Kafirin viscoelastic masses: Factors influencing their formation and rheological behaviour

By

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Submitted in partial fulfilment of the requirements for the Degree

PhD Food Science

In the Department of Consumer and Food Sciences
Faculty of Natural and Agricultural Sciences
University of Pretoria
South Africa

October 2018

DECLARATION

I hereby declare that this thesis submitted at the University of Pretoria for the award of PhD degree is my work and has not been submitted by me for a degree at any other University or Institution of Higher Education.

Segun Isaac Oguntoyinbo
October 2018

ABSTRACT

Kafirin viscoelastic masses: Factors influencing their formation and rheological behaviour

by

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Supervisor: Prof John RN Taylor

Co-supervisor: Dr Janet Taylor

Sorghum is the world's fifth most important cereal crop. It is a drought–tolerant and widely cultivated in Africa. Therefore, the use of locally produced sorghum in bread products would be advantageous to farmers and consumers in Africa. However, sorghum flour does not produce wheat flour-like doughs as kafirin, its prolamin storage protein does not exhibit viscoelastic properties like wheat gluten. Recently, kafirin viscoelastic mass formation by coacervation with water from glacial acetic acid has been reported. The objective of this study was to determine how various factors influence the functionality of kafirin viscoelastic masses with the aim of producing kafirin-based doughs with similar rheological characteristics as gluten-based doughs.

Firstly, the effects of various different extraction solvents on kafirin composition and structural conformation were investigated. All the kafirin preparations formed viscoelastic masses by coacervation, regardless of the extractant used. FTIR data indicated that kafirin viscoelastic mass formation did not depend on the secondary structure of the protein.

Secondly, the effects of kafirin and zein (maize prolamin) composition from various sources on their rheological properties were also investigated using stress-relaxation and dynamic rheological analyses. Kafirins and zeins from all sources studied formed viscoelastic masses. Kafirin masses were much firmer and had a much higher elastic component than zein masses, which had predominantly viscous flow properties. Both were softer than gluten. Maintenance of kafirin elasticity when stored appears to require γ-kafirin.

Thirdly, the effects of final acetic acid and protein concentration during the coacervation process were investigated, with the aim of producing food-compatible functional masses. Coacervation with reduction in the final acetic acid concentration down to 0.1% still allowed

formation of kafirin and zein viscoelastic masses with functionality retained when stored at $4^{o}C$ for an extended period; indicating an irreversible molecular change with dissolution in glacial acetic acid. A minimum of between 5 and 10% prolamin in glacial acetic acid was required for viscoelastic mass formation at low final acetic acid concentration (5%).

Kafirin displayed a similarly high elastic component to gluten, whereas zein exhibited more viscous flow properties. A model is proposed to explain these behaviours. When the masses are compressed, the force is sufficient to break hydrogen bonds but the strong covalent disulphide bonds will remain intact. During compression, zein masses will deform more than kafirin and more energy will be dissipated, whereas kafirin with its higher number of disulphide bonds will exhibit greater resistance to compression, and more energy will be stored. On removal of the force, the kafirin mass will release the stored energy and recover almost to its original shape, with hydrogen bond reformation. However, zein with its lower number of disulphide bonds, will release insufficient energy to return to its original shape.

Both kafirin and zein proteins may be composited to obtain a viscoelastic mass with the desired balance of elasticity and viscous flow properties as gluten.

DEDICATION

This thesis is dedicated to:

My lovely life partner: Yetunde Grace Oguntoyinbo

My lovely daughters: Favour Oguntoyinbo and Faith Oguntoyinbo

For their love, patience and unflinching support

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to my supervisor, Prof. J. R. N. Taylor for his constant support, timely comments, valuable advice, encouragement, constructive criticism and patience. I am highly privileged to be supervised by a great teacher.

I am also very grateful to my co-supervisor, Dr. Janet Taylor for her guidance, tireless assistance, encouragements, valuable advice, unlimited help, nurturing spirit and continual mentorship.

My appreciation also goes to my precious wife, Yetunde Grace Oguntoyinbo and my lovely daughters, Favour and Faith for their unflinching supports. Your encouragements and prayers sustained me when my knees were feeble. You stood by me when my hands were weak.

I would like to thank the academic staff, support staff and fellow postgraduate colleagues at the Department of Consumer and Food Sciences, University of Pretoria for your assistance and support.

I am grateful to Mr. Allan Hall and Dr. Udri Venter of the University of Pretoria's Laboratory for Microscopy and Microanalysis for assistance with the microscopy.

My special appreciation goes to the University of Pretoria for the UP Postgraduate Doctoral bursary.

I am also grateful to the Cereal and Malt Extract Company of South Africa for sponsoring my attendance at the International Sorghum Conference "Sorghum in the 21st Century", Cape Town, South Africa.

I would like to thank the Tertiary Education Trust Fund, Nigeria for sponsoring my postgraduate programme.

Above all, I return all glory to the Almighty God.

TABLE OF CONTENTS

DECLARATION ..... **i**

ABSTRACT ..... **ii**

DEDICATION ..... **iv**

ACKNOWLEDGEMENTS ..... **v**

TABLE OF CONTENTS ..... **vi**

LIST OF TABLES ..... **x**

LIST OF FIGURES ..... **xii**

1 INTRODUCTION **1**

2 LITERATURE REVIEW **3**

2.1 Chemistry and structure of kafirin and zein 3

2.1.1 Structural models for zein and kafirin 6

2.1.2 Molecular basis of the functionality of zein and kafirin in bioplastics and doughs 7

2.1.3 The effect of kafirin and zein conformation on dough formation 10

2.2 Chemistry, structure and functionality of the wheat gluten proteins 11

2.2.1 Molecular basis of the viscoelastic properties of wheat gluten 13

2.3 Research on the development and improvement of viscoelastic dough properties of zein and kafirin 16

2.3.1 Effects of dilute organic acids 16

2.3.2 Effect of defatting 19

2.3.3 Effects of oxidizing agents 19

2.3.4 Effects of different compositions of kafirin and zein 20

2.3.5 Co-protein concept 21

2.3.6 Modification of kafirin 21

2.4 Techniques for studying the viscoelastic properties of kafirin and zein 25

2.4.1 Rheological properties of dough 25

2.4.2 Confocal laser scanning microscopy (CLSM) 28

2.4.3 Fourier transform infrared (FTIR) spectroscopy 28

2.5 Conclusions....29

3 HYPOTHESES AND OBJECTIVES....31

3.1 Hypotheses....31

3.2 Objectives....32

4 RESEARCH....33

4.1 **PROPERTIES OF COHESIVE MASSES PREPARED FROM KAFIRIN ISOLATED WITH DIFFERENT SOLVENTS....33**

4.1.1 Abstract....33

4.1.2 Introduction....34

4.1.3 Materials and methods....35

4.1.3.1 Materials for kafirin preparations....34

4.1.3.2 Kafirin extraction with aqueous ethanol....34

4.1.3.3 Kafirin extraction with glacial acetic acid....34

4.1.3.4 Protein preparation purity and yield....35

4.1.3.5 Methods investigated to attempt to form kafirin cohesive mass....35

4.1.3.5.1 Formation of kafirin cohesive mass using the coacervation from solution in glacial acetic acid method....35

4.1.3.5.2 Kafirin preparation hydrated in acetic acid....35

4.1.3.5.3 Kafirin preparation hydrated in water....36

4.1.3.6 Electrophoresis of kafirin preparations....36

4.1.3.6.1 SDS-PAGE....36

4.1.3.6.2 2D-Electrophoresis....36

4.1.3.7 Fourier Transform Infrared spectroscopy (FTIR)....37

4.1.3.8 Stereomicroscopy....38

4.1.3.9 Statistical analyses....38

4.1.4 Results and discussion....39

4.1.4.1 Protein preparation purity and yield....38

4.1.4.2 Electrophoresis……41
4.1.4.3 Secondary structure of kafirin preparations and cohesive masses……..48
4.1.4.4 Appearance of the hydrated kafirin and cohesive masses……53

4.1.5 Conclusions.....56

4.1.6 References.....57

4.2 THE EFFECT OF KAFIRIN AND ZEIN COMPOSITION ON ASPECTS OF THE RHEOLOGICAL PROPERTIES OF KAFIRIN AND ZEIN VISCOELASTIC MASSES 62

4.2.1 Abstract.....62

4.2.2 Introduction.....63

4.2.3 Materials and Methods.....64

4.2.3.1 Sorghum lines for kafirin extraction……63
4.2.3.2 Prolamin preparations……63
4.2.3.3 Preparation of viscoelastic masses……...64
4.2.3.4 Stress-relaxation test (large deformation test)……65
4.2.3.5 Dynamic rheological analysis (small deformation test)……..65
4.2.3.6 Statistical analyses……...66

4.2.4 Results and discussion 67

4.2.4.1 Stress-relaxation and dynamic rheological analysis of kafirin viscoelastic masses……...66
4.2.4.1.1 Kafirin viscoelastic masses from kafirin extracted with different extraction solvents……...66
4.2.4.1.2 Kafirin viscoelastic masses from high protein digestibility sorghum lines……....75
4.2.4.1.3 Stress-relaxation of kafirin viscoelastic masses from kafirin and zein of different subclasses……..82

4.2.5 Conclusions.....86

4.2.6 References.....86

4.3 KAFIRIN AND ZEIN FORMATION: EFFECTS OF ACETIC ACID AND PROTEIN CONCENTRATIONS 90

4.3.1 Abstract 90

4.3.2 Introduction 91

4.3.3 Materials and Methods 93

4.3.3.1 Materials 92

4.3.3.2 Viscoelastic mass formation 92

4.3.3.3 Analyses 93

4.3.3.3.1 Microscopy 93

4.3.3.3.2 Rheology of viscoelastic masses 94

4.3.4 Results and discussion 95

4.3.4.1 Microscopy of masses formed at different final acetic concentrations 94

4.3.4.2 Rheological properties of masses formed at different acetic acid concentrations 96

4.3.4.3 Effect of protein concentration during coacervation 103

4.3.5 Conclusions 109

4.3.6 References 110

5 GENERAL DISCUSSION 114

5.1 Methodological considerations 114

5.2 Mechanisms to explain the rheological behaviour of kafirin viscoelastic masses of different composition and the differences in behaviour between kafirin and zein masses 116

5.3 Potential future work 118

6 CONCLUSIONS AND RECOMMENDATIONS 124

7 REFERENCES 126

8 PUBLICATIONS AND PRESENTATIONS BASED ON THIS RESEARCH 140

LIST OF TABLES

Table 2. 1 The amino acid content (mole % of amino acid) of the kafirins and zeins.....4

Table 2.2 Characteristics of the major groups of prolamins of wheat and sorghum (Shewry, 2002)…….. ……12

Table 4.1. 1 Effects of different extraction solvents on the purity and yield of kafirin preparations.....41

Table 4.1. 2 Effects of different kafirin extractants and treatments on the secondary structure of kafirin measured by FTIR as indicated by differences in the Amide I region 52

Table 4.2. 1 Stress-relaxation of kafirin viscoelastic masses from kafirins extracted with different solvents and after storage for 2, 8 and 16 days at 4^{o}C…….……69

Table 4.2. 2 Dynamic rheological analysis (small deformation) frequency-sweep test at 1 rad/s of kafirin viscoelastic masses from kafirins extracted with different solvents…….75

Table 4.2. 3 Stress-relaxation of kafirins from waxy and high protein digestibility sorghum lines on day 0 and after storage for either 8 or 16 days at 4^{o}C…….……..…….……77

Table 4.2. 4 Dynamic rheological analysis (small deformation) frequency-sweep test at 1 rad/s of kafirin viscoelastic masses from waxy and high protein digestibility sorghum lines…….…….82

Table 4.2. 5 Stress-relaxation behaviour of viscoelastic masses from kafirin and zein of different sub-class composition after repeated compression on day 0 and after storage for 2 and 16 days at 4^{o}C……..…….85

Table 4.3. 1 Effect of different final acid acetic concentrations on the stress-relaxation of kafirin and zein viscoelastic masses prepared by coacervation at day 0 and after storage at 4°C for 2, 8 and 16 days. Protein concentration in glacial acetic acetic 28.6% 99

Table 4.3. 2 Effect of protein concentration in glacial acetic acid during preparation of kafirin and zein viscoelastic masses by coacervation on their stress relaxation at day 0 and after storage at 4°C for 2, 8 and 16 days. Coacervation to a final acetic acid concentration of 5% 108

