaces, UP = undissolved protein

It seems the W Eth RM and W SA RM zein films had more spherical undissolved particles on their surfaces after casting compared to the surfaces of W Eth QPM zein and commercial zein films (marked UP, Figure 5.4B). Zein can aggregate to form micelle-like particles, and this is mainly driven by the amphiphilic nature of the prolamin (Kim et al., 2004; Kim and Xu, 2008). Kim et al. (2004) suggested that the degree of zein aggregation can be explained by the electrostatic interaction of molecules in a solvent. Li et al. (2012) showed that when commercial zein is dissolved in GAA, the α-zein peptides unfolds in the helical coil. However, no work has been published that shows the behaviour of β-zein or γ-zein peptides in GAA. Hence, it could be probable that the spherical particles observed on the surfaces of W Eth QPM zein, W Eth RM zein and W SA RM zein films were contributed by the presence of β-zein, unlike commercial zein which has predominant α-zein.

Zein molecular aggregation is likely to occur when the level of β-sheet structures is high (Mizutani et al., 2003). Beta-prolamins are rich in methionine content (Shukla and Cheryan, 2001). Table 5.3 showed that W Eth QPM zein had the lowest methionine content (0.84 g/ 100 g protein) compared to W Eth RM zein and W SA RM zein preparations (>1.50 g/100 g protein). Hence, it would be expected for W Eth QPM zein to form a few spherical protein particles on the film surfaces, as observed by SEM (Figure 5.4B), compared to the W Eth RM and W SA RM zein films. Alternatively, it could be possible that the GAA film casting temperature (30ºC) was relatively low compared to 70ºC that was used for casting films from aqueous ethanol. As a result, the low casting temperature may have contributed to the formation of spherical protein aggregates instead of protein unfolding.

As previously mentioned, the W Eth QPM zein films cast from aqueous ethanol were visually opaque, with lumps of undissolved protein within the film after casting (yellow circle, Figure 5.3A - top row films A). QPM zein may be comparable to total kafirin because both prolamins have high levels of γ-prolamin. Total kafirin has poor solubility in 70% (w/w) aqueous ethanol because it contains more of the hydrophobic γ-prolamin compared to total zein from regular maize (Duodu et al., 2003; Taylor et al., 2005). Anyango et al. (2011) reported that opaque films were formed when kafirin microparticles were pre-heated at high temperatures (75ºC and 100ºC) before casting into films. The authors suggested that the opaqueness of these films was due to reduced solubility of the heat-treated kafirin microparticles caused by disulphide crosslinking. Pre-heat treatments were not done in this

study. Therefore, the visual opaqueness of W Eth QPM zein films cast from aqueous ethanol is attributable to the poor solubility of this prolamin preparation in the casting solvent.

When zein films cast from GAA were stored at ambient temperature (25ºC) for 3 days, the W Eth QPM zein films became flexible, as similar in flexibility to polyvinyl chloride, whereas total zein and commercial zein films cast from GAA remained brittle. In fact, the W Eth QPM zein films could form an arc without breaking (Figure 5.5, A1 & A2).
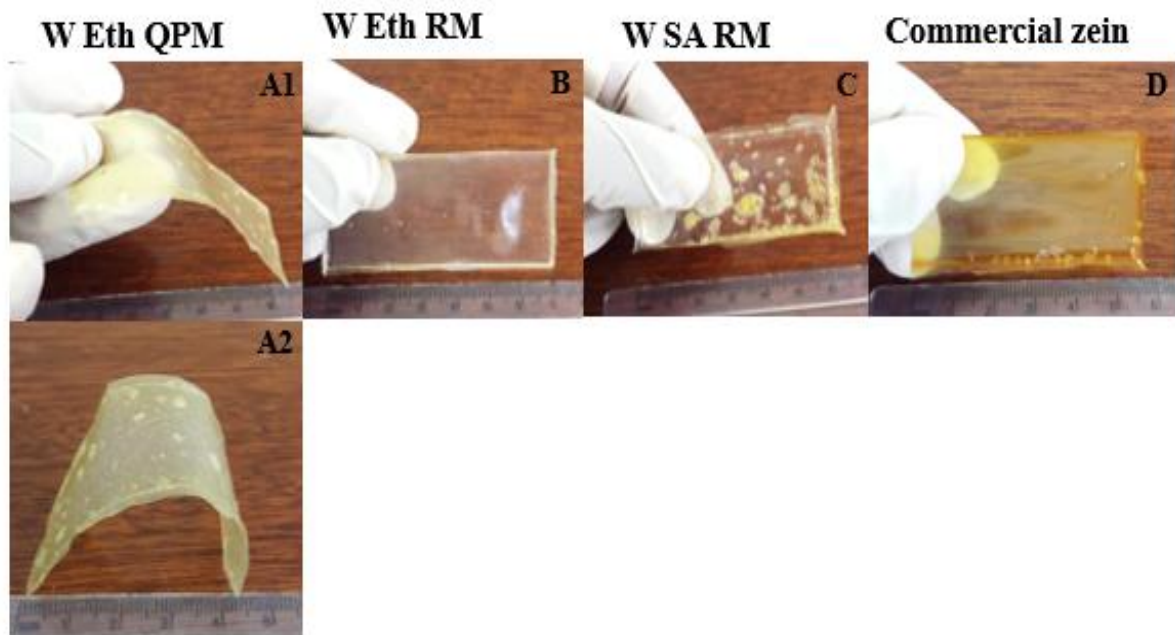
Figure 5.5: Effect of storing zein films cast from glacial acetic acid at 25ºC on the appearance and film flexibility. A1 & A2 = W Eth QPM zein film, B = W Eth RM zein film, C = W SA RM zein film, D = commercial zein film.

The W Eth QPM zein film is a novel finding because it is the first time a flexible prolamin film has been developed, without the inclusion of plasticisers. During storage, all zein films were exposed to the same conditions. The fact that only W Eth QPM zein films cast from GAA had formed a flexible film suggests that the W Eth QPM zein composition (subclasses present) had an effect on film flexibility.

As previously mentioned, the W Eth QPM zein preparation had high levels of γ-zein compared to other zein preparations (Table 5.3). Gamma-zein has a helical structure (Bicudo et al., 2018). Although not measured, it is possible to assume that the α-helical content of the W Eth QPM zein increased after dissolving the prolamin in GAA, similar to total kafirin (Elhassan et al., 2018). During film formation, the W Eth QPM zein α-helices probably aligned along the linear axis in an ordered manner. Since the relative humidity of the environment where the zein films were stored was high (approx. 100%), water molecules could have plasticised the W Eth QPM zein films by intercalating between the α-helices of the zein polymers. This enhanced mobility of polymers, consequently resulting in film flexibility. Unlike W Eth QPM zein which has high levels of γ-zein, the cysteine contents of W Eth RM zein, W SA RM zein and commercial zein preparations were low (Table 5.4). It is probable that the latter three zein preparations had a mixture of α-helical and β-sheet structures (King et al., 2016). Thus, it can be assumed that the α-helical contents of these three zein preparations were, however, not sufficient to enhance polymer mobility to produce flexible films. This probably explains why W Eth RM zein, W SA RM zein and commercial zein films remained brittle after storage. Since it was shown that W Eth QPM zein films could be cast from aqueous ethanol and GAA, the buffer uptake of all zein films cast from both aqueous ethanol and GAA was studied.

5.4.2.1.2 Effect of zein film type and casting solvent on the uptake of buffer solution

Zein films cast from either aqueous ethanol or GAA were immersed in sodium phosphate buffer (pH 6.8) for 12 h, with agitation. All zein films cast from aqueous ethanol absorbed similar ($p \geq 0.05$) amounts of buffer (Table 5.4). This result was not expected as it implies that the prolamin preparation type did not affect the amount of buffer that was absorbed by zein films. In contrast, the buffer uptake of the different zein films cast from GAA differed significantly ($p \leq 0.05$) from each other. The order of buffer uptake for films cast from GAA (least to most) was W Eth QPM zein < W SA RM zein < commercial zein < W Eth RM zein films. The buffer uptake values of the zein films cast from GAA ranged from 16 to 207% (Table 5.4). The fact that W Eth QPM zein films cast from GAA had absorbed the least amount of buffer was expected. This can be attributed to the high cysteine content of QPM zein (Table 5.3).

Table 5.4: Effects of casting solvent (70% w/w aqueous ethanol or glacial acetic acid) and zein preparation type (QPM zein, total zein and commercial zein) on the absorption of buffer by zein films.

Solvent used to cast zein films	Zein preparation type	Before buffer uptake			After buffer uptake			Increase in area of film after immersing films in buffer (%)	Buffer absorbed by zein films (%)
		Weight (mg)	Area (cm^2)	Surface density (mg/cm^2)	Weight (mg)	Area (cm^2)	Surface density (mg/cm^2)		
70% w/w aqueous ethanol	W Eth QPM zein	517.6^c ± 21.6	21.1^b ± 1.1	24.6^{ab} ± 0.8	732.9^{bc} ± 19.7	27.3^c ± 0.8	27.6^a ± 1.3	29.8^{bc} ± 5.9	41.7^{ab} ± 2.6
	W Eth RM zein	402.6^a ± 51.9	17.5^a ± 8.8	22.6^{ab} ± 1.6	646.9^{ab} ± 10.6	22.9^{ab} ± 1.6	28.0^a ± 1.3	36.7^c ± 17.9	62.8^b ± 25.0
	W SA RM zein	424.4^{ab} ± 13.7	18.5^a ± 0.3	23.0^{ab} ± 1.1	612.3^a ± 36.1	21.1^a ± 0.5	28.7^a ± 1.6	15.6^{ab} ± 4.0	44.2^{ab} ± 3.8
	Commercial zein	454.8^{ab} ± 14.8	17.9^a ± 0.5	25.5^b ± 1.4	781.7^c ± 33.3	21.2^a ± 0.5	36.0^b ± 2.5	17.3^{ab} ± 5.4	62.9^b ± 13.2
Glacial acetic acid (GAA)	W Eth QPM zein	584.0^d ± 3.0	22.0^b ±1.1	26.5^b ± 1.4	679.0^{ab} ± 8.0	24.5^{ab} ± 0.0	27.6^a ± 0.0	11.2^a ± 5.2	16.2^a ± 1.9
	W Eth RM zein	490.0^b ± 5.0	18.0^a ± 0.2	26.6^b ± 0.4	1503.0^e ± 83.0	45.2^d ± 1.0	34.3^b ± 2.0	144.5^e ± 7.5	207.0^d ± 13.8
	W SA RM zein	402.0^a ± 25.0	21.0^b ± 1.4	18.8^a ± 0.7	603.8^a± 23.1	24.6^{ab}± 1.3	25.1^a± 2.0	18.0^{ab}± 10.8	50.5^b±3.8
	Commercial zein	488.1^b ± 0.6	18.8^a ± 0.2	26.0^b ± 0.0	1089^d ± 12.0	37.7^c ± 3.0	29.0^a ± 3.0	101.6^d ± 17.6	123.0^c ± 2.6

