

Modification of the functional properties of zein and kafirin protein films by oxidation using hydrogen peroxide

by

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DECLARATION

I, **Sharon Njila** declare that the dissertation, which I hereby submit for the degree MSc Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:

DATE:

DEDICATION

This dissertation is dedicated to my parents, Strike Njila and Maureen Nolieth Taisi-Njila and my late brother-in-law Jethro Dzenga. My parents for not only raising and nurturing me but also for taxing themselves dearly over the years for my education and intellectual development, something they have never enjoyed. My late brother-in-law Jethro Dzenga, who supported my dream of studying at the University of Pretoria, who had been a source of motivation and strength during moments of despair and discouragement. Unfortunately Jethro Dzenga, you could not witness my success because death defeated you but I am forever grateful.

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- My siblings Dr. Tendai Njila and Sylvia Njila for always being there to listen to me and encouraging me when I was giving up
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ABSTRACT

Modification of the functional properties of zein and kafirin protein films by oxidation using hydrogen peroxide

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Maize and sorghum flours do not form viscoelastic doughs similar to wheat flour. However, isolated zein (maize prolamin) can form a viscoelastic dough when first cast into a film and then hydrated in water above its glass transition temperature (T_g). Wheat gluten functionality is strongly influenced by disulphide and dityrosine bonding. Furthermore, kafirin (sorghum prolamin) films are stabilised by disulphide bonds formed through cysteine oxidation. Thus, this study determined the effects of oxidising commercial zein (mainly α -zein), total zein (α -, β -, γ - and δ -zein) and total kafirin (α -, β -, γ - and δ -kafirin) with hydrogen peroxide on film functionality and dough formation.

Films were prepared by casting where the prolamins were denatured in 70% aqueous ethanol at 70⁰C, allowing the solution to cool to ambient temperature and then adding hydrogen peroxide (up to 150 mg/g protein) with and without copper catalyst and then evaporating the solvent off.

Increasing hydrogen peroxide concentration increased zein and kafirin film aqueous buffer uptake, but not with hydrogen peroxide plus copper catalyst. SDS-PAGE revealed disulphide bond polymerisation of total zein and total kafirin films prepared with hydrogen peroxide, but not with films from commercial zein. DSC showed an increase in T_g and heat capacity change of commercial zein, total zein and total kafirin films prepared with hydrogen peroxide. FTIR indicated an increase in α -helical conformation of commercial zein and total zein and an increase in β -sheet conformation of total kafirin with increase in hydrogen peroxide concentration, but not with commercial zein films. The change in total kafirin structure could have been due to kafirin containing more cysteine residues than total zein.

The dried films were manipulated with distilled water at 50°C into doughs. Commercial zein films prepared with hydrogen peroxide formed doughs but not the films prepared without hydrogen peroxide. All total zein film preparations formed doughs, whereas all total kafirin film preparations did not. Addition of hydrogen peroxide to commercial zein and total zein increased dough cohesiveness, extensibility and water holding capacity. The zein doughs were extensible when left below T_g for 1min. Inclusion of hydrogen peroxide in commercial zein and total zein promoted fibril formation as was seen under SEM and stereomicroscopy. However, no fibrils were formed with total kafirin. FTIR of commercial zein and total zein doughs indicated a slight increase in α -helical and an increase in β -sheet conformation with total kafirin aggregates.

Commercial zein, total zein and total kafirin films react differently with hydrogen peroxide treatment. Commercial zein contains few cysteine residues, hence the effects of hydrogen peroxide could be due to hydroxylation of amino acid side chains which would promote hydrogen bond formation. As hydrogen peroxide promotes disulphide bond crosslinking of total zein and kafirin, extensive crosslinking prevents dough formation from kafirin films. Therefore oxidation of zein and kafirin with hydrogen peroxide modifies the film and dough functional properties. However, further research is required on how to form doughs from total kafirin.

TABLE OF CONTENTS

DECLARATION	ii
DEDICATION	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT	v
LIST OF TABLES	x
LIST OF FIGURE	xi
1. INTRODUCTION	1
2. LITERATURE REVIEW	2
2.1. Chemistry of prolamin proteins.....	2
2.1.1. Zein and kafirin.....	2
2.1.2. Structure of α -zein and α -kafirin.....	3
2.1.3. Gluten.....	5
2.2. Functionality of prolamin proteins	7
2.2.1. Film formation	7
2.2.2. Dough forming properties.....	8
2.3. Research into the modification of the functional properties of zein and kafirin protein films by oxidation	12
2.3.1. Physical treatment.....	13
2.3.2. Chemical cross-linking	13
2.3.3. Enzymatic treatment	14
2.4. Research into modification of gluten and other prolamin functionality by oxidising agents	14
2.4.1. Application of oxidative cross-linking enzymes.....	14
2.4.2. Application of chemical oxidising agents.....	17
2.5. Improvement of zein and kafirin functionality by oxidation	18
2.6. Protein Structure Analytical Techniques for α -zein, total zein and total kafirin preparations	19
2.6.1. Fourier Transform Infrared (FTIR) spectroscopy.....	19
2.6.2. Differential Scanning Calorimetry (DSC)	22
2.7. Conclusions	24
3. HYPOTHESES AND OBJECTIVES	25
3.1. General Aim	25
3.2. Hypotheses	25

3.3. Objectives.....	26
4. EXPERIMENTAL.....	27
5. RESEARCH.....	28
Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the functional properties of the films	28
ABSTRACT.....	28
5.1. Introduction	29
5.2. Materials and Methods	31
5.2.1. Materials	31
5.2.2. Methods.....	31
5.3. Results and discussion.....	36
5.3.1. Moisture and protein contents of maize and sorghum meals, zein and kafirin protein preparations	36
5.3.2. Effects of preparation of commercial zein, total zein and kafirin films with hydrogen peroxide on prolamin solubility, and film physical quality and colour.....	38
5.3.3. Effects of preparation of commercial zein, total zein and kafirin films with hydrogen peroxide on film aqueous buffer uptake	44
5.3.4. Effect of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film SDS-PAGE pattern	46
5.3.5. Effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film protein secondary structure	49
5.3.6. Effect of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film T _g and enthalpy changes.....	53
5.4. Conclusions	57
Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on their dough forming properties	58
ABSTRACT.....	58
5.5. Introduction	59
5.6. Materials and Methods	60
5.6.1. Materials	60
5.6.2. Methods.....	60
5.7. Results and discussion.....	62
5.7.1. Effects of film preparation with hydrogen peroxide on commercial zein, total zein and kafirin dough and fibril formation.....	62
5.7.2. Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough protein secondary structure.....	69

5.8. Conclusions	73
6. GENERAL DISCUSSION	74
6.1. Critical review of methodologies applied	74
6.2. Discussion of research findings.....	76
6.2.1. Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the functional properties of the films.....	81
6.2.2. Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the dough forming properties	85
6.3. Way forward.....	86
7. CONCLUSIONS AND RECOMMENDATIONS	87
8. REFERENCES	88
9. APPENDIX.....	106

LIST OF TABLES

Table 5.1: Moisture content, protein content of commercial zein, maize and sorghum meal and extracted total zein and kafirin preparations.....	37
Table 5.2: Effects of preparation of commercial zein, total zein and kafirin films with hydrogen peroxide on protein solubilisation and film physical quality and colour	40
Table 5.3: Effect of preparation of commercial zein, total zein and kafirin films with hydrogen peroxide on film buffer uptake	45
Table 5.4: Effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film protein secondary structure.....	52
Table 5.5: Effects of preparation of commercial zein, total zein and total kafirin with hydrogen peroxide on film glass transition and enthalpy of changes.....	56
Table 5.6: Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough formation and extensibility.....	66
Table 5.7: Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough protein secondary structure.....	72
Table 6.1: Summary of the effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the properties of films and doughs formed	76

LIST OF FIGURE

Figure 2.1: Proposed structural model for α -zein by Argos et al. (1982).....	3
Figure 2.2: Proposed structural model for α -zein by Matsushima et al. (1997).....	4
Figure 2.3: Proposed structural model for zein by Bugs et al. (2004) showing α -helices, β -turns and random coils.....	5
Figure 2.4: Classification of wheat gluten proteins (Shewry et al., 1986).....	6
Figure 2.5: Proposed structure of HMW glutenin containing the repeat-rich β -spiral domain and α -helical ends (Shewry et al., 2000).	7
Figure 2.6: Possible mechanism for zein self-assembly from single molecules to nanospheres (Wang and Padua, 2012).	8
Figure 2.7: Effect of hydration on loop and train behaviour and the deformation of polymers during stretching to form an extended network (Belton, 1999).	10
Figure 2.8: Structure of dityrosine crosslink which brings together glutenin molecules (Tilley et al., 2001).	11
Figure 2.9: Confocal laser scanning Micrography showing large spherical shaped GMPs indicated by white arrows (Don et al., 2003).	12
Figure 2.10: Mechanism of glucose oxidase action in gluten dough forming disulphide bonds or dityrosine bonds (Vemulapalli and Hoseneey, 1998).	15
Figure 2.11: Mechanism of action of laccase to generate reactive radicals that oxidise tyrosine or cysteine to form crosslinks in doughs (Joye et al., 2009)	16
Figure 2.12: Mechanisms of action of ascorbic acid, potassium bromate and azodicarbonamide as oxidising agents (Joye et al., 2009).....	18
Figure 2.13: Illustration of IR absorbance spectrum of an aqueous protein.....	20
Figure 2.14: An illustration of the protein secondary structure of (kafirin film treated with 150 mg hydrogen peroxide/100 g protein) as detected by FTIR at different wavelength (From author's own work).....	21

Figure 2.15: Conformational changes that occur during heating of protein as observed on DSC thermograms, (Malvern Instruments, 2016)	22
Figure 2.16: The transition changes that occurs as the temperatures of the two cells are gradually increased in DSC (Malvern Instruments, 2016)	23
Figure 4.1: Experimental design to determine the effect of oxidation by hydrogen peroxide on the buffer uptake of films, secondary structure, thermal properties, dough formation, dough tensile properties and surface morphology of films and doughs	27
Figure 5.1: Effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film colour and aqueous buffer uptake	41
Figure 5.2: SEM showing the effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film surface structure, before and after immersion in buffer	43
Figure 5.3: Effect of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film SDS-PAGE pattern	48
Figure 5.4: Effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film protein secondary structure.	51
Figure 5.5: Effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film T_g and enthalpy changes	55
Figure 5.6: Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough formation and extensibility	65
Figure 5.7: Stereomicroscopy showing the effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the fibril formation.....	67
Figure 5.8: SEM showing the effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough formation and extensibility	68
Figure 5.9: Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough protein secondary structure.....	71

Figure 6.1: Model to explain the increased water uptake by commercial zein films promoted by addition of hydrogen peroxide during casting and the decrease in aqueous buffer uptake when copper catalyst is added.....83

Figure 6.2: Model to explain the increased aqueous buffer uptake by total zein and total kafirin films promoted by addition of hydrogen peroxide during casting and the decrease in aqueous buffer uptake with the inclusion of copper catalyst.84

1. INTRODUCTION

Bread consumption is increasing in sub-Saharan Africa. The increased demand for bread has resulted in increased importation of wheat, the expense of which has impacted on local economies (Tadesse and Straziuso, 2012). When wheat flour is mixed into a dough with water, a gluten protein complex is formed, which has unique viscous flow and elastic (viscoelastic) properties (Joye et al., 2009). These viscoelastic properties are responsible for the wheat dough's cohesiveness, elasticity and gas-holding properties that give wheat bread its typical open cellular crumb structure and high loaf volume (Veraverbeke and Delcour, 2002).

To address the socio-economic issues of the need for bread products without expensive wheat importation, an alternative is needed particularly in sub-Saharan Africa. Maize and sorghum are the major cereal crops in the region and are well suited for the climate and soil conditions. However, producing bread from maize and sorghum is problematic as their flours do not exhibit the viscoelastic properties of wheat flour doughs (Andersson et al., 2011). Maize and sorghum are difficult to hydrate and more hydrophobic than gluten (Oom et al., 2008). Zein the prolamin protein of maize, can be used to make a dough, under certain conditions. Hydrating commercial zein (essentially α -zein) in water above its glass transition temperature (T_g) (~ 28 °C) has shown to form a gluten-like viscoelastic dough (Lawton, 1992). Recently, King et al. (2016) found that by casting total zein (comprised of α -, β - and γ -zein) into bioplastic films before hydrating in water a viscoelastic dough can be formed.

The rheological properties of wheat doughs are greatly altered by the formation of disulphide bonds in gluten (Wieser, 2007). Because of this, dough improvers such as oxidising and reducing enzymes have been commonly used to improve the bread making quality of low protein wheat flour (Joye et al., 2009). There has, however, been little research done on the influence of using oxidising agents on improving dough quality of zein and kafirin. The aim of this study was to determine the effects of treatment of zein and kafirin with oxidising agent hydrogen peroxide on their film properties and subsequent dough formation.

2. LITERATURE REVIEW

In this literature review the chemistry, structure and functionality of zein and kafirin prolamin proteins will be examined. Developments in modifying prolamin protein functionality will be discussed. Specifically, the focus will be on research into oxidation of gluten, zein and kafirin with the aim of improving film and dough functionalities. The principles of important analytical methods that can be used in assessing prolamin protein structure and functionality and information obtained from them will be reviewed.

2.1. Chemistry of prolamin proteins

2.1.1. Zein and kafirin

Zein is the prolamin protein of maize. Zein has a close homology with kafirin which is the prolamin of sorghum (DeRose et al., 1989), hence they shall be considered together. Zein and kafirin comprise four subclasses that are classified according to their relative mass, amino acid sequence and solubility as α -, β -, γ - and δ -prolamins. In sorghum the δ -prolamin has only been identified from DNA sequencing but not at protein level (Belton et al., 2006). The most abundant are the α -prolamins, where α -kafirin accounts for about 80 percent of total prolamin. Kafirin has a high content of proline and amide nitrogen from glutamine and the two amino acids account for more than 30 % of the total residues in total kafirin fractions (Belton et al., 2006). Alpha-zein is divided into two classes with apparent molecular weights of 19 kDa, referred to as Z19 or α 2-zein and 22 kDa referred to as Z22 or α 1-zein (Shewry and Tatham, 1990). Alpha-prolamins are rich in glutamine, proline, alanine and leucine and contain blocks of repeated sequences (Belton et al., 2006). Alpha-zein consists of oligomers and monomers that can be extracted in aqueous alcohol and under reducing conditions.

Beta-zein has no repetitive sequences and comprises bands with apparent molecular weight of 14 kDa to 18 kDa that can be separated into different polypeptides (Shewry and Tatham, 1990). It is rich in methionine and cysteine, with cysteine being involved in disulphide bonding. The γ -prolamins are rich in cysteine and constitute approximately 12 % of the prolamins (Shewry and Tatham, 1990). They are the most hydrophobic of the prolamins in cereals and link to other prolamins by disulphide bonds (Belton et al., 2006). All classes of zein and kafirin monomers are small proteins with molecular weight less than 28 kDa (Taylor et al., 2013). A major difference between kafirin and zein is in their γ -prolamins, γ -zein has two tandem repeats whereas γ -kafirin does not have repeat sequences (Duodu et al., 2003).

Also β -zein contains seven cysteine residues, whereas β -kafirin contains ten cysteine residues (Belton et al., 2006).

2.1.2. Structure of α -zein and α -kafirin

The secondary structures of zein and kafirin are not yet clear. However, there are several proposed models by different authors. Argos et al. (1982), developed a model based on circular dichroism analysis to describe the structure of α -zein (Figure 2.1). In their model, zein proteins have a tightly coiled α -helical structure resulting mainly from the repetitive sequence which folds up to form a cylindrical shape. The α -helical structures are linked together by hydrogen bonds formed between glutamine residues in the turn regions promoting packaging within the protein body. Arrangement of the polar and hydrophobic residues on the helical structure allow for chemical interactions causing the rod-shaped zein molecule to assemble in molecular planes. This model has also been used to describe kafirin, as native kafirin is predominantly an α -helical protein (Gao et al., 2005).

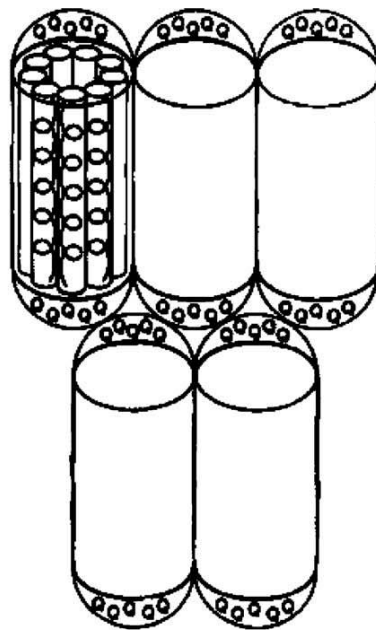


Figure 2.1: Proposed structural model for α -zein by Argos et al. (1982). The α -helices are arranged antiparallel to form a distorted cylinder. Hydrogen bond interactions around molecules in neighbouring planes is promoted by the glutamine residues (Q)

Garratt et al. (1993) developed a zein structural model which is similar to the model of Argos et al. (1982). Both models comprise three dimensional structure consisting of antiparallel packed helices. However, the models differ in the packing fashion. The model that was developed by Garratt et al. (1993), assumes that each repeat unit forms a single α -helix which are joined by glutamine turns or loops. This model is based on hydrophobic membrane propensities and tandem repeat units which form a helical wheel. It has a three dimensional structure with a series of antiparallel helices.

A different model was developed by Matsushima et al. (1997), using Small-angle X-ray scattering (SAXS) which indicated that a single tandem unit of α -zein has an average of 20 residues with α -zein having a majority of α -helical content and little β -sheet and 9 tandems, whereas Z22 had 10 tandems. The zein model more resembles an elongated structure than a globular structure, in which each tandem repeat unit forms a single α -helix joined by glutamine turns or loops (Figure 2.2). This results in the anti-parallel helices being linearly stacked in the direction perpendicular to helical axis.

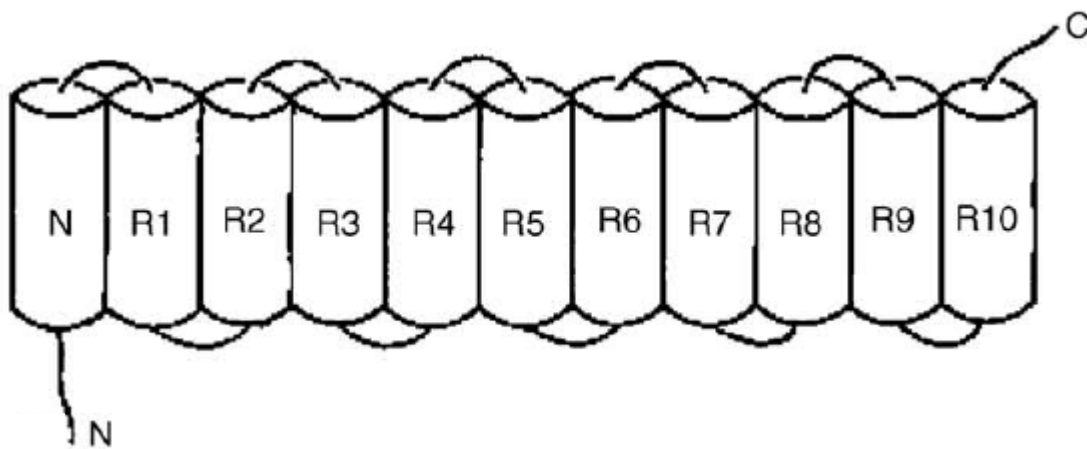


Figure 2.2: Proposed structural model for α -zein by Matsushima et al. (1997). Each of the tandem repeat units formed by a single α -helix is represented by the cylinder and glutamine turns or loops joined by the curve. On the c-axis the antiparallel helices of tandems repeats stack linearly in the direction perpendicular to the helical axis.

In the model developed by Bugs et al. (2004), α -zein has an extended hairpin structure, consisting of α -helix, β -sheet and turns folded back on itself (Figure 2.3). This model is based on the data obtained from circular dichroism, nuclear magnetic resonance and Fourier Transform infrared spectroscopy.

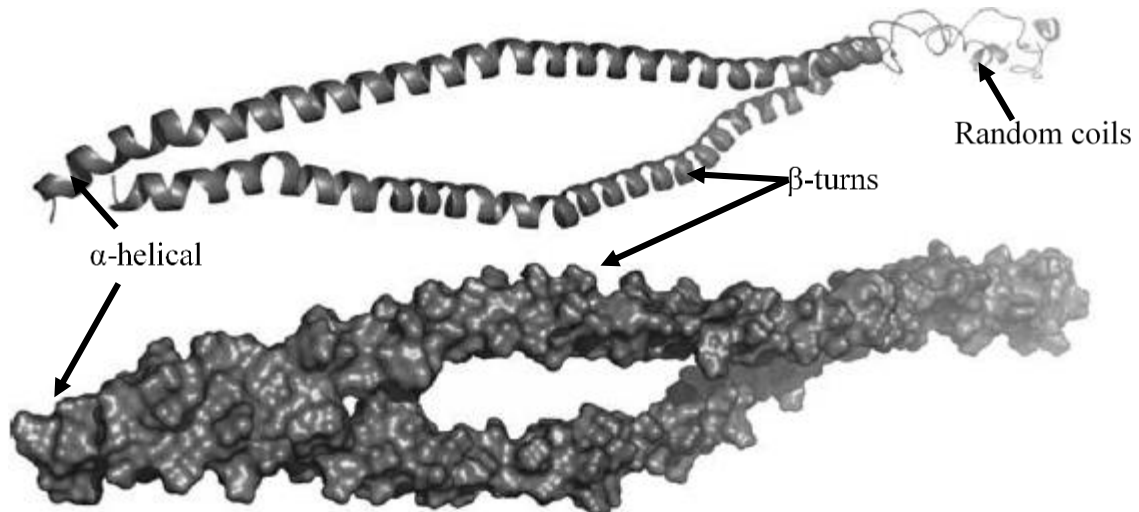


Figure 2.3: Proposed structural model for zein by Bugs et al. (2004) showing α -helices, β -turns and random coils.

2.1.3. Gluten

Gluten can be defined as the rubbery protein mass that is formed and remains when wheat flour is mixed with water then washed to remove starch granules and water soluble constituents (Wieser, 2007). Gluten gives the unique viscoelastic dough characteristics of wheat flour. Wheat gluten protein is divided into two main fractions, alcohol-soluble gliadins and the alcohol-insoluble glutenins. The two main gluten protein fractions can further be divided into three groups; the sulphur-rich, sulphur-poor and high molecular weight subunits (Figure 2.4) (Shewry and Tatham, 1990). The gliadin and glutenin fractions are characterised by high glutamine and proline contents and low contents of amino acids with charged side chains (Wieser, 2007).

The gliadin fraction is made up of proteins consisting of single polypeptide chains that are stabilised by hydrogen bonding and hydrophobic interactions and consist of four classes α -, γ - and ω -gliadins (Figure 2.4) (Wieser, 2007). The α/β -gliadins have six cysteine residues and the γ -gliadins have eight cysteine residues which are stabilised by intrachain disulphide bonds (Veraverbeke and Delcour, 2002). In contrast, ω -gliadin, which accounts for the majority of the total composition of the gliadins, belongs to the sulphur-poor subunits as it has fewer residues of cysteine and methionine (Shewry and Halford, 2002).

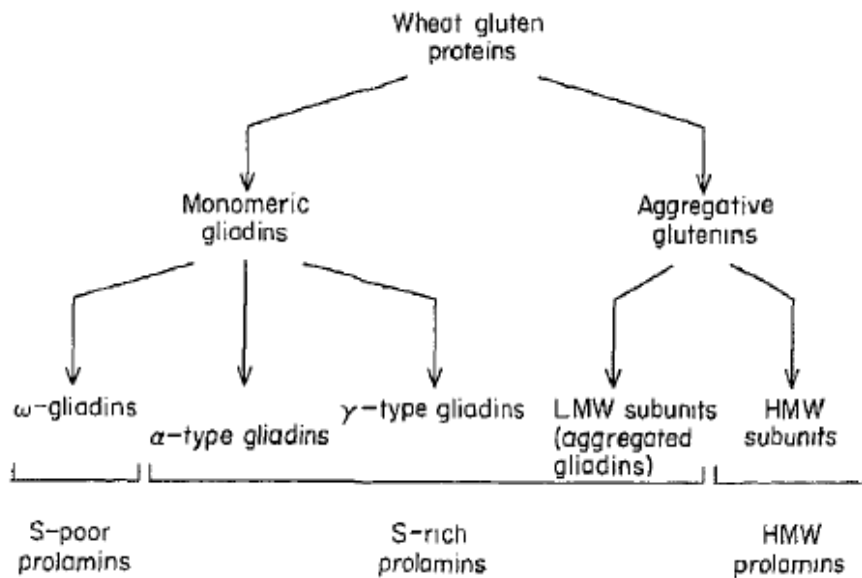


Figure 2.4: Classification of wheat gluten proteins (Shewry et al., 1986).

Glutenins are made up aggregated proteins linked by interchain disulphide bonds (Wieser, 2007). They are divided into low molecular weight (LMW) and high molecular weight (HMW) subunits. HMW glutenins are important in dough viscoelasticity but strangely they are relatively minor glutenin proteins.

The HMW subunits consist of a clear three structured domain with a non-repetitive domain at the *N*-terminal, a repetitive central domain at the *B*-terminal and a *C*-terminal domain with the *C*- and *N*-termini characterised by presence of cysteine residues (Wieser, 2007). The *C*-terminal comprises of exactly 42 amino acids, whereas, the *N*-terminal varies from 80 to 150 amino acids residues. The central domain has a repetitive sequences rich in glycine, glutamine and proline (Veraverbeke and Delcour, 2002).

Shewry et al. (2000) proposed a structural model for the HMW subunits. The central domain has an overlapping β -reverse turn that may form β -spiral secondary structure which contributes to gluten's elasticity, whereas the *C*- and *N*-terminal are predominantly α -helical (Figure 2.5). The cysteine residues in gliadins and glutenins play an important role in structure. They either form intrachain or interchain disulphide bonds (Veraverbeke and Delcour, 2002).