LIST OF FIGURES

Figure 2. 1 Sorghum protein body (Mesa-Stonestreet et al., 2010) 5

Figure 2. 2 Structural models for α-zein. A-Argos et al. (1982), B-Garratt et al. (1993), C-Matsushima et al. (1997), D- Bugs et al. (2004), E-Momany et al. (2006) (Taylor, 2008) 7

Figure 2. 3 Summary of the fibril formation process observed in the initiation, growth and propagation of amyloid fibrils (Erickson et al., 2012). 8

Figure 2. 4 Possible mechanism for zein self-assembly from single molecules to nanospheres and nanotubes (Wang and Padua, 2012). 9

Figure 2. 5 a The effects of hydration on the 'loop and train' behaviour of HMW subunits. (a) Low level of hydration - hydrogen bonds are mainly interchain (b) Intermediate level of hydration - some loops are formed (c) High level of hydration - many loops are formed (Belton, 1999). 14

Figure 2.5 b The 'loop and train'model. (a) The equilibrium configuration (b) small extension; only the loops are deformed (c) Large extension; loops are flattened and the interchain hydrogen bonds are broken so that chains slip over each other (Belton, 1999). 14

Figure 2. 6 (a-c) CSLM images of Glutenin Macropolymers dispersions versus various mixing times (Don et al., 2003). 16

Figure 2. 7 a Proposed changes in secondary structure of kafirin during dough (viscoelastic mass) formation (Elhassan, 2016) 23

Figure 2.7 b Kneaded kafirin dough (Elhassan, 2016) 24

Figure 4.1. 1 SDS-PAGE of kafirin preparations extracted with different solvents. A: SDS-PAGE under reducing conditions; B: SDS-PAGE under non-reducing conditions; lanes: MW: Molecular weight standards; lane 1: kafirin extracted with 70 % ethanol + 0.35 % acetic acid; lane 2: kafirin extracted with glacial acetic acid; lane 3: kafirin extracted with 70 % ethanol + 0.5 % sodium metabisulphite + 0.35 % acetic acid; lane 4: kafirin extracted with glacial acetic acid after 1 % sodium metabisulphite pre-soak……...42

Figure 4.1. 2 2D-PAGE band pattern of kafirin preparations extracted with different solvents. A: kafirin extracted with 70 % ethanol + 0.35 % acetic acid, B: kafirin extracted with glacial acetic acid, C: kafirin extracted with 70 % ethanol + 0.5 % sodium metabisulphite + 0.35 % acetic acid, D: kafirin extracted with glacial acetic acid after 1 % sodium metabisulphite pre-soak 46

Figure 4.1. 3 SDS-PAGE of kafirin preparations isolated with different solvents subjected to different treatments under non-reducing conditions, ...47

Figure 4.1. 4 FTIR of kafirin preparations and cohesive masses. A: Dry kafirin preparations, B: Kafirin preparations hydrated in water, C: Kafirin preparations hydrated in 33% (v/v) acetic acid, D: kafirin viscoelastic masses. EtOH: kafirin extracted with 70 % ethanol + 0.35 % acetic acid; GAA: kafirin extracted with glacial acetic acid; KESM: kafirin extracted with 70 % ethanol, 0.35 % acetic acid and 0.5 % sodium metabisulphite; GAA + KMS: kafirin extracted with glacial acetic acid with 1% sodium metabisulphite presoak; VWG: vital wheat gluten 49

Figure 4.1. 5 Effect of kafirin preparations on the morphology of kafirin cohesive mass by stereomicroscopy. 55

Figure 4.2. 1 Frequency sweep curve for storage (G') modulus of kafirin viscoelastic masses from kafirins extracted with different solvents. 73

Figure 4.2.2 Frequency sweep curve for storage (G') modulus of kafirin viscoelastic masses from transgenic high protein digestibility-high lysine line (TG-HD) and its null control (Null).....80

Figure 4.2. 3 Frequency sweep curve for storage (G') modulus of kafirin viscoelastic masses from waxy high protein digestibility lines (WHD1, WHD2, WHD3), non-waxy high protein digestibility line (NHD) and kafirin extracted with 70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid 81

Figure 4.3. 1 Stereomicroscopy (left, bar = 100 μm) and scanning electron microscopy (right, bar = 2 μm) of kafirin and zein cohesive masses formed at different final acetic acid concentrations. Solid arrows indicate thick bands of fibrils, dotted arrows indicate finer fibrils……..96

Figure 4.3. 2 Effect of final acetic acid concentration on the stress-recovery behaviour of kafirin and zein viscoelastic masses 102

Figure 4.3. 3.A Confocal laser scanning microscopy of kafirin and zein cohesive masses prepared by coacervation at different protein concentrations (bar = 200 μm) 105

Figure 4.3.3.B Scanning electron microscopy of kafirin and zein cohesive masses prepared by coacervation at different protein concentrations (bar = 2 μm) 106

Figure 4.3.4 Effect of final protein concentration in glacial acetic acid on the stress-recovery behaviour of kafirin and zein viscoelastic masses…… 107

Figure 5. 1 Model to explain the differences in kafirin and zein stress-recovery behaviour when compressed……...120

Figure 5. 2 Model to explain the stress-recovery behaviour of all kafirins extracted with different solvents, high α-kaifrn, kafirin fromTG-HD and its null control when compressed.....121

Figure 5. 3 Model to explain the stress-recovery behaviour of the kafirins minus γ-kafirin when compressed. 122

Figure 5. 4 Model to explain the stress-recovery behaviour of kafirins from waxy (W) and high protein digestibility (HD) WHD1, WHD2, WHD3, NHD when compressed...123

1 INTRODUCTION

The increased demand for bread has led to increased importation of wheat in the sub-Saharan Africa (Pingali, 2007). The climate in Africa is generally inappropriate for the cultivation of wheat, a temperate cereal (Tadesse and Straziuso, 2012). This has led to the quest for making gluten- free bread products from sorghum, a drought-tolerant cereal crop widely cultivated in the sub-Saharan Africa (Srinivas et al., 2009). However, kafirin, the sorghum prolamin is not an ideal function replacement for wheat in bread making due primarily to its relative hydrophobic nature when compared to gluten (Taylor and Belton, 2002). Notably, excellent successes have been made on the formation of viscoelastic doughs from commercial hydrated zein (from maize) in aqueous systems above its glass transition temperature (T_g) (Lawton, 1992; Schober et al., 2010). In fact, wheat-free breads of reasonable quality have also been produced (Schober et al., 2008; 2010). Some progress has been made in developing gluten-free dough-based products (Taylor et al., 2016b).

Many factors have been proposed to be responsible for the functionality of zein (Mejia et al., 2007; Schober et al., 2011; Erickson et al., 2012; Smith et al., 2014). Functional zein that was predominantly α-zein with low proportion of β+γ-zeins was essential for the formation of a stable viscoelastic material i.e. a material that maintains its viscoelastic characteristics over a period of time at a storage temperature below its hydrated glass transition temperature, which indicated that disulphide crosslinking was not desirable in the formation of zein viscoelastic mass (Schober et al., 2011). Non-covalent interactions i.e. hydrophobic interactions have also been indicated to play role in zein viscoelastic mass formation (Smith et al., 2014). The secondary structure of the protein is also thought to influence zein viscoelastic mass formation (Erickson et al., 2012). High extensibility of zein dough above its T_g has been attributed to high proportion of β-sheet conformation (Erickson et al., 2012). There was a transformation of α-helices into β-sheet conformation when commercial zein (predominantly α-zein) was mixed with water above its T_g (Mejia et al., 2007; 2012). These authors found that the viscoelastic properties of zein were related to the secondary structural changes from α-helices into β-sheet strands. However, the conformation of total zein viscoelastic mass (comprising α-, β-, γ-, δ-zein) formed with dilute acetic acid was mostly α-helical (King et al., 2016).

Previous attempts to form stable kafirin viscoelastic mass had not been successful. Recently, however, kafirin viscoelastic masses were formed by coacervation with water from glacial acetic acid (Elhassan et al., 2018; Taylor et al., 2018). The ‘mass’ in this context refers to the aggregation of prolamin proteins into fibrils through a coacervation process (Elhassan et al., 2018). When kneaded by hand, the fibrils formed a cohesive viscoelastic material referred to as viscoelastic mass. In this study, various factors that affect the functionality of kafirin viscoelastic mass were investigated. Therefore, the objective of this study was to determine how various factors influence the functionality of kafirin viscoelastic masses with the aim of producing kafirin-based doughs with similar rheological characteristics as gluten-based doughs.

2 LITERATURE REVIEW

This chapter will review the chemistry, structure and functionality of sorghum prolamins and wheat gluten. The basis of the functionality of zein and kafirin in bioplastics and doughs and the basis of viscoelastic elastic properties of wheat gluten will be examined. Research into the improvement of the functionality of zein and kafirin in dough-like systems will be reviewed. Since this research involves the use of different advanced techniques, their principles and applications will be reviewed.

2.1 Chemistry and structure of kafirin and zein

Kafirin is the sorghum prolamin storage protein. It is readily soluble in lactic and glacial acetic acids and aqueous solutions of higher alcohols such as tert-butanol (Taylor et al., 2005b). Kafirin is classified into α-, β-, γ- and δ-kafirin fractions based on their solubility, amino acid constituents and sequences, electrophoretic mobility, chemical reactions and molecular masses (Shull et al., 1991). The relative masses of kafirin range from about 15 kDa to almost 30 kDa as revealed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) when extracted and separated under reduced conditions (Belton et al., 2006). Kafirin is more hydrophobic than maize zein (Belton et al., 2006). The hydrophobicity of kafirin and zein is due to the presence of higher levels of hydrophobic amino acids (Landry and Moureaux, 1980).

Kafirin contains similar levels of proline, histidine, glutamine, cysteine, alanine and leucine to zein (Table 2.1) (Taylor and Belton, 2002). Both kafirin and zein contain little lysine. The major amino acids (mol %) in α-kafirin, the major sorghum prolamin, include glutamine, leucine, alanine, proline and cysteine (Shull et al., 1992). Alpha-kafirin is rich in non-polar amino acids. Beta-kafirin consists majorly of proline, glutamine, alanine, leucine, methionine and cysteine (Shull et al., 1992). Notably, βeta-kafirin is rich in sulphur-containing amino acids methionine and cysteine. This enables it to form either intra- or inter-molecular disulphide crosslinking resulting in oligomers and polymers (Belton et al., 2006). Beta-kafirin can act as a bridge or link between α_1- and γ-kafirins, resulting in polymers, which are too large to be extracted without reduction (El Nour et al., 1998). Gamma-kafirin is rich in proline, glutamine, histidine, cysteine and methionine (Taylor and Belton, 2002) (Table 2.1).

Table 2. 1 The amino acid content (mole % of amino acid) of the kafirins and zeins (Taylor and Belton, 2002)

Amino acid	α-Zein	α-Kafirin	β-Zein	β-Kafirin	γ-Zein	γ-Kafirin
Asn	5.3	6.0	2.5[a]	3.3[a]	0.0	0.0
Asp	0	0.4	Ng	Ng	0.0	0.0
Thr	2.8	4.0	2.5	4.6	4.4	4.7
Ser	6.9	6.0	5.0	4.6	3.9	5.2
Gln	20.7	24.6	18.1[b]	17.8[b]	14.7	11.9
Glu	0.8	0.4	Ng	Ng	1.0	1.0
Pro	8.9	7.7	8.8	9.7	25.0	23.3
Gly	0.8	1.6	8.8	6.8	6.4	8.8
Ala	13.8	14.9	13.8	13.4	4.9	5.7
Cys	0.4	0.4	4.4	4.9	7.4	7.8
Val	6.9	4.4	1.9	5.2	7.4	6.2
Met	2.0	0.8	11.3	5.7	0.5	1.0
Ile	4.5	5.6	0.6	2.3	2.0	2.6
Leu	17.1	15.3	10.0	12.0	9.3	8.3
Tyr	2.8	2.8	8.8	3.0	2.0	2.1
Phe	3.3	2.4	0.0	1.9	1.0	1.6
His	1.2	1.2	0.0	0.9	7.8	7.8
Lys	0.0	0.0	0.0	0.5	0.0	0.0
Arg	1.6	0.8	3.1	2.7	2.5	2.1
Trp	0.0	0.4	Ng	Ng	0.0	0.0

[a]Asn + Asp expressed as Asn
[b]Gln + Glu expressed as Gln
Ng-not given

Notably, γ-kafirin is richer in cysteine than any of the other kafirin sub-classes (Belton et al., 2006) and it is the only water soluble kafirin fraction under reducing conditions (Taylor et al., 1989).