Mean ± standard deviation of two analyses. Mean values in the same column with different superscript letters are significantly different at $p \le 0.05$.
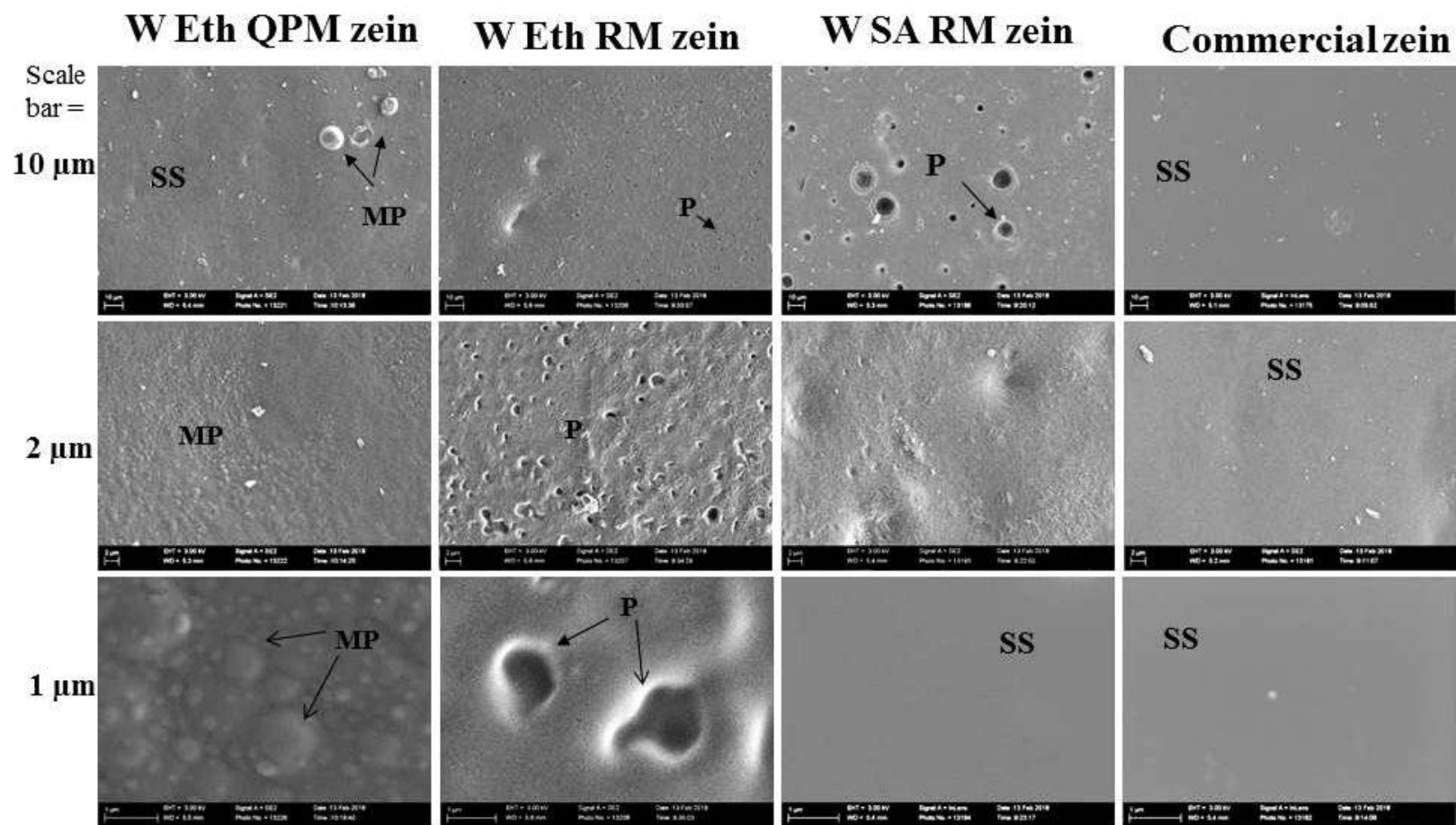
Figure 5.6A: SEM showing the surface morphology of different zein films (column 1 = zein from white QPM, column 2 = total zein from white l Ethiopian regular maize, column 3 = total zein from white South African regular maize, column 4 = commercial zein) prepared using **70% w/w aqueous ethanol,** after immersing films in 0.2 M sodium phosphate buffer (pH 6.8). SS = smooth surface, MP = microparticles, P = pores /holes on film surface
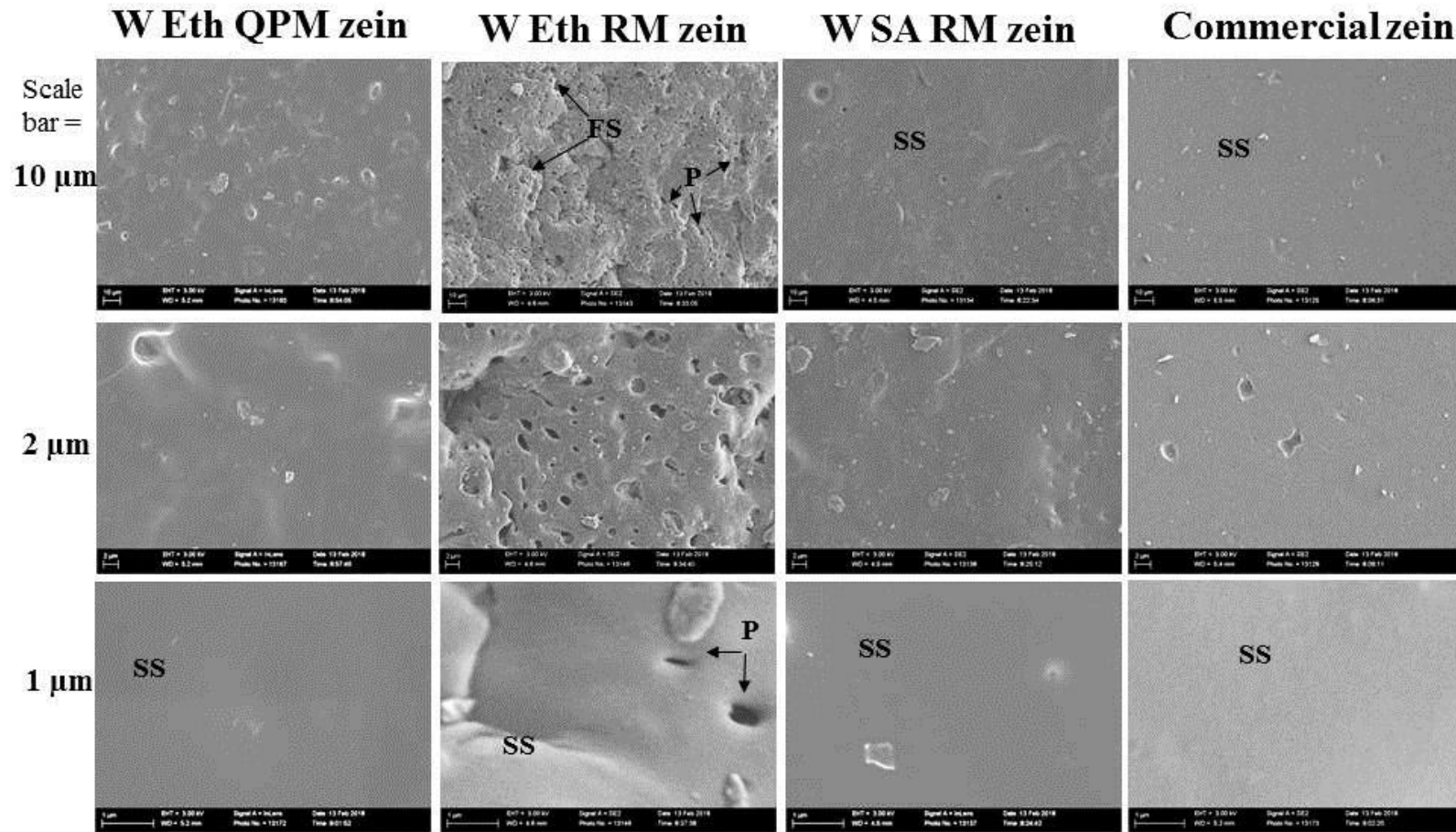
Figure 5.6B: SEM showing the surface morphology of different zein films (column 1 = zein from white QPM, column 2 = total zein from white Ethiopian regular maize, column 3 = total zein from white South African regular maize, column 4 = commercial zein) prepared using **glacial acetic acid**, after immersing films in 0.2 M sodium phosphate buffer (pH 6.8). SS = smooth surface, FS = folded surface, P = pores / holes on film surfaces, UP = undissolved protein

SEM revealed that the W Eth QPM zein films cast from aqueous ethanol, after buffer uptake, had spherical microparticles on their surfaces (Figure 5.6 A - marked MP) whereas the W Eth RM zein, W SA RM zein and commercial zein films cast from the same solvent had smooth surfaces. Formation of microparticles is dependent on the solubility of the protein in the solvent (Taylor et al., 2013). In aqueous phases, the solubility of proteins is reduced. Hence the protein precipitates out of solution, and this consequently results in the formation of microparticles. It is likely that the microparticles observed on the surface of W Eth QPM zein films in the current study were formed when the films came in contact with the buffer during immersion. This is because SEM of W Eth QPM zein films cast from aqueous ethanol (before buffer uptake) did not show evidence of microparticles (Figure 5.4A). Similar microparticles have been observed on the surfaces of kafirin films (Taylor et al., 2009). In the current study, the formation of microparticles in W Eth QPM zein films was possibly contributed by the partial solubility of the zein in aqueous ethanol, as previously mentioned.

The difference in buffer uptake between the zein films cast from aqueous ethanol and GAA can be attributed to the effect of casting solvent on the secondary structure of zein. When dissolved in GAA, zein polypeptides unfold in the coil region (Li et al., 2012). Protein unfolding increases the surface area for zein polypeptides to interact through hydrogen bonding, ionic bonding and possibly Van der Waals forces. Hence during buffer uptake, zein films cast from GAA possibly had more sites available where the buffer can form bonds with the polypeptides, consequently resulting in more buffer uptake. On the other hand, zein polypeptides only unfold slightly when the prolamin is dissolved in aqueous ethanol (Li et al., 2012). This limits the interaction of polypeptides with the buffer during immersion. Hence, it is proposed that the degree of zein unfolding influences the buffer uptake of films.

The W Eth QPM zein films cast from GAA absorbed the least amount of buffer, compared to the other zein films cast from GAA and the other prolamin films cast from aqueous ethanol. This result can be attributed to the high levels of cysteine-rich γ-zein, which can form disulphide crosslinkages (Duodu et al., 2003). When dissolved in GAA, it is likely that the γ-zein polypeptides of QPM unfolded and this enhanced the formation of more disulphide crosslinkages between the polypeptides chains. Crosslinking presumably brought the zein polypeptides together, consequently resulting in a compact protein network that would prevent the movement of liquids, while strengthening the film (Anyango et al., 2011).

5.4.2.2 Preparation of viscoelastic materials

5.4.2.2.1 Effects of water and acetic acid treatments on the functional properties of different zein preparations

Stereomicroscope showed that the W Eth QPM zein did not form viscoelastic materials with the distilled water and acetic acid treatments (Figure 5.7a-c). However, SEM showed that W Eth QPM zein had formed protein aggregates and spherical microparticles (approx. 10 μm diameter) with distilled water and the 5.4% acetic acid treatments (Figure 5.8A, marked PA or MP). When the concentration of acetic acid was increased to 33%, it seems the W Eth QPM zein aggregates fused to form a continuous layer with smooth surfaces (Figure 5.8Al, marked SS). Thus, it is likely that the solubility of W Eth QPM zein had increased as the concentration of acetic acid increased.

The W Eth RM zein preparation formed a protein aggregate that was not viscoelastic with the distilled water treatment (Figure 5.7d). Therefore, it is possible that the W Eth RM zein was simply hydrated in water. When the prolamin (W Eth RM zein) was prepared with 5.4% and 33% acetic acid, solutions were formed (Figure 5.7e-f). SEM, however, showed that the W Eth RM zein had formed distinct spherical microparticles (≈ 20 μm diameter) with the water treatment (Figure 5.8B). Upon addition of 5.4% and 33% acetic acids, the W Eth RM zein microparticles became smaller, and they fused to form a continuous uneven surface (Figure 5.8Ba-l). This observation possibly indicates that the solubility of the W Eth RM zein had increased as the concentration of acetic acid increased, similar to the W Eth QPM zein preparation (Figure 5.8A).

Stereomicroscopy showed that the W SA RM zein preparation formed solutions when prepared with the distilled water. Upon addition of 5.4% and 33% acetic acids, the W SA RM zein preparation became more viscous (Figure 5.7g-i). SEM showed that the W SA RM zein had formed distinct microparticles (≈ 20 μm diameter) with the water treatment. These microparticles fused into a continuous layer upon addition of acetic acid (Figure 5.8C), similar to the W Eth QPM zein and W Eth RM zein microparticles (Figure 5.8A-B).

Of all the zein preparations examined in the current study, only commercial zein formed viscoelastic materials when prepared with distilled water and 5.4% acetic acid treatments at 50°C, above the glass transition temperature (T_g) of hydrated zein (Figure 5.7j-k). The viscoelastic materials could be manually stretched by hand, similar to Sly et al. (2014) and

King et al. (2016). With the 33% acetic acid treatment, a solution was formed, probably indicating that the commercial zein preparation had dissolved fully in the solvent. SEM showed that the commercial zein viscoelastic mass prepared in distilled water had rough surfaces, whereas that formed in 5.4% acid treatment had fibrils (Figure 5.8D). This result was expected, as it showed that acetic acid improves the extensibility of zein viscoelastic materials, similar to what Sly et al. (2014) reported.