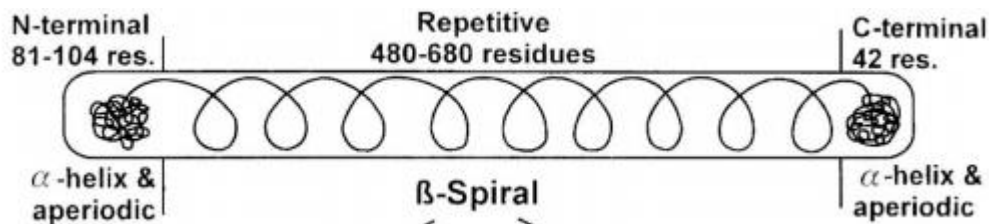


Figure 2.5: Proposed structure of HMW glutenin containing the repeat-rich β -spiral domain and α -helical ends (Shewry et al., 2000).

2.2. Functionality of prolamin proteins

Prolamin protein molecules can associate into different macropolymeric structures: films, nano- and microparticles, and most notably doughs, especially in the case of gluten and to a lesser extent zein (Taylor et al., 2016b). These structures are to some extent interchangeable. For example, Don et al. (2003) found that glutenin could exist in the form of microparticles, which they referred to as glutenin macropolymers (GMP) and King et al. (2016) found that total zein could form a dough after being cast into a film using glacial acetic acid.

2.2.1. Film formation

Zein and kafirin have excellent film forming properties. Their films are formed by controlled protein aggregation, in which, under suitable conditions, the proteins unfold and self-assemble into β -sheet rich structures (Taylor et al., 2013). The α -helical structure of α -zein initially unfolds and transforms into β -sheets (Figure 2.6A and 2.6B) which pack in opposite directions side by side (Figure 2.6C and 2.6D) into a long ribbon stabilised by hydrophobic interactions (Figure 2.6E) (Wang and Padua, 2012). The β -sheets formed will grow into nanospheres (Figure 2.6F), hence forming a three dimensional column. The cysteine-rich β -

and γ -prolamins have the ability to form disulphide linkages between or within the polypeptides, which serve to stabilise bioplastic materials (Taylor et al., 2013).

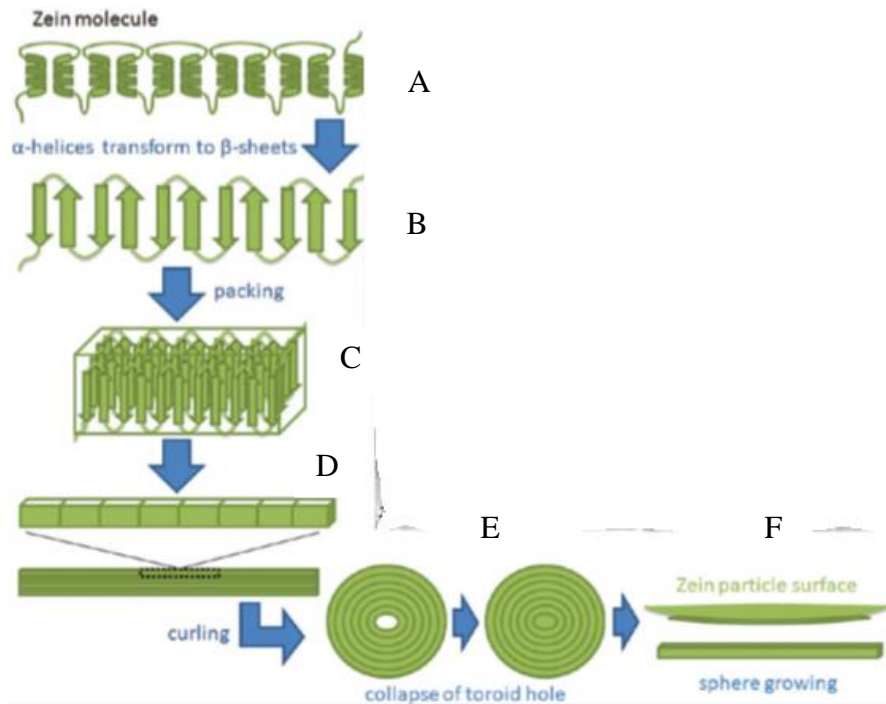


Figure 2.6: Possible mechanism for zein self-assembly from single molecules to nanospheres (Wang and Padua, 2012). A- α -helical structure of zein molecule, B- unfolding of α -helical structure into β -sheet, C- packing of antiparallel β -sheet, D- formation of a long ribbon, E- curling into a ring driven by hydrophobic interactions, F- formation of nanospheres due to addition of β -sheet rich structures.

2.2.2. Dough forming properties

Wheat gluten has the ability to form strong, cohesive and viscoelastic dough that retains gas to produce light aerated products such as bread (Wieser, 2007), whereas zein does not form such doughs at ambient temperature. Understanding the structural similarities and differences between viscoelastic polymers formed by gluten, zein and kafirin may give clues to how zein and kafirin can be manipulated to enable gluten-like viscoelastic properties.

Factors that contribute to this unique behaviour of gluten are related to its amino acid composition, structure and polymeric nature (Bansal et al., 2012). Zein and kafirin have a high helical content whereas glutenin is mainly β -spiral (Shewry et al., 2000).

In the grain, zein and kafirin are embedded in protein bodies which contributes to them being unavailable for dough fibril formation, whereas the gluten proteins are present in continuous phase (Bansal et al., 2012). Another important difference between zein and gluten viscoelastic systems is that zein lacks the slow dough relaxation time typical of wheat gluten (Mejia et al., 2007). This results in rapid relaxation and instability of the extended β -sheet alignment that occurs upon formation of a viscoelastic zein polymer. Gliadins contribute to extensibility and viscosity of dough, whereas glutenins are responsible for dough elasticity and strength (Veraverbeke and Delcour, 2002). Gluten elasticity is mediated by non-covalent interactions mainly intra- and inter-hydrogen bonds (Belton, 1999). Gliadins weaken the interactions between glutenin chains, hence acting as a plasticiser which increases dough viscosity (Veraverbeke and Delcour, 2002).

The HMW-glutenin subunits play a critical role in wheat gluten dough formation (Belton, 1999). There are important changes that occur in the secondary structure of HMW-subunits during dough formation. Gluten when mixed with water at ambient temperature undergoes glass transition which turns the protein into a polymer which favours the interaction with other gluten polymers (Mejia et al., 2007). In a doughy state, the β -sheet structure of HMW-subunits increases at the expense of β -turn and α -helical structures (Wellner et al., 2005). The HMW-subunit sulphhydryl groups promote disulphide-sulphydryl interchange that involves breakage and reformation of disulphide bonds which contribute to dough strength and stability. Hydrophobic interactions from the hydrophobic side chains together with hydrogen bonding also play an important role in stabilising gluten structure and in creation of the rheological properties and leavening of wheat dough (Veraverbeke and Delcour, 2002). The β -sheet structure and disulphide bridge formation seem to contribute to the stabilisation of the polymers during dough formation (Belton, 2005).

Several different theories have been developed to account for the elasticity of glutenins. Dough strength and elasticity are associated with the development of gluten's polymeric matrix, comprised of high molecular weight (HMW) and low molecular weight (LMW) subunits linked through intermolecular disulphide bonds (Shewry et al., 2000). Belton (1999), developed the 'loop and train theory' to explain the elasticity of glutenin. During dough formation, there is hydrogen bonding between glutenin chains forming a dense extended mass 'Trains' [Figure 2.7A (a)]. Hydration results in water-protein interactions forming loops [Figure 2.7A (b)]. There is a balance between protein-water interactions and protein-protein

interactions, hence the interchain hydrogen bonds will not be broken instantly [Figure 2.7A (c)]. Under stress, the water molecules are squeezed out of the trains, resulting in deformation of the loops as the glutenin molecules stretch out (**Figure 7B**).

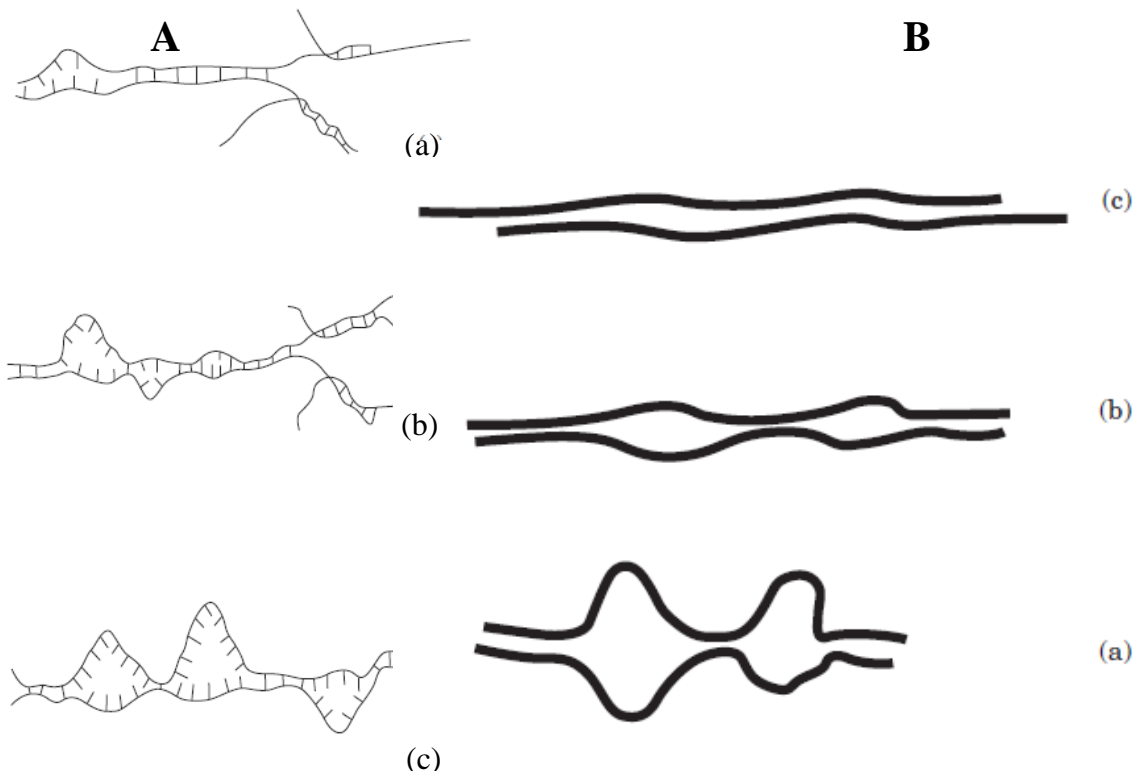


Figure 2.7: Effect of hydration on loop and train behaviour and the deformation of polymers during stretching to form an extended network (Belton, 1999). 5A (a) - hydrogen bonds are mainly interchain, 5A (b) -formation of loops due to hydration, 5A (c) - high levels of hydration forming many loops still maintaining interchain bonds. 5B (a) - a balance between protein-water interactions and protein-protein interactions, 5B (b) - slight extension resulting in formation of loops, 5B(c)-large extension loops are flattened and the interchain hydrogen bonds are broken so that chains slip over each other.

It has also been postulated that dityrosine bonds are involved in gluten elasticity within the HMW-subunits of glutenin (Tilley et al., 2001). HMW-subunits contain approximately 3-5% of tyrosine. Tyrosine residues are thought to form inter- and intra-molecular cross-linking of tyrosine amino acid residues in polypeptide chains (Figure 2.8). Bonds formed during mixing and baking of wheat dough contribute to the gluten network structure.

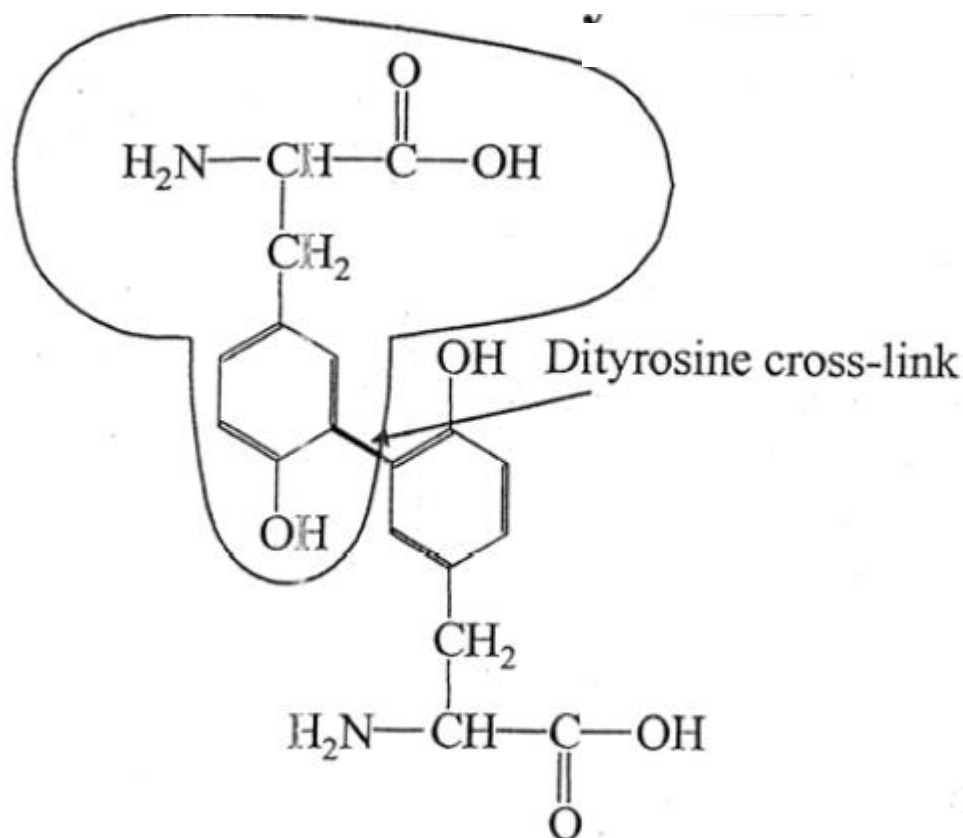


Figure 2.8: Structure of dityrosine crosslink which brings together glutenin molecules (Tilley et al., 2001).

In contribution to these theories for the molecular bonds of glutenin elasticity, Hamer et al. (2000) postulated that there is a strong correlation between the elastic properties of dough and the formation of Glutenin Macro Polymers (GMP). GMP is a gel formed by physical and chemical interactions. The GMP theory concludes that glutenin elasticity is at particle level (Figure 2.9) (Don et al., 2003). GMP consists of large structures of HMW and LMW subunits. Differences in rheological properties of GMP dispersions were investigated across several wheat flours of variable bread making qualities and were considered to be related to differences in HMW glutenin compositions across wheat varieties.

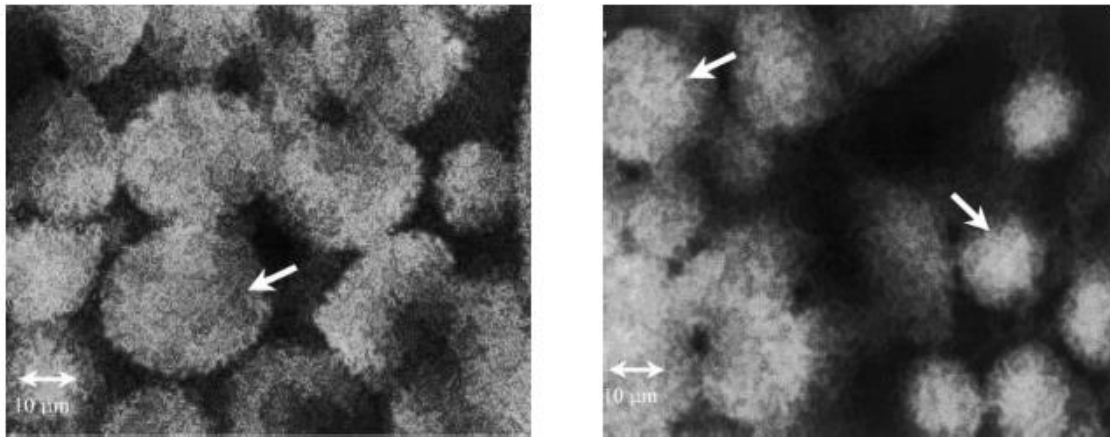


Figure 2.9: Confocal laser scanning micrography showing large spherical shaped GMPs indicated by white arrows (Don et al., 2003).

2.3. Research into the modification of the functional properties of zein and kafirin protein films by oxidation

There is intensified research and development to improve the functional properties of prolamin proteins using various types of modifications. Modification of protein film properties is necessary to improve the film functionality over a diverse range of applications requirements (Byaruhanga et al., 2005). The most promising way to improve cohesion, rigidity, mechanical strength and the barrier properties of films to water is through cross-linking. This is possible because of the presence of different functional groups of proteins, which have the ability to interact with a wide range of active compounds (Wittaya, 2012). Protein cross-linking can involve the formation of covalent bonds between polypeptide chains within a protein (intramolecular crosslinks) or between proteins (intermolecular crosslinks) (Gerrard, 2002). Modification methods that have been used to improve functional properties of protein can be categorised as: chemical, physical and enzymatic (Taylor et al., 2013).

2.3.1. Physical treatment

Physical treatment can result in protein cross-linking through the formation of disulphide and hydrophobic bonds (Taylor et al., 2013). Physical treatment includes microwave heating, heating and irradiation. Microwave heating of wet kafirin films resulted in increased tensile strength, tensile strength at break and decreased strains of the films due to the formation of intermolecular crosslinks (Byaruhanga et al., 2005).

Ionizing irradiation has been found to improve barrier and mechanical properties of protein-based films (Zhang and Mittal, 2010). Proteins can be converted to higher molecular weight aggregates through the formation of inter-protein cross-linking reactions and the formation of disulphide bonds (Wittaya, 2012). The hydroxyl and superoxide anion radicals that are generated by irradiation affect proteins by causing conformational changes and oxidation of amino acids. Lee et al (2003) found that γ -irradiation treatment on zein films resulted in increased elongation. The γ -irradiated zein films had reduced water vapour permeability and tensile strength. Soliman et al. (2009) also found that γ -irradiated zein films had reduced water vapour permeability compared to the control. This was attributed to disulphide bond formation.

2.3.2. Chemical cross-linking

Natural compounds such as amino acids can be used as non-toxic cross-linkers. Cysteine has been used to crosslink prolamin bioplastic by forming disulphide bonds (Wittaya, 2012). Cysteine residues play a vital role in prolamin films as they are involved in the formation of inter- and intramolecular disulphide bonds (Hernández-Muñoz et al., 2004ab). Cysteine could cleave intrachain disulphide bonds, hence promoting molecular rearrangements through disulphide-sulphydryl exchange reactions. These authors found that gliadin films cross-linked with cysteine had increased glass transition temperature, increased tensile strength, were less extensible and the films maintained their integrity in water compared to the non-treated gliadin films. This was due to a more rigid structure through formation of both inter- and intramolecular disulphide bonding.

2.3.3. Enzymatic treatment

Oxidising enzymes such as lipoxygenase, peroxidase and laccase (diphenol oxidase) can be used to improve the functional properties of protein bioplastic films by catalysing inter- and intra-cross linking (Wittaya, 2012). Apart from disulphide bonds, dityrosine bonds can also be formed (Tilley et al., 2001). Horseradish peroxidase acts on proteins in the presence of hydrogen peroxide it catalyses oxidation of tyrosine leading to formation of dityrosine (Stuchell and Krochta, 1994).

2.4. Research into modification of gluten and other prolamin functionality by oxidising agents

Many different modification methods have been used to improve gluten and gluten-free dough functionality, including the use of hydrocolloids, acids and bases, cross-linking by oxidising agents and enzymes, proteolysis, disulphide bond reduction and high pressure treatment (Taylor et al., 2016b). Protein oxidation can be defined as the covalent modification induced by a wide array of oxidising agents (Shacter, 2000). The focus of this present research is on oxidative treatments. This is primarily because gluten functionality is strongly influenced by disulphide bonding (Wieser, 2007). Gluten proteins are known to form different types of crosslinks and the only crosslink that has been found between HMW and LMW-GS is a disulphide bond. The gluten network is formed by polymerisation primarily through oxidation of the thiol groups and depolymerisation when there is interchange of the thiol group to sulphhydryl bond (Weegels et al., 1997). This results in the formation of a polymeric structure that gives an optimal gluten network, which confers dough machinability, good gas retention and fine bread crumb structure (Belton, 2005).

2.4.1. Application of oxidative cross-linking enzymes

Oxidising agents can be used to improve the dough quality of wheat flours with low protein content which naturally give weak doughs (Vemulapalli and Hoseneey, 1998). The main effect of oxidants in doughs appears to be the oxidation of free sulphhydryl groups to disulphide bonds which minimises sulphhydryl-disulphide interchange reactions and promotes cross-links between the protein molecules (Lagrain et al., 2007).

Exogenous oxidising enzymes such as glucose oxidase, laccase, tyrosinase, soybean lipoxygenase, glutathione oxidase and sulphhydryl oxidase can be used to improve dough strength and stability (Selinheimo et al., 2007). Laccase, tyrosinase, and peroxidase directly, and glucose oxidase and pyranose oxidase indirectly, through hydrogen peroxide production, catalyse oxidation of phenolic compounds present in both proteins and polysaccharides to form disulphide or dityrosine bonds (Selinheimo et al., 2007).

Glucose oxidase is the preferred alternative to chemical oxidising agents for bread improvement (Bonet et al., 2006). Glucose oxidase in the presence of oxygen catalyses the oxidation of β -D-glucose to D-gluconic acid and hydrogen peroxide (Figure 2.10a) (Joye et al., 2009). The hydrogen peroxide produced then oxidises the thiol groups of cysteine residues of gluten to form disulphide bonds (Figure 2.10b) (Rasiah et al., 2005) or dityrosine crosslinks (Tilley et al., 2001). The dityrosine is formed when hydrogen peroxide produced by glucose oxidase acts as a substrate for peroxidase in wheat flour which catalyses cross-linking through phenolic linkages (Figure 2.10c) (Rodriguez-Mateos et al., 2006). Glucose oxidase treatment of gluten dough can result in dry dough indicating that the active ingredient responsible for improving dough is hydrogen peroxide (Vemulapalli and Hoseneey, 1998).

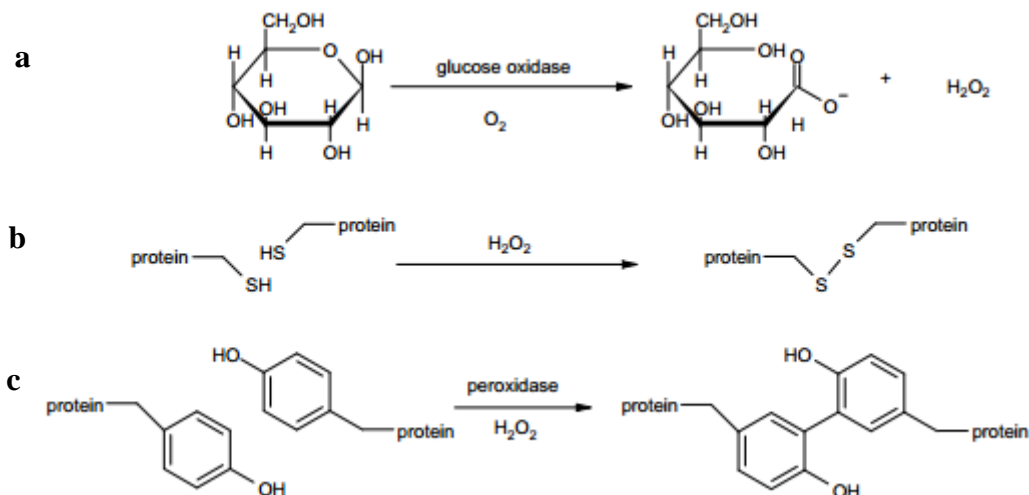


Figure 2.10: Mechanism of glucose oxidase action in gluten dough forming disulphide bonds or dityrosine bonds (Vemulapalli and Hoseneey, 1998).

Addition of glucose oxidase to wheat dough has also been found to result in increased dough water absorption, increased stability and higher loaf volume (Dagdelen and Gocmen, 2007). Gujral and Rosell (2004) investigated the effect of glucose oxidase on rice flour. It resulted in increased loaf volume and decreased crumb hardness. Glucose oxidase oxidises free sulphhydryl units giving disulphides linkages, resulting in stronger dough with resistance to mechanical shock and large loaf volume. On the contrary, glucose oxidase was found to have no effect on buckwheat bread (Renzetti and Arendt, 2009). This might have been due to low levels of sulphhydryl groups as most of the cysteine residues existed as disulphide linkages.

Laccase is a copper ion containing oxidising enzyme. Oxidation by laccase is through one-electron removal to form reactive radicals that can undergo further cross-linking of monomers and it can only oxidise tyrosine and cysteine (Figure 2.11) (Selinheimo et al., 2007). These authors treated wheat dough with laccase and there was improved loaf volume, increased resistance to deformation and decreased extensibility due to the formation of disulphide and dityrosine bonds by oxidation. Renzetti and Arendt (2009), also found that there was a significant increase in bread volume and crumb structure of oat bread treated with laccase.

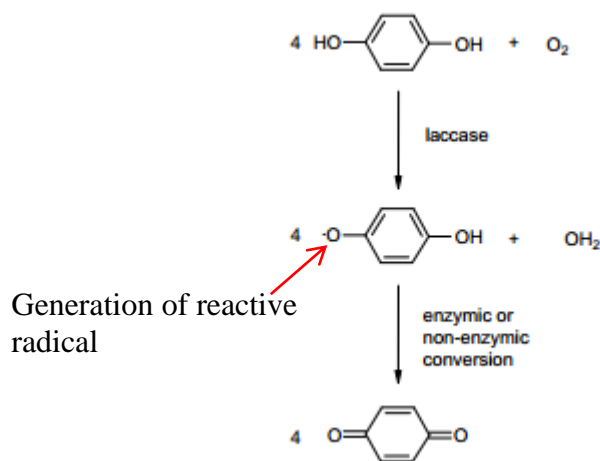


Figure 2.11: Mechanism of action of laccase to generate reactive radicals that oxidise tyrosine or cysteine to form crosslinks in doughs (Joye et al., 2009)

2.4.2. Application of chemical oxidising agents

Chemical oxidising agents that have been added to flour to improve the quality of gluten dough include potassium bromate, iodate, ascorbic acid, peroxides and azodicarbonamide (Joye et al., 2009). Ascorbic acid, also known as vitamin C, has a well-studied strengthening effect on dough that leads to a higher expansion of the dough (Faccio et al., 2012). The mechanism of the action of ascorbic acid in dough is not fully understood. However, it is postulated that ascorbic acid is firstly oxidised by the endogenous ascorbic acid oxidase or atmospheric oxygen to its stable dehydroascorbic acid, which is believed to be the actual dough improver (Figure 2.12a) (Faccio et al., 2012). Dehydroascorbic acid then oxidises sulphhydryl groups of cysteine to form intermolecular disulphide bonds (Nakamura and Kurata, 1997). As a result of the formation of disulphide bonds in the gluten network, ascorbic acid decreases the extensibility of the dough and helps increase its elasticity (Rouille et al., 2000).