Gamma-kafirin is found as oligomers and polymers. Both β- and γ-kafirins form intermolecular and intramolecular disulphide bonds and they are highly cross-linked (El Nour et al., 1998). All kafirin subclasses undergo polymerization. Alpha-kafirin polymerises through disulphide bonding with β- and γ-kafirins but it is found primarily as monomers and oligomers. El Nour et al. (1998) found that α_1-kafirin formed different oligomers of different sizes with γ-kafirin through disulphide bonds, while α_2-kafirin formed dimers or small oligomers. The polymerisation behaviour exhibited by these α-kafirins is due to the presence of one cysteine residue in α_2-kafirin and two cysteine residues in α_1-kafirin. The presence of only one cysteine residue in α_2-kafirin would prevent the possibility of further polymerisation, whereas α_1-kafirin with two free S-H groups enables an increase in polymer size.

In the sorghum grain, the kafirins are isolated in spherical protein bodies (Belton et al., 2006), which are embedded in a matrix of glutelin proteins and surrounded by starch granules (Mesa-Stonestreet et al., 2010). This makes them unavailable for participation in fibril and dough formation. The outer shell of these protein bodies contains mainly, the β- and γ-kafirins, which are highly cross-linked by intra- and inter-disulphide bonds, while the interior predominantly contains the α-kafirin (Mesa-Stonestreet et al., 2010) (Figure 2.1).

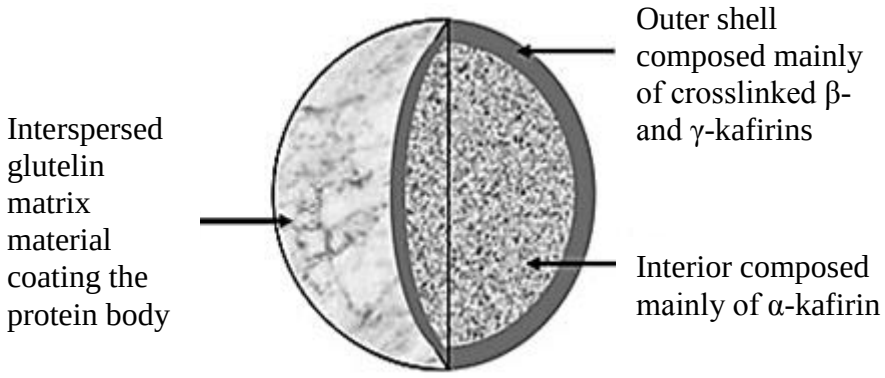

Figure 2. 1 Sorghum protein body (Mesa-Stonestreet et al., 2010)

2.1.1 Structural models for zein and kafirin

A ground breaking structural model for α-zein was proposed by Argos et al. (1982) comprising a group of antiparallel tightly coiled α-helical structures that are folded to form distorted cylinders (Figure 2.2A). The authors suggested that nine of these repetitive adjacent cylinders constituted a single polypeptide linked together by hydrogen bonds between hydrophilic glutamine residues in the turn regions. The hydrophobic amino acids are within the α-helices, while the polar amino acids are on the surface of the helices. The polar amino acids allow both intra- and intermolecular hydrogen bonding to the molecules in neighbouring planes.

This model was later modified and extended by Garratt et al. (1993) as a general model for all α-prolamins, including α-kafirin (Figure 2.2B). This structure was based on pairs of repetitive amino acid sequences which formed anti-parallel α-helices arranged hexagonally with alternating groups of hydrophobic and polar amino acids. Tatham et al. (1993) found that α-zein has extended conformations in the solid state and solution. Matsushina et al. (1997) also modified the Argos et al. (1982) model and suggested that the α-zein could consist of α-helices stacked linearly in the direction of long axis (Figure 2.2C). Each of the tandem repeat units formed by a single α-helix is represented by a cylinder and the helices are joined at the ends by glutamine-rich 'turns' or loops. Bugs et al. (2004) proposed a helical hairpin model comprising two anti-parallel α-helical structures of coiled coils forming a superhelical conformation with both hydrophobic and polar charged amino acids distributed along the helical surfaces (Figure 2.2D).

A model for Z19 α-zein comprises three interacting coiled-coil helix conformations with segments placed end to end (Momany et al., 2006) (Figure 2.2E). These helices include helix-1- Z19 with leucine and isoleucine residues (58-75 residues), helix-2-Z19 with leucine and isoleucine residues (76-98 residues) and helix-3-Z19 with leucine, valine and alanine residues (99-113 residues). The non-polar amino acid side chains form a hydrophobic face inside the triple super helix. This model also accommodates lutein, a natural carotenoid pigment of zein in the core of the triple helical segments and helps to stabilize the configuration

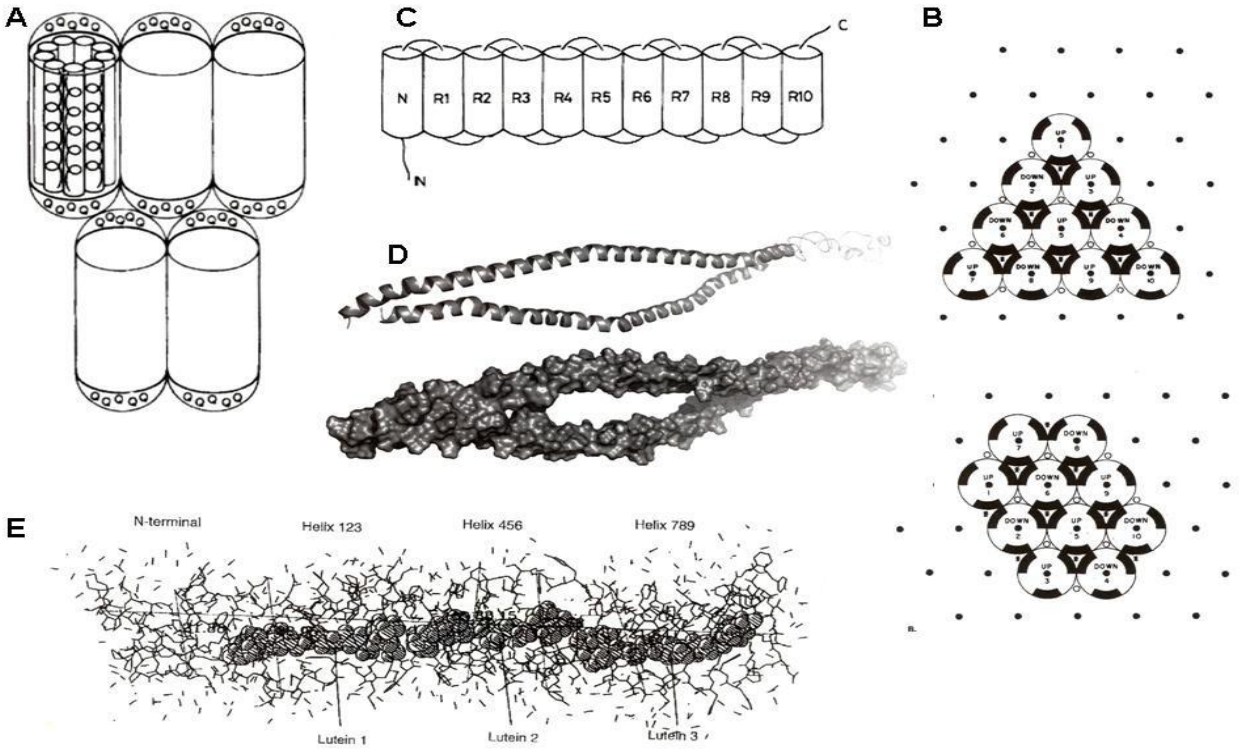

Figure 2. 2 Structural models for α-zein. A-Argos et al. (1982), B-Garratt et al. (1993), C-Matsushima et al. (1997), D- Bugs et al. (2004), E-Momany et al. (2006) (Taylor, 2008)

2.1.2 Molecular basis of the functionality of zein and kafirin in bioplastics and doughs

Kafirin and zein have the inherent ability or potential to form bioplastics (Taylor et al., 2005a). Films may be produced by controlled protein aggregation, such that under suitable conditions, the proteins are thought to unfold from α-helices and self-aggregate into β-sheets structures (Taylor et al., 2013). Zein tertiary structure consists of nearly equal amounts of hydrophilic and lipophilic residues (Wang et al., 2008). When the chain folds on itself, it forms a 'ribbon' of amino acids with hydrophobic and hydrophilic domains, which allows it to function as a polymeric amphiphile. In fact, the major driving force for self-aggregation of proteins is amphiphilicity (Wang et al., 2008). Protein molecules with both polar and non-polar groups tend to reduce any unfavourable interactions with water by forming aggregates in which the hydrophobic groups exclude water molecules whereas the hydrophilic groups interact with water (Wang et al., 2008). These authors found that when zein dissolved in aqueous ethanol solution was dried overnight at ambient temperature, spherical particles (spheres) were formed. This aggregation was attributed to the increasing polar medium, which resulted in hydrophobic protein-protein interactions induced by solvent evaporation.

When oleic acid was added to the zein solution, the zein spheres were transformed into sponge-like structures with empty cells. The formation of aggregates from zein-oleic acid solution was attributed to both hydrophobic interactions and hydrogen bonding. Furthermore, when chloroacetic acid was added to zein dissolved in aqueous ethanol solution, a smooth, thin and transparent film was formed. Chloroacetic acid decreased the radius of curvature to the point of forming smooth films. It was thought that zein molecules interacted with chloroacetic acid through hydrogen bonding, which prevented the formation of spheres.

The aggregation of prolamin proteins into fibrils has been suggested as an important step in dough formation (Lawton, 1992; Schober et al., 2010). Given the absence of a mechanism for zein's fibril formation, it was suggested by Taylor (2018) and Erickson et al. (2012) that the amyloid fibrillogenesis processes proposed by Rochet and Lansbury (2000) could be utilized to describe the transition of zein from soluble, globular aggregates to insoluble β-sheet rich fibrils during zein-based doughs formation (Figure 2.3). It was proposed that fibril formation could be initiated by the formation of unstable partially-ordered structures, which accumulate to form amorphous, soluble aggregates, thereby increasing the concentration of the misfolded protein. The researchers suggested that once a critical concentration of the amorphous, soluble aggregates was achieved, fibril formation process could proceed to form a β-sheet-rich, fibrous network through a nucleation dependent pathway.

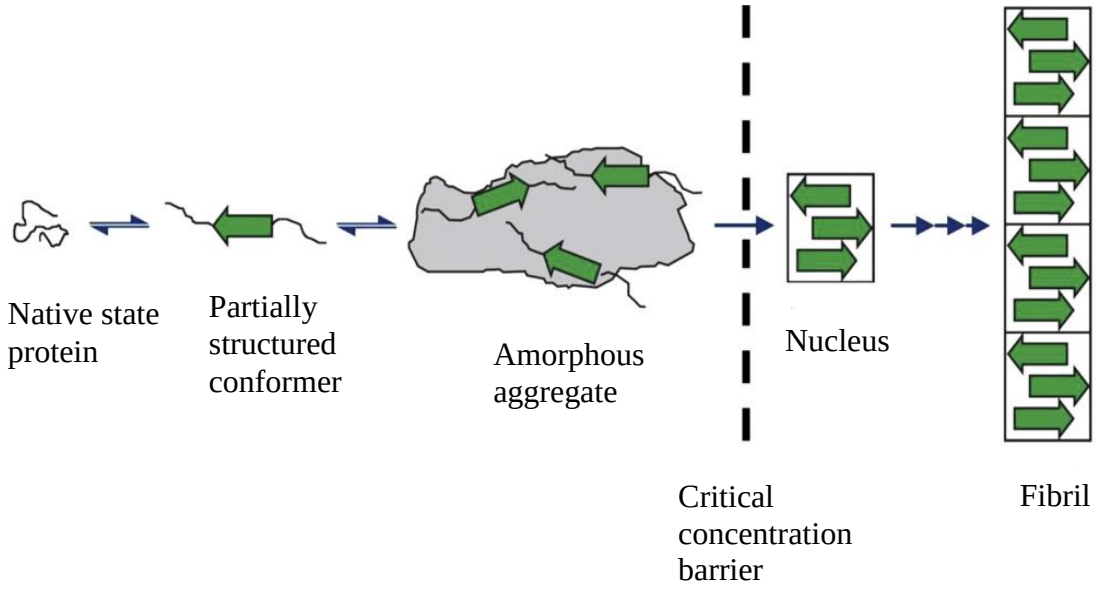

Figure 2. 3 Summary of the fibril formation process observed in the initiation, growth and propagation of amyloid fibrils (Erickson et al., 2012).