None of the total zein preparations used in the current study (W Eth QPM zein, W Eth RM zein and W SA RM zein) formed viscoelastic materials when prepared with either distilled water, 5.4% or 33% acetic acid. Since total zein preparations contain all zein subclasses (α-, β-, δ- and γ-), it was suggested that the presence of β-, δ- and γ-subclasses interfered with the formation of viscoelastic materials, unlike commercial zein which predominantly has α-zein subclass (Lawton, 1992). Also, it may be suggested that intermolecular hydrogen bonds, Van der Waals forces or covalent interactions were formed between the zein polypeptides and this disrupted protein aggregation between α-zein polypeptides.
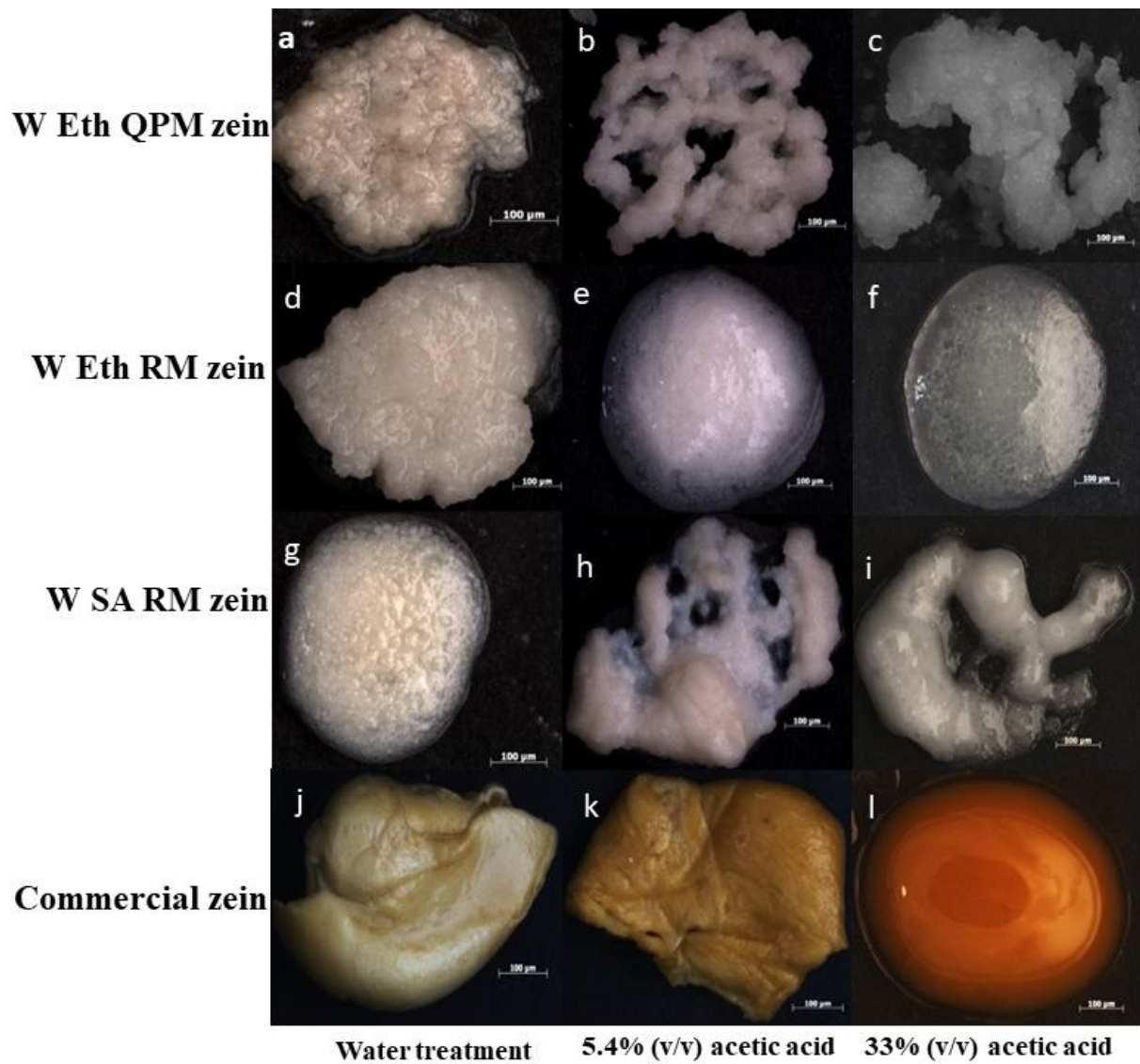
Figure 5.7: Stereomicroscopy showing the effect of mixing W Eth QPM zein, W Eth RM zein, W SA RM zein and commercial zein preparations with distilled water (column 1), 5.4% (v/v) acetic acid (column 2) and 33% (v/v) acetic acid (column 3) at 50°C.
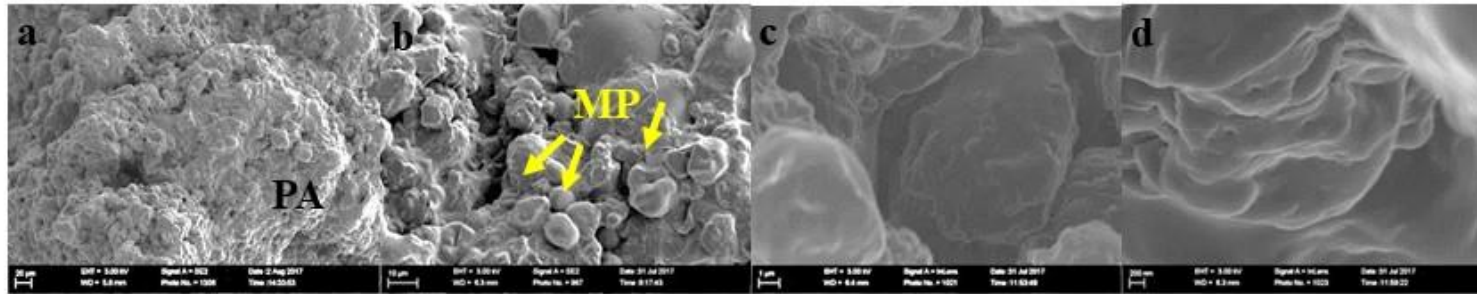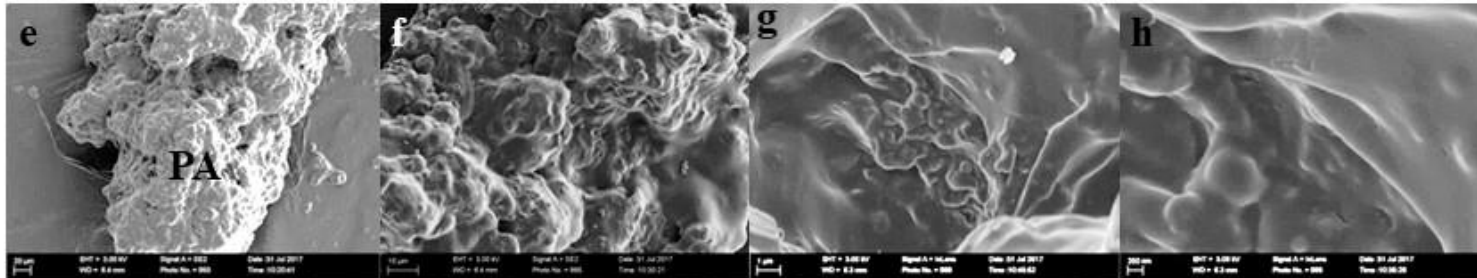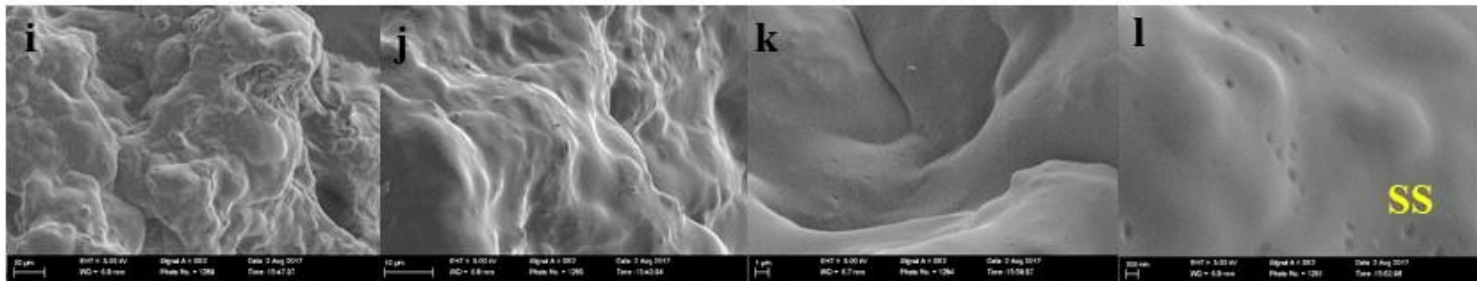
Figure 5.8A: Low to high resolution scanning electron microscopy (scale bars 20 µm, 10 µm, 1 µm and 200 nm) showing the effect of water, 5.4% and 33% (v/v) acetic acid treatments on the structure of W Eth QPM zein (total zein extracted from white Ethiopian QPM). PA = protein aggregation, MP = microparticles, SS = smooth surface.
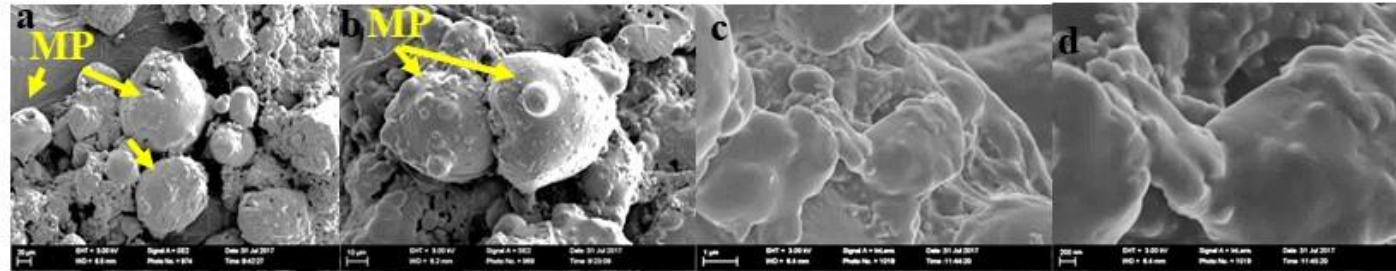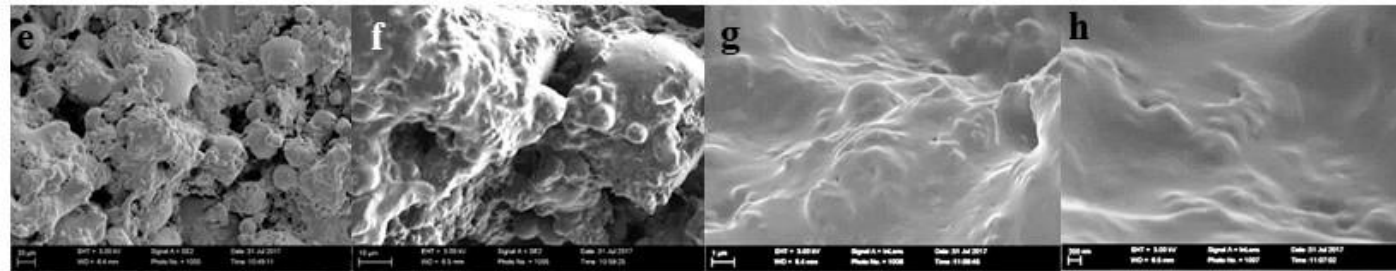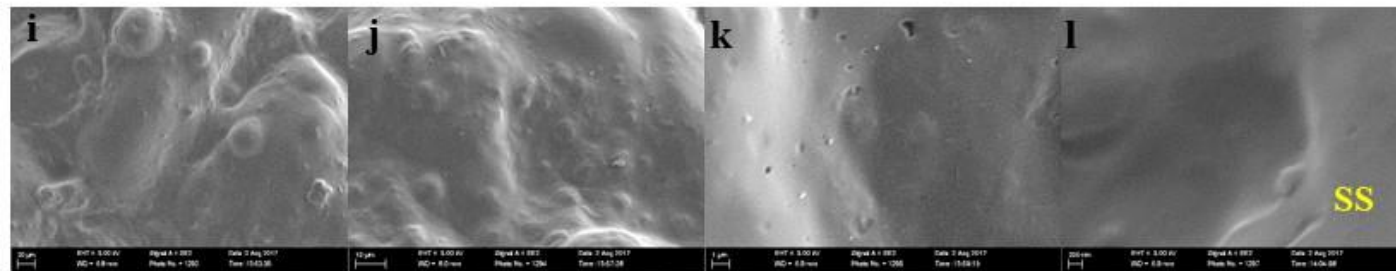
Figure 5.8B: Low to high resolution scanning electron microscopy (scale bars 20 μm, 10 μm, 1 μm and 200 nm) showing the effect of water, 5.4% and 33% (v/v) acetic acid treatments on the morphology of W Eth RM zein (total zein extracted from white Ethiopia regular maize). MP = microparticles, SS = smooth surface.
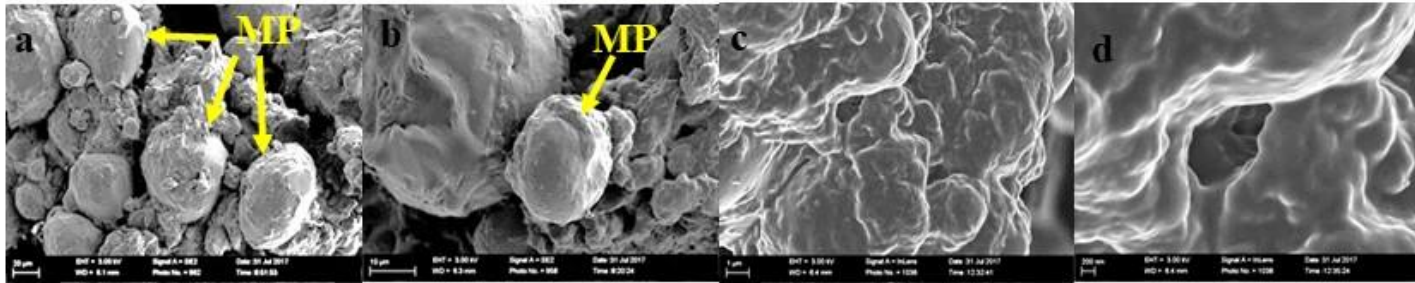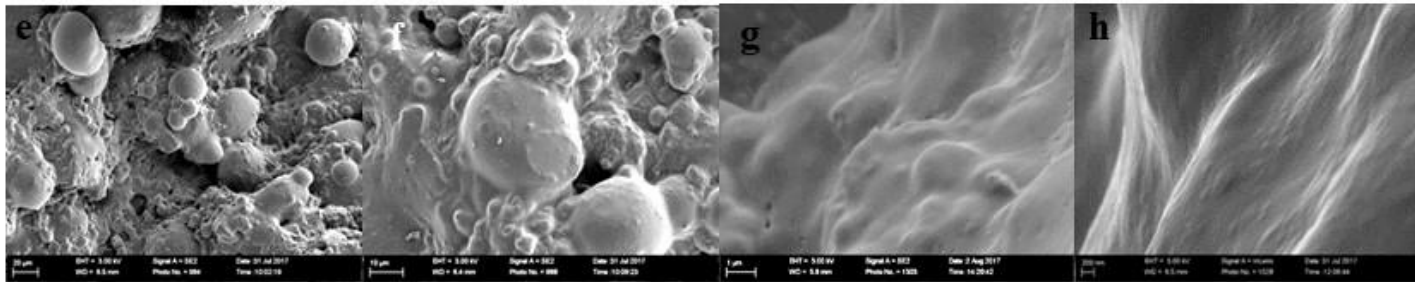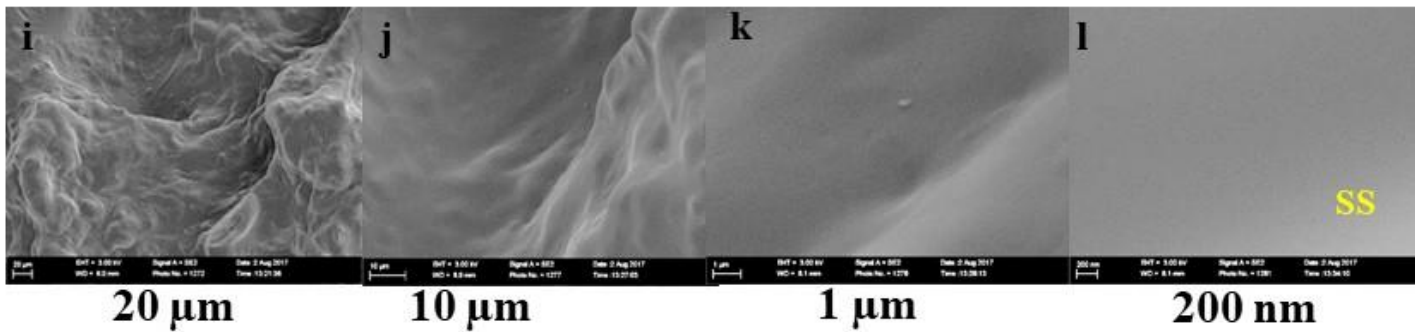
Figure 5.8C: Low to high resolution scanning electron microscopy (scale bars 20 μm, 10 μm, 1 μm and 200 nm) showing the effect of water, 5.4% and 33% (v/v) acetic acid treatments on the morphology of W SA RM zein (total zein extracted from white South African regular maize). MP = microparticles, SS = smooth surface.