Potassium bromate is a slow oxidising agent that is usually added at the last stage of fermentation (Lagrain et al., 2007). It is used to strengthen dough and hence it also improves loaf volume, crumb structure and texture (Joye et al., 2009). The mechanism of action of bromate is that it oxidises free sulphhydryl groups to give disulphide bonds and bromide (Figure 2.12b). Addition of potassium bromate to wheat flour was found to increase dough relaxation time (Dong and Hosney, 1995). The increase of relaxation time might be caused by a decrease of sulphhydryl groups in the dough. Increased relaxation is important as it allows relaxation of the stresses introduced during mixing, continued hydration of flour components, and redistribution of water (Dong and Hosney, 1995).

Another oxidising agent that is faster than potassium bromate is potassium iodate. Potassium iodate similarly oxidises free sulphhydryl groups to give disulphide bonds and iodide (Joye et al., 2009). However, when added in excess iodate has negative effects on dough as it favours oxidation of sulphhydryl groups. Azodicarbonamide, another oxidising agent also improves dityrosine crosslinks in bread (Tilley et al., 2001). Azodicarbonamide is a fast oxidant that quickly oxidises free sulphhydryl groups and itself is reduced to biurea (Figure 2.12C) (Joye et al., 2009). Azodicarbonamide improves gas retention and machinability of the dough, loaf structure and crumb texture.

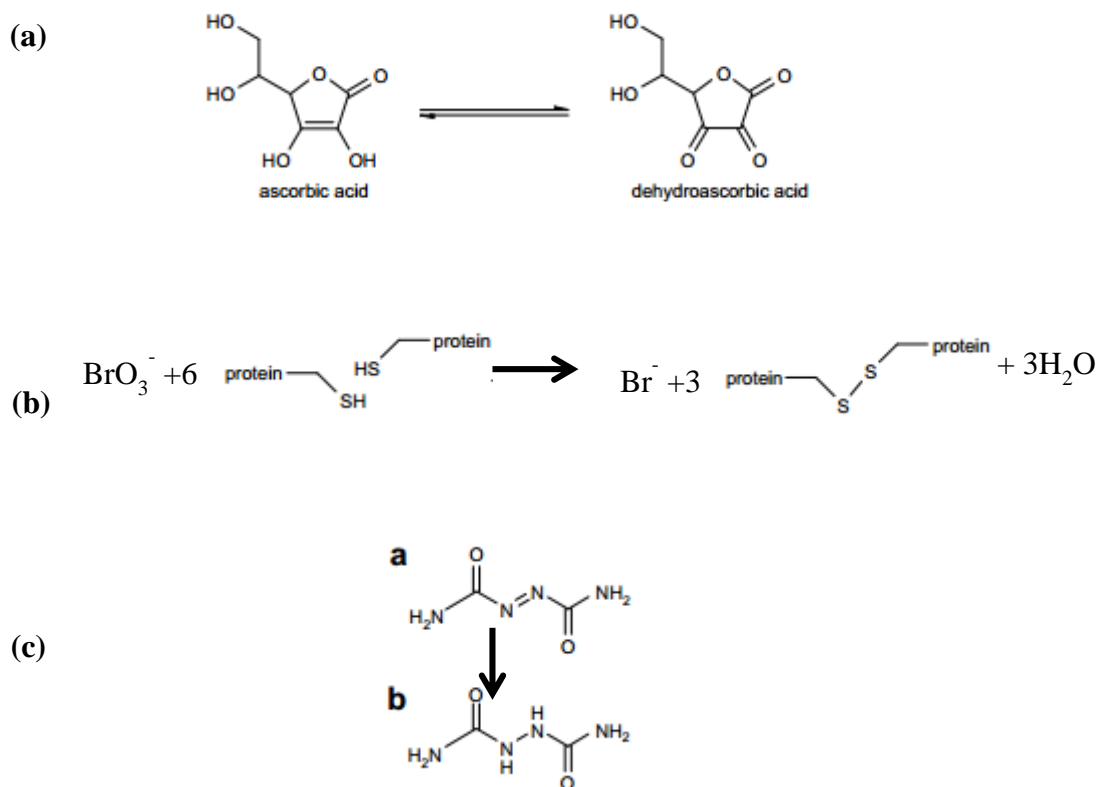


Figure 2.12: Mechanisms of action of ascorbic acid, potassium bromate and azodicarbonamide as oxidising agents (Joye et al., 2009). (a) dehydroascorbic acid, a relatively stable oxidant, (b) disulphide bond formation by bromate, (c) formation of biurea after oxidation by azodicarbonamide and the reduced biurea after oxidation

2.5.Improvement of zein and kafirin dough functionality by oxidation

Very little research has been done on the improvement of dough functionality of zein and kafirin by oxidation. Taylor et al. (2016a) investigated zein dough formation with hydrogen peroxide and horseradish peroxidase. They found that preparation of zein doughs with hydrogen peroxide changed their rheological properties. The zein dough prepared with hydrogen peroxide were soft, highly extensible even below the T_g of zein, however, the zein doughs prepared with horseradish peroxidase were slightly extensible. (Note- some of this particular research is discussed in this dissertation).

Renzetti and Arendt (2009) found that sorghum and maize dough modified with glucose oxidase has improved dough viscosity and produce higher volume and firm bread which did not collapse at the top. Glucose oxidase can promote protein polymerisation and networking (Renzetti and Arendt, 2009). Also, Smith et al. (2014) found that zein mixed with sodium iodate resulted in a dough that was softer and more extensible than the control.

2.6. Protein Structure Analytical Techniques for α -zein, total zein and total kafirin preparations

2.6.1. Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectroscopy is used in the characterisation of protein secondary structure. The principle of FTIR spectroscopy is based on the fact that bonds and groups of bonds vibrate at characteristic frequencies (Jung, 2000). Infrared radiation is used to excite vibration transitions in organic molecules. Proteins have fractions of secondary structural elements (α -helices and β -sheets) associated with a characteristic hydrogen bonding pattern between those groups. Each type of secondary structure gives rise to different frequencies at which amide bond vibrations occur, forming the basis of protein structure analysis (Figure 2.13) (Elkhalifa et al., 2009).

The Amide I absorption in the region 1600 and 1700 cm^{-1} contains contributions from C=O (approximately 80%). Amide II absorption in the region 1480 to 1575 cm^{-1} arises from N-H bending (approximately 60%) and a minor stretching vibration from C-N (approximately 40%) (Figure 2.14). The C=O, N-H and C-N stretch are present on every amino acid (Kötting et al., 2012). The Amide I region relies on only one of the amide functional groups in contrast to Amide II, and the Amide I absorption is often used as a good indicator to determine the protein secondary structure (Jackson and Mantsch, 1995).

In FTIR analysis, a spot on the specimen is subjected to a modulated infrared beam. The specimen's transmittance and reflectance of the infrared energy at different frequencies is translated into an infrared absorption plot consisting of reverse peaks (Jackson and Mantsch, 1995). Different secondary structures have different levels of hydrogen bonding with different strength, which results in specific electron densities in the C=O group, resulting in different Amide I frequencies. If β -sheets are present in the protein secondary structure, the strength of intramolecular β -sheets are weaker than the intermolecular hydrogen bonds, hence there is an increase in Amide I wavenumber (Figure 2.14) (Jackson and Mantsch, 1995).

The resulting FTIR spectral pattern is then analysed and matched with known signatures of identified materials (Vanecek and Poruba, 2002). FTIR is an accurate technique in that the reaction occurs in a one stage system $A \rightarrow B$. This results in the bands representing only the changes which occurred during the reaction as the vibrations from the bands that did not change during reaction will counteract each other ($B-A$) (insert Figure 2.13) (Kötting et al., 2012).

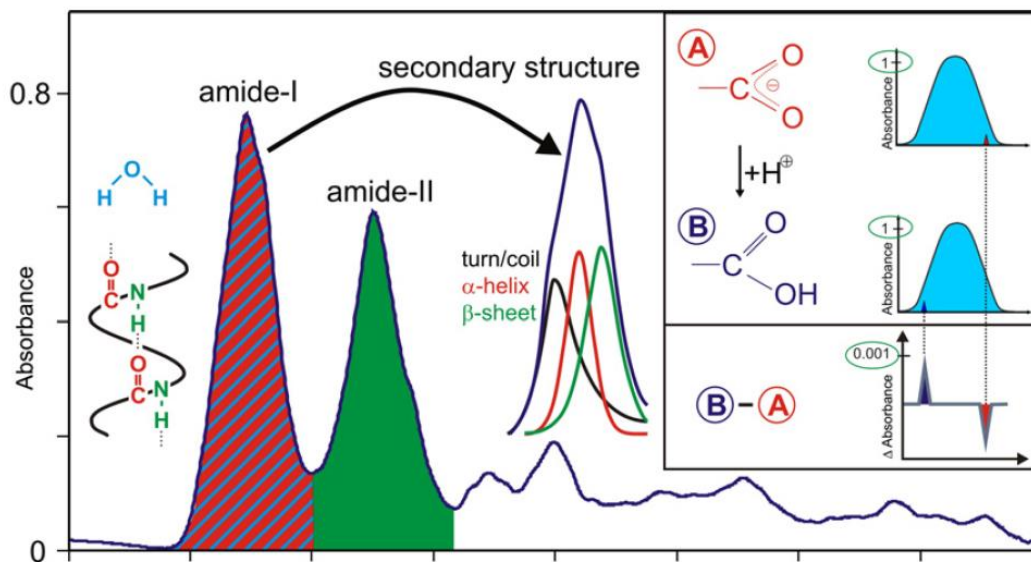


Figure 2.13: Illustration of IR absorbance spectrum of an aqueous protein. Red shows amide I C=O stretching vibration, blue shows the vibration of water and green indicates amide II C-n and N-H stretch vibrations. Top part of the insert shows two absorption spectra of a protein with protonation of a carboxyl group. Bottom part shows how the unchanged parts of the protein are cancelled out to show absorbance that only occurred during the reaction (Kötting et al., 2012).

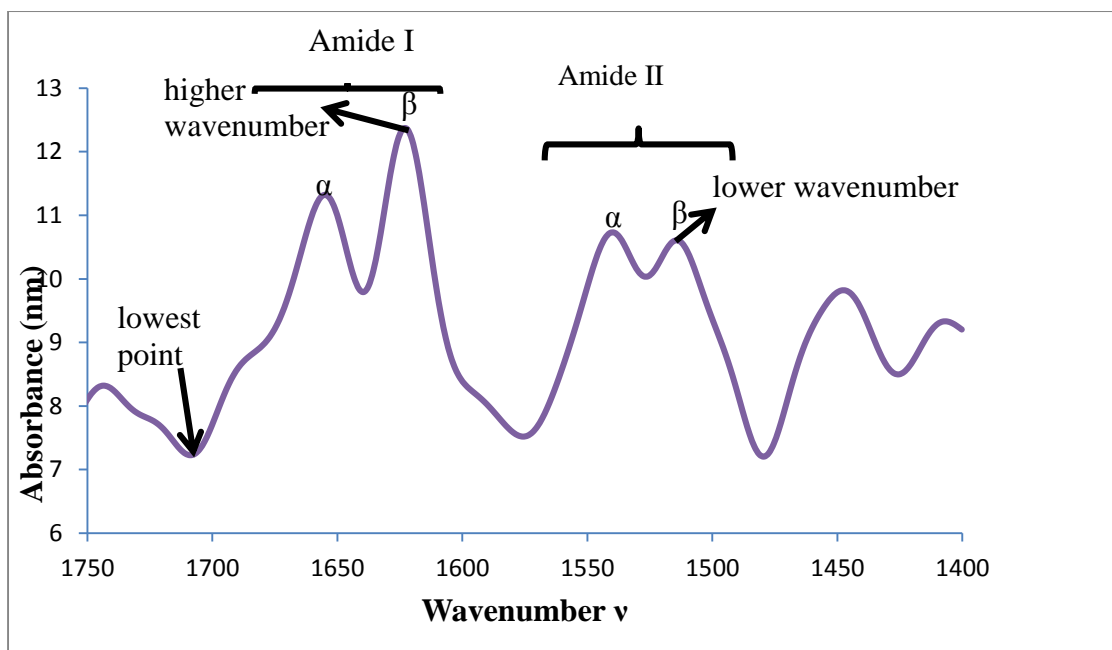


Figure 2.14: an illustration of the protein secondary structure of (kafirin film treated with 150 mg hydrogen peroxide/100 g protein) as detected by FTIR at different wavelength (From author's own work).

2.6.2. Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is a technique that allows the study of thermal changes in proteins for example protein unfolding, melting point, glass transition, carbohydrates and lipids (Höhne et al., 2013). A DSC system consists of two cells, a reference and a sample cell. The two cells are maintained at the same temperature. The reference sample is empty and the sample cell is filled with the sample. During analysis they are heated at a constant rate. Absorption of heat by the test that occurs when a substance for example, a protein unfolds causes a temperature difference (ΔT) between the cells, resulting in a thermal gradient. The difference between the two can be attributed to the presence of the substance under analysis (Sanchez-Ruiz, 1995). Data analysis is highly dependent on the assumption that both the reference and sample cells are constantly and accurately maintained at equal temperatures. With proteins, DSC measures the heat capacity of the unfolding of proteins as a function of heat (Lopez and Makhatadze, 2002). Protein folding or unfolding is accompanied by heat effects. It provides a direct estimate of the modes of protein unfolding. Proteins have well-defined structures that undergo thermally-induced conformational changes. Structural rearrangements result in the absorption of heat caused by the redistribution of non-covalent bonds (Figure 2.15).

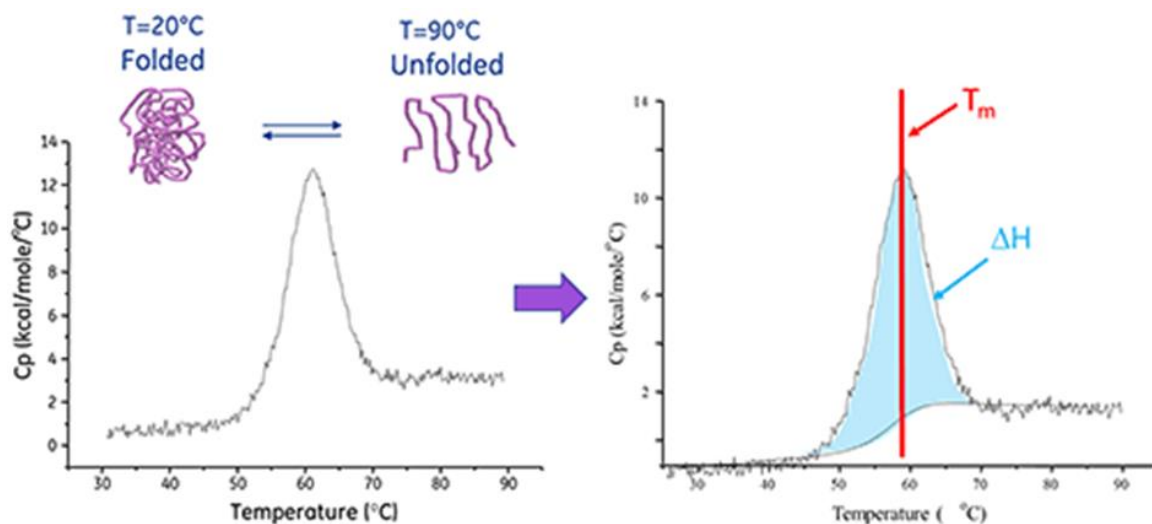


Figure 2.15: Conformational changes that occur during heating of protein as observed on DSC thermograms, (Malvern Instruments, 2016)

T_m-thermal transition midpoint

ΔH-enthalpy

DSC provides the heat capacity profile in the form of a differential thermogram (Spink, 2008). The area under the heat capacity profile represents the enthalpy of unfolding, transition temperature and the difference in heat capacities (ΔC_p) associated with the transition from the native and the unfolded states, the maximum thermal transition midpoint T_m , which defines the temperature dependence of enthalpy and entropy function and thus the temperature dependence of protein stability (Lopez and Makhatadze, 2002). The maximum heat capacity also shows the glass transition state (T_g). Glass transition is an important physical property of polymers as it helps to explain physiochemical behaviours of food polymers (Spink, 2008). The sharpness of the heat capacity profile also shows another characteristic, the effective enthalpy of transition referred to as the Van't Hoff enthalpy (ΔH_{vH}) (Figure 2.16). The ratio of enthalpy of unfolding and Van't Hoff enthalpy provides information about the mode of observed transition. A ratio equal to 1 indicates that the observed transition is a two state proceeding from the native to the unfolded state without a lot of intermediates (Lopez and Makhatadze, 2002). A ratio greater than 1 indicates that the transition is more complicated.

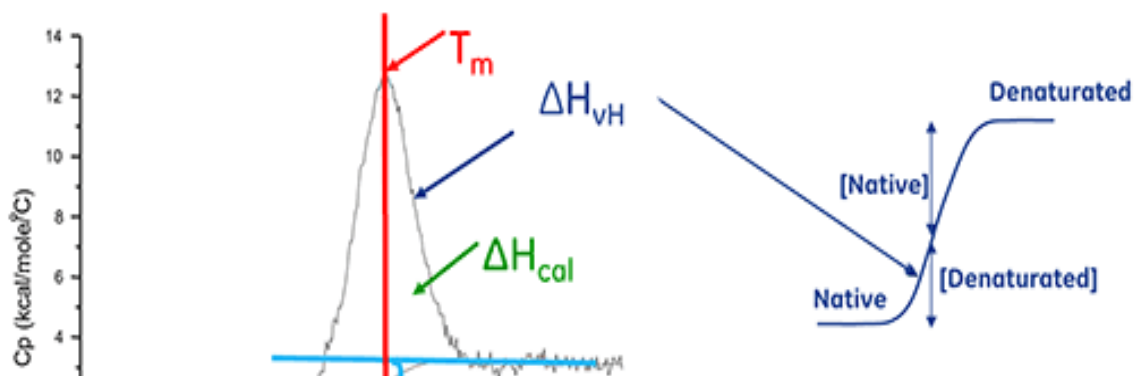


Figure 2.16: The transition changes that occurs as the temperatures of the two cells are gradually increased in DSC (Malvern Instruments, 2016)

T_m -thermal transition midpoint

ΔH_{vH} -the Van't Hoff enthalpy

ΔH_{cal} -calorimetric enthalpy

ΔC_p -heat capacity change

2.7. Conclusions

Disulphide and dityrosine bonds play a vital role in the functional properties of prolamin protein films. Cross-linking of zein and kafirin films increases their tensile strength, film stability of the films in water and reduces water vapour permeability. Zein has the ability to form viscoelastic doughs if it is held and manipulated above its hydrated glass transition temperature. This literature review provides evidence that oxidation is a promising approach to modify the functional properties zein and kafirin films. Cross-linking of maize and sorghum with enzymes has shown to give bread that resembles wheat bread with improved specific loaf volume, crumb structure and delays staling. Hydrogen peroxide liberated from glucose oxidase has shown to oxidise gluten dough. However, there has been little research on the improvement of dough functionality of commercial zein, total zein and kafirin using hydrogen peroxide as an oxidising agent. Thus, the purpose of this research is to investigate the effect of hydrogen peroxide treatment on zein and kafirin films with the aim of modifying their functional properties.

3. HYPOTHESES AND OBJECTIVES

3.1. General Aim

To improve the functional properties of commercial zein, total zein and total kafirin films and doughs by oxidation

3.2. Hypotheses

Hypothesis 1

Treating commercial zein, total zein and total kafirin with hydrogen peroxide will promote protein cross-linking through disulphide bonding.

Disulphide bonds are formed through oxidative coupling of two cysteine residues in which an oxidising agent accepts hydrogen atoms from the thiol groups of the cysteine residues, producing disulphide cross-links (Gerrard, 2002). Commercial zein is essentially α -zein and there are few cysteine residues to form disulphide bonds (Belton et al., 2006). However, total zein and total kafirin in addition to α -, contain β - and γ -subunits which are rich in cysteine (Shewry and Tatham, 1990) and are available to form disulphide cross-linkages.

Hypothesis 2

Oxidation of commercial zein, total zein and total kafirin films with hydrogen peroxide will reduce film water uptake.

Increased polymer chain interaction and a high degree of cross-linking tend to reduce the water vapour permeability of bioplastic films (Byaruhanga et al., 2005). Heat induced disulphide cross-linking of kafirin (Byaruhanga et al., 2007) and zein films (Guo et al., 2012) has been shown to reduce water vapour permeability of cast films. Oxidative induced disulphide cross-linking should have a similar effect to heat on cast films water vapour permeability.

Hypothesis 3

Treating commercial zein, total zein and total kafirin films during casting with hydrogen peroxide will enable the films to form cohesive, extensible doughs when hydrated with distilled water above their T_g .

Oxidising agents can be used to improve the dough quality of wheat flours with low protein which naturally give weak doughs (Vemulapalli and Hosney, 1998). Formation of disulphide bonds contribute to the stabilisation of the polymers during dough formation (Belton, 2005). Hydrogen peroxide is an oxidising agent and promotes the formation of disulphide cross-linkages and as such should promote dough formation of hydrated films.

3.3. Objectives

Objective 1

To determine the effect of hydrogen peroxide treatment on the formation of disulphide bonds in commercial zein, total zein and total kafirin films.

Objective 2

To determine the effect of hydrogen peroxide treatment on the functional properties of commercial zein, total zein and total kafirin films with the aim of improving the film water barrier properties.

Objective 3

To determine if forming commercial zein, total zein and total kafirin into films will enable the formation of cohesive, extensible doughs when the films are hydrated above the T_g of zein or kafirin.

Objective 4

To determine the effect of treating commercial zein, total zein and total kafirin films with hydrogen peroxide on the dough forming properties of the films.

4. EXPERIMENTAL

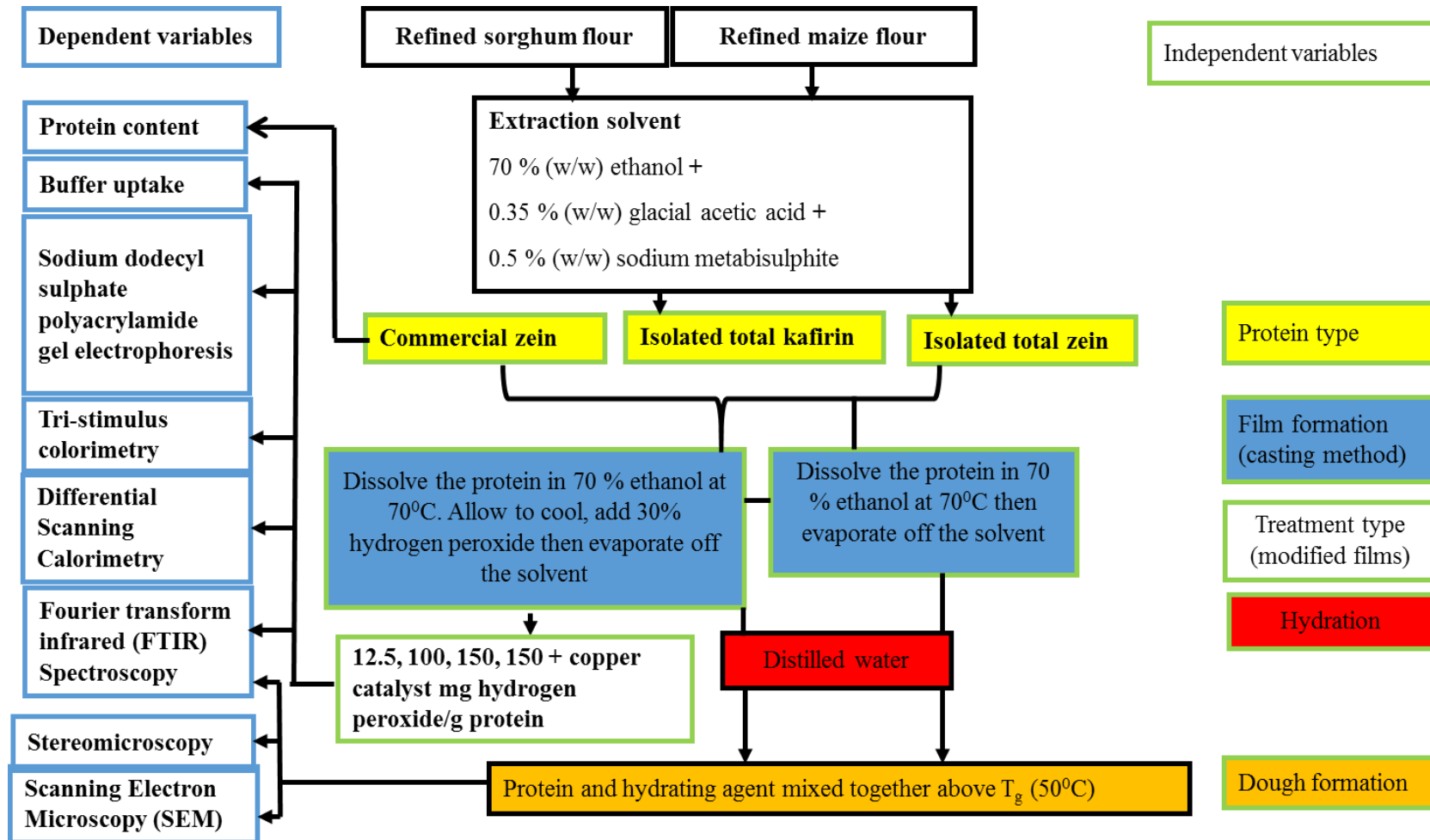


Figure 4.1: Experimental design to determine the effect of oxidation by hydrogen peroxide on the buffer uptake of films, secondary structure, thermal properties, dough formation, dough tensile properties and surface morphology of films and doughs

5. RESEARCH

Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the functional properties of the films

ABSTRACT

Protein bioplastics have inferior functional properties compared to synthetic plastics due to their protein complexity. Modification of protein bioplastic film properties is necessary to improve film functionality over a diverse range of applications. This study showed that oxidising commercial zein, total zein and total kafirin with hydrogen peroxide increased the aqueous buffer uptake by the films as well as film physical stability. However, addition of a copper catalyst with hydrogen peroxide reduced film buffer uptake. Film colour intensity increased with increase in hydrogen peroxide concentration. SEM indicated that preparation of films with hydrogen peroxide promotes protein interaction giving a compact even structure to the films. SDS-PAGE revealed disulphide bond polymerisation of total zein and total kafirin films prepared with hydrogen peroxide. However, there was no evidence of polymerisation of commercial zein films prepared with hydrogen peroxide. This is due to the essential absence of cysteine rich β - and γ -subclasses in commercial zein. The changes in the properties of commercial zein films with the inclusion of hydrogen peroxide were probably due to hydroxylation. FTIR indicated similarities in protein secondary structure of commercial zein and total zein films and an increase in α -helical conformation of commercial zein and total zein films with preparation with hydrogen peroxide. In contrast, there was an increase in β -sheet conformation of total kafirin films with hydrogen peroxide preparation. It is concluded that cross-linking of commercial zein, total zein and total kafirin films with hydrogen peroxide promotes the protein to protein interaction resulting in a continuous film network.