Wang and Padua (2012) proposed a three-step mechanism for zein self-assembly from single molecules to nanospheres. The authors theorised that the α-helices in the original zein solution would first transform into antiparallel β-sheet strands stabilized by glutamine-rich bridges through hydrogen bonds. In the second step, zein molecules in the form of antiparallel β-sheet strands would pack side by side to form a long ribbon. The process would be driven by the hydrophobic interactions generated between the sides of the β-sheets. In the third step, the long ribbon curls into a ring which continuously grows to form a sphere (Figure 2.4). Figure 2.4 describes a model for self-assembly of zein molecules into nano structures, according to Wang and Padua (2012). These authors had concluded that these spheres were the base of all other microstructures (Wang and Padua, 2010). Taylor, Anyango and Taylor et al. (2013) suggested that these nanostructures assemble to form microstructures of similar morphology to each other, which in turn aggregate to form similar macroscale structures, including fibrils. The aggregation of prolamin proteins into fibrils has been proposed as being an important step in dough formation (Lawton, 1992; Schober et al., 2010), and consequently would influence the rheological characteristics of the dough formed. Wang and Padua (2012) reported that the α-helices must first be transformed into β-sheet strands, a stretch of polypeptide chain, followed by unfolding of the strands into a chain of β-sheets with head-to-tail connections of glutamine bridges via hydrogen bonds and then coiling of the β-sheet chains into three dimensional polyhedral columns (Figure 2.4).

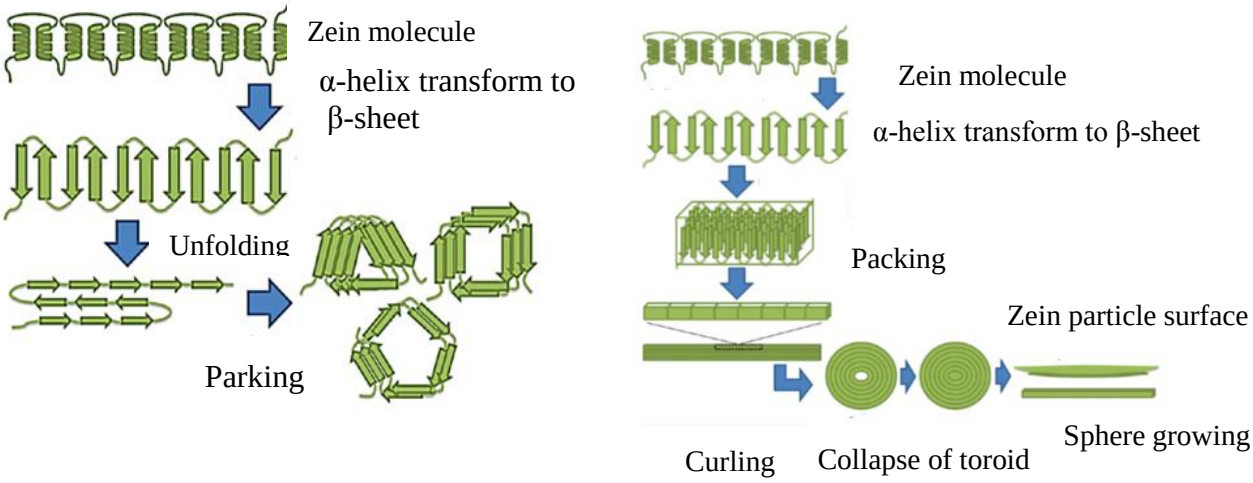

Figure 2. 4 Possible mechanism for zein self-assembly from single molecules to nanospheres and nanotubes (Wang and Padua, 2012).

2.1.3 The effect of kafirin and zein conformation on dough formation

It was established by Lawton (1992) that a gluten-like dough could be formed by mixing commercial zein and corn (maize) starch with warm water and dibutyl tartrate (as a plasticizer) at 35^{o}C, which is above zein's hydrated glass transition temperature of 29^{o}C (Schober et al., 2008). No viscoelastic doughs were formed below 25^{o}C. This is unlike hydrated gluten, which is rubbery at normal ambient temperatures and can form a dough (Hoseney et al., 1986). Zein-starch doughs mixed at 30^{o}C and 35^{o}C were very extensible (Lawton 1992). It can be hypothesised that this behaviour was due to there being fewer covalent bonds (disulphide bonds) present in zein to resist extension when compared to the extended gluten polymer or cross-linked gluten matrix in wheat dough.

Schober et al. (2011) stated that the dough forming properties of zein and kafirin with respect to their ability to aggregate in warm water into doughs or gluten-like substances depends on their composition and degree of hydrophobicity. Like gluten (see section 2.2), zein and kafirin consist of a number of subclasses, as described in section 2.1. However, the polypeptide monomers of zein and kafirin are much smaller than those of the wheat high molecular weight-glutenin subunits (HMW-GS) but they polymerise in a similar way, i.e. through disulphide cross-linking, involving the β- and γ-sub-classes, which are high in cysteine (Taylor et al., 2016). Gluten is less hydrophobic than zein (Duodu et al., 2003), which may explain why Schober et al. (2011) found that the zein aggregated into dough above its T_g, when it contained predominantly more of α-zein compared to β+γ zein. This observation was attributed to the fact that β- and γ-zeins are more hydrophobic than α-zein. Based on their findings, Schober et al. (2011) concluded that hydrophobic interactions are important for zein aggregation and disulphide bonds are detrimental. The authors showed that kafirin has a higher glass transition temperature than zein when hydrated (>40^{o}C). However, kafirin remained as a paste and did not aggregate in water to form a gluten-like mass over a range of elevated temperatures from 40 to 85^{o}C. This was not surprising as kafirin is more hydrophobic than zein and has a higher tendency to form disulphide linkages, as it contains more of the cysteine-rich subclasses (Emmambux and Taylor, 2009). As stated, both of these properties are detrimental to aggregate (dough) formation.

The dough forming properties of zein are also influenced by its secondary structure (Mejia et al., 2007). In its native state, the conformation of α-zein is 50-60% α-helical structure with β-

turns or random coil configuration comprising the remaining structure (Argos et al., 1982), while kafirin contains 54-59% α-helical conformation (Duodu et al., 2001). The viscoelastic properties of zein have been proposed to be related to the transformation of α-helices into β-sheet strands (Mejia et al., 2007). Schober et al. (2011) also found that secondary structure of zein plays a role in dough formation. These workers observed an increase in the more open β-sheet conformation with zein dough formation at 40^{o}C, with a corresponding decrease in α-helices. However, when the temperature was reduced to 25^{o}C, the proportion of the β-sheet also decreased. This is analogous to the situation with glutenin, where the elasticity of the high molecular weight glutenin subunits has been attributed to the formation of loose spirals of β-sheet structures (Shewry et al., 1992). However, the formation of glutenin "loops and trains" by hydrogen bonding as postulated by Belton (1999) is the most widely accepted theory for wheat gluten elasticity, although the author considered that β-sheet formation also plays a role (Belton, 2005).

2.2 Chemistry, structure and functionality of the wheat gluten proteins

Gluten comprises two main storage protein groups, namely gliadin and glutenin (Mejia et al., 2007). Gliadins are classified into α-, β-, γ- and ω-gliadins based on their mobility by gel electrophoresis at low pH (Wieser, 2007), while glutenins consist of high molecular weight glutenin subunits (HMWGS) and low molecular weight subunits (LMWGS). These units are linked together by interchain disulphide bonds, as oligomers and polymers (Don et al., 2003). HMW glutenin subunits can be categorized into two different types: the x-type with the MWs from 83-87 kDa and the y-type from 67-74 kDa. The disulphide crosslinks between HMWGS and LMWGS occur between a cysteine residue in a repetitive central domain of y-HMWGS and a cysteine residue in the C-terminal domain of LMW subunits (Wieser, 2007). These may be inter- or intra-chain disulphide bonds. HMWGS have greater influence on the viscoelastic properties of wheat dough than the LMWGS (Wieser, 2007). Gliadins have molecular weights that range between 30 kDa and 80 kDa (Goesaert et al., 2005). Gliadins are made up of proteins that comprise single polypeptide chains. The single polypeptide chains are stabilised by hydrogen bonding and hydrophobic interactions (Wieser, 2007). Gluten proteins are also classified into groups according to their amino acid composition and other characteristics (Shewry, 2002) (Table 2.2).

Table 2.2 Characteristics of the major groups of prolamins of wheat and sorghum (Shewry, 2002)

Species	Prolamin group	% Total fraction	Polymers or monomers	Subunit M_r	Partial amino acid composition (mol%)
Wheat	HMW prolamins	6-12	Polymers	65,000-90,000	30-35 Gln
	HMW subunit				10-16 Pro
					15-20 Gly
					0.5-1.5 Cys
	S-rich prolamins				
	LMW subunits	70-80	Polymers	30,000-45,000	30-40 Gln
	α-Gliadin		Monomers		15-20 Pro
	γ-Gliadin		Monomers		2-3 Cys
	S-poor prolamins				
	ω-Gliadin	10-20	Monomers	40,000-75,000	40-50 Gln
					20-30 Pro
					8-9 Phe
					0 Cys
Sorghum	α-Kafirin	80	Monomers	23,000-25,000	22 Gln
					9 Pro
					15 Ala
					1 Cys
					0.6 Met
	β-Kafirin	7-8	Polymers	16,000-20,000	18 Gln
					19 Pro
					13 Ala
					12 Leu
					5 Cys
					6 Met
	γ-Kafirin	9-12	Polymers	28,000	14 Gln
					23 Pro
					9 Gly
					9 Leu
					7 Cys
					1 Met

2.2.1 Molecular basis of the viscoelastic properties of wheat gluten

Both glutenins and gliadins play a part in the rheological properties of wheat flour dough. Hydrated gliadins are responsible for dough viscosity and extensibility (Wieser, 2007), through non-covalent interactions such as hydrogen bonding, Van der Waals forces, electrostatic and hydrophobic attractions (Shewry and Tatham, 1997). Hydrated glutenins, however, are more cohesive and elastic, and contribute to dough strength and elasticity (Wieser, 2007). The viscoelastic properties of wheat gluten have been proposed to be related to the presence of fibrous, β-sheet-rich protein networks (Wellner et al., 1996).

2.2.1.1 Loop and train theory

The 'Loop and train' theory proposed by Belton (1999) describes the elastic properties of glutenin. According to the author, the plasticity of wheat dough can be explained by the interprotein disulphide bonds but not dough elasticity (Belton, 1999). The Loop and Train theory is based on hydrogen bonding between glutenin polymers themselves and between the glutenin polymers and water. When wheat proteins are hydrated, many hydrogen bonds are formed, which are not broken when wheat gluten is stretched (Figure 2.5a). This results in unbonded mobile regions. Here, the interaction is between glutenin polymers and water (loops) and bonded regions (trains) where the interaction is between cysteine-cysteine of glutenin polymers through hydrogen bonding. As such, there is always a balance between protein-water and protein-protein interactions. Further stretching extends loops (Figure 2.5b) and then causes the protein molecules to slide over one another. The elastic restoring force is provided by the re-establishment of the loop-train equilibrium. When wheat dough is under stress, the water molecules are squeezed out and the trains between the glutenin molecules become longer, stretching out the individual glutenin molecules (Figure 2.5a).

The starting point for this model assumes that an isolated protein represents a long chain with a globular end of cysteine residue that is made sticky by high density of polar hydrogen bonding groups (Belton, 1999). HMW subunits contain a very high level of hydrophilic glutamine that has a very high capacity to form both intra- and inter-molecular hydrogen bonds. The author hypothesized that in the absence of water, the chains tend to bond to each other through hydrogen bonding to form a dense mass. As water is added, there is an increase in the number of water-protein hydrogen bonds formed. However, the large number of interchain hydrogen bonds ensures that it is unlikely that all the interchain hydrogen bonds break simultaneously.