Commercial zein
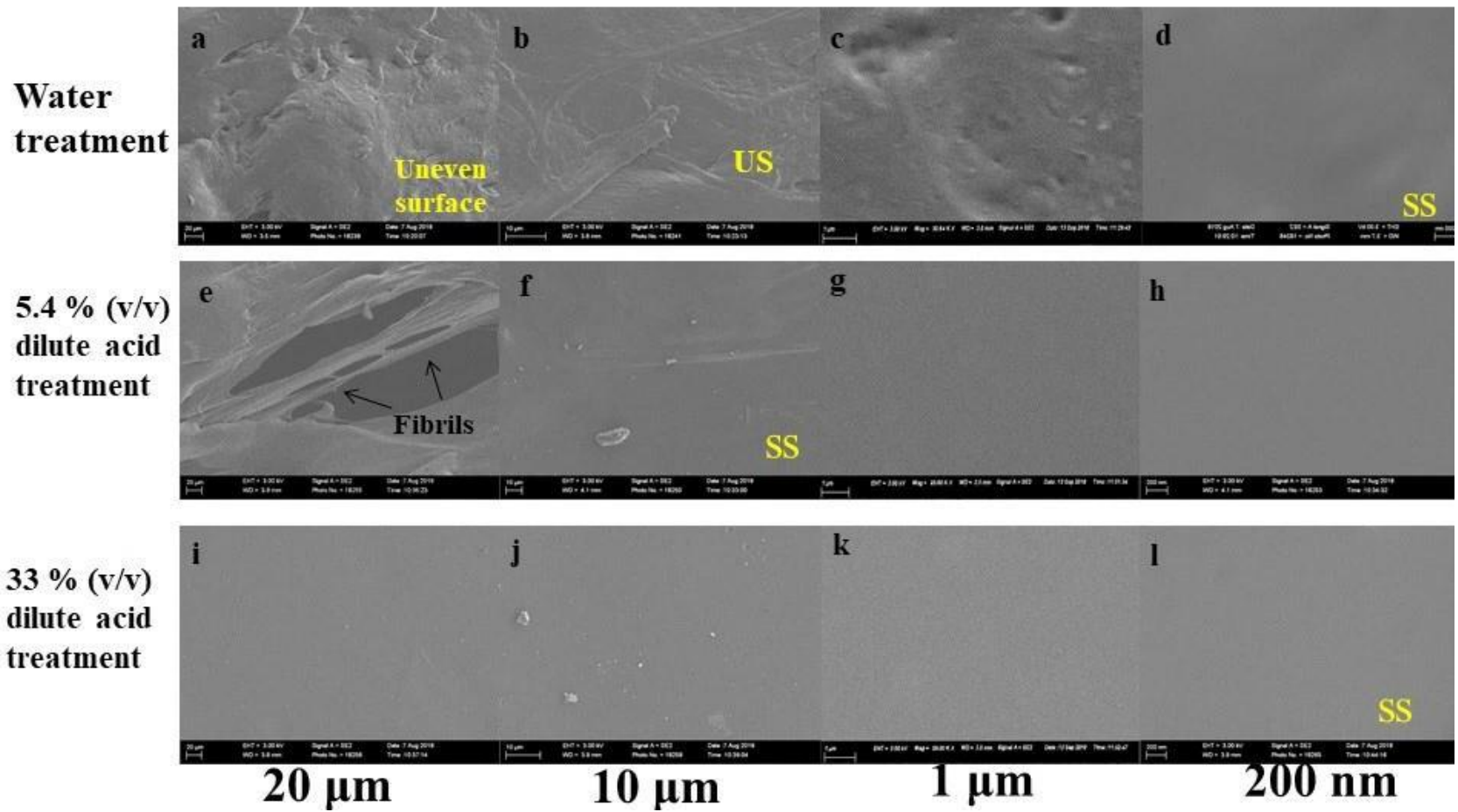
Figure 5.8D: Low to high resolution scanning electron microscopy (scale bars 20 μm, 10 μm, 1 μm and 200 nm) showing the effect of water, 5.4% and 33% (v/v) acetic acid treatments on the morphology of commercial zein. US = uneven surface, SS = smooth surface

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Since SEM had showed that all total zein preparations (W Eth QPM, W Eth RM and W SA RM) had formed spherical microparticles with water treatment and 5.4% acetic acid treatments (Figure 5.8A-C), it was thought that the zein preparations may have contained starch granules. All dry zein preparations were then tested for starch using the iodine test and the results were negative for all preparations. Hence, the formation of microparticles was probably due to two factors. Firstly, the concentration of protein in the solvent (distilled water or acetic acids) was high (approx. 33%), and this may have enhanced protein aggregation, consequently resulting in the formation of microparticles. Secondly, to ensure complete solubilisation, a spatula was used to mix the zein preparations after solvent addition. Thus, it is possible that high shear was applied during mixing and this consequently resulted in protein aggregation and the formation of protein microparticles instead of forming fibrils.

Taylor et al. (2018) proposed that T_g, β-sheet conformation, subclass composition and hydrophobicity are factors that influence the formation of viscoelastic materials from prolamin preparations. Lawton (1992) reported that hydrated commercial zein (predominantly α-zein) could form viscoelastic materials when heated above its T_g (25°C). In the present study, it is unlikely that T_g was the major determinant as to why viscoelastic materials were not formed from total zein preparations (W Eth QPM zein, W Eth RM zein and W SA RM zein), irrespective of treatment because all zein preparations were mixed with solvents at 50°C, above the T_g of hydrated zein (Lawton, 1992).

The findings obtained from the current study are similar to those of King et al. (2016), who could not form viscoelastic materials using total zein with distilled water under the same conditions as used in the present study. The authors, however, found that in distilled water (50°C), total zein preparations have a higher β-sheet content compared to when zein preparations are dry. Even though not investigated, it can be speculated that β-sheet content of the W Eth QPM zein, W Eth RM zein and W SA RM zein preparations used in the current study increased after dissolving the preparations in distilled water. A high β-sheet content is an indication of zein aggregation (Mizutani et al., 2003). In addition, Rochet and Lansburg (2000) suggested that the aggregation of proteins could take place when they undergo conformational changes that result in the unfolding of polypeptides, which subsequently increases the surface area for intermolecular interactions between polypeptides. Assuming that the β-sheet content of the total zein preparations had increased after dissolving in distilled water (as reported by King et al., 2016), it is possible that the zein polypeptides self-

assembled to form microparticles instead of fibrils that could be worked into a viscoelastic mass. King et al. (2016) suggested that β-sheet conformation was not directly responsible for the formation of viscoelastic materials. Hence, it is unlikely that β-sheet structures would contribute to the formation of viscoelastic materials.

In addition, Schober et al. (2011) and King et al. (2016) suggested that the γ-prolamin subclass is undesirable for viscoelastic mass formation because disulphide bond crosslinkages interfere with zein aggregation and formation of a protein network. Since all total zein preparations (W Eth QPM zein, W Eth RM zein and W SA RM zein) contained γ-zein in various amounts (Table 5.3), disulphide bond crosslinkages may have hindered the formation of viscoelastic materials. Therefore, it may be assumed that the zein composition, in terms of subclasses present, had a significant influence on the functionality of the prolamin during formation of viscoelastic materials. Apart from zein composition, the concentration of zein in the solvent could be another possible reason why viscoelastic materials were not formed. Oguntoyinbo et al. (2018) showed that a minimum of 5-10% protein in GAA, before the addition of water, is required to form a viscoelastic mass from total zein using the coacervation process. In the current study, the final concentration of protein in solution after mixing zein preparations with either distilled water or acetic acid was approx. 33%, nearly three times higher. Hence, it is likely that the high protein concentration also favoured protein aggregation, instead of fibrils.

Apart from dissolving protein in a solvent (distilled water, acetic acid) above the T_g of zein, an alternative way of making viscoelastic materials using the simple coacervation process of Taylor et al. (2018) was investigated.

5.4.2.2.2 Effect of glacial acetic acid on the morphology of zein preparations during the formation of viscoelastic materials using simple coacervation process.

Simple coacervation process can be used to make viscoelastic materials using total zein (Taylor et al., 2018) and total kafirin (Elhassan et al., 2018). During simple coacervation, the prolamin is first dissolved in GAA. Then water is added to precipitate the prolamin out of solution. Since both total kafirin and W Eth QPM zein have abundant γ-prolamin, it would be expected for W Eth QPM zein to form viscoelastic materials using the coacervation process. In the current study, zein preparations were first dissolved in GAA to give a concentration of

33% (w/v) protein. Of all the zein preparations, the W Eth QPM zein showed interesting properties because a viscous solution was formed (Figure 5.9a), whereas the other zein preparations (W Eth RM, W SA RM and commercial zein) formed runny liquids (Figure 5.9 b-d).
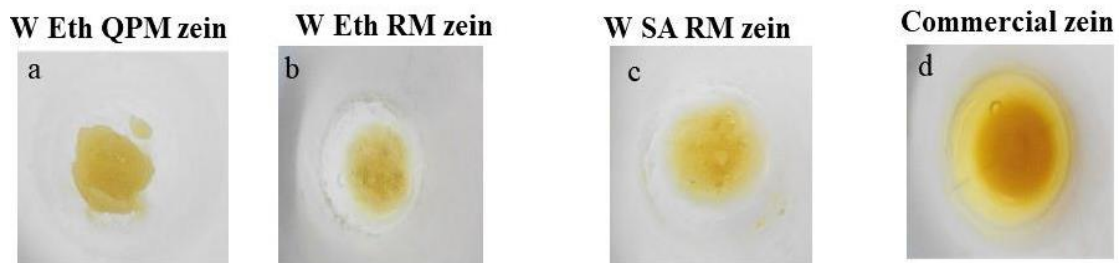
Figure 5.9: Images showing the solubility of zein preparations in GAA during coacervation process.

In an attempt to dilute the W Eth QPM zein viscous solution into a runny liquid, more GAA was then added to the prolamin to give a protein concentration of 11% (w/v), followed by heating to 50°C and continuous vortexing. The same amount of GAA was also added to the other zein preparations. The zein solutions were stored overnight (12 h) in the dark at 25°C and water was added the following day. Irrespective of the zein prolamin composition, all zein preparations, including W Eth QPM zein, formed colloids consisting of protein aggregates dispersed in solution after addition of water (the coacervation process). Light microscopy confirmed that the W Eth QPM zein and W Eth RM zein colloids had zein suspensions (marked PA - Figure 5.10), compared to the W SA RM zein and commercial zein colloids. Interestingly, SEM showed that the W Eth QPM zein colloids were composed of large irregular sphere-shaped protein aggregates (yellow arrows marked SP - Figure 5.10), whereas those of W Eth RM zein, W SA RM zein and commercial zein preparations had smaller spherical particles that fused into a continuous uneven film surface (second to fourth row images - Figure 5.10). The sphere-shaped particles were presumably microparticles that were formed when the W Eth QPM zein was mixed with GAA. The microparticles obtained in this current study (top row marked MP - Figure 5.10) were similar to total kafirin microparticles formed by Taylor et al. (2009) who used the coacervation technique and GAA as a solvent.
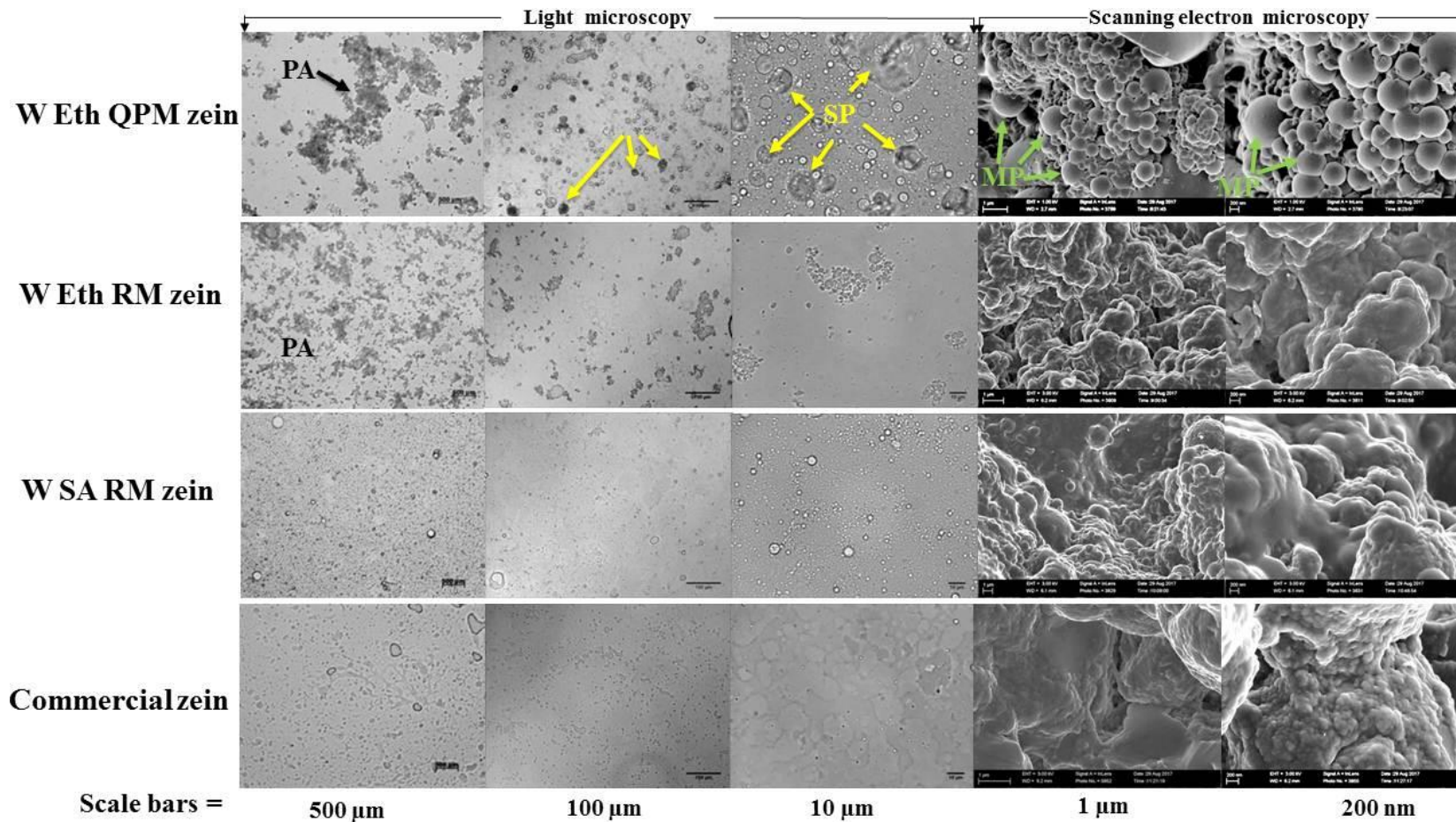
Figure 5.10: Light microscopy and scanning electron microscopy showing the effect of simple coacervation on the morphology of W Eth QPM zein, W Eth RM zein, W SA RM zein and commercial zein preparations during attempted formation of viscoelastic materials SP = spherical particles, MP = microparticles.