5.1.Introduction

Plant proteins are a good potential resource for the production of bioplastics. Bioplastic films have been made from cereal plant proteins (prolamins) such as wheat gluten (Hernández-Muñoz, et al., 2005), maize zein (Cuq et al., 1998), sorghum kafirin (Buffo et al., 1997) and barley hordein (Xia et al., 2011). However, there are very few commercial prolamins based bioplastics due to their inferior functional properties compared to the synthetic polymer plastics, in particular their poor barrier properties. The inferior functional properties of prolamins bioplastics are in part due to the complexity of prolamins, which consist of several classes of prolamins protein types (Taylor et al., 2013).

Zein, the prolamins protein of maize, has the more suitable properties for bioplastic films than wheat gluten due to its better barrier properties (Cuq et al., 1998). Furthermore, kafirin is more hydrophobic and cross-linked giving better film barrier properties than zein (Taylor et al., 2013). Zein films are formed through the development of hydrophobic, hydrogen and limited disulphide bonding between zein chains (Janjarasskul and Krochta, 2010). The tight bonding of the protein molecules through hydrogen bonds, hydrophobic interactions and the polar groups of amino acids results in a brittle material in the dry state, which, however, has high moisture absorption.

Many researchers have investigated methods to improve prolamins bioplastic functionality and some success has been achieved. Amino acids have a large number of reactive side chains (NH-, COO-, S-) that are susceptible to physical, chemical or enzymatic modification, covalent cross linking of polypeptide chains and provide a means for improving the physical integrity of these networks and consequently enhancing the functionality of the resulting films (Balaguer et al., 2011). Protein film functional properties such as tensile strength and stability when immersed in water can be modified by physical treatments such as heat treatment (Byaruhanga et al., 2007), chemical treatments such as glutaraldehyde cross-linking (Anyango et al., 2011) or enzymatic treatments such as by transglutaminase (Larré et al., 2000). Thin kafirin films treated with glutaraldehyde were found to show reduced water uptake (Anyango et al., 2011). Emmambux et al. (2004), found that kafirin films cross-linked with sorghum condensed tannins and tannic acid had reduced water vapour permeability compared to the unmodified film.

A closely related avenue which requires investigation is the use of oxidising agents to improve zein and kafirin film properties. Total zein and total kafirin have a high content of cysteine residues found in β - and γ -subclasses. The cysteine residues are easily oxidised to form disulphide bonds (Byaruhanga et al., 2006). Such covalent cross-linking of polypeptide chains is potentially a valuable mechanism for increasing the strength of tri-dimensional protein networks and providing greater physical integrity in aqueous media. Disulphide or sulphhydryl group-containing proteins can undergo sulphhydryl-disulphide interchange reactions under specific environmental conditions, introducing new intra and intermolecular cross-links (Taylor et al., 2013). Therefore the objective of this study was to improve the functional properties of commercial zein, total zein and total kafirin films by oxidation using hydrogen peroxide.

5.2. Materials and Methods

5.2.1. Materials

In this research the following protein preparations were used: commercial zein, total zein and total kafirin (where total indicates that the protein preparations contained all the prolamin classes in the proportions that are present in the grain). Total zein and total kafirin were extracted in the laboratory. Total zein was extracted from refined white maize flour (Spar Super maize meal, Pride Milling, Vorsterskroon, South Africa) that had been milled to a maximum of 0.5 mm particle size using a laboratory hammer mill. Kafirin was extracted from a mixture of two very similar tannin-free white tan-plant sorghum varieties PANNAR PEX 202 and 606. The grain was first decorticated to an 80% extraction rate using an abrasive dehuller. Commercial zein predominately (α -zein) was obtained from Sigma-Aldrich, Johannesburg, South Africa (Sigma product code 3625).

5.2.2. Methods

5.2.2.1. Extraction of total zein and total kafirin

Total zein and total kafirin were extracted as described by Da Silva and Taylor (2004), with slight modifications. The extraction solvent consisted of 70% (w/w) absolute ethanol containing 0.5% (w/w) sodium metabisulphite and 0.35% (w/w) acetic acid. The milled grain (500 g) was weighed into a plastic bucket (5 L) with a tight fitting lid containing a central small hole for the paddle rod of an electric stirrer. The extractant was added and extraction was carried out at $70 \pm 0.2^\circ\text{C}$ for 1 h with vigorous stirring. The supernatant containing the denatured prolamin protein was recovered by centrifugation at 1000 g for 5 min at 25°C . The extracted milled maize or sorghum was washed with a further 500 g of the extractant and centrifuged. The combined clear supernatant was removed by decanting into a shallow stainless steel tray and the ethanol was allowed to evaporate in a fume hood at ambient temperature ($\leq 25^\circ\text{C}$). On evaporation of the ethanol a thin yellow layer was formed over the protein precipitate which was broken up to allow further evaporation.

The protein precipitate was washed using chilled distilled water ($\sim 8^\circ\text{C}$) and filtered under vacuum using a Buchner funnel through two layers of Whatman No. 4 filter paper and air dried at ambient temperature in a fume hood. The recovered total zein or total kafirin preparations were milled into a fine powder with an air-cooled knife-type laboratory mill,

iodine test was done to check for starch contamination. The recovered total zein and total kafirin were stored in airtight zip-lock bags at 10°C.

5.2.2.2. Analyses

5.2.2.2.1. Protein and moisture contents

The protein content of the protein preparations was determined by a Dumas combustion method, Approved Method 46-30 (AOAC International, 2000) using a conversion factor ($N \times 6.25$).

The moisture content of the protein preparations was determined by Approved Method 44-15A, Air oven method (AACC International, 2000).

5.2.2.2.2. Film formation

Zein and total kafirin films were prepared using a casting method as described by Emmambux et al. (2004), but without adding a plasticiser. Prolamin protein preparation (equivalent to 1.2 g 100% pure protein) was mixed with 8.8 g 70% (w/w) aqueous ethanol in a 100 ml Erlenmeyer flask. A magnetic stirrer was added to the flask and the flask closed with foil. The weight of the flask and contents was recorded. The flask was heated to 70°C on a stirrer hotplate. After 10 minutes had elapsed, the flask was reweighed and absolute ethanol was added to the flask to replace that lost by evaporation. The film forming solution was allowed to cool to ambient temperature. Then 30% (w/w) hydrogen peroxide was added and stirred with a magnetic stirrer for 1 min (to allow interaction of protein with hydrogen peroxide). Concentrations of hydrogen peroxide investigated were 0, 12.5, 100 and 150, 150+ copper catalyst mg hydrogen peroxide/100 g protein. Aliquots of the film forming solution (3 g) were weighed into rectangular silicone trays (69 × 28 mm) and gently swirled to coat the bottom of the trays. The trays were placed on a level surface (checked with a spirit level) in an oven (not forced draft) at 50°C overnight. The dry films were carefully removed from the trays and stored in airtight zip lock bags at 10°C.

5.2.2.2.3. Colour determination

The colour of the films subjected to the various concentrations of hydrogen peroxide was determined by tri-stimulus colorimetry method using a Minolta colorimeter (Chromameter-CR-400, Konica Minolta Sensing, Tokyo, Japan). The colour meter was calibrated against a standard white reference tile (L = 90.67, a = - 0.8, b = 1.7) prior to analysis. Crushed film from each treatment was placed in a petri dish and the colour treatment was analysed.

5.2.2.2.4. Film aqueous buffer uptake

Films (69 x 28 mm) were immersed in a closed container containing 0.2 M sodium phosphate buffer (pH 6.8) for 12 h in an oven at 39°C. After 12 h, the films were removed from the buffer and the surface water carefully blotted with a paper towel before photographing and weighing. Percentage aqueous buffer uptake of the films was calculated as follows:

$$\% \text{ aqueous buffer uptake} = \frac{(\text{mass of film after immersion} - \text{initial dry mass of film})}{\text{initial dry mass of film}} \times 100$$

5.2.2.2.5. Scanning Electron Microscopy

The surface morphology of the films was analysed using a Zeiss Gemini Ultra plus Field Emission SEM (Zeiss, Oberkochen, Germany). The films were dried in a desiccator containing silica gel for 72 h. Film (5mm x 5 mm) was cut using a sharp knife and mounted on aluminium stubs using double-sided carbon tape and coated with carbon using Emitech K950X carbon coater (Ashford, UK) and viewed at 1 kV.

5.2.2.2.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The film compositions were characterised by SDS-PAGE, performed under reducing and non-reducing conditions, essentially as described by Byaruhanga et al. (2006). For reducing conditions 3% (w/v) mercaptoethanol and SDS were used in the denaturation buffer. Gels were loaded with constant protein using 10 µl of protein solution and an Invitrogen Mark 12 (Invitrogen Life Technologies, Carlsbad, CA) Unstained protein standard was used for

molecular weight estimation. Electrophoresis was carried out on 4-12% Bis-Tris (BT) gradient gels (NuPAGE Novex Gels), mounted on a X Cell SureLock™ vertical electrophoresis unit (Invitrogen, Life Technologies). Electrophoresis was conducted at a constant voltage of 200 V, 80 mA and 10 Watts. The gels were stained with Coomassie Brilliant Blue R250 overnight. After destaining, the band pattern was recorded using a flatbed scanner.

5.2.2.2.7. Fourier Transform Infrared Spectroscopy (FTIR)

Protein secondary structure of films was determined by FTIR spectroscopy essentially as described by Anyango et al. (2011). Films were first crushed using a pestle and mortar and dried in a desiccator containing silica gel for 72 h. Samples were analysed with a Vertex 70v FT-IR spectrophotometer (Bruker Optik, Ettlingen, Germany) using 64 scans, 8 cm⁻¹ bandwidth and an interval of 1 cm⁻¹ in the Attenuated Total Reflectance (ATR) mode over the frequency range 400-4000 cm⁻¹. Four replicates were analysed for each treatment. The FTIR spectra were Fourier deconvoluted using Lorentzian filter with a resolution enhancement factor of 2 and 8 cm⁻¹ bandwidth. A Rubberband correction of 64 baseline points was used for correcting FTIR absorbance spectra. The equation below was used to determine the relative proportions of α -helical conformations with an assigned peak height at ≈ 1650 cm⁻¹ and the β -conformation with a peak height at ≈ 1620 cm⁻¹. The relative proportions of α -helical and β -sheet conformation as well as the α/β ratio were calculated:

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

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

$$\alpha:\beta \text{ ratio} = \frac{\% \alpha\text{-helical conformation}}{\% \beta\text{-sheet conformation}}$$

Where:

Abs α -helix peak = absorbance at ≈ 1650 cm⁻¹ after baseline correction for commercial zein, total zein and total kafirin films

Abs β -sheet peak = Absorbance at ≈ 1620 cm⁻¹ after baseline correction for commercial zein, total zein and total kafirin films

5.2.2.2.8. Differential Scanning Calorimetry (DSC)

DSC analysis of films was performed by the method of Luo et al. (2011), with some modification, using a Mettler Toledo HP DSC827^e Differential Scanning Calorimeter (Schwerzenbach, Switzerland). Films were first crushed using a pestle and mortar then dried in a desiccator containing silica gel for 72 h. Dry crushed film (15 mg) was accurately weighed into 100 μ L DSC aluminium pan and hermetically sealed. The instrument was calibrated using pure indium and a sealed empty pan was used as reference. DSC scans were performed at a heating rate of 10°C/min from 25-280°C under nitrogen (40 bar) and a 60 ml/min flow. The data were analysed using Mettler Toledo STAR^e software version 9.20.

5.2.2.2.9. Statistical analysis

All experiments were carried out in triplicate unless otherwise stated. IBM SPSS software was used to analyse data. Effects of commercial zein, total zein and total kafirin films preparation with hydrogen peroxide on the film functional properties were analysed by one-way analysis of variance (ANOVA) and compared at 95 % confidence level using Fisher's Least Significance Difference test (LSD).

5.3.Results and discussion

5.3.1. Moisture and protein contents of maize and sorghum meals, zein and kafirin protein preparations

The commercial zein had the lowest moisture content of the prolamin protein preparations (Table 5.1). Commercial zein is thermally dried (Anderson and Lamsal, 2011). Hence, its low moisture content, whereas the laboratory prepared total zein and total kafirin were air-dried at ambient temperature. Commercial zein also had the highest protein content. The higher protein content of commercial zein was probably because it was extracted from corn gluten which is high in protein (minimum of 60% protein dry basis) (Shukla and Cheryan, 2001). The lower protein contents of the total zein and total kafirin was because they were extracted from partially refined cereal grain which is much lower in protein and hence the preparations contained higher levels of impurities. The maize and sorghum meals had protein contents of approx. 9% and 10%, respectively. This is in agreement with the literature as the protein content of maize ranges from 8 to 11% (Mesfin and Shimelis, 2013) and sorghum ranges from 7 to 15% (Dicko et al., 2006).

Table 5.1: Moisture content, protein content of commercial zein, maize and sorghum meal and extracted total zein and kafirin preparations

Material	Moisture content (g/100 g as is)	Protein content (g/100 g as is)	Protein content (g/100 g dry basis)
Commercial zein	2.40 ^a ± 0.28 ¹	89.76 ^d ± 0.23	92.44 ^e ± 0.31
Total zein preparation	8.08 ^d ± 0.08	82.89 ^c ± 0.48	88.63 ^d ± 1.05
Total kafirin preparation	7.49 ^b ± 0.22	69.22 ^b ± 0.58	74.83 ^c ± 0.26
Refined maize meal	7.85 ^c ± 0.17	8.35 ^a ± 0.12	8.95 ^a ± 0.15
Refined sorghum meal	13.00 ^e ± 0.70	8.49 ^a ± 0.03	10.01 ^b ± 0.04

^a Mean values in columns with different superscript letters differ significantly (p < 0.05)

¹ Mean values ± standard deviation n=3

5.3.2. Effects of preparation of commercial zein, total zein and kafirin films with hydrogen peroxide on prolamin solubility, and film physical quality and colour

When the film-forming solutions were left to cool at ambient temperature it could be seen that commercial zein had completely dissolved in ethanol to give a clear dark yellow solution (Table 5.2). In contrast, the total zein and total kafirin only partially dissolved in aqueous ethanol, presumably due to their more hydrophobic nature (Duodu et al., 2003). However, addition of hydrogen peroxide to the film-forming solution improved the solubility of the total zein and total kafirin. This was probably due to the formation of new intermolecular bonds that favoured the solubilisation of the proteins (for detailed explanation see section 6.2).

The surfaces of commercial zein films prepared without hydrogen peroxide were rough and had cracks and voids in them (Figures 5.1 and 5.2A). SEM revealed the presence of fibrils in the cracks (Figure 5.2, boxed in red). The presence of fibrils can be an indication of the coexistence of the proteins in the form of films and fibrils. Fibrils are an indication of dough formation (Sly et al., 2014). The inclusion of hydrogen peroxide when preparing the commercial zein films resulted in films that were smooth with no voids but had loose flakes (Figure 5.2B, C). However, as the concentration of hydrogen peroxide increased the film matrix became more compact, dense, smooth and flaked when observed by SEM (Figure 5.2D, E).

Total zein films prepared without hydrogen peroxide contained white spots (Figure 5.2F). The white spots were possibly starch particles, which could have been contaminants in the protein preparation. However, they were in too tiny proportion to be detected by the iodine test, which was performed directly after extraction of total zein. The surfaces of the films were rough (Figure 5.1B). Inclusion of hydrogen peroxide during film preparation resulted in films that were smoother (Figure 5.1B). SEM showed presence of fibrils in total zein films prepared with hydrogen peroxide, as with those in commercial zein films prepared without hydrogen peroxide (Figure 5.2, boxed in red). This may be explained by the observed better solubility of total zein when prepared with hydrogen peroxide.

Total zein films prepared with hydrogen peroxide plus copper catalyst were very rough and folded (Figures 5.1B and 5.2J). This was probably as a result of extensive intermolecular bonding. Total kafirin films prepared without hydrogen peroxide were rough on the surface (Figures 5.1C and 5.2K). This was because kafirin did not completely dissolve in the aqueous ethanol, as is explained above. Inclusion of hydrogen peroxide in total kafirin film-forming solution resulted in films that were compact, dense and smoother. This was possibly due to increased protein to protein interaction resulting in a compact matrix.

Furthermore, film preparation with hydrogen peroxide greatly improved the flexibility of all the films. This is in agreement with the findings by Parris and Coffin (1997) of an increase in zein film flexibility with cross-linking with various reagents. Protein-based films are brittle due to their molecular complexity (Arcan and Yemenicioğlu, 2011). Yang et al. (1996), explained that zein films in particular are brittle when they are prepared in alcohol solutions which does not permit the protein molecules to unfold, resulting in bulky side groups preventing the polypeptides from associating and aligning in an ordered film matrix. Addition of hydrogen peroxide probably promotes crosslinking of the proteins (see section 5.3.4 Figure 5.3 and section 6.2).

Commercial zein, total zein and total kafirin film preparation with hydrogen peroxide also increased the lightness (high L) of the films (Table 5.2, Figure 5.1). The increase in film lightness with hydrogen peroxide was probably due to bleaching of the pigments. Hydrogen peroxide is an oxidant of many organic compounds and has been used as a bleaching agent in cereal flours (Delcros et al., 1998). Total kafirin films were darker and more red (lower L and higher a) than the commercial zein and total zein films. This was due to the presence of proanthocyanin-type pigments in extracted kafirin (Da Silva and Taylor, 2005).

Table 5.2: Effects of preparation of commercial zein, total zein and kafirin films with hydrogen peroxide on protein solubilisation and film physical quality and colour

Film formulation (mg H ₂ O ₂ /g protein)	Prolamin solubility at ambient temperature ^b	Film physical quality ^c			Film colour	
		Surface texture	Flexibility	L* (white-black)	a* (red-green)	b* (yellow-blue)
Commercial zein						
0	Clear dark yellow liquid	4	5	78.8 ^a ±1.3 ¹	-2.00 ^a ±0.7	41.8 ^c ±1.3
12.5	Clear yellow liquid	1	5	81.1 ^b ±0.4	-6.90 ^d ±0.2	44.9 ^{cd} ±2.0
100	Very watery clear yellow liquid	1	2	82.8 ^c ±0.1	-7.30 ^d ±0.1	46.8 ^d ±0.4
150	Very watery, clear faint yellow liquid	1	1	87.5 ^d ±0.7	-5.80 ^c ±0.3	30.2 ^b ±3.4
150 + copper catalyst	Turned white cloudy on adding copper catalyst and hydrogen peroxide	1	1	88.5 ^e ±0.1	-4.90 ^b ±0.2	22.8 ^a ±1.7
Total zein						
0	Clear white liquid, particles of undissolved protein	5	5	82.3 ^a ±0.3	-3.10 ^{bc} ±0.0	16.1 ^c ±0.5
12.5	Watery clear white liquid, tiny particles of undissolved total zein	4	5	85.6 ^{bc} ±0.5	-3.20 ^c ±0.0	11.5 ^b ±0.7
100	Watery clear white liquid, all protein dissolved	4	3	87.2 ^c ±0.1	-2.80 ^{abc} ±0.1	8.2 ^a ±0.3
150	Watery clear white liquid, all protein dissolved	2	2	87.2 ^c ±0.6	-2.80 ^{abc} ±0.1	7.9 ^a ±0.5
150 + copper catalyst	Very watery almost clear liquid with all protein dissolved	2	2	87.8 ^{cd} ±0.1	-3.10 ^{bc} ±0.0	7.9 ^a ±0.0
Total kafirin						
0	Very viscous solution, tiny particles of undissolved protein	5	5	69.7 ^a ±0.6	2.80 ^c ±0.2	13.7 ^b ±0.6
12.5	Cream, viscous solution, more viscous on cooling	5	3	83.5 ^b ±0.1	-1.50 ^a ±0.2	19.4 ^d ±0.1
100	Slightly white and viscous liquid	1	2	83.7 ^b ±0.1	-1.80 ^{ab} ±0.1	16.7 ^{cd} ±1.9
150	Less viscous white liquid	1	2	84.2 ^{bc} ±0.2	-2.30 ^b ±0.1	16.6 ^{cd} ±0.8
150 + copper catalyst	Turned cloudy on adding copper catalyst and hydrogen peroxide	1	2	87.8 ^d ±0.4	-2.30 ^b ±0.1	11.7 ^a ±0.7

^a Mean values in columns with different superscript letters differ significantly (p< 0.05) ¹Mean values ± standard deviation n=3

^b The solubility changes of the prolamin casting solvent was observed visually upon adding hydrogen peroxide and stirring at ambient temperature

^c Surface texture (1-smooth 5-rough) Flexibility (1- flexible 5-brittle)

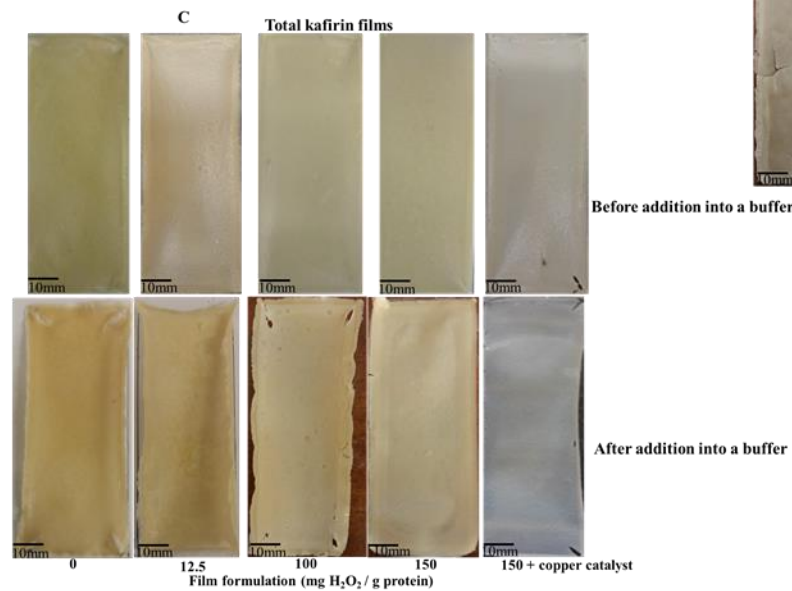
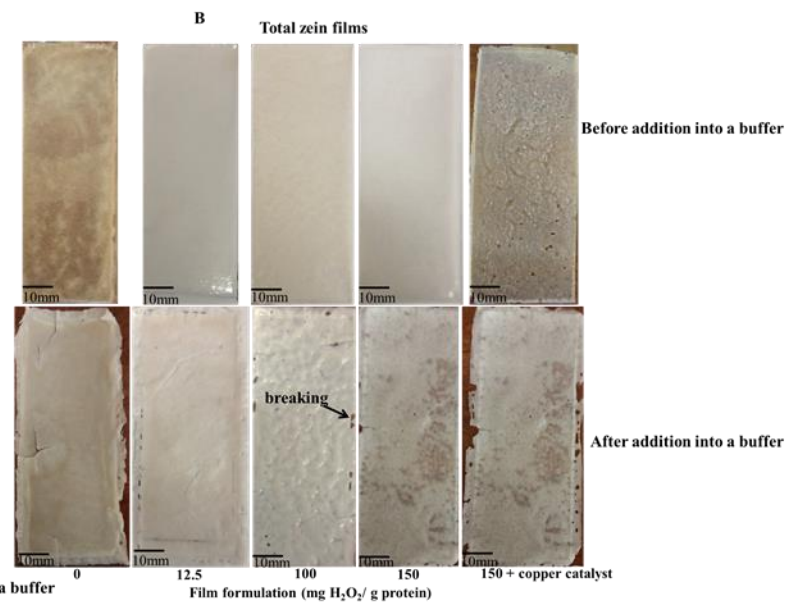
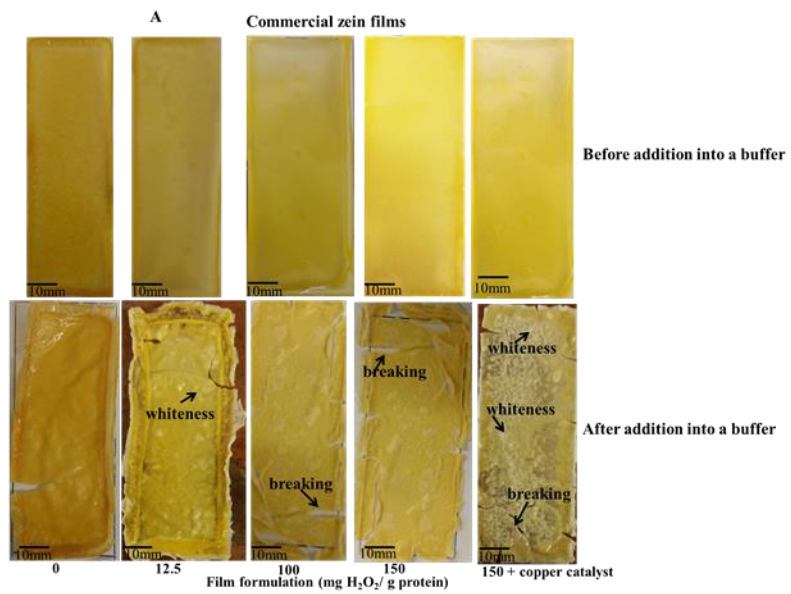
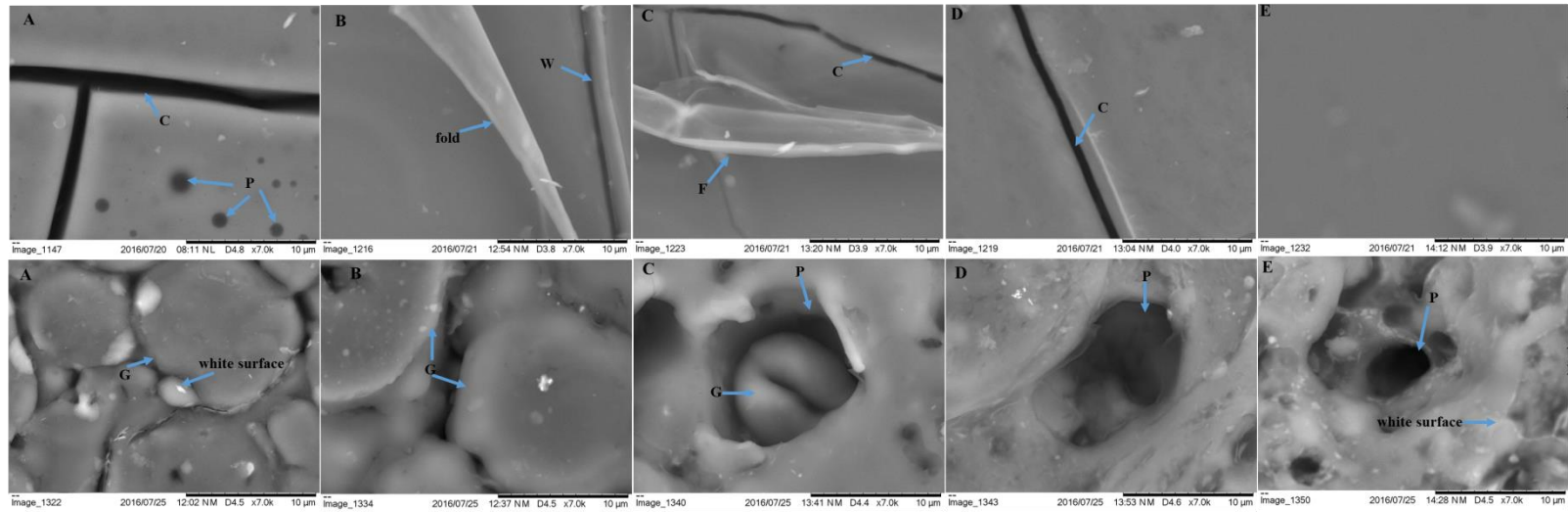