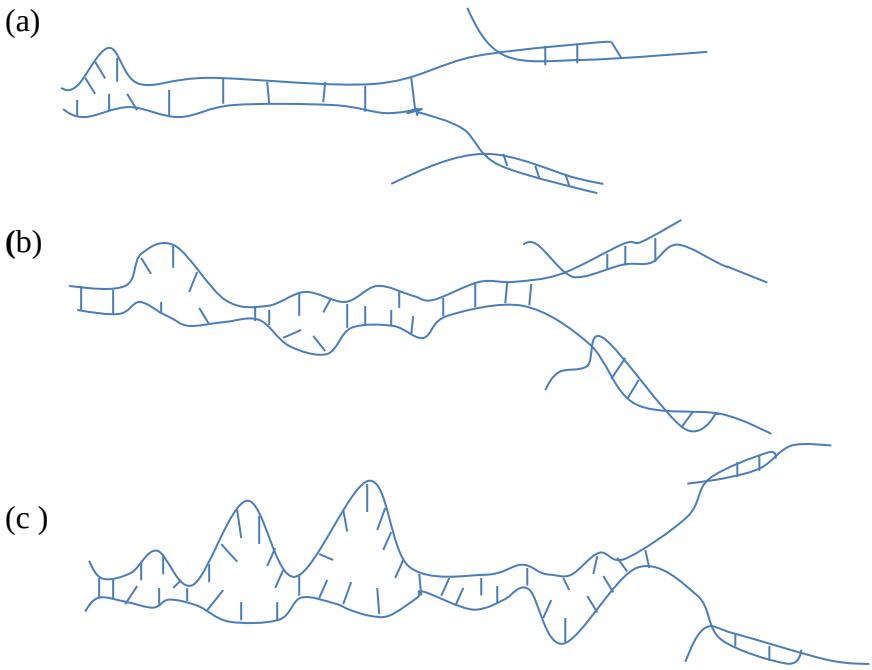

Figure 2. 5 a The effects of hydration on the 'loop and train' behaviour of HMW subunits. (a) Low level of hydration - hydrogen bonds are mainly interchain (b) Intermediate level of hydration - some loops are formed (c) High level of hydration - many loops are formed (Belton, 1999).

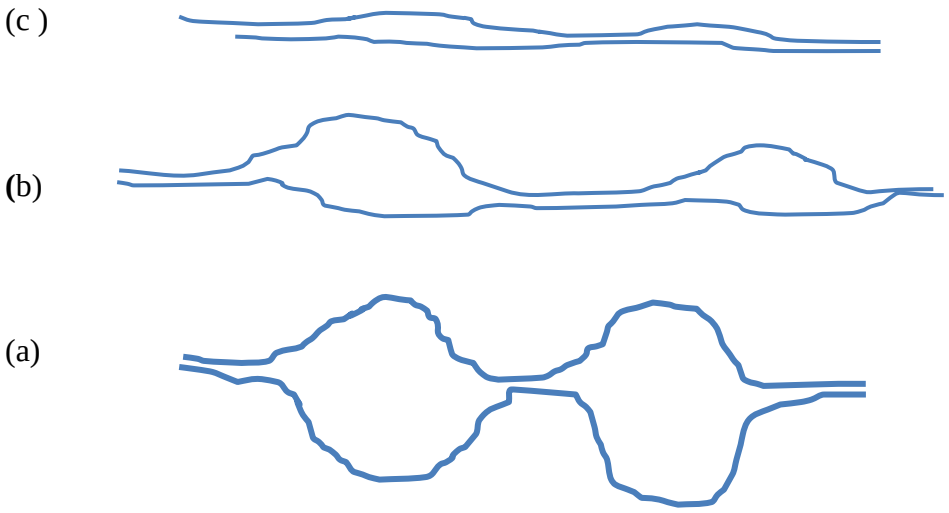

Figure 2.5 b The 'loop and train'model. (a) The equilibrium configuration (b) small extension; only the loops are deformed (c) Large extension; loops are flattened and the interchain hydrogen bonds are broken so that chains slip over each other (Belton, 1999).

2.2.1.2 Disulphide and dityrosine bond formation

Cross-linking of the gluten proteins in wheat dough influences its viscoelastic properties (Tilley et al., 2001). It has been proposed that the development of di- and isodi-tyrosine bonds in wheat dough during mixing and baking contributes to the structure of the gluten network (Tilley et al., 2001). These authors observed that when oxidizing agents such as ascorbic acid, azodicarbonamide and potassium bromate were added to wheat dough, cross-linked structures of dityrosine and isodityrosine were formed. It has been found that these cross-links between the proteins of wheat gluten induce changes in its mixing characteristics such as dough development time, dough softening time, peak time and dough stabilizing time (Takasaki et al., 2005). In contrast however, Pena et al. (2006) observed that although there was an increase in the formation of dityrosine derivatives when potassium bromate was added to wheat dough, the rheological properties of gluten were not in any way influenced. These authors reported that dityrosine crosslinks appeared to play no significant role in the structure of wheat proteins, or in the structure of gluten. Furthermore, they were of the opinion that the disulphide-sulphydryl bridges that formed between cysteine residues were the main linkages in the formation of the gluten network and therefore were mainly responsible for the rheological properties of the dough.

2.2.1.3 Glutenin macropolymers

Don et al. (2003) found that the rheological properties of wheat flour dough were strongly related to the presence and properties of very large glutenin protein aggregates stabilized by both chemical (disulphide bond) and physical interactions. The authors found that gluten proteins isolated as an SDS insoluble gel-layer formed large spherical protein particles (0.5-50 μm), which they called Glutenin Macropolymers (GMP). They proposed that the GMPs were responsible for the elasticity of gluten dough. During dough mixing, there was a decrease in the average size and volume of glutenin macropolymer particles (Figure 2.6). This was thought to be a fundamental step in dough development. It has been similarly suggested that the gluten network is a particulate in nature and that physical interactions at the mesoscopic (0.1-100 μm) level affect dough properties (Lefebvre, 2000). In support of this concept, the quantity of GMP has been shown to be an indicator of wheat flour quality (Weegels et al., 1996) and bread-making quality (Pritchard, 1993).

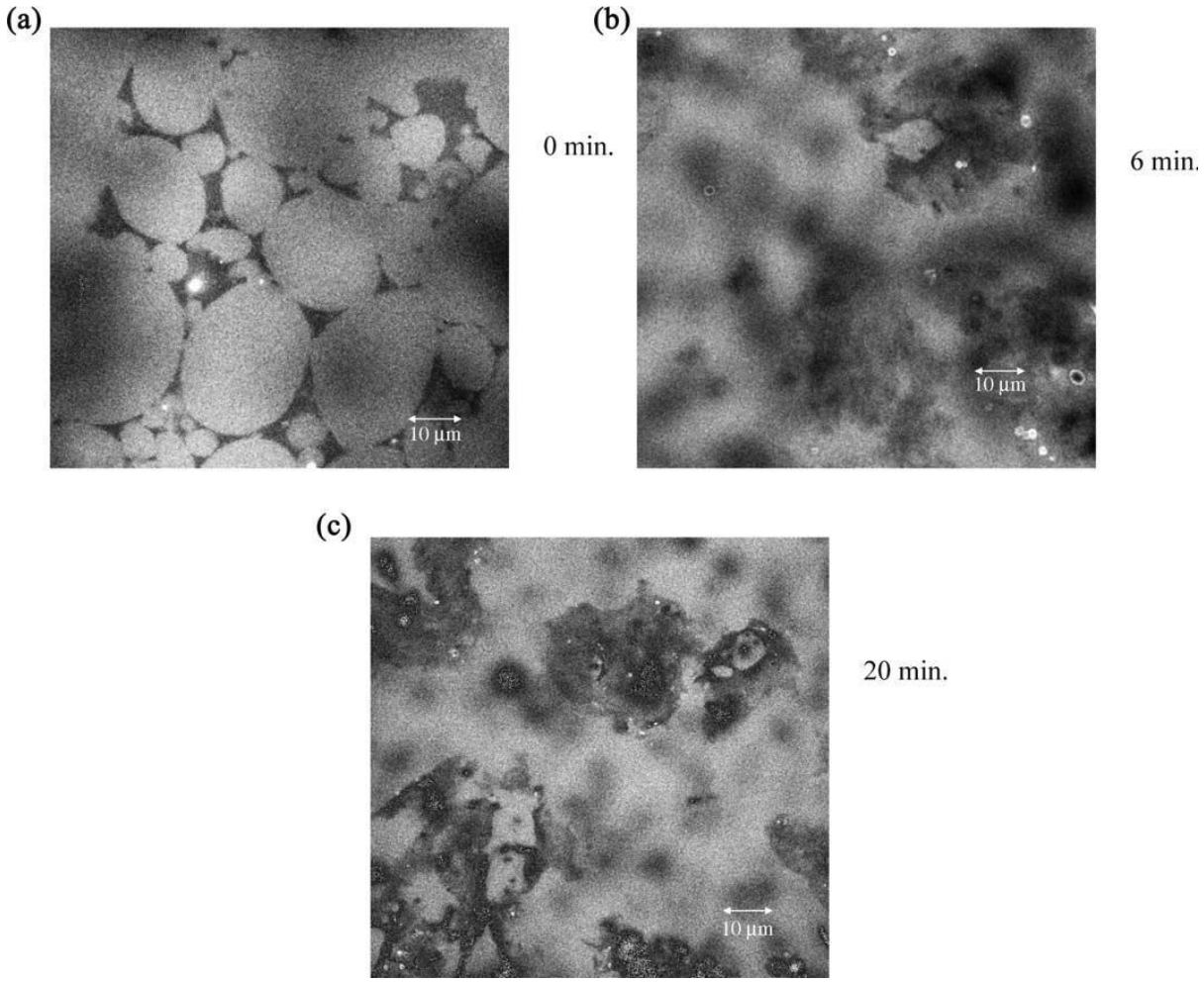

Figure 2. 6 (a-c) CSLM images of Glutenin Macropolymers dispersions versus various mixing times (Don et al., 2003).

2.3 Research on the development and improvement of viscoelastic dough properties of zein and kafirin

2.3.1 Effects of dilute organic acids

Various methods have been studied to improve zein and kafirin dough functionality. Oom et al. (2008) investigated whether the zein-dough formation phenomenon achieved by mixing zein above its T_g could occur with kafirin (comprising α- and γ-kafirin) resins (masses plasticised with oleic acid and lactic acid). Contraction flow measurements indicated that both commercial zein and kafirin resins had the desired rheological properties for leavened baked products. Extensional viscosity and strain hardening of both kafirin and zein resins were found to be similar to those of gluten. However, the kafirin resins rapidly became stiff,

which was attributed to the higher levels of disulphide bonding due to the presence of γ-kafirin.

Sly et al. (2014) investigated the characteristics of commercial zein doughs (viscoelastic masses) formed dilute acetic acid or lactic acids. Dough formation in these dilute organic acids resulted in highly extensible but weak zein doughs. When the concentrations of the organic acids were increased from 0.7% to 5.4%, the cohesion was maintained but there was a substantial decrease in the dough strength. These changes were attributed to the uniformity of the dough and the linear orientation of the zein fibrils, as revealed by confocal laser scanning microscopy. It was found that cohesive doughs formed with zein-maize starch and zein-rice flour could hold some air and inflate a dough bubble, as measured by alveography. Zein-maize starch dough prepared with the lowest acid concentration of 0.7% lactic acid had the most similar alveograph stability (P) and distensibility (L) to wheat flour dough of 75.4 and 81.6 versus 103.2 and 79.6, respectively. The P/L ratio of these zein starch doughs was less than 1 regardless of the type of starch or acid concentration. The P/L ratio is indicative of the elastic resistance and extensibility ratio of the flour (Rosell et al., 2001) and is this case indicated a high dough extensibility and low elastic resistance (Sly et al., 2014).

Sly et al. (2014) also found that as the concentration of organic acids increased, the relative proportion of α-helical conformation also increased compared to zein mixed with water. The increase α-helical conformation was attributed to deamination. Zhang et al. (2011) investigated the effects of mild acid or base (0.0005-0.002 M HCl or NaOH) on commercial zein in water at 20^{o}C (below its T_g) and found that there were secondary structural changes, including a decrease in β-sheet and β-turns. These changes were attributed to mild deamidation of glutamine residues, which would result in the formation of glutamate or glutamic acid. However, as stated, some authors attribute the high extensibility of zein dough above its glass transition temperature to high proportion of β-sheet structure (Erickson et al., 2012).