As previously stated, the W Eth QPM zein had formed a more viscous solution upon addition of GAA compared to other zein preparations, and this can be attributed to the formation of disulphide bonds involving the cysteine-rich γ-zeins. Prolamin proteins with high cysteine content are more likely to polymerise through disulphide bonding (El Nour et al., 1998). Also, when the degree of polymerisation is high, prolamin proteins become less functional during formation of viscoelastic materials (Taylor et al., 2018). In the current study, the final concentration of protein in GAA before addition of water (coacervation) (approx. 11%) is similar to the minimum concentration of protein in GAA (5-10%) that is required to enable fibril formation using coacervation process (Oguntoyinbo et al., 2018). Therefore, it would have been expected for fibrils to be formed. Instead, as previously described, all zein preparation, including W Eth QPM zein, had formed colloids and this was unexpected. Despite the correct protein concentration, it is likely that high shear applied when mixing zein in GAA with a spatula, after adding more GAA, could have disrupted fibril formation resulting in formation of microparticles.

5.4.2.3 Firmness of zein and kafirin preparations in glacial acetic acid

5.4.2.3.1 Effects of glacial acetic acid and storage time on the functional properties of different zein and kafirin preparations

The firmness of all zein preparations was investigated because the W Eth QPM zein preparation had formed a viscous solution upon addition of GAA, during the coacervation process (see section 5.4.2.2.2). Three different kafirin preparations (total kafirin, kafirin without β-kafirin and kafirin without γ-kafirin) were included as controls because it was thought that various levels of β- and γ-kafirin subclasses would influence disulphide bonding, which may contribute towards the firmness of prolamin solutions.

The zein and kafirin preparations were dissolved in glacial acetic acid (GAA), under non-reducing (i.e. without the addition of 5% 2-mercaptoethanol (BME)) and under reducing conditions (with the addition of 5% BME to GAA) until the final concentration of protein in solvent was 33% (w/v). Of all the zein and kafirin preparations that were investigated under non-reducing conditions, only total kafirin formed a gel. The remaining zein and kafirin preparations formed either opaque colloids (W Eth QPM zein, W Eth RM zein, W SA RM zein, kafirin without γ- kafirin) or clear runny liquids (commercial zein and kafirin without β-kafirin).

The first ever study on kafirin was conducted by Johns and Brewster (1917) who discovered the protein. They observed that it gelled more readily in hot ethanol when compared to zein. Using zein, Nonthanum et al. (2012) and Nonthanum et al. (2013) reported that zein preparations containing high amounts of γ-zein displayed a measurable gelation and that they required less time to form gels, when compared to zein comprising predominantly α-zein (commercial zein). Based on the findings of Nonthanum et al. (2012) and Nonthanum et al. (2013), it is proposed that γ-prolamin plays a key role in zein and kafirin gelation. Apart from the work done by Johns and Brewster (1917), kafirin gelation has not been studied systematically. Also, no work has been reported on the gelling properties of QPM zein.

Since kafirin contains more γ-prolamin compared to zein (Duodu et al., 2003), it is likely that formation of a total kafirin gel is attributable to disulphide bonding. It was assumed that the gelation of total kafirin took place when the firmness of the material increased sharply from 2.02 N to 9.61 N after 96 h storage (Figure 5.11A). The considerable increase in firmness of

the total kafirin material could be an indication that more hydrogen bonds, ionic bonds, and disulphide bonds were being formed during storage. In contrast to total kafirin, which is rich in γ-prolamin, the W Eth QPM zein, which also has abundant γ-zein (Figure 5.2A), did not form a gel under non-reducing conditions. This result was not expected because γ-prolamin can polymerise through disulphide bonding (Duodu et al., 2003). Since total kafirin is considered more hydrophobic that zein, it is assumed that the difference in functionality between total kafirin and W Eth QPM zein was due to the difference in hydrophobicity.

Table 5.5: Effects of storage time (h) and prolamin type (zein or kafirin) on the firmness of prolamin materials prepared under non-reducing and reducing conditions, using glacial acetic acid (GAA) as the main solvent.

Solvent conditions	Storage time (h)	Zein preparations				Kafirin preparations		
		W Eth QPM zein	W Eth RM zein	W SA RM zein	Commercial zein	Total kafirin	Kafirin without γ-kafirin	Kafirin without β-kafirin
	2	$0.02^a \pm 0.00$	$0.02^a \pm 0.01$	$0.03^{ab} \pm 0.01$	$0.02^a \pm 0.01$	$0.05^{ab} \pm 0.04$	$0.02^a \pm 0.01$	$0.02^a \pm 0.00$
Non-reducing	24	$0.03^{ab} \pm 0.00$	$0.22^{abc} \pm 0.00$	$0.06^{ab} \pm 0.00$	$0.02^a \pm 0.01$	$0.69^{defg} \pm 0.43$	$0.27^{abcd} \pm 0.01$	$0.01^a \pm 0.00$
	48	$0.06^{ab} \pm 0.02$	$0.31^{abcd} \pm 0.04$	$0.08^{ab} \pm 0.00$	$0.01^a \pm 0.00$	$0.56^{cdef} \pm 0.25$	$0.33^{abcd} \pm 0.03$	$0.02^a \pm 0.00$
	96	$0.27^{abc} \pm 0.01$	$0.55^{cdef} \pm 0.05$	$0.90^{fg} \pm 0.00$	$0.14^{abc} \pm 0.14$	$2.02^i \pm 0.59$	$0.35^{abcd} \pm 0.08$	$0.02^a \pm 0.06$
	144	$0.44^{bcdef} \pm 0.01$	$0.97^g \pm 0.06$	$0.75^{efg} \pm 0.53$	$0.06^{ab} \pm 0.06$	$9.61^j \pm 0.31$	$1.57^h \pm 0.56$	$0.01^a \pm 0.00$
	2	$0.55^{abcde} \pm 0.00$	$0.02^a \pm 0.00$	$0.03^{ab} \pm 0.01$	$0.01^a \pm 0.00$	$0.02^a \pm 0.01$	$0.03^a \pm 0.00$	$0.02^a \pm 0.00$
	24	$1.26^{bcdef} \pm 0.03$	$0.05^{ab} \pm 0.20$	$0.04^{ab} \pm 0.02$	$0.01^a \pm 0.00$	$0.04^{ab} \pm 0.02$	$0.22^{abc} \pm 0.15$	$0.01^a \pm 0.00$
Reducing	48	$1.31^{cdefg} \pm 0.10$	$0.03^{ab} \pm 0.30$	$0.06^{ab} \pm 0.02$	$0.01^a \pm 0.00$	$0.21^{abc} \pm 0.11$	$1.66^{efg} \pm 2.15$	$0.02^a \pm 0.00$
	96	$3.42^h \pm 0.20$	$0.17^{abc} \pm 0.20$	$0.25^{abc} \pm 0.53$	$0.02^a \pm 0.00$	$0.29^{abcd} \pm 0.11$	$1.12^{bcdefg} \pm 1.17$	$0.02^a \pm 0.00$
	144	$5.14^i \pm 0.09$	$1.47^{defg} \pm 0.56$	$2.13^{fg} \pm 0.86$	$0.19^{abc} \pm 0.00$	$2.45^{gh} \pm 0.50$	$5.44^i \pm 2.29$	$0.02^a \pm 0.01$

Mean ± standard deviation, n = two. Mean values in the same treatment (reducing or non-reducing conditions) with different superscript letters differ significantly at $p \leq 0.05$.
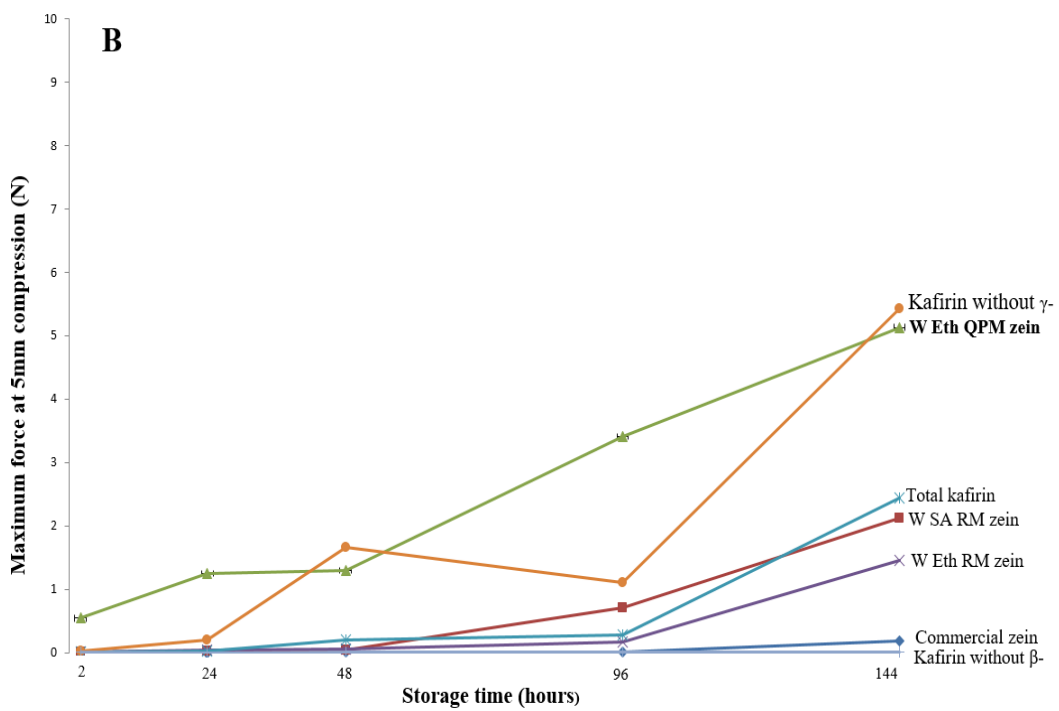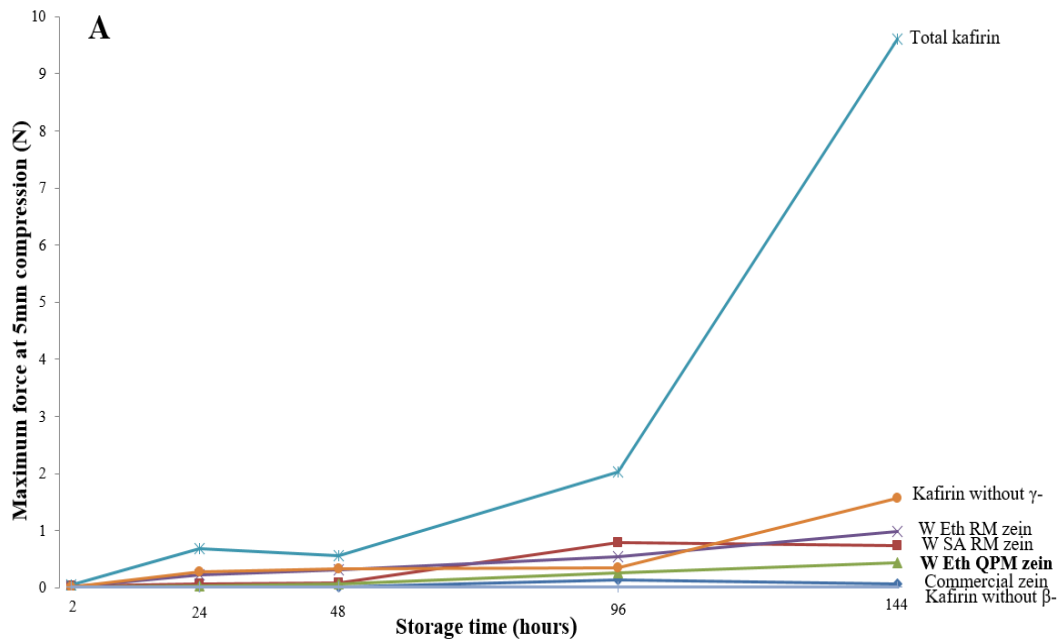
Figure 5.11: Effects of storage time (h) and prolamin type (zein or kafirin) on the firmness of prolamin materials prepared under (A) non-reducing conditions and (B) reducing conditions using glacial acetic acid (GAA) as the main solvent.