Figure 5.1: Effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film colour and water uptake

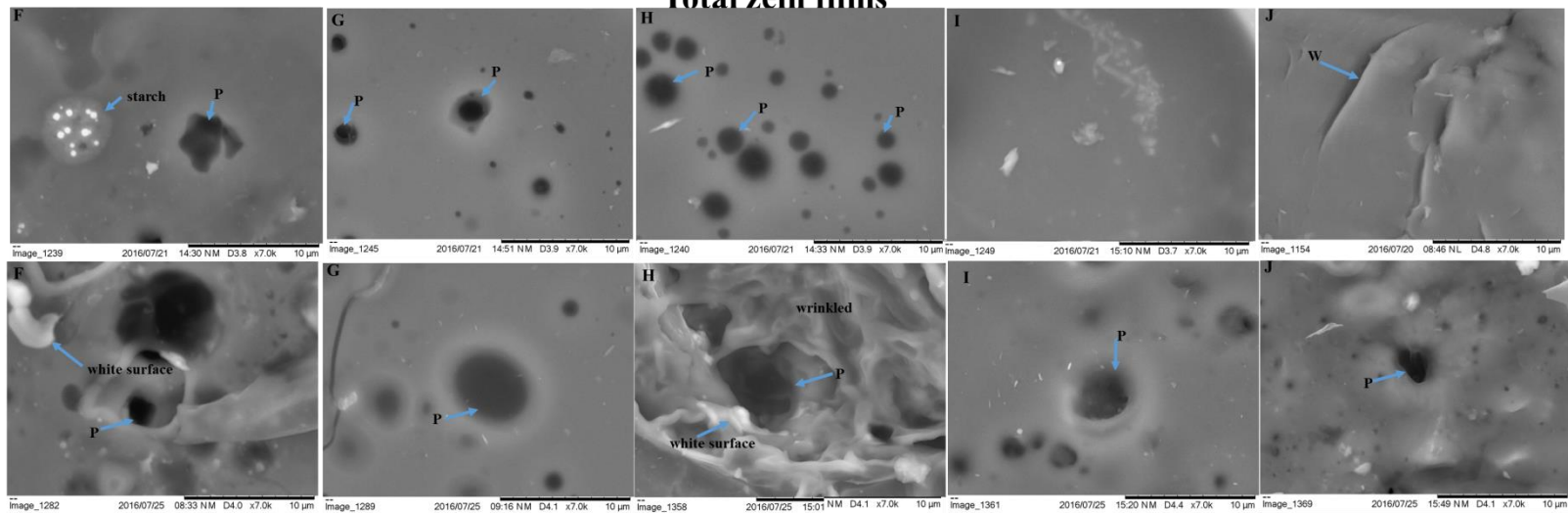
Commercial zein films



Before addition into buffer

After addition into buffer

Total zein films

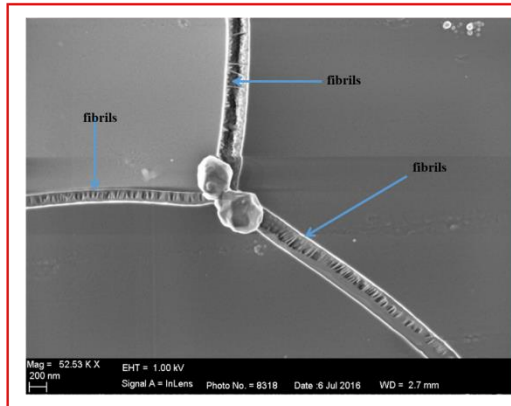
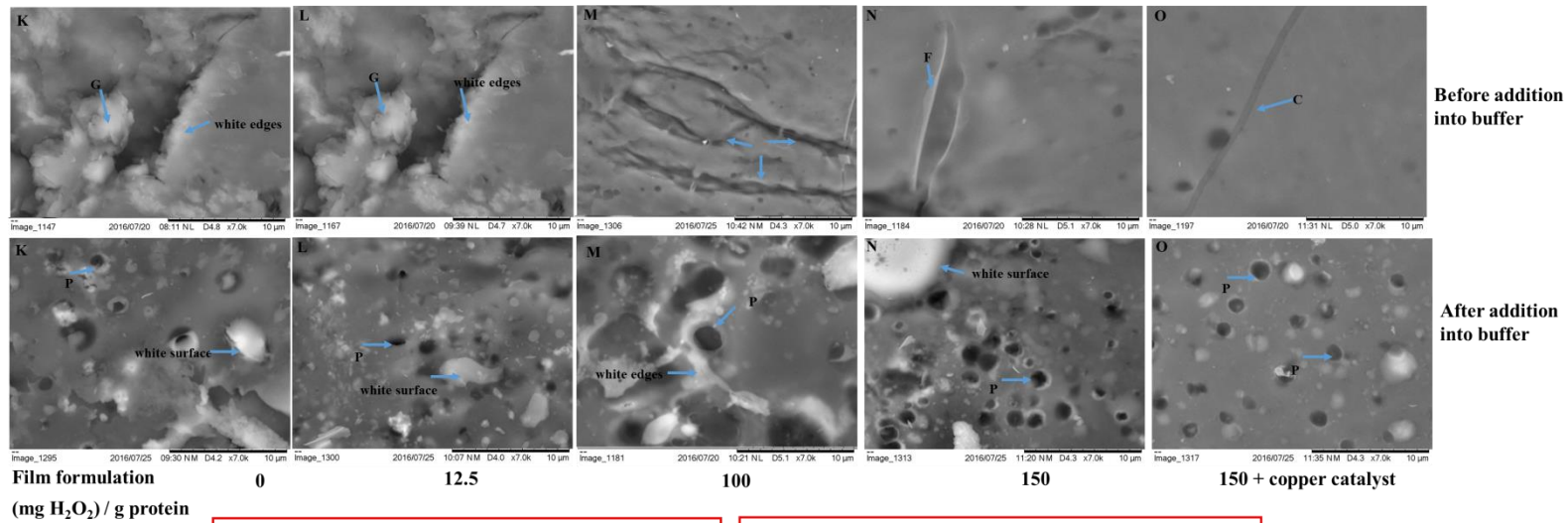


Before addition into buffer

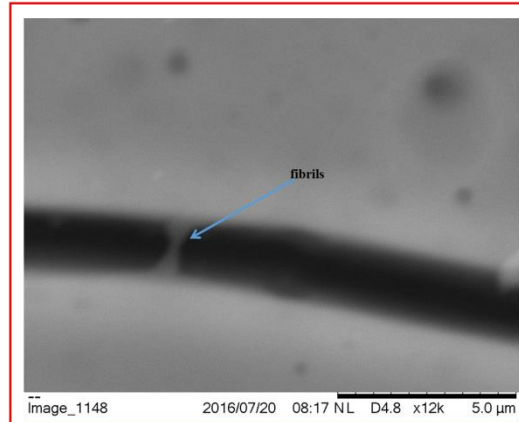
After addition into buffer

Film formulation (mg H₂O₂) / g protein 0 12.5 100 150 150 + copper catalyst

Total kafirin films



Commercial zein films prepared without hydrogen peroxide observed at high resolution



Total zein films prepared with 12.5 mg hydrogen peroxide observed at lower resolution

Figure 5.2: SEM showing the effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film surface structure, before and after immersion in buffer
C-crack, F-flakes, G-globular like structure, P-pores

Boxed in red shows presence of fibrils observed at high and lower resolution

5.3.3. Effects of preparation of commercial zein, total zein and kafirin films with hydrogen peroxide on film aqueous buffer uptake

There was an increase in buffer uptake of commercial zein, total zein and total kafirin films with an increase in the concentration of hydrogen peroxide (Table 5.3). This is contrast to the findings of Anyango et al. (2011), who observed a reduction in water uptake of kafirin films after treating them with glutaraldehyde. Byaruhanga et al. (2007), also found a reduction in water uptake of kafirin films after heat treatment, which was due to the induced disulphide bond formation. The difference in the findings could be due to hydroxylation as explained in section 6.2.

However, when films were prepared with hydrogen peroxide plus copper catalyst buffer uptake was reduced (Table 5.3). Commercial zein film buffer uptake decreased by 9.6%, total zein film uptake by 12.9% and total kafirin uptake by 7.9% compared to films prepared with 150 mg/g hydrogen peroxide alone. The decrease in buffer uptake was possibly due to increased reaction of hydrogen peroxide with commercial zein, total zein and total kafirin protein functional groups, resulting in fewer active sites to interact with water, as is explained in section 6.2.

Increasing the concentration of hydrogen peroxide during preparation of commercial zein, total zein and total kafirin films reduced the physical stability of the films in the buffer (Figure 5.1A, B, indicated by arrows, and Figure 5.2 (SEM)). The decrease in physical stability was possibly due to formation of higher molecular weight polymers (see section 5.3.4), which would have greater resistance to expansion during buffer uptake, hence causing the films to break. The whiteness observed on the surface of the commercial zein, total zein (Figure 5.2A, 5.2F, indicated by arrow), and total kafirin films (Figure 5.2K-O) after immersion in buffer is probably due to protein that was not actually formed into a film structure and was washed off.

Table 5.3: Effect of preparation of commercial zein, total zein and kafirin films with hydrogen peroxide on film buffer uptake

Film formulation (mg H ₂ O ₂ /g protein)	Before immersion in buffer				After immersion in buffer			
	Film weight (mg)	Film area (cm ²)	Film thickness (µm)	Film density (mg/cm ²)	Film weight (mg)	Film area (cm ²)	Increase in film surface area (%)	Film aqueous buffer uptake (%)
Commercial zein								
0	353.9 ^b ±0.3 ¹	19.3 ^a ±0.1	78.0 ^a ±0.7	18.3 ^b ±0.01	532.5 ^a ±0.5	23.0 ^a ±0.1	19.2 ^a ±0.1	50.4 ^a ±0.3
12.5	355.2 ^b ±0.2	19.3 ^a ±0.2	80.8 ^b ±0.6	18.4 ^b ±0.01	551.6 ^c ±1.1	27.4 ^c ±0.4	42.0 ^c ±0.5	55.3 ^c ±0.3
100	357.1 ^b ±0.5	19.3 ^a ±0.1	81.1 ^b ±0.8	18.5 ^b ±0.02	564.1 ^d ±2.5	30.4 ^d ±0.2	57.5 ^d ±1.1	57.9 ^c ±0.8
150	346.8 ^a ±0.6	19.3 ^a ±0.2	81.4 ^b ±0.4	17.9 ^a ±0.08	562 ^e ±2.3	30.8 ^e ±0.3	59.6 ^e ±2.0	62.1 ^d ±1.0
150 + copper catalyst	355.7 ^b ±0.7	19.3 ^a ±0.1	80.6 ^b ±0.2	18.4 ^b ±0.08	542.4 ^b ±0.5	26.3 ^b ±0.5	36.3 ^b ±0.5	52.5 ^b ±0.4
Total zein								
0	436.1 ^b ±0.7	18.9 ^a ±0.3	76.0 ^a ±0.5	22.6 ^b ±0.1	642.9 ^b ±2.1	23.6 ^a ±0.5	24.9 ^a ±0.7	47.41 ^a ±0.3
12.5	451.6 ^d ±0.4	19.3 ^a ±0.1	81.2 ^d ±0.3	23.4 ^c ±0.1	681.4 ^c ±2.2	27.0 ^c ±0.9	39.9 ^c ±0.6	50.9 ^c ±0.7
100	436.5 ^b ±0.2	18.9 ^a ±0.2	79.8 ^c ±0.4	22.6 ^b ±0.2	691.7 ^d ±1.1	28.4 ^d ±1.0	50.3 ^d ±0.8	58.4 ^d ±0.6
150	443.8 ^c ±0.7	19.3 ^a ±0.0	83.1 ^c ±0.1	23.0 ^c ±0.1	716.5 ^e ±0.7	30.0 ^e ±0.9	55.4 ^e ±0.4	61.5 ^e ±0.4
150 + copper catalyst	359 ^a ±0.7	18.8 ^a ±0.4	79.3 ^b ±0.3	18.5 ^a ±0.1	530.4 ^a ±2.1	24.8 ^b ±0.3	32.0 ^b ±0.3	48.6 ^b ±0.3
Total kafirin								
0	367.4 ^c ±0.4	18.7 ^c ±0.3	79.6 ^b ±0.2	19.5 ^d ±0.1	531.3 ^b ±0.6	21.2 ^a ±0.2	13.4 ^a ±0.6	44.6 ^a ±0.76
12.5	355.2 ^b ±0.8	18.3 ^b ±0.4	80.1 ^b ±0.1	19.2 ^c ±0.2	531.5 ^b ±0.8	22.7 ^b ±0.7	24.0 ^c ±0.7	49.6 ^b ±0.3
100	368.5 ^d ±0.4	18.8 ^c ±0.3	82.3 ^c ±0.8	19.7 ^d ±0.1	570.0 ^d ±0.8	24.4 ^c ±0.3	29.8 ^d ±0.4	54.7 ^d ±0.1
150	354.1 ^b ±0.3	19.1 ^c ±0.2	78.9 ^a ±0.1	18.5 ^b ±0.2	564.5 ^c ±0.4	27.0 ^d ±0.1	41.4 ^e ±0.4	59.4 ^e ±0.2
150 + copper catalyst	320.3 ^a ±0.5	18.1 ^a ±0.1	83.4 ^d ±0.1	17.2 ^a ±0.4	485.3 ^a ±0.5	21.0 ^a ±0.3	16.0 ^b ±0.5	51.5 ^c ±0.3

^a Mean values in columns of the same sample with different superscript letters differ significantly (p< 0.05).

¹Mean values ± standard deviation, n=3

5.3.4. Effect of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film SDS-PAGE pattern

SDS-PAGE under reducing conditions of commercial zein films prepared without hydrogen peroxide revealed two distinctive bands and have been identified as α_1 - and α_2 -zein (Tatham et al., 1993) with molecular weights of approximately 22 kDa and 19 kDa, respectively (Figure 5.3B, track 1). This confirms that commercial zein was essentially α -zein as no bands corresponding to β -zein (M_r 17-19 kDa) and γ -zein (M_r 27 kDa) (Paraman and Lamsal, 2011) were observed. There was no evidence from the SDS-PAGE, either under non-reducing or reducing conditions, that disulphide cross-linking of commercial zein occurred with hydrogen peroxide treatment (Figure 5.3A and 5.3B, track 1-5). As indicated, commercial zein lacks β - and γ -zein and this results in it having low levels of cysteine residues for substantial disulphide bonding. Hence, it appears that oxidation of commercial zein by hydrogen peroxide does not lead to disulphide cross-linking.

SDS-PAGE under reducing conditions of total zein films prepared without hydrogen peroxide (Figure 5.3B, track 6) showed an additional band of 14 kDa compared to commercial zein. This band can be assigned as β -zein. Beta-zein exists in polymeric form (Paraman and Lamsal, 2011), hence it was not seen under non-reducing conditions (Figure 5.3A, track 1 circled in orange). However, with SDS-PAGE under non-reducing conditions it can be seen that there was a decrease in intensity of the total zein monomer bands with the films prepared with hydrogen peroxide (Figure 5.3A, track 8-10 circled in green). This decrease in monomer band intensity is evidence of polymerisation. Additionally, the increase in monomer band intensity with SDS-PAGE under reducing conditions (Figure 5.3B, track 9-10 circled in green) confirms that the polymerisation observed under non-reducing conditions was due to formation of disulphide cross-links.

SDS-PAGE under reducing conditions of kafirin films prepared without hydrogen peroxide clearly shows the presence of γ -, α_1 -, α_2 - and β -kafirin subclasses, with α_1 -kafirin being predominant (Figure 5.3B, track 11). This band pattern is in agreement with the kafirin study of El Nour et al. (1998). With SDS-PAGE under non-reducing conditions the monomer band intensity was decreased for kafirin films prepared with hydrogen peroxide at high

concentration (≥ 100 mg/g protein) (Figure 5.3A, track 13-15 circled in yellow). This indicates formation of highly polymerised kafirin polymers which were too large to migrate into the separating gel. Similar results were found by Anyango et al. (2011), who observed the disappearance of monomer bands when kafirin films were cross-linked with a high concentration of glutaraldehyde. Byaruhanga et al (2006), also found polymerisation of kafirin films as a result of heat-induced disulphide cross-links. The polymerisation of total zein and total kafirin prepared with hydrogen peroxide observed with SDS-PAGE is due to the fact that, total zein and total kafirin contain β - and γ -kafirin subclasses, which are rich in cysteine and are easily oxidised to form inter and intramolecular disulphide bonds (Mesa-Stonestreet et al., 2010). However, the fact that the intensity of the monomer bands for the high concentration of hydrogen peroxide treatments with SDS-PAGE under non-reducing conditions was still lower than at the low concentration of hydrogen peroxide treatments with SDS-PAGE under reducing conditions indicates that not all the cross-links could be cleaved by mercaptoethanol (Figure 5.3B, tracks 14-15 circled in yellow). This was possibly because of the presence of non-disulphide cross-links. Duodu et al. (2002), also found the presence of polymerised kafirin with SDS-PAGE under reducing conditions and this was attributed to inaccessible disulphide and non-disulphide cross-linking formed during wet heat treatment of kafirin.

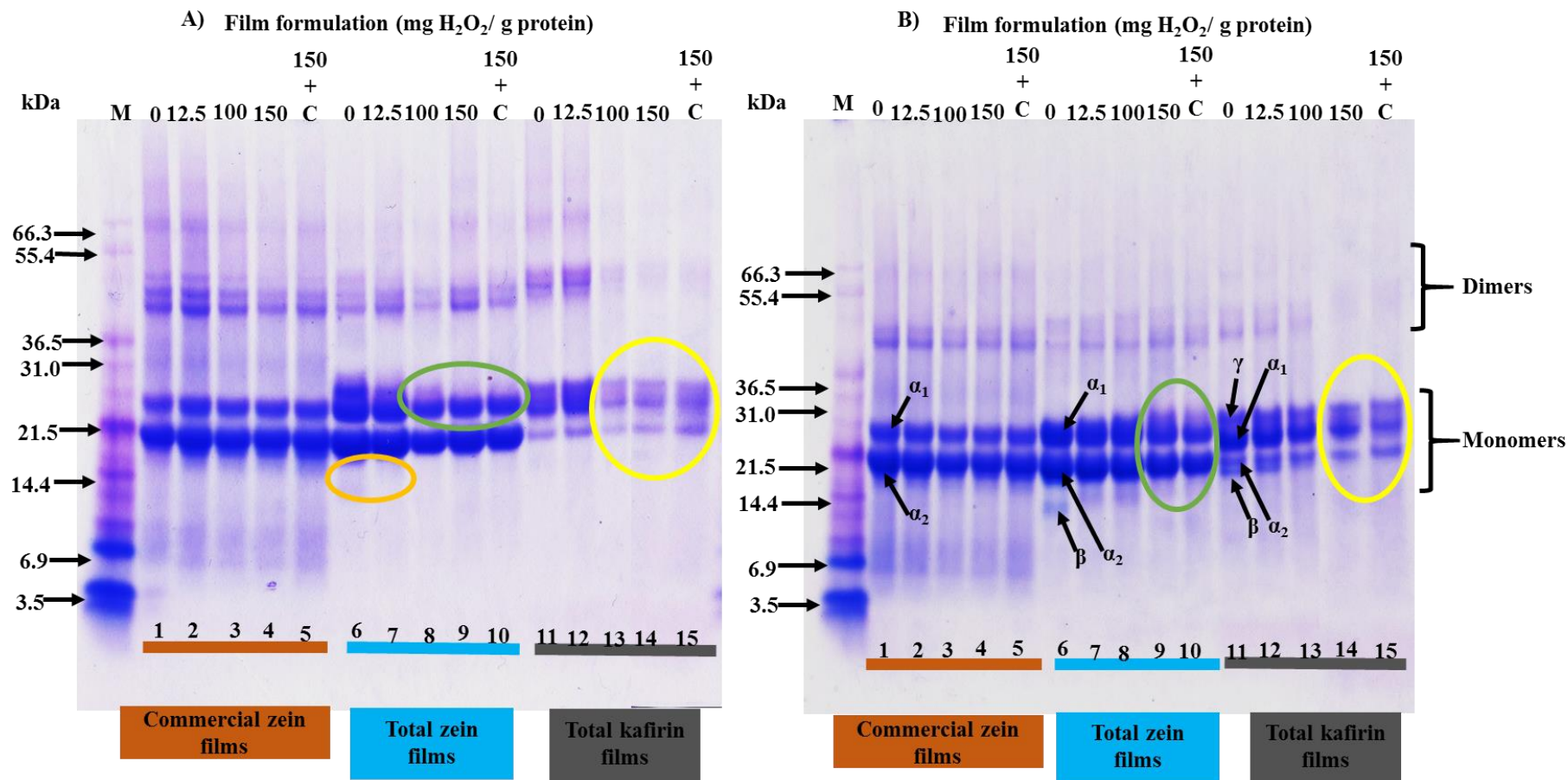


Figure 5.3: Effect of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film SDS-PAGE pattern

M-Molecular weight marker,

A)-Non-Reducing conditions

B)-Reducing conditions

150 + C-(150 (mg H₂O₂/ g protein) + copper catalyst)

5.3.5. Effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film protein secondary structure

The effects of hydrogen peroxide on the protein secondary structure of the films were investigated using FTIR spectroscopy. With kafirin films, the deconvoluted FTIR spectra frequency in the 1700-1600 cm^{-1} has been identified as the Amide I region and in the frequency 1575-1475 cm^{-1} as the Amide II region (Gao et al., 2005). With kafirin in the Amide I region, the peak at around wavenumbers 1650 and 1620 cm^{-1} has been assigned to α -helices (Duodu et al., 2001). Protein random coils also contribute to absorption in this region (Singh, 2000). With kafirin, the peak around wavenumber 1620 cm^{-1} has been assigned to intermolecular β -sheets (Duodu et al., 2001). The shoulder at 1680-1690 cm^{-1} (Figure 5.4 indicated by the arrow) and peaks at this wavenumber have been assigned to β -turns (Byaruhanga et al., 2006). The Amide II region has a lower protein conformational sensitivity than the Amide I region (Kong and Yu, 2007). As explained, the Amide I absorption contains contributions from C=O \approx 80% and a minor stretching vibration from C-N, whereas Amide II absorption arise from N-H bending \approx 60% and C-N stretching \approx 40% (Jackson and Mantsch, 1995). Hence, the Amide I rather than Amide II region is very often used as the indicator in studies of the secondary structure of prolamins (Mejia et al., 2007). For this reason, the Amide II data will not be discussed.

The FTIR spectra of commercial zein and total zein films (Figure 5.4A and B, respectively) indicate that their protein secondary structure was more similar to each other than that of the total kafirin films (Figure 5.4C). This is possibly due to the fact that kafirin was more polymerised than commercial zein and total zein, as shown by SDS-PAGE (section 5.3.4, Figure 5.3). Commercial zein films prepared without hydrogen peroxide were predominantly α -helical (86%) with a small proportion of β -sheet conformation (Figure 5.4A, Table 5.4). These FTIR spectroscopy data for commercial zein films agree with King et al (2016) and Hsu et al. (2005) who found that the secondary structure of commercial zein films was predominantly α -helical. There was a slight but significant ($p < 0.05$) increase ($\sim 2.8\%$) in the α -helical conformation with an increase in hydrogen peroxide (Table 5.4). Total zein films prepared without hydrogen peroxide showed an almost equal ratio of α -helical to β -sheet conformation, with the α -helical conformation being slightly higher (Figure 5.4B, Table 5.4). Preparation of total zein films with 150 mg hydrogen peroxide/g protein caused very small but significant ($p < 0.05$) increase of 1.2% in the α -helical conformation (Table 5.4). Sun et al. (2016) also found an increase in the α -helical conformation and random

coils of heated zein powder. In this present work, clear peaks of α -helical and β -sheet conformation were observed in total zein films prepared without hydrogen peroxide but the clarity of the peaks declined with an increase in the concentration of hydrogen peroxide in the films (Figure 5.4B, circled in brown). Perhaps, the increase in α -helical conformation was also an indication of slight increase in random coils.

Total kafirin films prepared without hydrogen peroxide had an equal proportion of α -helical and β -sheet conformation (Figure 5.4C circled in yellow, Table 5.4). This is not in agreement with Belton et al. (2006), who found that kafirin is predominantly α -helical. These differences are probably because kafirin films were analysed in this current work instead of kafirin powder as analysed by Belton et al. (2006). In fact, Byaruhanga et al. (2006), found kafirin films to be predominantly β -sheet conformation. Therefore it is likely that films are more stable in the β -sheet conformation than in the α -helical conformation. The fact that kafirin films prepared without hydrogen peroxide did not break up in the buffer (Figure 5.1) is thus consistent with the FTIR data. With the kafirin films prepared with hydrogen peroxide there was a small but significant ($p < 0.05$) increase in relative β -sheet conformation by approximately 2.1% (Figure 5.4C, Table 5.4).