King et al. (2016) dissolved commercial zein or total zein (comprising α-, β-, and γ-zein) in glacial acetic acid heated at 35^{o}C and 65^{o}C for 10 min with continuous, rapid stirring. The protein solutions were dried under a fume hood and thereafter at 50^{o}C to form films. When the zein films were hydrated in warm water at 50^{o}C above the zein's T_g, viscoelastic "doughs" were formed. Total zein that had not been made into a film remained as a slurry when hydrated under the same conditions. The authors found, for the first time, that it was

possible to form a viscoelastic dough (alternatively referred to as a viscoelastic mass) with total zein. However, the total zein dough was less extensible compared to commercial zein dough. This was attributed to cross-linking through disulphide bonding. Representative stress-strain curves of the effects of zein type, film formation and the effect of removing residual glacial acetic acid from films, showed the non-rinsed total zein film dough and the rinsed commercial zein (mostly α-zein) film dough exhibited a higher elastic type component compared to wheat gluten dough, with a similar viscous flow component. Commercial zein dough prepared with water (not made into a film first) had a similar elastic type component to wheat gluten but was more extensible. There was an increase in the relative proportion of β-sheet conformation (α: β ratios 0.90-0.97:1) when both the commercial zein and total zein preparations and their films were hydrated in distilled water to form doughs or slurry (when a dough did not form). However, the non-rinsed (residual acetic acid still present) commercial zein film dough was found to contain predominantly α-helical structure (α: β ratio 1.12:1). The authors concluded that zein dough formation did not depend on the proportion of β-sheet conformation present. It was suggested that the acetic acid treatment caused chemical changes in the zein which enabled the zein to better interact with water molecules and enabled dough formation.

The most recent and successful attempt to form a dough from kafirin was reported by Elhassan et al. (2018), where kafirin was dissolved in warm glacial acetic acid above its T_g. On hydration with water by a patented process of coacervation (Taylor and Taylor, 2010), viscoelastic masses (doughs) were formed (Figure 2.7b). The authors found that there was a slight increase in the proportion of β-sheet conformation in the dough compared to dry kafirin (Figure 2.7a). The change in conformation was attributed to the resulting hydrogen bonding between kafirin-water molecules. The authors suggested that the Loop and Train theory for glutenin elasticity of Belton (1999) could be applied to interpret the formation of viscoelastic dough from kafirin. It was assumed that the kafirin β-sheet conformation formed a loose spiral polymer structure, similar to the glutenin polymer model (Shewry et al., 1992), which enhanced hydrogen bonding between water and kafirin molecules. The loose spiral β-sheet structure was assumed to be in the hydrated loop region according to the Loop and Train theory. Elhassan et al. (2018) proposed that the rheological characteristics of sorghum dough were influenced by the changes in kafirin composition. Transgenic sorghums of high protein digestibility had stronger kafirin "dough" when compared to their null controls. It was

attributed to the reduction of γ-kafirin subclass in the transgenic sorghum lines, as γ-kafirin is the most hydrophobic of the kafirin subclasses (Duodu et al., 2003).

2.3.2 Effect of defatting

Schober et al. (2010) found that defatted zein+starch aggregated in water above zein's T_g formed a cohesive dough, while undefatted zein+starch failed to aggregate and fell apart upon stretching. The authors attributed this effect to surface lipid removal, which could enhance the aggregation and cohesiveness of zein particles surfaces. Johansson et al. (2012) also investigated the influence of the surface lipids in commercial zein supplemented with hydroxypropyl methylcellulose (HPMC) on the microstructural and rheological properties of a gluten-free dough and found a slight increase in protein-protein interactions and quicker particle aggregation with defatted zein during zein+starch dough formation. Frequency sweep testing showed that the two different doughs formed with undefatted zein and defatted zein were more elastic than viscous in the range of frequencies used. The authors also found that the dough from defatted zein had a slightly higher storage modulus (G') when compared to dough from undefatted zein. The slightly lower G' for the undefatted zein was attributed to the plasticizing effect of the lipids present.

2.3.3 Effects of oxidizing agents

Taylor et al. (2016a) found that commercial zein "dough" formed in hydrogen peroxide solution was softer and far more extensible than zein dough formed in water, even when the dough was cooled below the T_g of hydrated zein. Zein doughs treated with hydrogen peroxide retained water below the T_g of hydrated zein, whereas there was rapid loss of water from doughs prepared with water. The improved water holding of zein doughs formed in hydrogen peroxide solution was proposed to be due to the hydroxylation of the hydrophobic amino acid residues with aliphatic side chains in zein such as leucine, isoleucine, valine and alanine. It was suggested that the hydroxylation increased water uptake through hydrogen bonding. This was supported by the finding that the presence of hydrogen peroxide did not result in any observable polymerisation through disulphide bonding, which indicated that there was no substantial oxidative cross-linking of the commercial zein. This was attributed to the presence of just one terminal cysteine residue (chain terminator) in the commercial zein (α-zein) (Belton et al., 2006), which prevented further disulphide bonding. In contrast, the major role

of hydrogen peroxide in wheat dough is to initiate both inter- and intra-molecular disulphide bonding (Primo-Martin et al., 2003) and to enhance dityrosine cross-linking activity (Rodriguez-Mateos et al., 2006). . When oxidative enzymes like glucose oxidase and L-amino acid oxidase enzymes were used as wheat flour improvers, the hydrogen peroxide produced induced an important modification to the gluten proteins that was related to the formation of HMW polymers, which reinforced the gluten network (Bonet et al., 2006; Manu and Rao, 2011). The activity of glucose oxidase is to catalyse the oxidation of glucose into hydrogen peroxide and gluconic acid (Rasiah et al., 2005). When glucose oxidase was applied to maize and sorghum breads, it improved the loaf volume and reduced top collapsing of the loaf (Renzetti and Arendt, 2009). Also, hydrogen peroxide, and hydrogen peroxide plus horseradish peroxidase have been found to increase the dityrosine concentration of wheat dough, resulting in increased dityrosine crosslinking (Takasaki et al. 2005). In the presence of hydrogen peroxide, tyrosine probably interacts with the endogenous peroxidases to form dityrosine (Rodriguez-Mateos et al., 2006). Since dityrosine has been found to be partially responsible for the elastic and insoluble properties of gluten (Rodriguez-Mateos et al., 2006), the addition of hydrogen peroxide to kafirin may improve its viscoelastic properties in the same way as has been found with commercial zein (Taylor et al., 2016a).

2.3.4 Effects of different compositions of kafirin and zein

As stated previously, Schober et al. (2011) found that functional zein comprised mainly α-zeins very low levels of β- and γ-zeins, while non-functional zein contained high levels of β- and γ-zeins. These authors found that α-zein isolated with 70% ethanol aggregated into a viscoelastic dough at 40^{o}C, while α-kafirin isolated with 83% isopropanol aggregated into cohesive mass in water at 55^{o}C, but kafirin isolated with 70% ethanol did not aggregate. However, the aggregated kafirin cohesive mass immediately became stiff and hence had very poor dough properties. The better performance of kafirin isolated with 83% isopropanol relative to the kafirin isolated with 70% ethanol was attributed to higher hydrophobicity of the 83% isopropanol. Hence, extraction method affects the composition of kafirin, which in turn influences the ability of kafirin to form a viscoelastic dough. The authors suggested that hydrophobic interactions were critical to viscoelastic functionality of both kafirin and zein, and that disulphide cross-linking was not desirable. This was supported by later work by Smith et al. (2014) who found that the addition of salts (NaCl and Na_2SO_4) affected α-zein's surface hydrophobicity, which in turn affected its ability to form viscoelastic masses.

2.3.5 Co-protein concept

The co-protein concept involves the addition of a secondary protein source to a non-wheat prolamin protein in order to fundamentally change its structure and rheological properties (Erickson et al., 2012). To make zein more gluten-like for breadmaking, Mejia et al. (2012) utilized HMW glutenin as a co-protein to change the structure and improve the viscoelastic properties of the zein mass. The co-protein increased and stabilized the β-sheet protein conformation, to a level similar to that of wheat gluten. The addition of a small amount of co-protein to zein resulted in the zein polymer having a balance of viscous flow and elastic rheological components similar to wheat gluten. Using a squeeze flow test, the authors found that that the addition of a small amount of HMW glutenin to commercial zein resulted in a higher resistance to the deformation applied and slower relaxation time than for zein alone. Also, the addition HMW glutenin to the zein resulted in a relaxation curve that closely resembled wheat gluten. Similar results were also found by Fevzioglu et al. (2012), where the addition of a small amount of HMW glutenin (5% and 10%) to zein resulted in a substantial change in its viscoelastic properties, with a phase angle (the ratio of the viscous modulus to elastic modulus (G"/G')) close to gluten. In related work on co-proteins, Goodall et al. (2012) added 18% vital wheat gluten to sorghum high protein digestibility-high lysine (HDHL) flour. The authors found that dough from sorghum line of high protein digestibility exhibited higher resistance to extension when compared to the normal sorghum.

2.3.6 Modification of kafirin

The low digestibility of wet-cooked kafirin has been attributed to extensive cross-linking involving the β- and γ-kafirins, which prevents the digestive protease enzymes from hydrolysing the kafirin protein body proteins (Oria et al., 1995; Duodu et al., 2003). The HDHL sorghum mutant, previously mentioned, is more digestible because the easy-to-digest α-kafirin in the protein bodies is exposed to the digestive protease (Oria et al., 2000). This is due to the fact that in the HDHL mutant the shape of the protein bodies are altered from spherical to invaginated, resulting in a change in the location of γ-kafirin from the periphery to the protein body interior. As stated, the viscoelastic properties of composite dough and bread made from HDHL sorghum-wheat composite flour was investigated by Goodall et al. (2012). Normal sorghum flour revealed a significant reduction in strain hardening at a 60%

sorghum flour substitution, unlike HDHL sorghum which maintained a similar level of strain hardening compared to wheat flour. The authors found that dough from HDHL sorghum line had higher resistance to extension when compared to the normal sorghum. Furthermore, the loaf volumes for HDHL sorghum-wheat flour dough were higher compared to normal sorghum-wheat composites at substitution above 30% and up to 56% sorghum flour, with the largest difference at 42%. This clearly indicates that the endosperm protein matrix structure has direct effects on the dough and bread quality.

Various modification methods have been investigated in order to develop and improve the viscoelastic dough properties of zein and kafirin, including the use of dilute organic acids, removal of fat, the use of oxidizing agents, effects of different compositions of kafirin and zein, addition of co-protein as well as modification of kafirin. However, no-one to date had produced a stable viscoelastic dough-like material from kafirin due to its higher hydrophobicity than zein and its greater degree of disulphide cross-linking (Schober et al., 2011; Emmambux and Taylor, 2009). Hence, the need for this research.

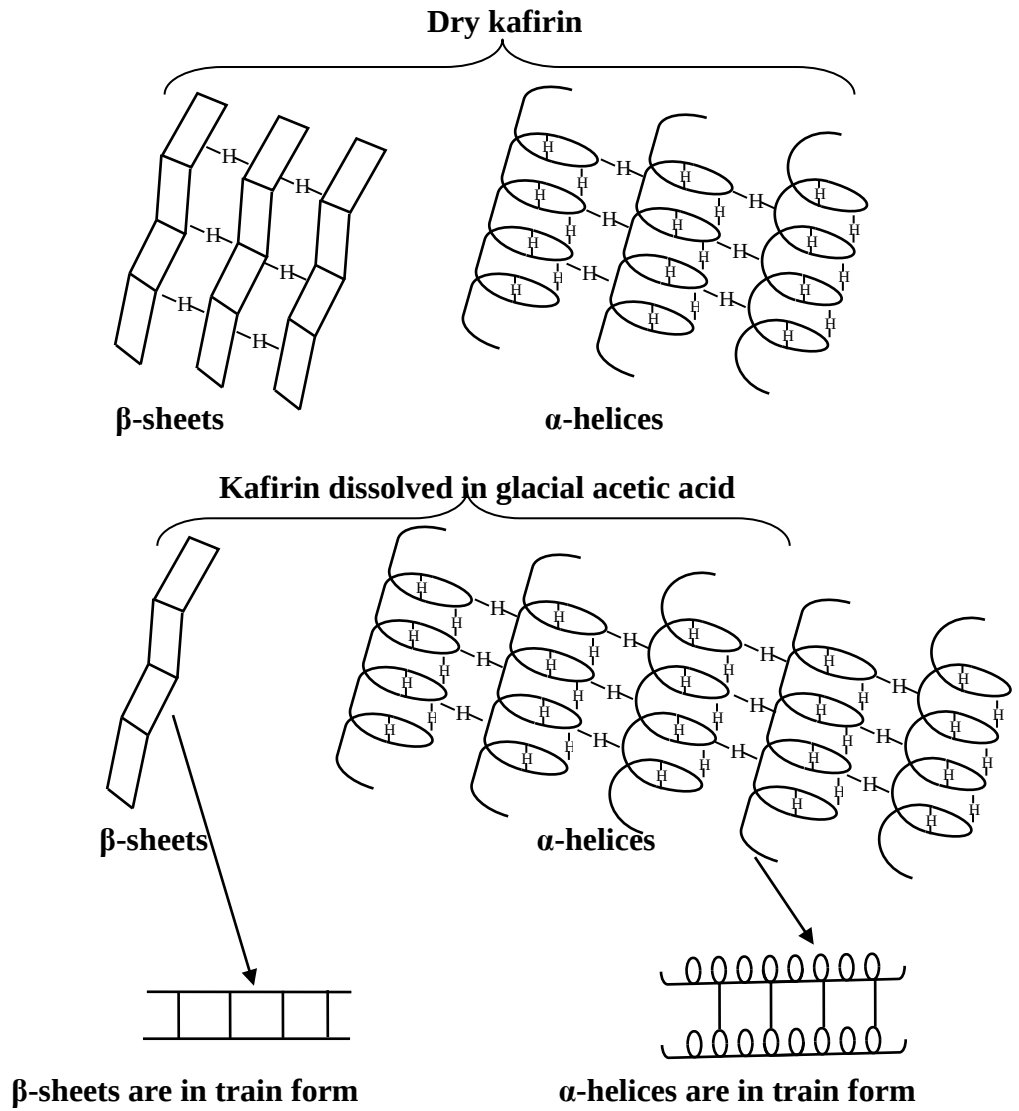

Figure 2. 7a Proposed changes in secondary structure of kafirin during dough (viscoelastic mass) formation (Elhassan, 2016)