The kafirin without γ-kafirin material prepared under non-reducing conditions was nearly three times firmer (1.57 N) compared to the W Eth QPM zein material (0.44 N) prepared under the same condition (Table 5.5, Figure 5.11A). This result was unexpected because it had been suggested that disulphide bonding plays a crucial role in the formation of prolamin gels. However, this result was probably influenced by two factors, namely the composition of the prolamin in terms of subclasses present and the effect of GAA on the secondary structure of the protein's secondary structure.

The firmness of the commercial zein and kafirin without β-kafirin materials that had been prepared under non-reducing conditions (i.e. in the absence of BME) hardly changed throughout storage (< 0.06 N), whereas the firmness of other zein and kafirin preparations increased gradually over storage time (Table 5.5, Figure 5.11A). This observation may be attributed to the subclass composition of the prolamins. Commercial zein consists of predominantly α-zein polypeptides (King et al., 2016), which are stabilised by weak hydrogen bonds. In kafirin, the α2- and γ-kafirin subclasses act as chain terminators (El Nour et al., 1998). They prevent the formation of large polypeptides by forming dimers and small polypeptides instead. Thus, the presence of high levels of α- and γ-kafirin may explain why the firmness of kafirin without β-kafirin materials remained constant over storage.

The finding that only total kafirin formed a gel under non-reducing conditions, whereas W Eth QPM zein did not was not anticipated. It is, therefore, possible that the difference in the gel-forming properties between the two preparations (total kafirin and W Eth QPM zein) was related to their relative hydrophobicity.

The kafirin without γ-kafirin preparation contains less γ-kafirin subclass compared to other subclasses (Elhassan et al., 2018). In contrast, the W Eth QPM zein preparation had more γ-zein compared to other subclasses (Table 5.3). When dissolved in GAA, the proportion of β-sheet structures in the kafirin without γ-kafirin preparation presumably increases (Anyango et al., 2013). Although not measured, it can be assumed that the α-helical content of the W Eth QPM zein preparation also increases after dissolving prolamin in GAA, similar to total kafirin (Elhassan et al., 2018). Compared to helical structures, β-sheets have an open structure that probably enables the polypeptides to interact with other polypeptides or solvent (Nowick, 2008). In the case of kafirin without γ-kafirin, it can be assumed that the β-sheet component had a large surface area to enhance the formation of intermolecular bonds between kafirin polypeptides and the solvent. This may possibly result in the formation of a

protein network that can be stabilised by hydrogen bonds, ionic bonds or Van der Waals forces (Nowick, 2008). For the W Eth QPM zein preparation, the α-helical structures possibly limited polymer-polymer interactions or polymer-solvent interactions. Hence, few intermolecular hydrophobic interactions could be formed between the zein polymer chains. This could explain why the firmness of the W Eth QPM zein material was lower compared to that of kafirin without γ-kafirin material.

The above results showed that only total kafirin formed a gel under non-reducing conditions and this finding is attributed to disulphide bonding between γ-kafirin. Hence, 5% of BME was added to GAA form a GAA-BME solvent solution. After inclusion of BME, the significant finding was that only W Eth QPM zein and kafirin without γ-kafirin preparations formed gels, whereas the other zein and kafirin preparations formed viscous, opaque or runny liquids. The formation of gels under reducing conditions was unexpected. However, using total zein prepared with 70% aqueous ethanol, Nonthanum et al. (2012) reported similar findings of forming zein gels after the inclusion of 5% BME. The authors attributed this result to protein unfolding. During protein unfolding, protein polypeptides unfold to form scrambled structures and possibly a protein network that is stabilised by disulphide bonds, hydrogen bonds, ionic bonds or Van der Waals forces (Chang, 1997). Therefore, it is likely that protein unfolding is the reason why the W Eth QPM zein and the kafirin without γ-kafirin preparations formed gels only under reducing conditions.

Total kafirin did not form gels after inclusion of BME. This result was expected because BME prevents the formation of disulphide bonds between cysteine-rich γ-subclasses (Clark, 2001). The firmness of the total kafirin materials prepared under reducing conditions, however, was similar to the W Eth RM zein and W SA RM zein materials prepared under the same conditions (Table 5.5). Probably, these total zein preparations (W Eth RM zein and W SA RM zein) had lower cysteine contents. Thus, BME had little effect on the preparations. However, the presence of all subclasses and their interaction within the GAA-BME solvent solution, after reduction of proteins may be a possible reason why the firmness of W Eth RM zein and W SA RM zein materials were similar to that of total kafirin

Neither commercial zein nor kafirin without β-kafirin preparations formed gels after inclusion of BME (Table 5.5; Figure 5.11). Also, the firmness of these materials did not

increase during storage, and this observation is similar to that observed with both materials under non-reducing conditions (Figure 5.11). As commercial zein is very low in γ-zein, the BME presumably had little effect. For kafirin without β-kafirin preparation, BME could have reduced the intermolecular disulphide bonds that were formed between the γ-kafirin polypeptides. However, the fact that kafirin without β-kafirin preparation contains α2- and γ-kafirin subclasses which form dimers and monomers instead of polymers (El Nour et al., 1998) could be a possible reason why this preparation did not form gels under either reducing and non-reducing conditions.

Under reducing conditions, BME is expected to prevent polymerisation through breaking intermolecular disulphide crosslinkages formed between protein polypeptides (Clark, 2001), hence enabling polypeptides to unfold. Protein unfolding increases the surface area for solvation, allowing the protein polypeptides to form more polymer-polymer interactions (Li et al., 2012). The formation of gels under reducing conditions was not expected and this could have been due to the particular subclass prolamin composition. W Eth QPM zein had a higher cysteine content (Table 5.3) compared to other zein preparations.

With the GAA-BME treatment, it is possible that the BME reduced intermolecular disulphide bonds formed between the QPM γ-zein polypeptides, consequently resulting in protein unfolding that enhances mobility/movement of polymers within the solution. As a result of increased mobility, the polypeptides probably formed an entangled scrambled structure and a protein network that was stabilised by hydrogen or hydrophobic interactions (Nonthanum et al., 2012), thus resulting in the W Eth QPM zein gel formation. It can be assumed that kafirin without γ-kafirin also formed gels under reducing conditions, by a similar mechanism.

5.5 Conclusion

It seems the composition of prolamin preparations (subclasses present) has a great effect on their functionality. QPM zein films cast from aqueous ethanol or from glacial acetic acid (GAA) are visually more opaque compared to commercial zein or total zein (from regular maize) films cast from the same solvent. This is attributable to the presence of high levels of hydrophobic γ-zein that consequently results in the partial solubility of the QPM zein in the casting solvent. QPM zein films cast from GAA form highly flexible films after storage and this again is attributable to their high γ-zein content and the solubilising effect of GAA on the prolamin, during film formation. However, QPM zein films cast from aqueous ethanol form microparticles on the surface after immersion, and this is attributable to partial solubility of prolamin in casting solvent. QPM zein did not a form viscoelastic materials with either distilled water, acetic acids or coacervation treatments. Despite the treatment, it is likely that high shear applied when the prolamin was dissolved in a solvent contributed to protein aggregation instead of fibril formation. Interestingly, QPM zein formed a viscous solution in GAA, and this was attributed to disulphide crosslinking between γ-zein. After inclusion of reducing agent in GAA, only QPM zein and kafirin without γ-kafirin formed gels, probably due to protein unfolding and formation of a scrambles structure. Apart from prolamin composition, it may be possible that prolamin gelation is contributed by complex factors.

6. GENERAL DISCUSSION

The general discussion will give a critical review of the methodologies used to determine the functional properties of the four different zein types (W Eth QPM zein, W Eth RM zein, W SA RM and commercial zein). Then, the important findings obtained from the current study will be evaluated. Lastly, recommendations for future work on QPM zein functionality will be proposed.

6.1 Critical review of major methodologies used

Amino acid analyses and electrophoresis (SDS-PAGE) were used to characterise the zein preparations used in the current study. In addition to these techniques, Fourier Transform Infrared (FTIR) spectroscopy could have been performed. FTIR shows the secondary structural composition of a protein by estimating its content of α-helical, β-sheet and other conformations (Forato et al., 2003; Bicudo et al., 2005; Belton et al., 2006). During FTIR, protein absorbs infrared radiation. This results in the vibrational transition of molecules to produce an infrared absorption spectrum that can be analysed. A protein's infrared absorption spectra consists of nine Amide bands with vibrational contributions from both amino acid side chains and protein backbone. Of these bands, Amide I and Amide II bands are normally used to determine the secondary structure of the protein (Gallagher, 2009). FTIR provides information relating to the bond parameters, bond angles, redox state of the protein, the chemical structure of vibrating group and conformation of the protein's secondary structure (reviewed by Barth, 2007). In, for example, research into bioplastic film formation and the formation of viscoelastic masses, FTIR has been used to follow secondary structural changes (e.g. α-helical to β-sheet conformations) that occur in prolamin proteins (Gao et al., 2005; Mejia et al., 2007; Sessa et al., 2008; Kötting et al., 2011). Understanding the changes that occur on the secondary structure of QPM zein, may provide useful evidence that could be used to explain why the functionality of QPM zein differs from regular or commercial zein.

Zein films were cast from GAA and 70% aqueous ethanol, using the casting method described by Taylor et al. (2005), without the inclusion of plasticisers. The amount of buffer absorbed by the zein films was determined by using a method described by Anyango et al. (2013), with modifications. Films were immersed in sodium phosphate buffer (pH 6.8) for 12 h to determine the amount of buffer absorbed by the different zein film types. It was

problematic to measure the precise area of all the zein films manually, as the films were either fragile, irregularly shaped and in some cases folded after immersing in the buffer. A better solution could be measuring the area of films using a planimeter (also known as platometer) because it can measure the area of two-dimensional shapes (Kubo et al., 1999). The differences between the weights of the films, before and after immersion was used to determine the buffer uptake. After immersion, it was difficult to remove the surface buffer consistently using a tissue. Hence, this affected the film weight, leading to inaccuracy and large standard deviations. The standard deviation could be reduced by analysing more samples.

Total zein viscoelastic materials can be formed when the prolamin is dissolved in GAA, followed by addition of water to precipitate the protein out of solution (coacervation process) (Taylor et al., 2018). However, in this current study, none of the zein preparations used formed viscoelastic materials using the coacervation process. Instead, all zein preparations formed colloids and this was not expected. In the current study, the final concentration of protein in GAA (before addition of water) was approx. 11%. As this concentration of protein in GAA was similar to the minimum protein concentration (5-10%) that is required to form fibrils (Oguntoyinbo et al., 2018), it would have been expected for fibril formation to take place. Therefore, it is likely that shear applied while mixing the zeins in GAA with a spatula before addition of water (coacervation), in a cylindrical tube, was too high and this may have disrupted fibril formation. Instead of using a stirrer and cylindrical tubes, it would be better to mix the prolamin and GAA in a beaker using a magnetic stirrer. When a magnetic stirrer is used, shear applied during mixing may be low, thus allowing fibril formation to take place.

When SEM was used to study the morphology of the zein colloidal suspensions formed after coacervation, all zein preparations had formed aggregates (Figure 5.10). For SEM, the zein colloids were washed with distilled water to remove the residual acetic acid. This action reduced the concentration of acetic acid while increasing the pH and simultaneously favouring protein aggregation. Furthermore, a drop of the zein colloid was then mounted directly on the double-sided tape, then air-dried in the desiccator for 3 days before the carbon coating. It is probable that during air drying, microparticles were formed via an evaporation-induced self-assembly mechanism, similar to what Wang and Padua (2012) described. Instead of air drying, freeze drying would have been a better method. This is because freeze drying does not alter the structure of protein being dried, and the protein hardly shrinks during drying (Shukla, 2011).

The compression test method was used to measure the firmness of zein and kafirin preparations prepared in GAA, under reducing and non-reducing conditions. After mixing with the solvent, the zein and kafirin preparations were transferred into small rigid cylindrical plastic lids (12.3 mm diameter x height 13.2 mm). The lids were placed in a beaker, and the beaker was placed in an air-tight zip-lock type plastic bag (length 400 mm x 300 mm width), then stored in the dark at 25˚C. During storage, however, some zein preparations became dry. This observation was unexpected since the small rigid cylindrical plastics lids had been placed in zip-lock type plastic bags to prevent evaporation of the solvent during storage. The drying of zein preparations could be because the solvent had evaporated to reach equilibrium inside of the zip-lock type plastic bag. Instead of using zip-lock type plastic bags that have a lot of space in them, a better solution would be to use smaller, airtight and closely fitting containers that would cover the rigid cylindrical plastic lids during storage.

6.2 Important research findings

6.2.1 Summary of the research findings
Table 6.1: Summary of the effects of prolamin type (W Eth QPM zein, W Eth RM zein, W SA RM zein, commercial zein, total kafirin, kafirin without β- kafirin and kafirin without γ-kafirin) on the functionality of prolamins with respect to the formation of bioplastic films, viscoelastic materials and gelation.