As stated, total zein and total kafirin both contain β - and γ -classes but the effect of the hydrogen peroxide on their protein secondary structure was different in that total zein films prepared with hydrogen peroxide showed slightly increased α -helical conformation, whereas total kafirin films prepared with hydrogen peroxide showed slightly increased β -sheet conformation (Figure 5.4B and C, respectively). This was possibly due to the fact that kafirin contains more cysteine residues than total zein (Belton et al., 2006). Hence, it has a greater propensity to polymerise than total zein films. It was, however, expected that oxidation by hydrogen peroxide would increase the β -sheet if there had been cross-links through disulphide bond formation. However, since SDS-PAGE (section 5.3.4, Figure 5.3) showed no evidence of disulphide bond formation with commercial zein films prepared without hydrogen peroxide, the fact that there was no increase in β -sheet conformation is consistent.

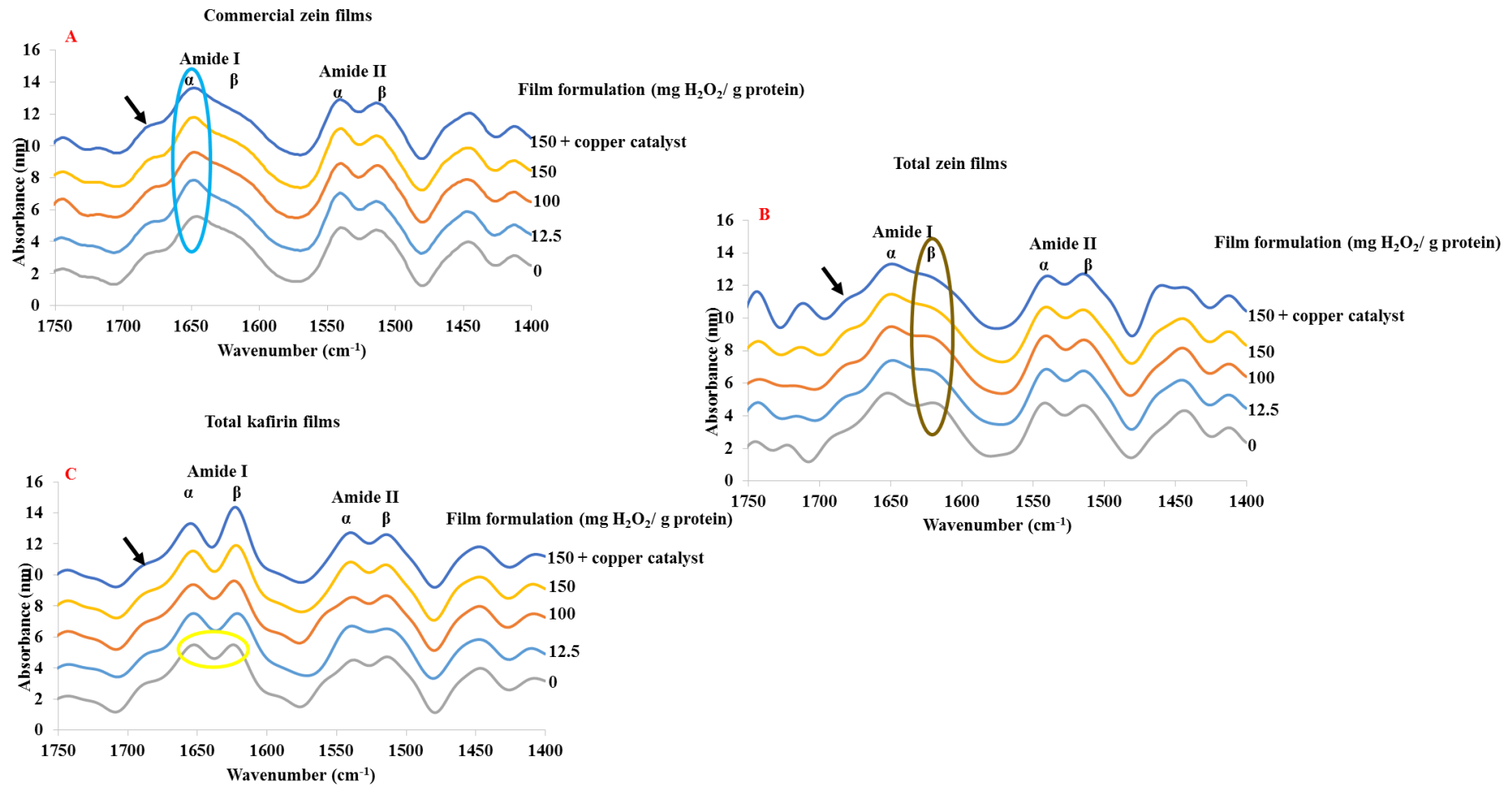


Figure 5.4: Effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film protein secondary structure. Each curve represents an average of two independent experiments where each sample was subjected to 64 scans

Blue ring- an increase in α -helical conformation

Brown ring- a decrease in clear peak of β -sheet conformation

Yellow ring- \approx α -helical: β -sheet conformation

Table 5.4: Effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film protein secondary structure as determined by FTIR spectra of the Amide I region

Prolamin	Film formulation (mg H₂O₂/g protein)	α-helix wavenumber (cm⁻¹)	β-sheet wavenumber (cm⁻¹)	Relative α-helical conformation (%)	Relative β-sheet conformation (%)	Ratio (α:β)
commercial zein	0	1644	1623	85.5 ^a ± 0.6 ¹	14.5 ^c ± 0.6	5.89 ^a ±0.6
	12.5	1648	1623	86.0 ^a ± 0.5	14.0 ^c ± 0.5	6.14 ^a ±0.5
	100	1649	1621	87.8 ^b ± 0.9	12.2 ^b ± 0.9	7.20 ^b ±0.2
	150	1648	1625	88.3 ^b ± 0.4	11.7 ^b ± 0.4	7.55 ^b ±0.6
	150 + copper catalyst	1648	1621	89.5 ^c ± 1.7	10.5 ^a ± 1.7	8.52 ^c ±1.7
Total zein	0	1652	1624	53.7 ^a ± 0.1	46.3 ^c ± 0.1	1.16 ^a ± 0.04
	12.5	1649	1624	54.1 ^a ± 0.2	45.9 ^c ± 0.2	1.18 ^a ± 0.01
	100	1649	1622	54.0 ^a ± 0.8	46.0 ^c ± 0.8	1.17 ^a ± 0.04
	150	1649	1625	54.9 ^b ± 0.6	45.1 ^b ± 0.6	1.21 ^a ± 0.04
	150 + copper catalyst	1648	1621	55.6 ^c ± 0.5	44.4 ^a ± 0.5	1.25 ^a ± 0.07
Total kafirin	0	1653	1624	50.1 ^c ± 0.5	49.9 ^a ± 0.5	1.00 ^c ± 0.02
	12.5	1655	1624	48.8 ^b ± 0.2	51.2 ^b ± 0.2	0.95 ^b ± 0.01
	100	1653	1624	48.8 ^b ± 0.3	51.2 ^b ± 0.3	0.95 ^b ± 0.01
	150	1653	1622	48.0 ^b ± 0.6	52.0 ^b ± 0.5	0.92 ^b ± 0.2
	150 + copper catalyst	1654	1623	45.1 ^a ± 0.9	54.9 ^c ± 0.9	0.82 ^a ± 0.2

^a Mean values in columns with different superscript letters differ significantly (p< 0.05), n=4

¹Mean ± Standard Deviation, each sample was subjected to 64 scans each

5.3.6. Effect of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film T_g and enthalpy changes

The T_g of commercial zein films prepared without hydrogen peroxide was 154.5°C (Table 5.5). This is considerably higher than the 135.4°C found by Sessa et al. (2007) which was for films made from zein extracted from white maize. However, Magoshi et al. (1992) found a similar T_g of 160°C for purified zein but Madeka and Kokini (1996) found a much lower T_g of 139°C for Sigma (commercial) zein powder, that is not for films prepared from Sigma zein. These differences are presumably a consequence of both the chemical composition and physical form of the zein analysed. There was an increase in the T_g of the commercial zein films prepared with hydrogen peroxide (Figure 5.5, Table 5.5). The heat capacity change (ΔC_p) is the amount of energy a unit of matter can hold (Tabor, 1991). The ΔC_p measures protein stability (Betz, 1993). There was a progressive significant increase ($p < 0.05$) in ΔC_p with increasing hydrogen peroxide concentration (Table 5.5). This increase is probably an indication of increased intermolecular bonding, hence more energy was required to pull the molecules apart. However, preparation of commercial zein films with hydrogen peroxide plus copper catalyst resulted in a decrease in both the T_g and ΔC_p (see section 6.2).

The T_g of total zein films prepared without hydrogen peroxide was 155.0°C (Table 5.5). There was an increase ($\sim 16.7^\circ\text{C}$) in the T_g of the total zein films prepared with hydrogen peroxide. This is in agreement with the finding of Hernández-Muñoz et al. (2005), who found an increase in the T_g of gliadins due to covalent cross-linking. The ΔC_p of total zein films prepared with hydrogen peroxide also progressively increased ($p < 0.05$). However, inclusion of the copper catalyst in the preparation of total zein films with hydrogen peroxide resulted in a decrease in the T_g and an increase in ΔC_p (see section 6.2).

The T_g of total kafirin films prepared without hydrogen peroxide was 178.9°C which was higher than that of commercial zein and total zein films. This is in agreement with Adebowale et al. (2011) who found no T_g for laboratory prepared kafirin up to a temperature of 120°C. This is probably due to disulphide cross-linking. In support of this, Anyango et al. (2013), found that γ -kafirin has a very high T_g (270°C). There was a slight increase in the T_g of total kafirin films prepared with hydrogen peroxide (Table 5.5).

This is consistent with the results of Emmambux et al. (2004) who reported an increase in T_g of kafirin films with increasing levels of tannin cross-linking.

The observed multiple transitions in commercial zein and total kafirin films prepared with high concentration of hydrogen peroxide ≥ 100 mg/g protein (Figure 5.5) could be a result of the complexity of these proteins. Cross-linking restrains internal rotation along macromolecular chain which reduces chain mobility and increases chain rigidity, which in turn increases T_g (Hernández-Muñoz et al., 2005). Therefore it is expected that hydrogen peroxide will reduce molecular mobility as result of increasing intermolecular disulphide bonding in total zein and total kafirin. The absence of a trend between T_g and concentration of hydrogen peroxide with the commercial zein films is consistent with the SDS-PAGE findings that there was no observable polymerisation. Thus, the increase in T_g of commercial zein could have been a result of increased molecular entanglement.

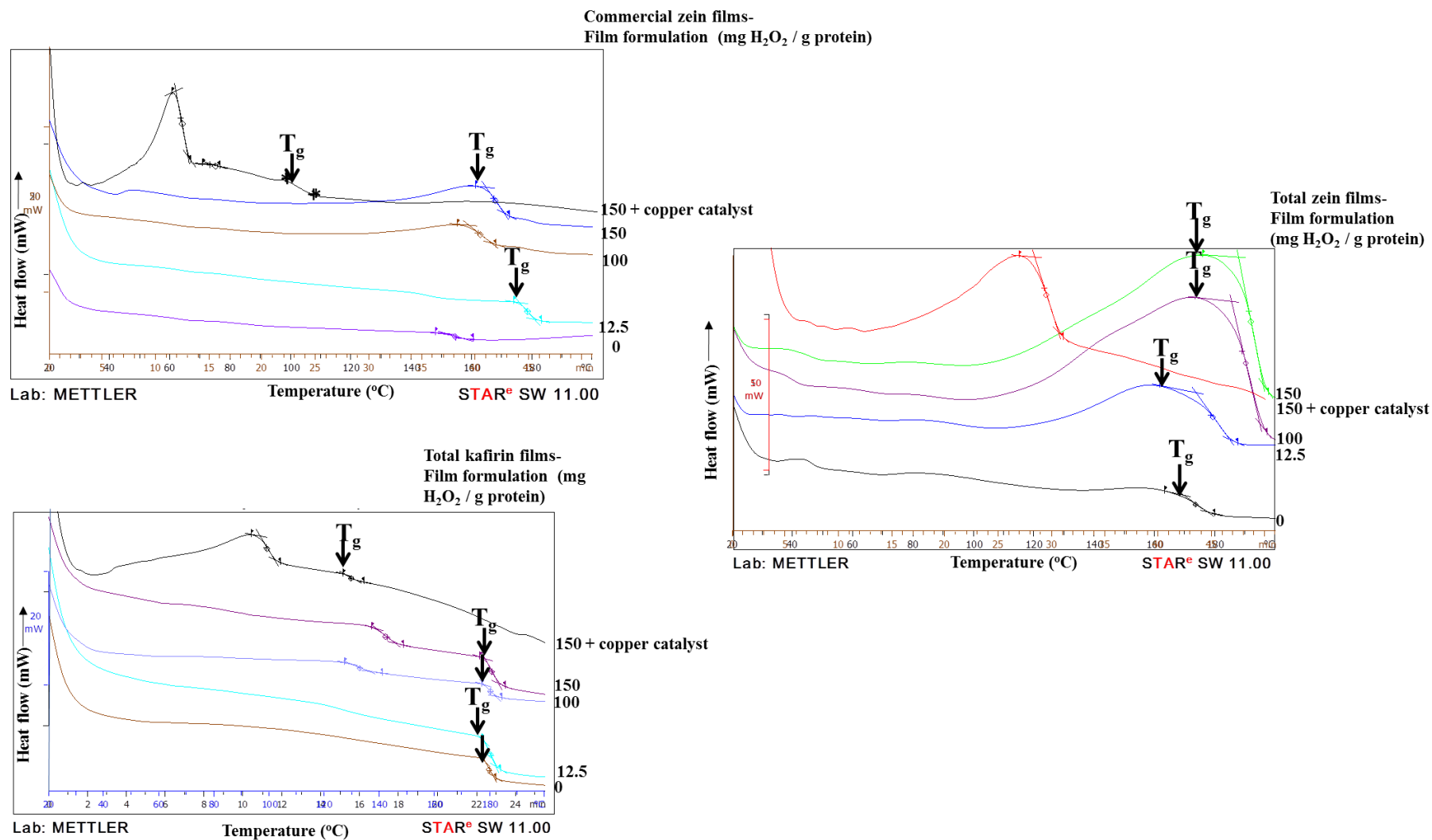


Figure 5.5: Effects of preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide on film T_g and enthalpy changes

Table 5.5: Effects of preparation of commercial zein, total zein and total kafirin with hydrogen peroxide on film glass transition and enthalpy of changes

Film formulation (mg H ₂ O ₂ /g protein)	T _{onset1} (°C)	T _{g1} (°C)	ΔC _{p1} (J/G) (°C)	T _{onset2} (°C)	T _{g2} (°C)	ΔC _{p2} (J/G) (°C)
Commercial zein						
0	No transitional change	No transitional change	No transitional change	150.1 ^b ±0.3 ¹	154.5 ^b ±0.1	0.219 ^a ±0.0
12.5	No transitional change	No transitional change	No transitional change	175.5 ^e ±0.6	177.6 ^e ±0.3	0.877 ^b ±0.01
100	No transitional change	No transitional change	No transitional change	159.0 ^c ±0.2	161.7 ^c ±0.1	0.881 ^b ±0.01
150	No transitional change	No transitional change	No transitional change	162.8 ^d ±0.2	166.5 ^d ±0.5	1.06 ^c ±0.2
150 + copper catalyst	70.74±0.4	75.56±0.2	7.27±0.4	110.8 ^a ±0.1	118.0 ^a ±0.3	0.236 ^a ±0.5
Total zein						
0	No transitional change	No transitional change	No transitional change	152.0 ^b ±0.04	155.0 ^b ±0.2	0.261 ^a ±0.0
12.5	No transitional change	No transitional change	No transitional change	154.9 ^c ±0.2	159.8 ^c ±0.4	0.838 ^b ±0.1
100	No transitional change	No transitional change	No transitional change	166.2 ^d ±0.1	169.4 ^d ±0.1	2.35 ^c ±0.5
150	No transitional change	No transitional change	No transitional change	168.5 ^e ±0.01	171.7 ^e ±0.4	2.57 ^c ±0.1
150 + copper catalyst	No transitional change	No transitional change	No transitional change	125.8 ^a ±0.2	128.1 ^a ±0.2	3.39 ^d ±0.1
Total kafirin						
0	No transitional change	No transitional change	No transitional change	177.3 ^b ±0.01	178.9 ^b ±0.0	0.547 ^b ±0.04
12.5	No transitional change	No transitional change	No transitional change	176.7 ^b ±0.05	178.9 ^b ±0.0	1.19 ^c ±0.1
100	130.7 ^b ±0.4	134.2 ^b ±0.2	0.219 ^a ±0.4	176.8 ^b ±0.5	179.3 ^b ±0.4	0.821 ^c ±0.1
150	138.5 ^c ±0.7	142.1 ^c ±0.0	0.500 ^b ±0.7	177.0 ^b ±0.0	179.4 ^b ±0.6	1.58 ^d ±0.7
150 + copper catalyst	95.5 ^a ±0.0	105.0 ^a ±0.0	3.00 ^c ±0.0	125.5 ^a ±0.7	129.0 ^a ±0.2	0.217 ^a ±0.0

^a Mean values in columns with different superscript letters differ significantly (p< 0.05), n=4

¹Mean ± Standard Deviation, each sample was subjected to 64 scans each

5.4. Conclusions

Hydrogen peroxide has the potential to modify the zein and kafirin film properties. Preparation of commercial zein, total zein and total kafirin films with hydrogen peroxide increases aqueous buffer uptake of the films, physical stability and T_g and ΔC_p . Commercial zein, total zein and total kafirin films undergo different oxidative reactions with hydrogen peroxide. SDS-PAGE of total zein and total kafirin shows evidence of disulphide bond formation, whereas there was no evidence with commercial zein. Therefore the improvement in the functional properties of the films is probably due to protein entanglement caused by hydroxylation and disulphide bond formation. However, when a copper catalyst is included with hydrogen peroxide there is a decrease in aqueous buffer uptake, T_g and ΔC_p of the films. These decreases when a copper catalyst is included are possibly due to copper ions substituting hydroxyl groups during hydroxylation reducing film aqueous buffer uptake as well as forming spaces within the protein hence a reduction in T_g and ΔC_p .

Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on their dough forming properties

ABSTRACT

Maize and sorghum have the potential to replace wheat in bread in bread making. Films made from commercial zein prepared with hydrogen peroxide were found to form doughs, but films prepared without hydrogen peroxide did not. However, total zein films prepared with and without hydrogen peroxide formed doughs, whereas total kafirin films prepared with and without hydrogen peroxide formed aggregates but not doughs. Stereomicroscopy and SEM showed that hydrogen peroxide promoted fibril formation in commercial zein and total zein doughs. However, hydrogen peroxide had no effect in fibril formation in total kafirin aggregates. FTIR of commercial zein and total zein doughs showed a slight increase in the α -helical conformation of the doughs with an increase in hydrogen peroxide concentration, whereas an increase in hydrogen peroxide concentration increased β -sheet conformation in total kafirin aggregates. Hydrogen peroxide treatment greatly improved commercial zein and total zein dough extensibility, cohesiveness and water holding capacity. Total kafirin is hydrophobic in nature hence, difficult to fully hydrate therefore could not form a dough with or without preparations of the films with hydrogen peroxide.

5.5.Introduction

Gluten is the main structure-forming protein in wheat flour, and is responsible for the elastic characteristics of dough, and contributes to the appearance and crumb structure of many baked products (Gallagher et al., 2004). Gluten functionality can be strongly influenced by disulphide and dityrosine bonding (Hanft and Koehler, 2005). However, gluten-free doughs lack the extensibility, viscosity, elasticity, cohesiveness and water absorption capacity (Houben et al., 2012) of gluten-containing doughs. Zein, the maize prolamin, when hydrated above its T_g forms a viscoelastic dough (Lawton, 1992). There has been some progress in the development of gluten-free dough-based products (Taylor et al., 2016b). Methods that have been used to improve the dough rheological properties of gluten-free dough include addition of hydrocolloids (Schober et al., 2008), co-protein (Mejia et al., 2007), chemical treatment with organic acids (Sly et al., 2014) and addition of oxidative enzymes (Gujral and Rosell, 2004). Sly et al. (2014), found that chemically treating zein with dilute acetic and lactic acids resulted in an extensible dough that formed uniform fibrils. It has also been found that rice flour treated with glucose oxidase had improved dough elasticity which improved loaf volume and bread texture (Gujral and Rosell, 2004). Glucose oxidase has been used as a dough improver in weak wheat flours (Joye et al., 2009). The hydrogen peroxide produced from glucose oxidase promotes inter- and intra-molecular crosslinking in wheat bread doughs. Protein crosslinking is a way of modifying the protein functionality and simultaneously increasing its applications in different processes (Bonet et al., 2006). Oxidation induces the formation of disulphide bonds by coupling of two cysteine residues (Oom et al., 2008) and dityrosine crosslinks (Tilley et al., 2001). Covalent cross-linking of proteins modifies protein functionality as the formation of a protein network is a key factor in enhancing gluten-free cereal performance in bread making (Sciarini et al., 2012).

This research follows the findings of an increase in film aqueous buffer uptake after oxidation (section 5.3.3). Furthermore, there has been little reported research on improving dough properties of zein and kafirin with hydrogen peroxide. Therefore the objective of this study was to determine the effect of film preparation with hydrogen peroxide on dough formation of commercial zein, total zein and total kafirin.

5.6. Materials and Methods

5.6.1. Materials

Commercial zein, total zein and total kafirin films prepared as described in section 5.2.2.2.2 were used to make doughs.

5.6.2. Methods

5.6.2.1. Dough formation

The doughs were prepared as described by Schober et al. (2010), with some modifications. Crushed films (0.5 g protein equivalent) and 1.8 mL distilled water were weighed into separate 40 mL plastic centrifuge tubes. These were pre-warmed to 50°C in a water bath. On reaching temperature, distilled water and crushed films were mixed. The suspension was vortexed at high speed for 30 s and then manipulated into dough using a spatula, ensuring that all protein was incorporated into a dough or aggregate (where a dough did not form). The centrifuge tubes were held in the water bath at 50°C for 1 h and any dough formed was manually stretched and reshaped at 15 min intervals. After 1 h, the doughs formed were stretched by hand and photographed. The doughs formed were stretched to a point before they broke and the distance was measured and recorded as the dough extensibility. When a dough did not form, water was removed from the aggregates by decanting before they were photographed.

5.6.2.2. Analyses

5.6.2.2.1. Water holding capacity

The doughs and aggregates (0.5 g) were left at ambient temperature (25°C) which was below the hydration temperature (50°C) for 1 min. After 1 min the doughs and aggregates were placed on a double tissue paper and pressed between the thumb and index finger. The degree of tissue paper wetness was recorded.

5.6.2.2.2. Stereomicroscopy

The doughs (0.5 g) were stretched out thinly (~15 mm × 5 mm × 1 mm) over a glass slide and viewed using a Discovery V20 Stereomicroscope (Zeiss, Goettingen, Germany). The doughs were viewed with a field view of 3.5 mm, 1.8 µm resolution and 64 µm depth of the field at x80 magnification and photographed.

5.6.2.2.3. Scanning Electron Microscopy

The surface morphology of the doughs and aggregates were analysed using Zeiss Gemini Ultra plus Field Emission SEM (Zeiss, Oberkochen, Germany). A cube of dough or protein aggregate (approx. 5 mm across) was cut using a sharp knife and mounted on aluminium stubs using double-sided carbon tape and coated with carbon using Emitech K950X carbon coater (Ashford, UK) and viewed at 1 kV.

5.6.2.2.4. Fourier Transmission Infrared Spectroscopy (FTIR)

Doughs and aggregates were analysed as described in section 5.2.2.2.7.

5.6.2.2.5. Statistical analysis

All experiments were carried out in triplicate unless otherwise stated. IBM SPSS software was used to analyse data. The effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on their dough functional properties was analysed by one-way analysis of variance (ANOVA) and compared at 95% confidence level using Fisher's Least Significance Difference test (LSD).

5.7. Results and discussion

5.7.1. Effects of film preparation with hydrogen peroxide on commercial zein, total zein and kafirin dough and fibril formation

Commercial zein films prepared without hydrogen peroxide did not form a dough when hydrated in distilled water at 50°C (Figure 5.6). This is in contrast to the findings of King et al. (2016), who found that an extensible zein dough could be formed from commercial zein films. This difference could be due to the solvent used for casting the films. In this work aqueous ethanol was used unlike glacial acetic acid which was used by King et al (2016). Glacial acetic acid dissolves commercial zein more completely than aqueous ethanol Li et al. (2012). Furthermore, Taylor et al. (2005) found glacial acetic acid to dissolve prolamin proteins better at a lower temperature than aqueous ethanol. When viewed by SEM commercial zein aggregates showed numerous globular like structures (Figure 5.8A, indicated by arrows). These are probably evidence of proteins associating after hydration where the hydrophilic domains had become exposed and the hydrophobic sides remained shielded, thus preventing full hydration to form a dough.

It was, however, found that inclusion of hydrogen peroxide in the preparation of commercial zein films cast with aqueous ethanol enabled dough formation (Figure 5.6, Table 5.6). The doughs were flexible when left at ~25°C for 1min which is below T_g of hydrated zein (~28°C) (Lawton, 1992). This was probably due to the increased water holding capacity of the films that the doughs were made from which enabled a plasticising effect by the water even below the T_g of commercial zein. Furthermore, the extensibility of the doughs progressively increased with hydrogen peroxide concentration. Film preparation with hydrogen peroxide promoted fibril formation in commercial zein doughs as viewed by stereomicroscopy (Figure 5.6B-E). On stretching the doughs, the fibrils aligned in the direction of stretching (Figure 5.6B-D (stereomicroscopy)). Fibril formation is believed to be of vital importance in zein dough formation, cohesiveness and extensibility (Lawton 1992, Schober et al., 2011).