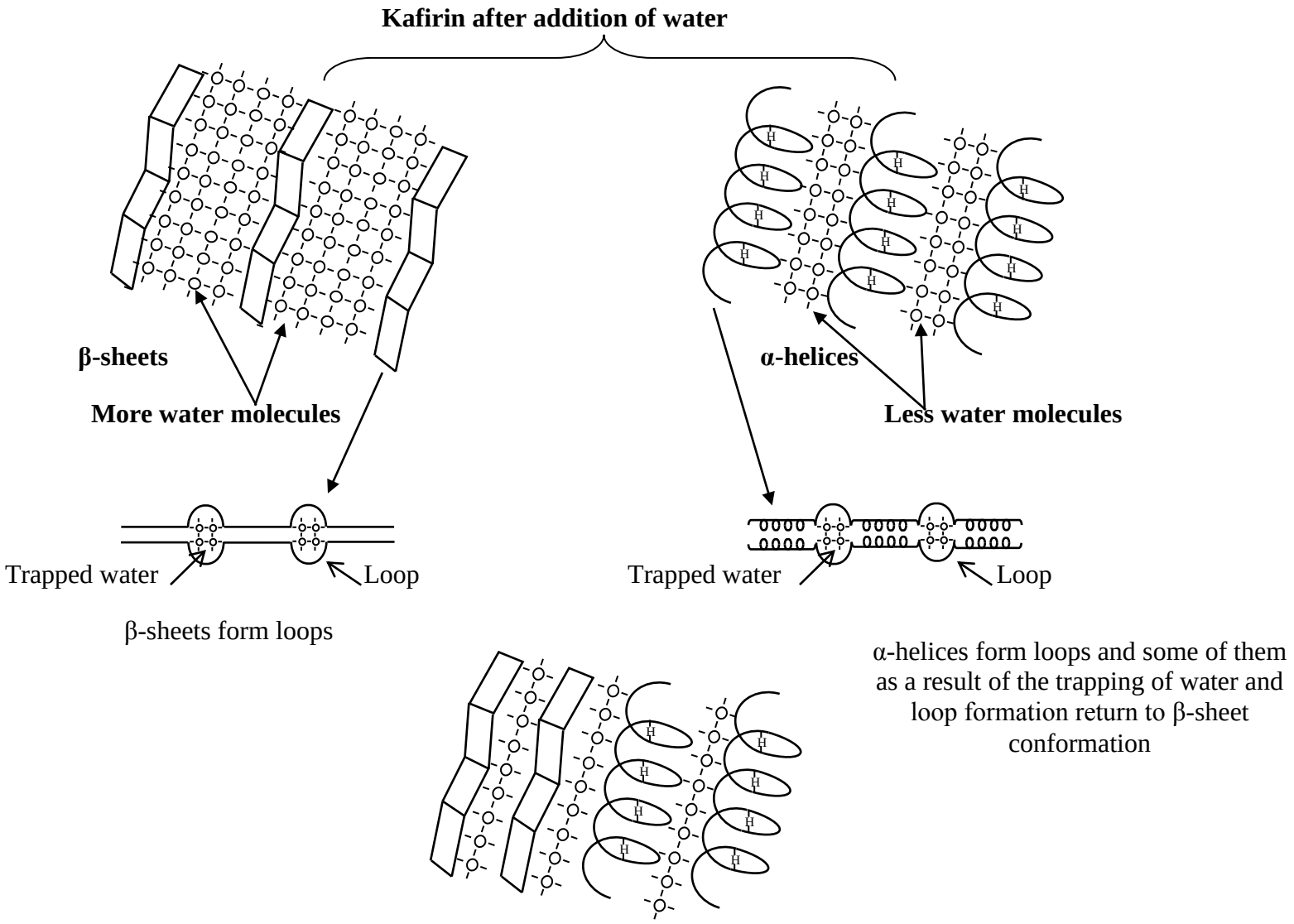

Figure 2.7 b Kneaded kafirin dough (Elhassan, 2016)

2.4 Techniques for studying the viscoelastic properties of kafirin and zein

2.4.1 Rheological properties of dough

Rheology, the study of flow and deformation of matter, is used to investigate complex systems such as dough-like systems (Petrofsky and Hoseney, 1995). Several factors affect the rheology of dough during and after dough mixing,.notably relaxation of the stresses induced during mixing, hydration of flour components and redistribution of water (Khatkar et al., 1995). Of critical importance to their functionality is the fact that wheat flour doughs exhibit both elastic and viscous flow behaviour (Belton, 1999; Upadhyay et al., 2012). In rheology, the elastic component of a material is measured as the storage modulus (G'), which is ability of the material to store energy. The viscous component is accounted for as the loss modulus (G") (Abang Zaidel et al., 2010). The loss modulus is the ability of a material to dissipate energy. The ratio of the viscous modulus to elastic modulus (G"/G') is the tangent of the phase angle, commonly referred to as the loss tangent (tan δ). The loss tangent (tan δ) of an ideal or perfect elastic material is equal to zero (0) since in an ideal elastic material there is no viscous dissipation of energy (Belton, 2005). The phase angle of a viscoelastic material must be within the limits of $0 < \delta < 90o$. Also, if the G' > G" (tan $\delta < 1$), then the material is taken to be predominantly gel- or solid-like. However, if G' < G" (tan $\delta > 1$) then the material is considered to be predominantly liquid like (Upadhyay et al., 2012). In a typical dough rheology experimental range, when the G' is greater G", it is an indication that the dough exhibits a viscoelastic behaviour with solid-like characteristics (Upadhyay et al., 2012).

Techniques used for measuring dough rheology are usually categorised according to the type of strain imposed (comprising compression, extension, shear and torsion) and the relative magnitude of the imposed deformation of the material (involving small or large deformation) (Dobraszczyk and Morgenstern, 2003). Small deformation involves deformation where only a small percentage of deformation is applied to the material in order not to break its structure (Angioloni and Collar, 2009). Small deformation testing also involves deforming a material when a small percentage deformation is required to break the material, and is often performed by fundamental tests (Tabilo-Munizaga and Barbosa-Ca'novas, 2005). In contrast, large deformation refers to deformation of a material to the point of permanent structural change (Angioloni and Collar, 2009).

Traditionally, the main rheological techniques used in cereal science have been grouped into descriptive empirical techniques and fundamental measurements. Descriptive empirical techniques are measured using instruments such as the Mixograph, Extensigraph, Farinograph and Alveograph. The Mixograph is used to measure the resistance of dough to dough mixing. The extensigraph measures resistance of the dough to uniaxial extension. The Farinograph is a revolving blade-type dough mixer attached to dynamometer for recording torque, while the Alveograph measures the biaxial extensibility of dough. The Alveograph uses air pressure to inflate a thin sheet of dough, simulating the bubbles that cause dough to stretch during proofing. These instruments are employed to measure large deformation of dough (Tronsmo et al., 2003). The descriptive empirical techniques are usually used as 'single point' tests, in which a single parameter is selected randomly from a large range of data (Hyun et al., 2001). These descriptive empirical techniques depend on the type of instrument, size and geometry of the test sample, and the specific conditions under which the test is carried out. In contrast, fundamental measurements include small deformation dynamic shear oscillation, small and large deformation shear creep and stress relaxation, large deformation extensional measurements and flow viscometry) (Dobraszczyk and Morgenstern, 2003).

In the stress-relaxation test (large deformation type test), the material is subjected to instantaneous deformation, which can be done by compression, extension or shear (Tabilo-Munizaga and Barbosa-Ca'novas, 2005). During the stress-relaxation test, the deformation or strain is kept constant throughout the test while the stress is monitored as a function of time. Stress relaxation has, for example, been used to provide information on permanent crosslinking in doughs (Ziegler and Rizvi, 1989), the effects of different chemical and enzymatic additives on dough baking quality (Wikstrom and Eliasson, 1998).

Dynamic oscillation rheology (small deformation type test) is a fundamental rheological technique that applies sinusoidally oscillating stress or strain with time and measuring the resulting response (Dobraszczyk and Morgenstern, 2003). The dynamic oscillation method involves simultaneous measurement of both elastic and viscous moduli. The dynamic oscillation method does not damage the test sample during analysis, which allows multiple measurements to be performed as the strain, frequency or temperature is varied. Rheometers

are instruments used for dynamic oscillatory testing. The Anton Paar Physica MCR Rheometer was used in this study for measuring both the elastic and viscous flow characteristics of kafirin and zein viscoelastic masses. During testing, the viscoelastic mass or dough is held between two parallel plates, where the lower plate is immobile or fixed and the upper plate is oscillatory. Other parameters that can be determined in addition to the storage modulus (G') and the loss modulus (G") include the complex modulus (G*). The Complex modulus can provide information on the strength of the material (Fevzioglu et al., 2012). $G^* = [(G')^2 + (G")^2]^{1/2}$.

Several different types of rheological tests can be performed. Probably the most useful with respect to understanding dough rheological properties is the frequency sweep test, where the frequency is varied, while the amplitude of the deformation (or shear-stress amplitude) is kept constant. This provides information about changes in the viscoelastic properties of the polymer network at different observation times (Ortolan et al., 2017). Another useful test is the temperature sweep test, where the temperature is increased, while maintaining constant frequency and strain. This can be used to simulate the baking process at constant frequency, i.e. to estimate the changes that would occur in dough during baking.

With regard to wheat doughs, the source of gluten has a significant effect on their rheological behavour. For example, Kim et al. (2008) investigated the fundamental rheological properties of hard wheat flour doughs of different strength as a function of mixing and time. Using small deformation dynamic tests, the authors found that during the initial dough resting period the complex modulus decreased and the phase angle also decreased for under-mixed doughs, whereas both the complex modulus and phase angle increased for over-mixed doughs. Concerning doughs made with non-wheat prolamin proteins, Mejia et al. (2012) found that "dough" prepared from zein plus a small amount of high molecular weight glutenin (as co-protein) had a higher resistance to deformation at 23^{o}C, unlike zein dough without the co-protein. Gluten dough also showed a slower relaxation rate, and sustained force while the constant strain was being applied. However, at 35^{o}C, zein dough with high molecular weight glutenin had a relaxation rate that resembled that of gluten dough. Oom et al. (2008) performed dynamic measurements in shear on zein resin and found that it maintained equal viscous and elastic components.

Concerning large deformation testing, the tensile properties of zein doughs prepared with both lactic and acetic acids were evaluated using a Kieffer rig by Sly et al. (2014), a miniature Extensigraph-type instrument. These authors found that the zein doughs were all highly extensible and reached the maximum extension on the Kieffer rig without breaking. These findings were similar to those of Lawton (1992), who found that zein-starch dough mixed at 30^{o}C and 35^{o}C extended throughout the entire length of Extensigraph without breaking.