Prolamin preparation type	Film formation	Viscoelastic mass formation	Gel formation
W Eth QPM zein	QPM zein films could be cast from 70% aqueous ethanol and GAA. Irrespective of casting solvent, QPM zein films were more opaque compared to other zein films cast from the same solvent.	QPM zein did not form viscoelastic materials when prepared with distilled water or acetic acids at 50˚C. However, protein aggregates and microparticles (approx. 10 μm diameter) were formed with water and 5.4% acetic acid treatments.	QPM zein formed a viscous solution when the prolamin was dissolved in GAA under non-reducing conditions.
	QPM zein films cast from GAA formed flexible films after storage without the inclusion of plasticizers.	QPM zein formed a more viscous solution when the prolamin was dissolved in GAA before addition of water (coacervation).	QPM zein formed gels only after inclusion of 5% 2-mercaptoethanol to GAA (reducing conditions). Similar findings have been reported by Nonthanum et al. (2012).
	QPM zein films cast from GAA absorbed the least amount of buffer when compared to zein films cast from GAA or other prolamin films cast from aqueous ethanol.	QPM zein formed a colloid with zein suspensions when the prolamin was dissolved in GAA, followed by addition of water (the coacervation process).	
	QPM zein films cast from aqueous ethanol formed microparticles on the surface after immersing in the buffer		
W Eth RM zein	W Eth RM zein films cast from GAA absorbed the highest amount of buffer when compared to other zein films cast from GAA.	W Eth RM zein did not form viscoelastic materials with any treatments (water, acetic acid or coacervation). However, microparticles (approx. 20 μm diameter) were formed with the water and 5.4% acetic acid treatments.	W Eth RM zein did not form gels under either reducing or non-reducing conditions

Table 6.1 Continued: Summary of the effects of prolamin type (W Eth QPM zein, W Eth RM zein, W SA RM zein, commercial zein, total kafirin, kafirin without β- kafirin, kafirin without γ- kafirin) on the functionality of prolamins with respect to the formation of bioplastic films, viscoelastic materials and gelation.

Prolamin preparation type	Film formation	Viscoelastic mass formation	Gel formation
W SA RM zein	W SA RM zein films cast from both solvents had gel-like aggregates on the surface after casting.	W SA RM zein did not form viscoelastic materials with any treatments (water, acetic acid or coacervation). Microparticles (approx. 20 μm diameter) were formed with water and 5.4% acetic acid treatments.	W SA RM zein did not form gels under reducing (i.e. with inclusion of BME) or non-reducing conditions (i.e. without inclusion of BME).
Commercial zein	Commercial zein films cast from GAA and aqueous ethanol were smooth and consistent, compared to other zein films cast from the same solvent.	Commercial zein only formed viscoelastic materials when prepared with water and 5.4% acetic acid treatments.	Commercial zein did not form gels under either reducing or non-reducing conditions. Similar findings have been reported by Nonthanum et al. (2012).
Y Eth QPM zein	Not performed	Not performed	Not performed
Y SA RM zein	Not performed	Not performed	Not performed
Total kafirin	Not performed	Not performed	Total kafirin only formed a gel under non-reducing conditions. Findings similar to the original work of Johns and Brewster (1917).
Kafirin without γ-kafirin	Not performed	Not performed	Kafirin without γ-kafirin only formed gels under reducing conditions.
Kafirin without β-kafirin	Not performed	Not performed	Did not form gels under either reducing or non-reducing conditions.

6.2.2 Properties of films

Table 6.2 shows the properties of W Eth QPM zein films cast from 70% ethanol solution and GAA.

Table 6.2: Properties of zein films cast from 70% aqueous ethanol solution and GAA.

Film functional property	Effect of prolamin type	Reasons for difference in film functionality
Clarity	W Eth QPM zein films cast from both aqueous ethanol and GAA were opaque, with what appeared like small lumps of undissolved protein or air bubbles within the films after casting.	The opaqueness of W Eth QPM zein films cast from aqueous ethanol is attributable to the partial solubility of the prolamin during casting, whereas that of the W Eth QPM zein cast from GAA is attributable to the formation of air bubbles when W Eth QPM zein was dissolved in GAA.
	W Eth RM zein films and commercial zein films cast from aqueous ethanol and GAA were transparent.	Prolamin preparations had completely solubilised in casting solvents.
	W SA RM zein films cast from both aqueous ethanol and GAA had gel-like aggregates on the surface of their films after casting.	Formation of gel-like aggregates was perhaps influenced by the methionine content, which was the highest in W SA RM zein compared to other zein preparations (Table 5.3). Methionine-rich prolamins (e.g. β-zein) can be unstable and prone to gelation (Pomes, 1971).
Flexibility	W Eth QPM zein films cast from GAA were flexible after storage, whereas all the other zein films cast from GAA were brittle. All zein films cast from aqueous ethanol were brittle on storage.	Film flexibility of W Eth QPM zein was probably due to the higher α-helical content of the W Eth QPM zein (>80%) compared with the other zein preparations (<60%) (King et al., 2016; Bicudo et al., 2018). This may have allowed entrapment of more water molecules between the α-helices resulting in a higher degree of plasticization, enhanced mobility of the polymers and greater film flexibility. See the film model (Figure 6.1).

Table 6.2 Continued: Properties of zein films cast from aqueous ethanol solution and glacial GAA.

Film functional property	Effect of prolamin type	Reasons for difference in film functionality
Buffer uptake and area of zein films after immersion	The buffer uptake of all zein films cast from aqueous ethanol were similar. In contrast, buffer uptake of zein films cast from GAA differed significantly from each other.	The difference in buffer uptake between the zein films cast from aqueous ethanol and GAA treatments is attributable to the casting solvents. When zein proteins are dissolved in aqueous ethanol, the polypeptides do not unfold to the same extent as in GAA (Li et al., 2012). As a result of the lower degree of unfolding, it is likely that there was limited interaction between zein polypeptides and buffer. Thus, less buffer was absorbed by zein films cast from aqueous ethanol, when compared to prolamin films cast from GAA.
	W Eth QPM zein films cast from GAA absorbed the least buffer when compared to other zein films cast from the same solvent (GAA). This value was lower than the buffer uptake of any of the prolamin films, including W Eth QPM films cast from aqueous ethanol.	W Eth QPM zein had a higher cysteine content compared to the other zein preparations (Table 5.4). Low buffer uptake of W Eth QPM zein films cast from GAA is attributable to protein unfolding in the GAA (Li et al., 2012), which could have enabled more disulphide crosslinking between the polypeptides. The resultant crosslinking increased the strength of the films and decreased the buffer uptake (Muhiwa et al., 2017).
	QPM zein films cast from aqueous ethanol formed microparticles on the surface of the film only after immersing in buffer. The area of the W Eth QPM zein films (after immersing) increased enormously compared to W SA RM zein and commercial zein films cast from the same solvent.	W Eth QPM zein may be considered more hydrophobic than other zein preparations due to its higher level of hydrophobic γ-zein (see Figure 5.2). W Eth QPM zein could have partially dissolved in the aqueous ethanol and when exposed to buffer, microparticles were formed. The increase in area of the W Eth QPM zein films cast from aqueous ethanol, after immersion, is attributable to the formation of and hydration of microparticles when immersed in buffer.

6.2.3 Discussion of major findings

6.2.3.1 Clarity of films: opaqueness of W Eth QPM zein films

The opaqueness of the W Eth QPM zein films is attributable to the prolamin composition (subclasses) and the partial solubility of the prolamin in the film casting solvents. Similar to total kafirin (Duodu et al., 2003), it is likely that the W Eth QPM zein is more hydrophobic than commercial zein or total zein (from regular maize) because of its high γ-zein content (Table 5.3). The hydrophobic nature of the W Eth QPM zein preparation could have made it more difficult to dissolve in aqueous ethanol during film formation. This is because lumps of undissolved zein were observed within the film after casting (Figure 5.3A, film A).

When the W Eth QPM zein preparation was dissolved in GAA, a more viscous solution was formed and a considerable amount of foaming was observed. It is likely that in GAA, the W Eth QPM zein polypeptides unfolded and air bubbles were entrapped within the viscous solution. The air bubbles could not escape from the GAA during oven drying. Thus, the presence of air bubbles within the W Eth QPM zein films could be the reason why these films were more opaque compared to other zein films cast from the same solvent (GAA) (Figure 5.3B, film I).

6.2.3.2 Flexibility of W Eth QPM zein films cast from GAA

The W Eth QPM zein films cast from GAA were highly flexible and could form an arc without breaking (Figure 5.5), whereas the films cast from the other zeins using the same solvent remained stiff after storage. This appears to be the first time that the formation of a flexible film has been demonstrated using prolamin protein alone, i.e. without the inclusion of a plasticiser.

Zein films are normally brittle and to form flexible films, plasticisers are required (Lawton, 2004). The arrangement of polymers in a protein matrix (random/ordered) influences the mobility of polymers and the flexibility of bioplastic films (Athamneh et al., 2008; Kaur et al., 2013). Plasticisers are small molecular weight molecules that embed themselves between polymer chains (Krotcha, 2002). This action reduces the hydrogen bonding between protein polymers chains, allowing the polymers to move in opposite directions with adjacent protein chains, resulting in film flexibility. In the current study, plasticisers were not used. Therefore,

the effect of the casting solvent (GAA) on the protein secondary structure and the composition of the zein protein (in terms of subclasses), will be considered. SDS-PAGE showed that W Eth QPM zein was more abundant in the γ-zein subclass when compared to other zein preparations (Figure 5.2A). It is known that the solvent in which γ-zein are dissolved in affects the conformation of zein subclasses (Bicudo et al., 2018). For example, γ-zein dissolved in sodium dodecyl sulphate (SDS) plus the reducing agent dithiothreitol (DTT) was found to be approximately 87% α-helical, whereas γ-zein in H_2O/DTT was only 55% α-helical. Hence, it can be assumed that the W Eth QPM zein dissolved in GAA was mainly α-helical. King et al. (2016) showed that the α-helical conformation of commercial zein and total zein (from regular maize) in GAA were both approx. 58%, whereas that of total kafirin, which contains more of the γ-subclass when treated with GAA, was significantly higher at 73% (Elhassan et al., 2018). Total kafirin is comparable to W Eth QPM zein because both are rich in γ-prolamin.

It is proposed that during film formation using GAA as a casting solvent, the α-helical structures of the W Eth QPM zein prolamin would unfold and the molecules would arrange themselves linearly along their linear axes, and stack on top of each other (Fig. 6.1A). According to Li et al. (2012), solvation in GAA results in protonation of zein and causes partial unfolding or swelling of α-zein, mainly in the α-helical region. Partial unfolding exposes the greater area for solvation and hydration of the zein surface. Considering that all zein films had been stored under the same conditions (25°C, 3 d) when the relative humidity of the environment was high (≈ 100%), the water molecules would have bonded onto the zein surface by hydrogen bonding and plasticized the W Eth QPM zein resulting in flexible films, as found (Figure 6.1A). Commercial zein and total zein films, with less α-helical conformation, would also unfold in GAA and during film formation, the zein molecules with their α-helical and β-sheet structures would be arranged along their linear axes (Figure 6.1B). However, in contrast to the W Eth QPM zein, the water molecules would only plasticise some parts of the film where the α-helical structures were adjacent to each other. As a result, there would be fewer sites available for hydrogen bonding between the zein and water molecules due to the reduced proportion of α-helical regions. Hence, the films remained brittle.
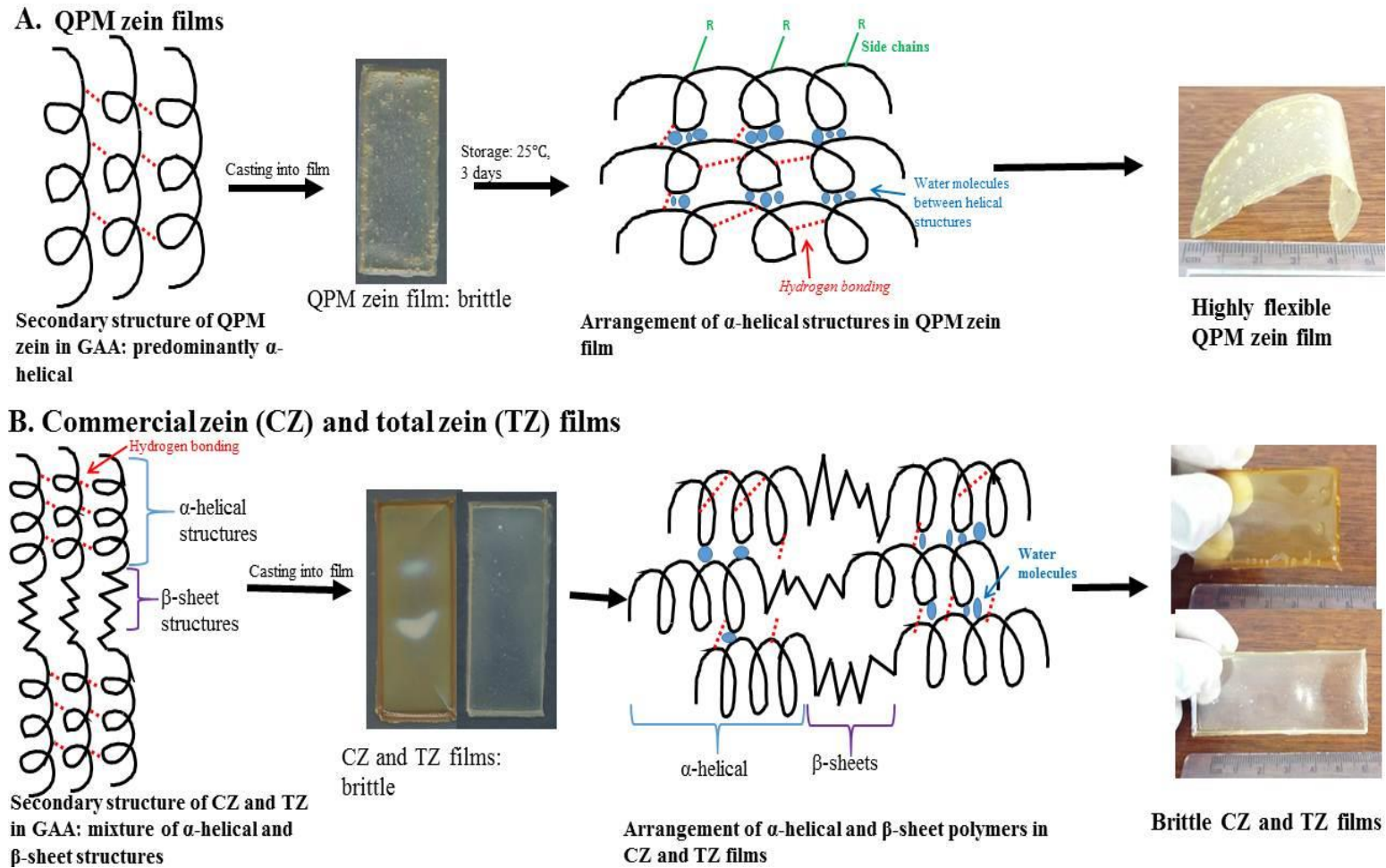
Figure 6.1: Hypothetical model to explain why QPM films cast from GAA were flexible, whereas (CZ) commercial zein and (TZ) total zein films cast from the same solvent remained brittle after storage. A = W Eth QPM zein; B = commercial zein and total zein films.