Casting films from total zein and then manipulating the dried film powder in distilled water at 50°C (above zein's T_g) resulted in dough formation (Figure 5.6). This is in apparent contrast to the work of King et al. (2016), who found that total zein films did not form a dough in water. The difference in results may be due to the apparent presence of starch granules in the total zein preparation used in this present work (Figure 5.2F). The starch could have helped in dough

formation by promoting hydration. Total zein doughs made from films prepared without hydrogen peroxide were, however, extremely soft, only slightly extensible as they exhibited little cohesiveness when stretched. In contrast, total zein doughs made from films prepared with hydrogen peroxide had greatly improved dough cohesiveness and extensibility (Figure 5.6). The increase in dough cohesiveness and extensibility could be due to the formation of disulphide bonds as observed by SDS-PAGE (section 5.3.4, Figure 5.3) and other cross-links such as dityrosine bonds. Dough extensibility increased slightly with increasing hydrogen peroxide concentration (Table 5.6). Total zein doughs formed from films prepared with hydrogen peroxide had improved water holding capacity when left below 25°C for 1 min compared to total zein doughs formed from films prepared without hydrogen peroxide. Total zein doughs made from films prepared without hydrogen peroxide had few fibrils and these were not well developed (Figure 5.7F (stereomicroscopy), Figure 5.8F (SEM)) but preparation with hydrogen peroxide promoted the transformation of total zein particles into numerous fine fibrils which were aligned in the direction of stretching (Figure 5.7G-J (stereomicroscopy), 5.8G-J (SEM)). Fibril formation progressively increased with an increase in the concentration of hydrogen peroxide. The formation of fibrils was highest in doughs made from films prepared with hydrogen peroxide plus copper catalyst (Figure 5.7J (stereomicroscopy), 5.8J (SEM)). Hydrogen peroxide clearly promoted total zein protein aggregation and crosslinking, probably through oxidation, thereby allowing for the development of extensive fibrous protein network as observed by SEM (Figure 5.8).

Kafirin films prepared with hydrogen peroxide only formed protein aggregates not doughs (Figure 5.7). This is consistent with the work of Oom et al. (2008), who found that kafirin when mixed with starch could not form a dough even at elevated temperatures and that kafirin only formed a dough when plasticised into a resin using oleic acid, which quickly stiffened below its T_g . This was attributed to high levels of disulphide bonding in kafirin as seen by SDS-PAGE (Figure 5.3). Kafirin's inability to form doughs is additionally probably a consequence of its unusually hydrophobic nature (Duodu et al., 2002), which would result in exclusion of water and prevent hydration and plasticisation. Goodall et al. (2012) also found that kafirin from normal sorghum would not form a dough when hydrated, whereas isolated kafirin from high protein digestibility, high lysine sorghum when mixed with wheat gluten formed a dough under the same conditions. Total kafirin film aggregates did not show presence of any fibrils with any of the film treatments (Figure 5.7K-O (stereomicroscopy), 5.8K-O (SEM)). This was probably due to the hydrophobic nature of total kafirin which prevented adequate hydration.

SEM showed that the globular structures of the different prolamins varied in size (Figure 5.8 (SEM)). Commercial zein globular structures were small and uniform (Figure 5.8A (SEM)), whereas total kafirin globular structures were large and non-uniform (Figure 5.8K-O (SEM)). This again is probably indicative of the total kafirin not being adequately hydrated to form a continuous dough matrix, as discussed above.

Addition of a copper catalyst together with hydrogen peroxide greatly increased the extensibility of the commercial zein and total zein doughs formed (Figure 5.6). Doughs prepared from commercial zein were more extensible compared to total zein doughs, despite the evidence of disulphide bonds in total zein films. Schober et al. (2011) proposed that hydrophobic interactions rather than disulphide bonds are the key to gluten-like functionality of zein and kafirin. They suggested that disulphide bonds could have a negative effect and might prevent protein molecule aggregation. Therefore the hydrophobic nature of kafirin and further cross-linking by disulphide bonding brought about by oxidation with hydrogen peroxide might have prevented the kafirin film powder from forming a dough.

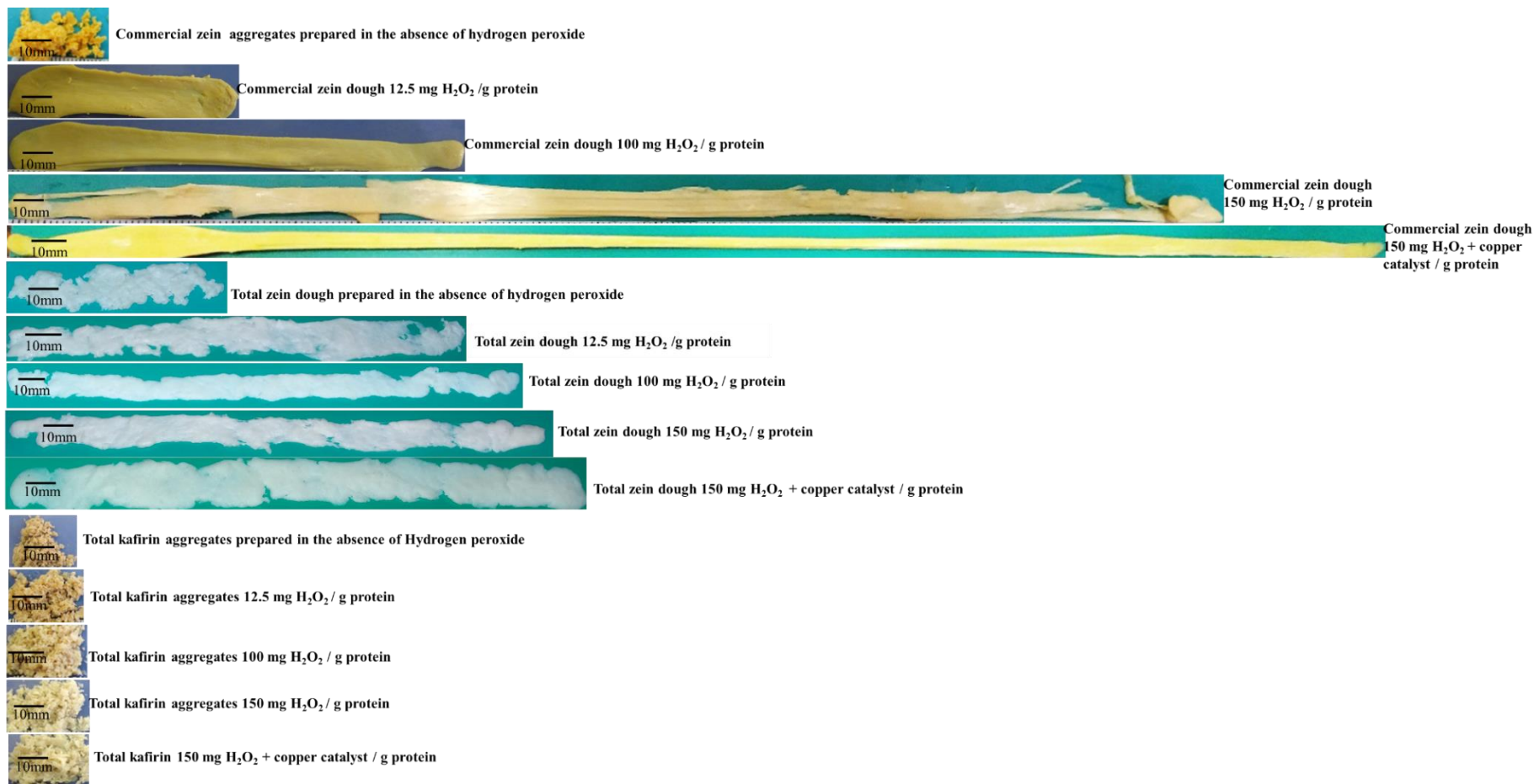


Figure 5.6: Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough formation and extensibility

Table 5.6: Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough formation and extensibility

Prolamin	Film formulation (mg H₂O₂/g protein)	Dough formation from films	Extensibility (mm)	Extensibility factor^{b c}	Water holding capacity^d
Commercial zein^b	0	No	No dough formed	Not applicable	Tissue paper was completely dry
	12.5	Yes	55.1 ^a ± 0.1 ¹	Not applicable	Tissue paper was slightly wet
	100	Yes	94.2 ^b ± 0.3	1.7	Tissue paper was wet
	150	Yes	210.0 ^c ± 0.3	3.8	Tissue paper was wet
	150 + copper catalyst	Yes	270.4 ^d ± 0.4	4.9	Tissue paper was wet
Total zein^c	0	Yes	56.2 ^a ± 0	Not applicable	Tissue paper was wet
	12.5	Yes	92.1 ^b ± 0.2	1.6	Tissue paper wet
	100	Yes	101.4 ^c ± 0.1	1.8	Tissue paper wet
	150	Yes	105.6 ^d ± 0.1	1.9	Tissue paper was wet
	150 + copper catalyst	Yes	112.1 ^e ± 0.4	2.0	Tissue paper wet
Total kafirin	0	No	No dough formed	Not applicable	Tissue paper was completely dry
	12.5	No	No dough formed	Not applicable	Tissue paper was dry
	100	No	No dough formed	Not applicable	Tissue paper was wet
	150	No	No dough formed	Not applicable	Tissue paper was completely wet
	150 + copper catalyst	No	No dough formed	Not applicable	Tissue paper was completely wet

^a Mean values in columns with different superscript letters differ significantly (p < 0.05)

^b Extensibility factor was calculated using the length (mm) of lowest concentration when a dough was formed. E₁/E₂ where E₁= extensibility of dough of a higher concentration (mm), E₂=extensibility of commercial zein dough with 12.5 mg H₂O₂/g protein

^c Extensibility factor was calculated using length of total zein dough without hydrogen peroxide. E₁/E₂ where E₁= extensibility of dough of a higher concentration (mm), E₂=extensibility of total zein dough without hydrogen peroxide

^d Water holding capacity was measured by placing the doughs/aggregates on a double tissue paper and pressing it between the thumb and index finger. The degree of tissue wetness was measured as slightly wet, wet, completely wet, dry and completely dry

¹ Mean ± Standard Deviation, n=3

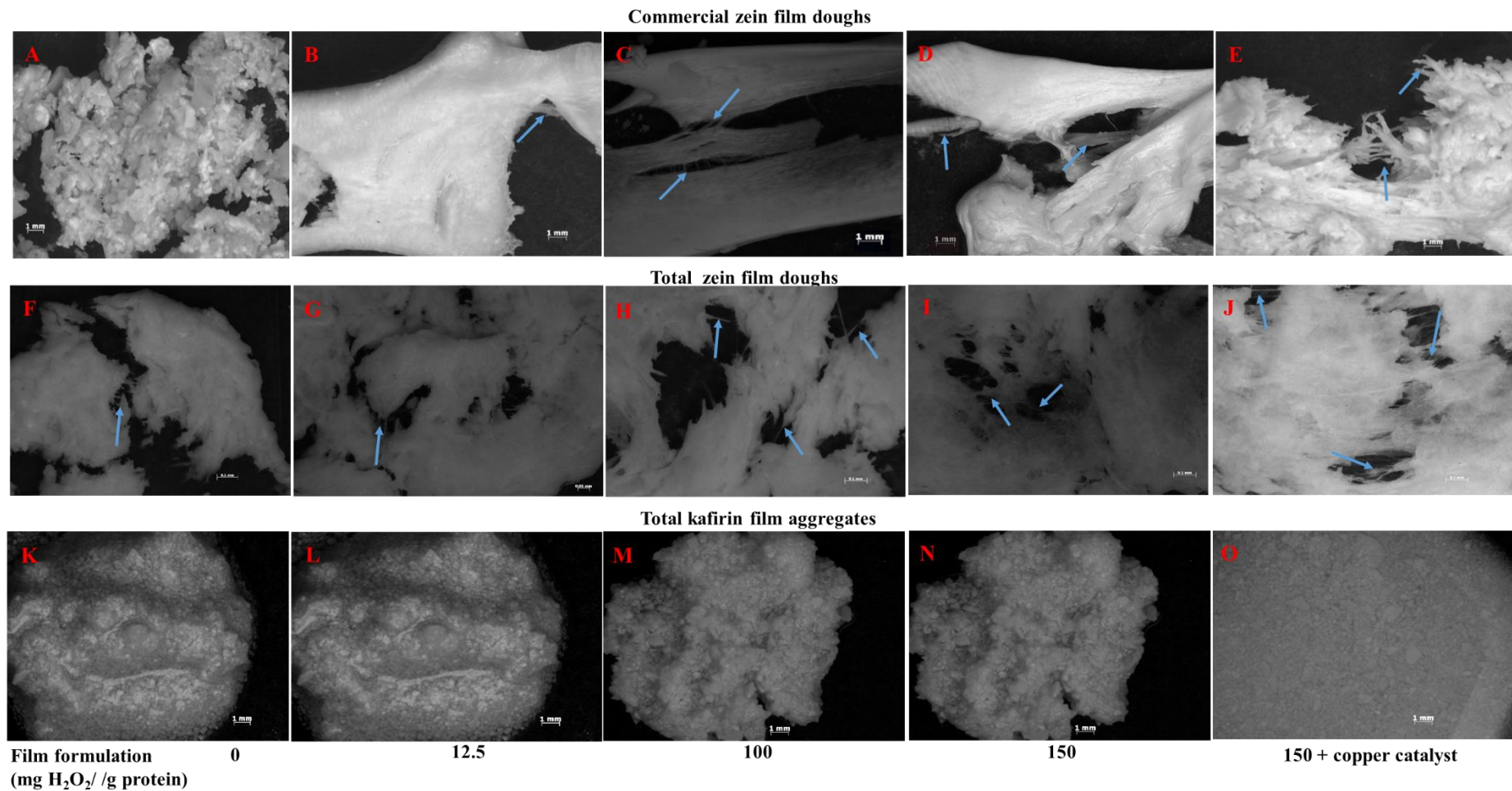
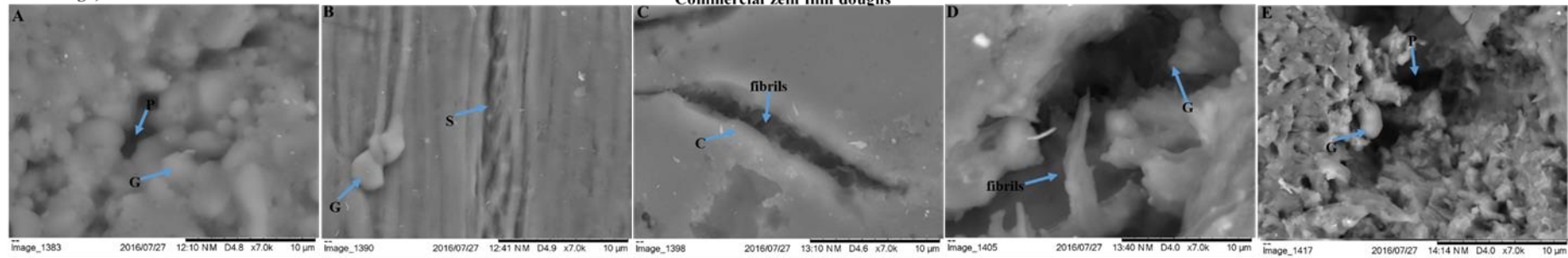


Figure 5.7: Stereomicroscopy showing the effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the fibril formation

Commercial zein film aggregates (did not form dough)

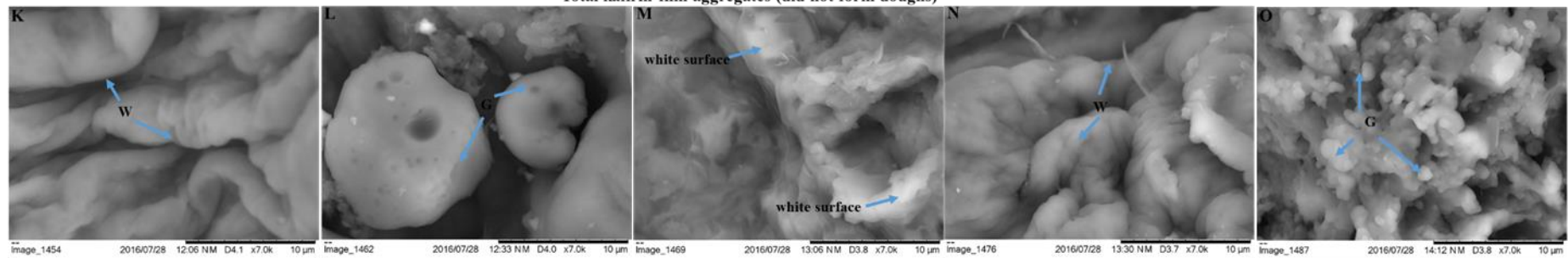
Commercial zein film doughs



Total zein film doughs



Total kafirin film aggregates (did not form doughs)



Film formulation
(mg H₂O₂) / g protein

0

12.5

100

150

150 + copper catalyst

Figure 5.8: SEM showing the effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough formation and extensibility
G-globular like structures, S-aligned fibrils, W-wrinkled

5.7.2. Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough protein secondary structure

The effects of film preparation with hydrogen peroxide on commercial zein, total zein and total kafirin doughs protein secondary structure were interpreted as described in section 5.3.5. The FTIR spectra of commercial zein and total zein doughs were similar to each other with more α -helical conformation than that of total kafirin aggregates (Figure 5.9).

Aggregates of commercial zein prepared from films cast without hydrogen peroxide had more α -helical than β -sheet conformation (Figure 5.9A). When compared to the FTIR spectra of commercial zein films without hydrogen peroxide there was a 28% decrease in the α -helical conformation (Tables 5.4 and 5.7, respectively). Furthermore, the commercial zein aggregate peaks shifted to lower frequencies (from 1644-1626 cm^{-1} to 1641-1616 cm^{-1}) (Figure 5.9A, circled in red, Table 5.7). This shift could be an indication of an increase in β -sheet conformation as Anyango et al. (2012) found a shift in the frequencies of kafirin microparticles and attributed it to an increase in β -sheet conformation. Commercial zein doughs had a slightly higher α -helical conformation than β -sheet conformation (Figure 5.9A, Table 5.7). However, in comparison to the commercial zein films prepared with hydrogen peroxide from which the doughs were made, there was a decrease in the α -helical conformation ($\sim 35\%$) with an increase in the β -sheet conformation. This is in agreement to the findings of Mejia et al. (2007) who found an increase in the β -sheet structure of zein after hydration at 35°C. Wellner et al. (2005) also found that in wheat dough formation the β -sheet conformation increased with a decrease in α -helical conformation and β -turns. The α -helical conformation of commercial zein doughs slightly increased ($p < 0.05$) with an increase in the concentration of hydrogen peroxide used in preparing the films from which the doughs were made (Table 5.7).

Total zein doughs showed an almost equal ratio of α -helical to β -sheet conformation (Figure 5.9B, Table 5.7). The α -helical conformation of total zein films decreased slightly by $\sim 2\%$ when manipulated into dough. Preparation of total zein films with hydrogen peroxide resulted in no significant change ($p \geq 0.05$) in the α -helical and β -sheet conformation of their doughs (Table 5.7). However, preparation of total zein films with hydrogen peroxide plus copper catalyst increased the α -helical conformation of their doughs (Figure 5.9B).

Total kafirin aggregates formed from total kafirin films prepared without hydrogen peroxide were predominantly β -sheet in conformation (Figure 5.9C, circled in purple). The proportion of β -sheet conformation in the total kafirin aggregates was slightly higher than in total kafirin films prepared without hydrogen peroxide (Tables 5.4 and 5.7). There was 7% increase in the β -sheet conformation of total kafirin aggregates formed from films prepared with hydrogen peroxide compared to the films (Table 5.7). The predominantly β -sheet conformation of total kafirin aggregates as found by FTIR is in agreement with the observation of protein aggregates by SEM (Figure 5.8K-O) because protein aggregation is associated mostly with the presence of β -sheet conformation (Mizutani et al., 2003).

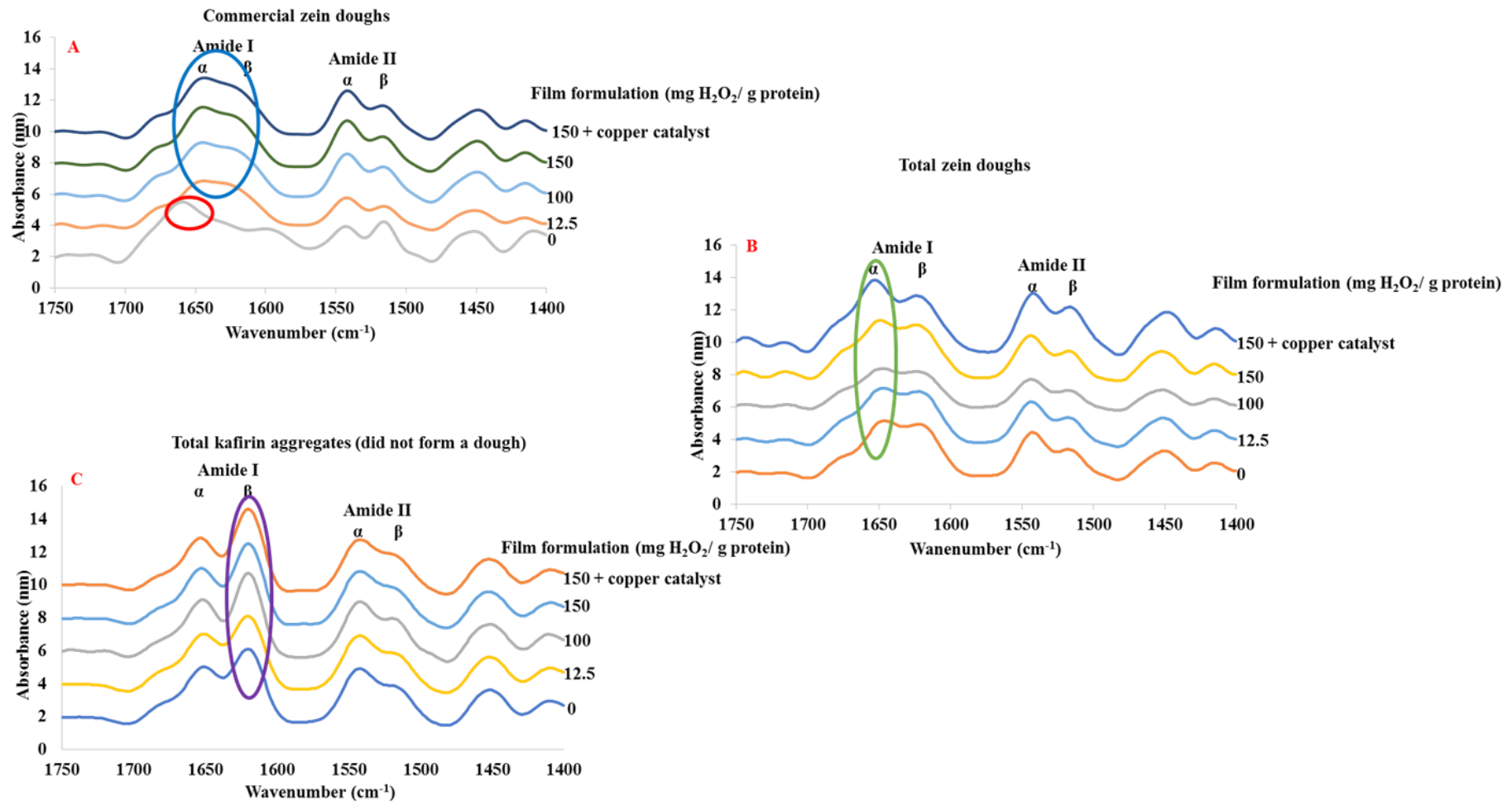


Figure 5.9: Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough protein secondary structure. Each curve represents an average of four independent experiments where each sample was subjected to 64 scans.

Red ring- shift of the peak frequency Blue and green ring-a slight increase in α -helical conformation

Purple ring- an increase in β -sheet conformation

Table 5.7: Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on dough protein secondary structure determined by FTIR spectra of the Amide I region

Zein type	Film formulation (mg H₂O₂/g protein)	α-helix wavenumber (cm⁻¹)	β-sheet wavenumber (cm⁻¹)	Relative α-helical conformation (%)	Relative β-sheet conformation (%)	Ratio (α:β) (1:X)
Commercial zein	0	1641	1616	57.7 ^c ± 0.5 ¹	42.3 ^a ± 0.5	1.36 ^b ± 0.2
	12.5	1644	1628	51.3 ^a ± 0.1	48.7 ^c ± 0.1	1.05 ^a ± 0.01
	100	1644	1627	52.0 ^a ± 0.3	48.0 ^{bc} ± 0.3	1.08 ^a ± 0.01
	150	1644	1625	53.4 ^b ± 0.6	46.6 ^b ± 0.6	1.15 ^a ± 0.02
	150 + copper catalyst	1644	1625	53.2 ^b ± 0.3	46.8 ^b ± 0.3	1.14 ^a ± 0.01
Total zein	0	1646	1622	51.6 ^a ± 0.2	48.4 ^c ± 0.1	1.06 ^a ± 0.01
	12.5	1646	1622	51.5 ^a ± 0.2	48.5 ^c ± 0.2	1.06 ^a ± 0.01
	100	1645	1624	52.2 ^a ± 0.1	47.8 ^c ± 0.1	1.09 ^a ± 0.01
	150	1647	1624	52.4 ^a ± 0.4	47.6 ^c ± 0.4	1.10 ^a ± 0.02
	150 +copper catalyst	1652	1623	56.6 ^b ± 0.5	43.4 ^a ± 0.5	1.30 ^a ± 0.02
Total kafirin	0	1650	1620	44.6 ^d ± 0.5	55.4 ^a ± 0.5	0.81 ^b ± 0.01
	12.5	1651	1621	43.1 ^c ± 1.1	56.9 ^b ± 1.1	0.76 ^a ± 0.02
	100	1651	1621	40.6 ^b ± 0.2	59.4 ^c ± 0.2	0.68 ^a ± 0.01
	150	1652	1619	41.0 ^b ± 0.2	59.0 ^c ± 0.2	0.69 ^a ± 0.01
	150 +copper catalyst	1652	1616	39.8 ^a ± 0.8	60.2 ^d ± 0.8	0.66 ^a ± 0.02

^a Mean values in columns with different superscript letters differ significantly (p < 0.05), n = 4

¹ Mean ± Standard Deviation, each sample was subjected to 64 scans each

5.8. Conclusions

Hydrogen peroxide has the potential to improve zein dough functionality because oxidative cross-linking of commercial zein and total zein during film formation promotes dough formation and enhances the dough cohesiveness and extensibility. In fact, zein doughs prepared from oxidised films are cohesive, can hold water for long when left below 25°C for 1min, are highly extensible and retain their extensibility at 25°C. The improved cohesiveness and extensibility of total zein doughs is probably due to formation of disulphide bonds which helps in the formation of a continuous protein network, as indicated by microscopy. However, total kafirin films prepared with hydrogen peroxide do not form a dough, probably due to kafirin being particularly hydrophobic. Although many challenges still remain, oxidative modification of zein dough properties is a step forward in the development of gluten-free doughs.