6.2.3.3 Buffer uptake and swelling of W Eth QPM zein films

The W Eth QPM zein films cast from GAA absorbed the least amount of buffer when compared to other zein films cast from GAA or other prolamin films cast from aqueous ethanol. The low buffer uptake of these W Eth QPM zein films is attributable to disulphide crosslinking between the γ-zein polypeptides. Mixing zein in the GAA results in protein unfolding through the breaking of intra-molecular hydrogen bonds and protonation on the zein surface (Li et al., 2012). Protein unfolding increases the surface area for solvation and enables the polymer to form more interactions with other polymer chains or solvents through hydrogen bonding, ionic bonds or Van der Waals forces. In the case of W Eth QPM zein films cast from GAA, it is likely that during film formation the protein polypeptides unfolded on the α-helical region and they aligned on the same linear axis, stabilised by intermolecular hydrogen bonding and disulphide crosslinking. Since the W Eth QPM zein had high levels of cysteine, more intermolecular disulphide crosslinkages between the zein polymers may have been formed. This would bring the zein polymers closer together, thus creating a compact protein network structure and a barrier that limited the movement of liquids during immersion. Hence, less buffer would be absorbed into these films during immersion, and this would explain why W Eth QPM zein films cast from GAA swelled the least.

 When dissolved in GAA, total zein and commercial zein preparations contain a mixture of α-helix and β-sheet structures (King et al., 2016) (see Figure 6.1 for proposed structure of total zein and commercial zein in GAA). As described above, GAA results in unfolding on the α-helical region of the zein protein. Therefore, when dissolved in GAA, total zein and commercial zein, with their lower α-helical content, would unfold less than W Eth QPM zein. Consequently, these films would be able to bind less water in these regions compared to W Eth QPM zein films.

All zein films when cast from aqueous ethanol had similar buffer uptake. When zeins are dissolved in aqueous ethanol, they do not unfold to the same extent as in GAA (Li et al., 2012). This would result in less buffer absorption due to the limited interaction between zein polypeptides and buffer.

6.2.3.4 Formation of microparticles and colloids

Only commercial zein formed viscoelastic materials when prepared in distilled water and 5.4% acetic acid at 50ºC, above the T_g of zein. When zein is heated above its T_g, its reactivity increases and the polymers change into a rubbery state (Lawton, 1992; Erickson et al., 2012). This enables the commercial zein to aggregate, forming a viscoelastic mass that can be stretched. When the acetic acid concentration was increased to 33%, none of the zein preparations used in the current study, including commercial zein, formed viscoelastic materials and this is similar to what was reported by Taylor et al. (2018). SEM showed that in distilled water, the W Eth QPM zein had formed protein aggregates and small spherical microparticles (approx. 10 μm diameter), whereas W SA RM zein and W Eth RM zein preparations had formed larger microparticles (approx. 20 μm diameter). Upon addition of acetic acid, the microparticles fused to form a continuous film with smooth surfaces (Figure 5.8B-D). This shows that zein dissolves better in acetic acid compared to water, as suggested by Sly et al. (2014).

When the different zein preparations were dissolved in GAA before coacervation, the W Eth QPM zein formed a very viscous solution, whereas other zein preparations formed normal runny solutions. More GAA was added to dissolve the W Eth QPM zein viscous solution into a runny liquid, and the same amount of GAA was also added to the other zein preparations until the final concentration of protein in GAA was 11%. Upon addition of water, all zein preparations formed colloids. The concentration of prolamin in GAA used in the current study (approx. 11%), before addition of water, was similar to a minimum of 5-10% protein in the GAA that is required for fibril formation to take place (Oguntoyinbo et al., 2018). Therefore, it would have expected for viscoelastic materials to be formed. Light microscopy showed that W Eth QPM zein and W Eth RM zein colloids had more numerous zein suspensions when compared to W SA RM zein and commercial zein colloids. SEM revealed that all colloids had formed protein aggregates (Figure 5.10). The formation of protein aggregates is attributable to the action of washing the colloids with distilled water. This was done to remove the residual acetic acid, before viewing using SEM. There is evidence that decreasing the acetic acid concentration during coacervation changes the secondary structure of the protein (i.e. reduces the amount of α-helical structure and increases the amount of β-sheet structure) (Elhassan et al., 2018). Although not measured, it may be assumed that the β-sheet content of all zein preparations, including W Eth QPM zein, increased after coacervation. A high level of β-sheet structures is an indication of zein

molecular aggregation (Mizutani et al., 2003). Hence, it is likely that a high β-sheet content contributed to the formation of protein aggregates on water addition, instead of fibril formation.

6.2.3.5 Gel forming properties of QPM zein

The W Eth QPM zein and kafirin without γ-kafirin preparations formed gels under reducing conditions (i.e. after inclusion of 5% 2-mercaptoethanol (BME) in GAA). Similar to the findings obtained from the current study, Nonthanum et al. (2012) reported that total zein prepared in 70% aqueous ethanol formed gels in the presence of 5% BME. Gel formation can be attributed to protein unfolding. Protein unfolding is a 2-step process whereby the reducing agent breaks the disulphide bonds between cysteine-rich oligomers, allowing the proteins to unfold and then form a protein network of scrambled structures that are stabilised by intermolecular hydrogen bonds and or hydrophobic interactions (Chang, 1997; Nonthanum et al., 2012). In the current study, the formation of W Eth QPM zein and kafirin without γ-kafirin gels was perhaps influenced by the composition (subclasses present) of the zein and kafirin preparations.

Two-dimensional PAGE electrophoresis showed that W Eth QPM zein had abundant 27 kDa γ-zein and higher molecular weight γ-zein of approx. 55 kDa (Shull et al., 1991) (Figure 5.2A). In the presence of the GAA-BME solvent solution, the BME may have reduced the intermolecular disulphide bonds formed between the cysteine-rich 27 kDa γ-zein or 55 kDa γ-zein within the zein material. Reduction of disulphide bonds by BME, and the protonation of zein by GAA (Li et al., 2012) possibly enabled the protein polypeptides to unfold and increased their mobility in the solution. The more mobile polymers would then be able to form an entangled polymeric network "scrambled structure" that could be stabilised by hydrogen bonds and or hydrophobic interactions, resulting in gel formation. The kafirin without γ- kafirin material did form gels under reducing conditions. The preparation did not have γ-kafirin present but did contain β-kafirin, which is also rich in cysteine and so can form inter- and intra-molecular disulphide bonds. Here the BME would reduce the disulphide bonds formed between the β-kafirin and other kafirin sub-classes enabling the protein to unfold and become more mobile as described for the QPM zein. Again in a similar way to W Eth QPM zein, a polymeric network would then be formed stabilised by hydrogen and or hydrophobic interactions resulting in gel formation.

Under the same reducing conditions, commercial zein (predominantly α-zein) did not form gels, and this is in agreement with the findings of Nonthanum et al. (2012). Without γ-zein, the BME would have no effect, and so no gel would be formed. Kafirin without β- kafirin did not forma gel under reducing conditions, possibly because α2- and γ-kafirin act as chain terminators (El Nour et al., 1998), forming only dimers or small polypeptides, which were insufficient in length to form protein entanglements needed for gel formation. Total kafirin formed a gel under non-reducing conditions but did not form a gel under reducing conditions. This results suggest that gelation of total kafirin, under non-reducing conditions was influenced by intermolecular disulphide bonding between γ-kafirin and the other kafirin subclasses. The W SA RM zein and W Eth RM zein preparations did not form gels under either reducing or non-reducing conditions, and this may be attributable to their low cysteine-content.

6.3 Way forward

QPM was developed to improve the nutritional status of some of the people in developing countries where protein-deficiency diseases are prevalent (Eshetie, 2017). In a country where QPM is produced commercially and used to manufacture food products, the high protein by-products of these industries, e.g. milling and brewing, could be used to extract QPM zein. In developed countries, QPM has potential use as an energy source for the production of bioethanol. Distiller's dried grains solubles (DDGS), the by-product of bioethanol production has a high protein content, and it is predominantly used as a source of animal feed (Swiatkiewicsz and Koreleski, 2008). Instead of using DDGS as animal feed it could be used as a raw material to extract zein. This QPM zein, with its superior film functional properties in terms of low water uptake and flexibility without the inclusion of plasticisers, could be a useful raw material when forming bioplastic films. Such bioplastic films could be used to produce biodegradable zein-based packaging materials (Corradini et al., 2014). This would reduce the amount of packing material made from non-renewable resources and reduce environmental pollution (Marsh and Bugusu, 2007), as synthetic polymers are largely non-biodegradable and are often not economically recyclable. QPM zein could also be used to produce coatings for climacteric fruits, such as pears and avocados to maintain the quality and extend the shelf-life (Buchner et al., 2011; Taylor et al., 2016c). In the pharmaceutical industry, QPM zein films could have applications as carriers of drugs that can be transported to specific organs within the gastrointestinal tract (e.g. anticancer therapeutics) (Taylor et al., 2014). However, before this can happen, research is needed to determine whether QPM zein films are safe for use in humans and animals.

QPM zein did not form viscoelastic materials using the coacervation process. However, QPM zein has the interesting property that it can form gels under reducing conditions. QPM zein gels may have applications in the food industry as stabilisers and gelling agents. Additionally, potential biomedical use could include implants for drug release systems. Again, safety remains a major concern since QPM zein films have not been tested using animal models or human studies.

7. CONCLUSIONS AND RECOMMENDATIONS

Amino acid analysis confirmed that zein from white and yellow QPM had high cysteine contents compared to zeins from regular maize counterparts. Furthermore, SDS-PAGE, under reducing conditions, revealed that the W Eth QPM and Y Eth QPM zein preparations have abundant γ-zein compared to other zein preparations.

QPM zein can be cast into bioplastic films. Concerning their functional properties, W Eth QPM zein films cast using either aqueous ethanol or GAA as solvents are visually more opaque compared to total zein or commercial zein films cast from the same solvent. This is probably a result of the partial solubility of QPM zein in the casting solvents during film formation, and may be attributed to the high levels of hydrophobic γ-zein. An important finding is that the W Eth QPM zein films cast from GAA from highly flexible films without the inclusion of plasticizers, which can be bent into an arc without breaking, whereas other films from other zeins cast from the same solvent remained brittle throughout storage. This is attributable to the high α-helical content of QPM zein, which possibly increases after the prolamin is dissolved in GAA. Furthermore, QPM zein films have better barrier properties, as they absorb less buffer compared to other films cast from other zeins using the same solvent. Flexible QPM zein films have potential in pharmaceutical applications such as for controlled drug release.

QPM zein does not form viscoelastic materials when prepared with distilled water or acetic acid as solvents at 50°C, or coacervated out of glacial acetic acid solution using distilled water (coacervation process). It is probable that during preparation of viscoelastic materials using either treatment, high shear was applied when the prolamin (QPM zein) was mixed with the solvent. This may have disrupted formation of fibrils, while promoting protein aggregation and formation of microparticles.

An important observation made during the attempted formation of viscoelastic materials using coacervation is that W Eth QPM zein forms a viscous solution when dissolved in GAA compared to other zein preparations. This was probably due to disulphide crosslinking between the abundant γ-zein polypeptides. After inclusion of reducing agent (5% 2-mercaptoethanol), only W Eth QPM zein and kafirin without γ-kafirin form gels, indicating that disulphide bonding is not a major factor that influences zein gelation. In fact, it appears that a complex interplay of factors such as the prolamin composition (subclasses present), the

presence or absence of reducing agent in the solvents contribute towards gelation of prolamins. Gelation of QPM zein should be further investigated as a potential functional food ingredient (gelling agent, filler or thickener). In addition, QPM zein gels could have potential uses in medical applications as implants. Animal and human studies need to be undertaken to ensure that the QPM zein gel production does not pose any health threats/problems.

Overall, this research has shown that QPM zein has unique functional properties when compared to commercial zein and total zein extracted from regular maize. Research into determining the secondary structure of QPM zein after preparing the prolamin under various treatments is required.

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9. APPENDIX

Conference presentation:

Baloyi, J. T., Taylor, J. and Taylor, J. R. N., 2018. Comparing the functional properties of kafirin and isolated quality protein maize (QPM) zein with respect to gel and film formation. Poster presentation at The International Sorghum Conference, Sorghum in the 21st Century. Century City Conference Centre, Cape Town, South Africa, 9-12 April 2018.