6. GENERAL DISCUSSION

This discussion first critically evaluates the major methodologies applied during the research. A proposed mechanism for cross-linking of commercial zein, total zein and total kafirin films with hydrogen peroxide and its relation to the aqueous buffer uptake of the films and dough formation of commercial zein and total zein films will then be discussed. Lastly, suggestions for further development based on these research findings will be made.

6.1. Critical review of methodologies applied

In the casting technique, prolamin films are obtained by drying of a solution consisting of protein and a volatile solvent. The interaction between the physiochemical and the drying temperature and relative humidity is important in respect of the quality of the film therefore must be controlled properly (Tapia-Blácido et al., 2005). It was found to be more difficult to peel the films from the silicone pans during summer compared to the winter season. The films that were cast in summer were rough and curled up during drying, and were difficult to peel off the tray, whereas those cast in winter were smooth, remained intact when in contact with the surface of the casting silicone tray and were easy to peel off. This is probably because of the higher relative humidity in summer which caused the top surface of the dry films to take up moisture from the atmosphere, leading to the curling up.

It is well known that zein when hydrated and mixed with water above its T_g ($\sim 28^\circ\text{C}$) can form cohesive and extensible doughs (Lawton, 1992). However, it was difficult to maintain the temperature of the zein doughs above zein's T_g during the analyses. This made stretching of the doughs for observation by stereomicroscopy to view the fibrils difficult as the doughs quickly stiffened below their T_g . Thus, cooling of doughs could not be prevented as the instruments were operated at ambient temperature (approx. 25°C). Temperature changes can affect zein's physicochemical properties. For example, zein dough protein secondary structure has been observed to change drastically from β -sheet to α -helix with a drop in temperature from 35°C to 25°C (Mejia et al., 2007).

Glass transition is a very important property of amorphous food polymers where the materials change in their thermo-mechanical properties at a specific temperature or temperature range (Abiad et al., 2009). DSC measures the changes in heat flow as a function of temperature as well as phase transition (He et al., 2004). Interpretation of the prolamin T_g data was difficult because the prolamin protein preparations were comprised of different classes, hence multiple transition occurred. To improve the sensitivity in order to detect low energy changes, increasing heating rate from 10°C/min to 20°C/min was investigated. This, however, decreased the ability to resolve transitions that occurred which were close in temperature and hence the analyses had to be performed at the heating rate of 10°C/min.

A related problem was that DSC cannot optimize both sensitivity and resolution in a single experiment (Ma et al., 1990). A technique that could have been used is Modulated DSC (MDSC). This is a modification of conventional DSC that increases resolution and sensitivity to detect weak transition or multiple transitions occurring at the same temperature (Reading et al., 1994). MDSC has two heating profiles a linear heating rate that provides information similar to standard DSC and modulated heating rate that permits the simultaneous measurement of the sample's heat capacity. MDSC has the following advantages over conventional DSC: separation of overlapping transitions, improved sensitivity for detecting weak transitions, accurate measurement of polymer initial crystallinity, improved sensitivity and resolution in a single experiment (Verdonck et al., 1999).

6.2. Discussion of research findings

Table 6.1: Summary of the effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the properties of films and doughs formed

Prolamin	Treatment	Effect on film properties	Effect on film dough formation	Effect on dough properties	Explanation
Commercial zein	Hydrogen peroxide	No disulphide and non-disulphide bonds formed			Alpha-zein has few cysteine residues, which are essential for disulphide bond formation through oxidation
		Increased film aqueous buffer uptake			An increase in hydroxyl groups due to hydroxylation of the hydrophobic amino acids in commercial zein increases hydrogen bonding with water. Thus, increases aqueous buffer uptake.
			Promotion of dough formation	Dough extensibility, cohesiveness, softness and water holding capacity were greatly increased	Increased aqueous buffer uptake by the films improves hydration promoting dough formation. Water acts as a plasticiser, hence increases extensibility, cohesiveness and softness of doughs.
			Promotion of fibril formation		Destabilisation of the native folding of the protein in which the conditions favour hydrogen bonding with the polypeptides results in fibril formation. Hydroxylation of commercial zein increase hydrogen bonding hence, the formation of fibrils.
		An increase in the α -helical conformation			Hydroxylation of commercial zein increases the secondary structure stability hence an increase in α -helical conformation.

Table 6.1: Summary of the effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the properties of films and doughs formed

Prolamin	Treatment	Effect on film properties	Effect on film dough formation	Effect on dough properties	Explanation
Commercial zein	Hydrogen peroxide		An increase in α -helical conformation		
		Increased the T_g and ΔC_p			Increase in hydroxyl groups promotes protein-to-protein interaction which increase the stability of the films reducing the tendencies to disintegrate, hence an increase in T_g and ΔC_p .
	Hydrogen peroxide + copper catalyst	Reduced film aqueous buffer uptake			Copper ion interacting with the protein during oxidation repels water. Also copper catalyst promotes more protein-protein interactions through hydrogen bonding, hence reducing polar sites available to bond with water.
				Dough extensibility, cohesiveness, softness was greatly increased. Water holding capacity was reduced below T_g	The reduced water holding capacity below T_g was due to insufficient hydration because of extensive hydroxylation which promotes protein-protein interaction.
			Numerous fibrils were formed		
		Decrease in T_g			Copper ions forming bonds with the proteins acts as a discontinuity, hence decrease the T_g
Total zein	Hydrogen peroxide	Disulphide bond formation through oxidation			Total zein contains cysteine residues in the β -zein which is essential for disulphide bond formation through oxidation.

Table 6.1: Summary of the effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the properties of films and doughs formed

Prolamin	Treatment	Effect on film properties	Effect on film dough formation	Effect on dough properties	Explanation
Total zein	Hydrogen peroxide	Increased film aqueous buffer uptake			During formation of disulphide bonds there is also hydroxylation taking place. Interaction of the hydroxyl groups with water increases the aqueous buffer uptake
				Increase dough extensibility, cohesiveness and water holding capacity	Increased aqueous buffer uptake by the films improves hydration promoting dough formation. Water acts as a plasticiser
			Promotion of fibril formation		
		Slight increase in the α -helical conformation			
			An increase in α -helical conformation		
		T_g and ΔC_p increased			Cross-linking restrains internal rotation along macromolecular chain, reduces chain mobility and increases chain rigidity, which increases T_g and transition enthalpy (Hernández-Muñoz et al., 2005)
	Hydrogen peroxide + copper catalyst	Greatly reduced aqueous buffer uptake.			

Table 6.1: Summary of the effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the properties of films and doughs formed

Prolamin	Treatment	Effect on film properties	Effect on film dough formation	Effect on dough properties	Explanation
Total zein	Hydrogen peroxide + copper catalyst		Fibril formation greatly increased	Dough extensibility and cohesiveness increased. However, the water holding capacity was reduced below T_g .	Decrease in aqueous buffer uptake was due to extensive cross-linking that hinders hydrogen bonding between the protein and water. Decreased water holding capacity of the dough when handled below T_g was due to low film hydration
Total zein	Hydrogen peroxide + copper catalyst	Decrease in T_g			
Total kafirin	Hydrogen peroxide	Disulphide bond formation through oxidation			Disulphide bond formation due to the presence of cysteine residues in β - and γ -kafirin
		Film aqueous buffer uptake progressively increased			Hydrogen peroxide promotes oxidation through disulphide bond formation and hydroxylation. The hydroxyl groups interact with water to increase aqueous buffer uptake. However, even with the increase in aqueous buffer uptake kafirin is too hydrophobic in nature which makes it difficult to hydrate.
			No dough or fibril formation	Not applicable	Kafirin is hydrophobic in nature, hence difficult to fully hydrate for dough formation
		The β -sheet conformation increased	The β -sheet conformation of aggregates increased		Kafirin has more cysteine residues than total zein, hence, a higher degree of polymerisation resulting in an increase in β -sheet conformation.

Table 6.1: Summary of the effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the properties of films and doughs formed

Prolamin	Treatment	Effect on film properties	Effect on film dough formation	Effect on dough properties	Explanation
Total kafirin	Hydrogen peroxide + copper catalyst	Greatly decreased the aqueous buffer uptake of total kafirin films.			
		T_g and ΔC_p decreased			

6.2.1. Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the functional properties of the films

Treating commercial zein, total zein and total kafirin with hydrogen peroxide increased the aqueous buffer uptake of the films (section 5.3.3). Addition of oxidising agents can form both disulphide and dityrosine cross links in wheat gluten (Wieser, 2007). However, SDS-PAGE of commercial zein (section 5.3.4 Figure 5.3) showed no evidence of disulphide or non-disulphide covalent bonds because commercial zein is essentially α -zein and there are few cysteine or tyrosine residues to form disulphide or dityrosine bonds (Oom et al., 2008). As stated (section 5.3.3), the increase in aqueous buffer uptake of the prolamin films can be attributed to the hydroxylation of the aliphatic side chains dominant in the α -subunits of commercial zein, total zein and total kafirin.

Hydroxylation is an oxidative process that introduces a hydroxyl group (-OH) into an organic compound (Tsukada, 2012). Oxidation of proteins with hydrogen peroxide can also result in formation of hydroxyl and carbonyl groups on hydrophobic amino acid residues with aliphatic side chains such as leucine, isoleucine, valine and alanine (Xu and Chance, 2007). Figure 6.1 illustrates the possible mechanism of action of hydrogen peroxide with commercial zein, total zein and total kafirin, leading to an increase in aqueous buffer uptake. Initially, the prolamin protein is a densely packed structure of polypeptides with α -helical conformation (Figure 6.1a). These α -helices are characterized by hydrophilic and hydrophobic sides due to a typical repeating sequence of amino acids with different polarities (Hoffman, 2012). Proteins undergo conformational transition in response to different changes such as increased temperature (Hennink and Van Nostrum, 2012). During film formation the tightly α -helical structure partially unfolds and the reactive groups of the hydrophobic amino acid residues (alanine, isoleucine, leucine and proline) are exposed. When hydrogen peroxide is added it dissociates to give reactive hydroxyl groups which then oxidise the hydrophobic amino acid residues introducing hydroxyl groups on them (Figure 6.1b). When the oxidised zein and kafirin films are immersed in water there is protein-water interaction through hydrogen bonding (Figure 6.1c) resulting in the observed increase in aqueous buffer uptake of the films.

Inclusion of hydrogen peroxide in total zein and total kafirin film solution improved the solubility of the protein. The high dielectric constant (41.1) of 70% ethanol indicates that it is hydrophilic (Taylor et al., 2005), hence total zein and total kafirin hydrophobicity decreased, probably due to hydroxylation making the proteins easily dissolve in ethanol.

However, SDS-PAGE of total zein and total kafirin films showed evidence of disulphide and non-disulphide bonds (section 5.3.4 Figure 5.3). The sulphhydryl groups of the cysteine present in β -subunits of total zein and total kafirin can be oxidised to disulphide bonds (Byaruhanga et al., 2005). When hydrogen peroxide is included in total zein and total kafirin film forming solutions, it readily oxidises the cysteine residues to form disulphide bonds (Figure 6.2). Non-disulphide bonds that can be formed include dityrosine bonds. Hydrogen peroxide catalyses oxidation of tyrosine to form dityrosine (Stuchell and Krotchta, 1994). This shows that different oxidative reactions occurred between hydrogen peroxide and the prolamins. When a copper catalyst is included the aqueous buffer uptake is reduced. It is postulated that the reduction in aqueous buffer uptake when a catalyst is included could be due to extensive hydroxylation that promotes protein-protein interaction rather than protein-water interaction as well as some hydroxyl groups being substituted by copper ions making the films less hydroxylated compared to the films without a catalyst (Figure 6.1d).

The FTIR spectroscopy data (section 5.3.5) showed that inclusion of hydrogen peroxide does not change the existing conformation of the protein but only reinforces the already existing structure.

Factors that affect the T_g of polymers include their molecular weight, chain branching, crystallinity and degree of cross-linking (Hernández-Muñoz et al., 2005). T_g reflects the molecular mobility of a biopolymer (Ringe and Petsko, 2003). The increase in T_g and ΔC_p of the films (section 5.3.6) with the inclusion of hydrogen peroxide was presumably due to increased intermolecular entanglement. However, inclusion of copper catalyst reduced the T_g and ΔC_p of the films. Despite the copper catalyst giving more cross linking effects (Figure 6.1), it is postulated that copper ions form bonds with the proteins creating large spaces within the protein (Figure 6.1d and 6.2d), which then results in decreased T_g and ΔC_p .

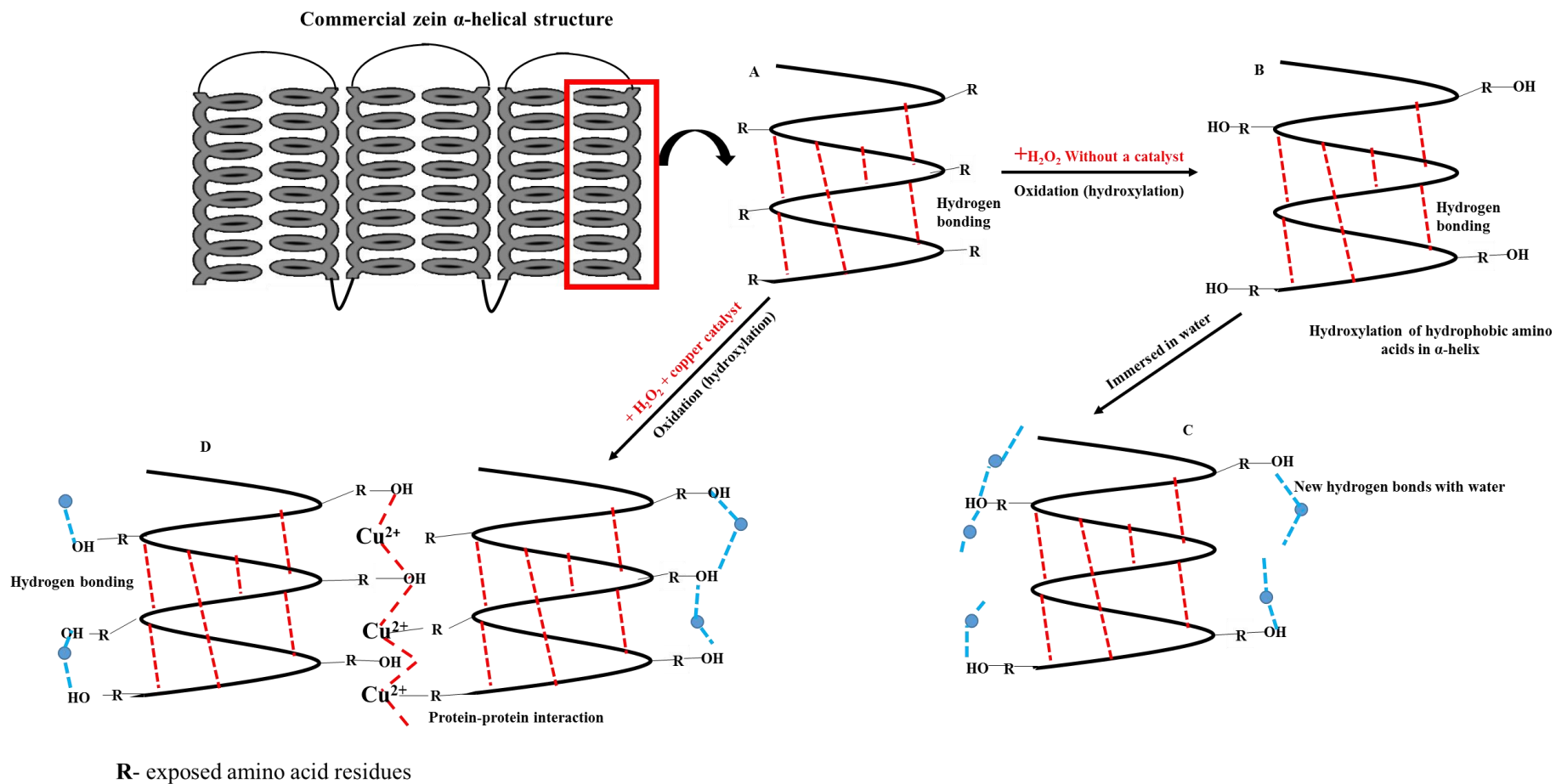


Figure 6.1: Model to explain the increased water uptake by commercial zein films promoted by addition of hydrogen peroxide during casting and the decrease in water uptake when copper catalyst is added.

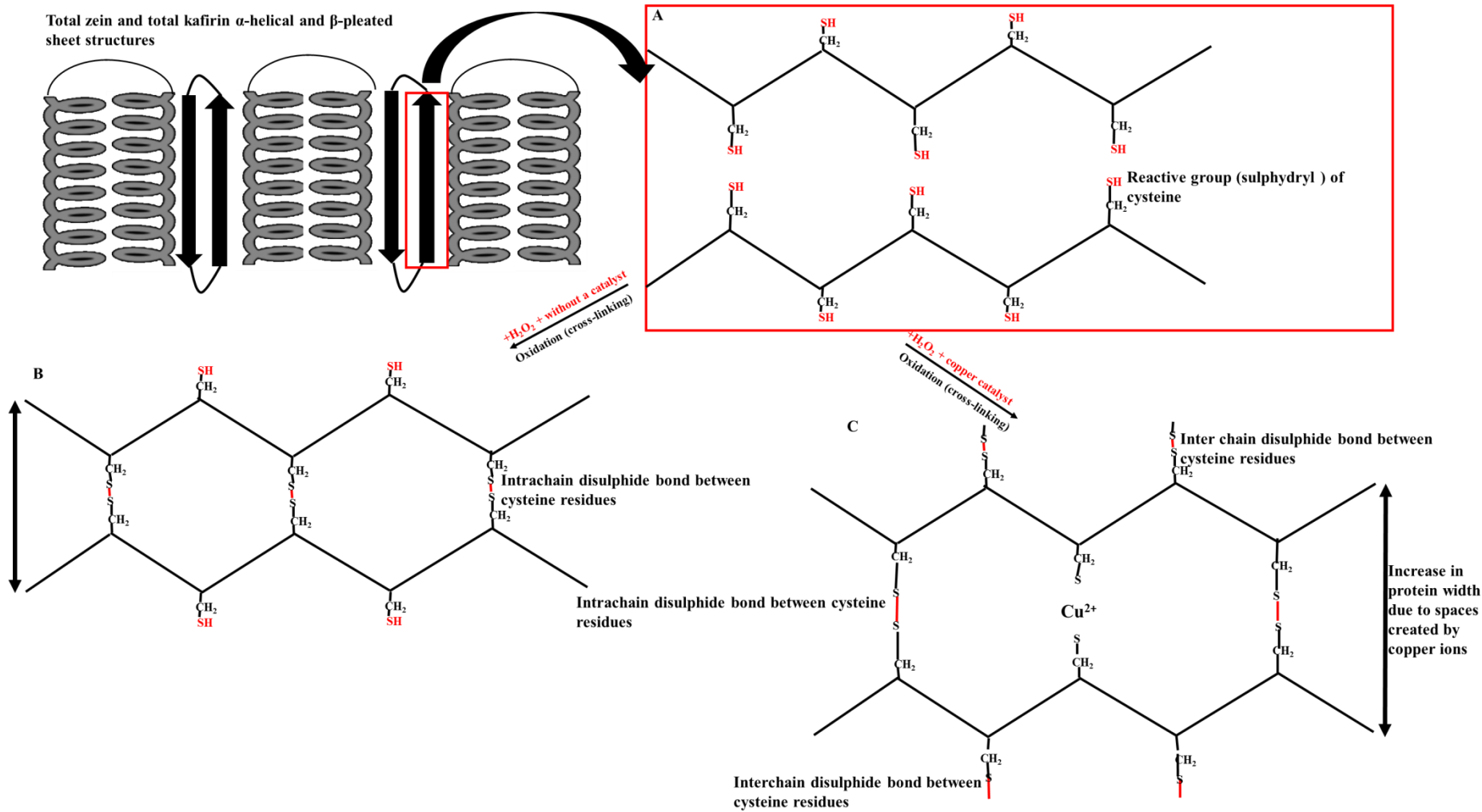


Figure 6.2: Model to explain the increased water uptake by total zein and total kafirin films promoted by addition of hydrogen peroxide during casting and the decrease in water uptake with the inclusion of copper catalyst.

6.2.2. Effects of commercial zein, total zein and total kafirin film preparation with hydrogen peroxide on the dough forming properties

The increase in film aqueous buffer uptake due to oxidation of the proteins by hydrogen peroxide improved the hydration of the films which enabled formation of doughs. Commercial zein films did not form doughs when hydrated in water above zein T_g , whereas films that had been oxidised with hydrogen peroxide were able to form doughs. This confirms that oxidation of commercial zein by hydrogen peroxide was responsible for dough formation. Total zein films prepared without hydrogen peroxide formed soft, only slightly cohesive and extensible doughs. However, oxidation of total zein films improved extensibility and cohesiveness of the dough. The improved cohesiveness and extensibility of total zein doughs with addition of hydrogen peroxide was probably due to formation of disulphide bonds. Formation of disulphide bonds improves the strength of gluten doughs (Wieser, 2007). A wheat gluten network is formed by polymerisation after oxidation of the thiol groups and there is interchange of the thiol groups to sulphhydryl bonds (Weegels et al., 1997). This results in the formation of a polymeric structure that gives an optimal gluten network which confers dough machinability, good gas retention and fine bread crumb structure (Belton, 2005). Lawton (1992) proposed that the zein-starch doughs were not as strong as wheat flour dough due to fewer intermolecular cross-links.

In this present work, it was found that there was an increase in fibril formation in commercial zein and total zein doughs with an increase in concentration of hydrogen peroxide (section 5.7.1 Figure 5.7). Fibril formation is initiated through deviation from the protein's native state forming unstable intermediates within the system (Erickson et al., 2012). These authors developed a model for fibril formation in zein doughs based on the self-assembly of amyloid fibrils, as observed in Alzheimer's disease. Amyloid-type fibril formation has also been exhibited in non-amyloidogenic proteins under partial denaturing conditions (Chiti et al., 1999).

Amyloid fibril formation occurs when the native folding of a protein is destabilized under conditions in which non-covalent interactions, in particular hydrogen bonding, within the polypeptide chain remain favourable (Erickson et al., 2012). In this present study hydrogen bonding in commercial zein film and total zein film doughs appeared to be essential to form extensive fibrils.

That SDS-PAGE under reducing conditions (section 5.3.4 Figure 5.3) did not show induced polymerisation of commercial zein, may not mean that any disulphide or dityrosine covalent bonds were not formed. Commercial zein contains one cysteine residue near the terminal (Oom et al., 2008). Therefore disulphide or dityrosine bonds could have been formed that contributed to dough formation but due to low concentration were not visible by SDS-PAGE. Despite the increase in aqueous buffer uptake, evidence of disulphide bonds and increase in β -sheet conformation, total kafirin films could not form doughs. This was probably due to the hydrophobic nature of kafirin as it is difficult to hydrate (Duodu et al., 2003).

Notably, in this present study, the FTIR spectroscopy did not indicate any increase in β -sheet conformation associated with the formation of zein fibrils. Therefore it is likely that in this study protein-water interactions played an integral part in the orientation of commercial zein and total zein molecules into fibrils allowing their aggregation into doughs.

6.3. Way forward

This study has shown that oxidation of commercial zein, total zein and total kafirin by hydrogen peroxide alters the functional properties of the films by increasing their aqueous buffer uptake and improving physical stability of the films in an aqueous medium. Thus, this increases the potential applications of such films as hydrogels in biomedical application such as drug and cell carriers (Ni and Dumont, 2017). Oxidation of commercial zein and total zein films by hydrogen peroxide also promotes their formation into cohesive and extensible doughs when hydrated in distilled water.

These findings create interesting avenues for further investigation. For example, it would be important to investigate the use of other food-grade oxidising agents such as carbamide and azodicarbonamide (Code of Federal Regulations, 2016) as this is important for human consumption.

7. CONCLUSIONS AND RECOMMENDATIONS

Oxidation of zein and kafirin proteins by hydrogen peroxide modifies their functional properties, in particular it increases the aqueous buffer uptake of their films. The increase in film aqueous buffer uptake appears to be due to hydroxylation of the proteins, hence the second hypothesis that oxidation of films with hydrogen peroxide would reduce film aqueous buffer uptake as result of formation of disulphide and dityrosine bonds must be rejected.

However, total zein and total kafirin films do show evidence of disulphide bond formation. This is probably as a result of the cysteine residues in their β - and γ -prolamins, whereas, there is no evidence of disulphide bond formation in films from commercial zein, which is predominantly α -zein. This indicates that different oxidative reactions occur between hydrogen peroxide and prolamins depending on the classes present. Oxidation of commercial zein, total zein and total kafirin with hydrogen peroxide results in the formation of a compact film protein structure as observed by microscopy and an increase in T_g and ΔC_p . This indicates protein entanglement in the films either by hydroxylation or disulphide bond formation.

The increase in the film aqueous buffer uptake after oxidation appears to improve hydration of commercial zein and total zein, which in turn promotes dough formation. However, total kafirin does not form a dough after oxidation, probably because it is difficult to hydrate.

Hydrogen peroxide modifies zein dough rheological properties especially cohesiveness, extensibility and water holding capacity. Oxidation favours formation of fibrils, probably due to deviation of the proteins from their native structure after new intermolecular bond formation. It can be concluded that formation of disulphide bonds is not involved in dough formation in zein or kafirin. The research aim of this study to modify the functional properties of commercial zein, total zein and total kafirin films and doughs by oxidation has been achieved.

This work has created interesting avenues for further investigation. Increase in the film aqueous buffer uptake after oxidation opens new applications, for example where prolamin hydrogel films could be used to encapsulate drugs in order that they are slowly released in the body. Additionally, although many challenges still remain, oxidative modification of zein rheological properties represents a further step forward in development of technology to enable zein to replace gluten in dough systems.

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9. APPENDIX

Publications and presentations from this research

Scientific paper:

Taylor, J. R. N., Johnson, S. K., Taylor, J., Njila, S. and Jackaman, C. (2016a). Oxidation of commercial (α -type) zein with hydrogen peroxide improves its hydration and dramatically increases dough extensibility even below its glass transition temperature. *Journal of Cereal Science* 70: 108-115.

Conference presentations:

Njila, S. (2017). Modification of the functional properties of zein and kafirin protein films by oxidation using hydrogen peroxide. Oral presentation at the 2nd New Voice Symposium Cereal Science and Technology (CST-SA), 11th of May 2017, Pretoria, South Africa.