2.4.2 Confocal laser scanning microscopy (CLSM)

CLSM provides images with better resolution compared to conventional light or fluorescence microscopy and it also can produce optical sections through a three dimensional specimen (Dürrenberger et al., 2001). These can be processed into a three-dimensional image. In CLSM, a laser light beam emitted by the laser system (excitation source) passes through a pinhole aperture situated in a conjugate plane (confocal), and the laser beam is then reflected by a dichromatic mirror and scanned across the sample in a well-defined focal plane (Paddock, 1999). Secondary radiations that are emitted from points on the sample (in the same focal plane) reflect back through the dichromatic mirror and are focused as a confocal point at the detector (a photomultiplier tube) pinhole aperture. Fluorescent dyes may be applied to the specimen before analysis to identify specific chemical components in the specimen. CLSM is mostly used in the fluorescence mode, which allows thick specimen to be viewed with ease unlike with conventional transmitted light microscopy. CLSM also minimises artifacts in the images compared to light microscopy because it requires little sample preparation. CLSM has been used to identify fine protein fibrils in zein doughs (Schober et al., 2008, 2010; Johansson et al., 2012; Sly et al., 2014; King et al ., 2016).

2.4.3 Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy is primarily used to characterise protein secondary structure. It measures the absorption peaks that correspond to the frequencies of vibrations between the bonds of the atoms of the protein (Jung, 2000). Each frequency has its corresponding secondary structures depending on the solvent used. Beta-sheet, β-turn, random coils and α-helical secondary structures of proteins can be detected and quantified using FTIR spectroscopy (Kong and Yu,

2007). The polypeptide and protein units produce up to nine characteristic infrared absorption bands, which include Amide A, B and I-VII (Kong and Yu, 2007). The most important bands in the infrared spectra of polypeptides and proteins are the Amide I and II regions (Pelton and McLean, 2000). These absorption regions arise from the amide bonds that link the amino acids. The Amide I vibration mode is predominantly a C=O stretching vibration coupled with the contributions from in-plane N-H, CN stretch, CCN deformation modes (Duodu et al., 2001). Normally, only the Amide I is usually considered for analysing the secondary structure of protein in viscoelastic systems as well as in solution because the frequencies of vibration of each secondary structural component of these proteins have been found to correlate with the frequencies of the Amide I bands (Kong and Yu, 2007). The Amide II region is considered less reliable for protein secondary structure determination because it is more sensitive to hydration, protein-solvent interactions (Wellner et al., 1996).

Deconvolution of the IR spectra is often performed to aid the interpretation of the data. Deconvolution refers to the removal of the intrinsic shape of the absorption spectra (Jackson and Mantsch, 1995). Different deconvoluted frequencies have been assigned approximately to secondary structure of proteins from the Amide I region (Kong and Yu, 2007). These include 1620-1642 cm-1 (β-sheet), 1691-1699 (β-sheet), 1640-1648 cm-1 (random coil), 1650-1658 cm-1 (α-helix) and 1665-1690 cm-1 (β-turns). The authors found that when zein was mixed at 35^{o}C, a viscoelastic system was formed by a structural rearrangement that favoured the formation of β-sheet structures.

Many researchers have utilized FTIR spectroscopy in an effort to determine the secondary structures of kafirin and zein.This is because their secondary structure appears to influence their functionality. For example, Mejia et al. (2007) found that the secondary structure of zein influenced its dough forming properties.

2.5 Conclusions

Despite the fact that kafirin and zein have the inherent functional ability or potential to form gluten-like doughs, progress towards forming a wheat gluten-like viscoelastic mass (dough)

from kafirin has been slow and challenging. Much progress has been recorded on the formation of gluten-like viscoelastic dough from α-zein. However, except for the recent progress by Elhassan et al. (2018) and Taylor et al. (2018), which formed part of the research reported in this thesis, the formation of a gluten-like viscoelastic dough kafirin below its glass transition temperature had not been successful. This is perhaps because kafirin is more hydrophobic in an aqueous system and more cross-linked than zein. As stated, sorghum is one of the major cereal crops grown in sub-Saharan Africa. Therefore, its utilization in wheat-free bread could be a way of providing a market for the farmers in the region and reducing wheat import. The mechanisms through the means of models to explain the rheological behaviour of kafirin viscoelastic masses have not been reported. Also, it is not totally clear enough whether it is essential for the α-helix to transform into β-sheet during dough formation. All these have to be investigated.

3 HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

i. Kafirin preparations extracted using different solvents, viz 70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid, 70% ethanol + 0.35% acetic acid, glacial acetic acid after 1% sodium metabisulphite pre-soak, and glacial acetic acid will form kafirin viscoelastic masses with different functionality when subjected to the simple coacervation process of Elhassan et al. (2018). The different solvents will extract kafirin with different spectra of subclasses because of their differing hydrophobicity and reducing power (Schober et al., 2011).

ii. Kafirins and zein comprising different subclasses will form viscoelastic masses that possess different rheological properties, depending on their subclass composition, when subjected to the simple coacervation process of Elhassan et al. (2018). There is extensive homology between kafirin and zein (De Rose et al., 1989) but to date, researchers have had little success in forming viscoelastic masses from kafirin. Schober et al. (2011) found that only α-kafirin was able to achieve partial functionality. In contrast, commercial zein (mainly α-zein) readily forms a highly extensible viscoelastic mass in warm water (Sly et al., 2014; King et al., 2016). However, both kafirin and zein containing all the subclasses (α-, β- and γ-) remained as a slurry when treated with warm water and did not aggregate into a cohesive mass (Schober et al., 2011; King et al., 2016).

iii. There will be a minimum concentrations of acetic acid and of protein needed for kafirin and zein to form fibrils and viscoelastic masses by simple coacervation. Taylor et al. (2009) formed microparticles from kafirin by simple coacervation, where kafirin was dissolved in glacial acetic acid and water added, changing the solvent concentration and precipitating out the kafirin in the form of microparticles. It was found that different final acetic acid concentrations resulted in materials of different forms, i.e. microparticles (5.4% acetic acid) or films (21.6% acetic acid). Prolamin protein biomaterials are all thought to be formed by controlled protein aggregation (Bolder et al., 2006). Depending on the interactions between the prolamin monomeric polymers and the solvent, different structures can be formed. The type of protein aggregation and thus the form the material takes depends on a number of factors which may include; the concentration of the protein, temperature, pH, salt concentration, salt type, and type of solvent added (Krebs et al., 2007). Thus, a high final

concentration of acetic acid would be necessary to form fibrils and viscoelastic masses due to the high hydrophobicity of kafirin.

3.2 Objectives

The overall objective of this study was to determine how various factors influence the functionality of kafirin viscoelastic masses with the aim of producing kafirin-based doughs with similar rheological characteristics as gluten-based doughs.

Specific objectives:

1 To determine the effects of various different extraction solvents (70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid, 70% ethanol + 0.35% acetic acid, glacial acetic acid after 1% sodium metabisulphite pre-soak, and glacial acetic acid) on the formation and functionality of kafirin viscoelastic masses using the coacervation from solution in glacial acetic acid method.

2 To determine the effects of kafirin and zein sub-class composition on the rheological properties of kafirin and zein viscoelastic masses formed using the coacervation from solution in glacial acetic acid method.

3 To determine the effects of final acetic acid and protein concentration on the formation and functionality of kafirin and zein viscoelastic masses produced using the coacervation from solution in glacial acetic acid process.

4 RESEARCH

4.1 PROPERTIES OF COHESIVE MASSES PREPARED FROM KAFIRIN ISOLATED WITH DIFFERENT SOLVENTS

4.1.1 Abstract

The properties of kafirin cohesive masses prepared from kafirins isolated with different solvents were investigated. Cohesive masses were formed from kafirins isolated with aqueous ethanol or glacial acetic acid with or without reducing agent through coacervation by water addition from a solution of kafirin protein in glacial acetic acid. All the kafirin preparations formed cohesive masses regardless of the solvent used for the kafirin extraction or difference in the secondary structure. Change in the secondary structure of kafirin proteins from α-helical to β-sheet conformation may not be critical for the formation of cohesive mass. Formation of stable cohesive masses can be attributed to the glacial acetic acid enabling complete solvation, protonation, and partial unfolding of the kafirin molecules, which are thought to be present in solution mainly as monomers (single polypeptide chains).

4.1.2 Introduction

Sorghum is a drought-tolerant cereal crop widely cultivated in the sub-Saharan Africa (Srinivas et al., 2009). Production of Sorghum accounts for 42.7 % of total world production (FAOSTAT, 2014). The use of locally produced sorghum in bread products would be advantagteous in the sub-Saharan Africa and have the potential to reduce the quantity of expensive wheat imports. However, the commercial utilization of sorghum in the production of gluten-like doughs is greatly limited by the inherent inertness of its prolamin storage protein, kafirin, which does not exhibit the same viscoelastic properties in normal dough systems as wheat gluten (Taylor et al., 2014). Kafirin is contained in granular-type protein bodies of the grain starchy endosperm (Taylor et al., 1984), which makes it unavailable for participation in dough fibril formation (Taylor et al., 2014). This is unlike gluten proteins which are present in a continuous matrix (Shewry, 1999). Furthermore, kafirins are more difficult to hydrate and more hydrophobic than wheat gluten (Taylor and Belton, 2002). Belton (1999) reported that this difficulty might be related to their high proportion of α-helical structure, in contrast to gluten which has a high level of β-sheet and β-turn structure.

The behaviour of kafirin (comprising α- and γ-kafirin) resins plasticised with oleic and lactic acids was investigated by Oom et al. (2008). It was found that the kafirin resins formed were viscoelastic. However, the kafirin resins rapidly became stiff. This was attributed to high levels of disulphide crosslinking of cysteine-rich γ-kafirin. Schober et al. (2011) attempted to form a viscoelastic substance with α-kafirin isolated with 83% isopropanol. The resulting aggregated cohesive mass immediately became stiff due to its poor dough forming properties. The most recent successful attempt on kafirin viscoelastic mass formation was reported by Elhassan et al. (2018). Kafirin extracted with aqueous ethanol with reducing agent was dissolved in warm glacial acetic acid. On addition of water, a viscoelastic mass was formed. This achievement provides a platform for more developmental research on gluten-free dough formation.

Hence, the focus of this research was to determine whether kafirin cohesive masses formed with kafirin isolated using various different solvents will possess different functional properties.

4.1.3 Materials and methods

4.1.3.1 Materials for kafirin preparations

The material used for the experiment was a mixture of two very similar tan-plant, non-tannin white sorghum cultivars PANNAR PEX 202 and 606.

4.1.3.2 Kafirin extraction with aqueous ethanol

Extraction of kafirin with aqueous ethanol was carried out using the method described by Taylor et al. (2005b) with modifications. Clean whole grains were decorticated by abrasion with a sorghum dehuller (Rural Industries Promotion Company, Botswana) until ≈21% grain by weight was removed. This resulted in the removal of the pericarp and most of the germ. The decorticated sorghum grain was milled using a laboratory hammer mill (Falling Number 3100, Huddinge, Sweden) fitted with a 500 μm opening screen. The milled sorghum grain (500 x *g*) was mixed vigorously with 70% (w/w) aqueous ethanol containing 0.5% (w/w) sodium metabisulphite and 0.35% (w/w) acetic acid at 70˚C for 1 h to extract total kafirin (containing β- and γ-kafirins as well as α-kafirin). The mixture was centrifuged at 1000 x *g* at 25^{o}C for 5 minutes to recover the kafirin containing supernatant. The residue (pellet) was washed further with 500 x *g* of the extractant and centrifuged. The two separate supernatants recovered from each centrifugation process were combined and the ethanol was evaporated off in a fume hood at ambient temperature. The precipitated protein was recovered by filtration under vacuum and air-dried at 25^{o}C. This extraction was also performed without the inclusion of reducing agent (sodium metabisulphite) (Taylor et al., 2005b), to extract kafirin with high level of α-kafirin but low levels of β- and γ-kafirins.

4.1.3.3 Kafirin extraction with glacial acetic acid

Kafirin was also extracted with glacial acetic acid using the method described by Taylor et al. (2005b). Milled decorticated sorghum grain (200 g) was pre-soaked in 1% sodium metabisulphite solution for 16 h at 25^{o}C to break the disulphide crosslinks between the polymerised kafirins and to extract γ-kafirin. The sodium metabisulphite solution containing γ-kafirin was collected by vacuum filtration, dialyzed against distilled water and then freeze-dried. Glacial acetic acid (1000 x *g*) was added to the pellet and mixed for 1 h with vigorous stirring at 25^{o}C. The filtrate was recovered under vacuum and the pellet was washed with 500 x *g* of extractant. The filtrates were combined and the extractant (acetic acid) was evaporated in a fume hood. Kafirin was precipitated by adjusting the pH to 5 with 30% (w/v) sodium

hydroxide at 25°C. The precipitated kafirin with high level of α-kafirin but low levels of β- and γ-kafirins was air-dried at 25°C after washing with cold distilled water (~8 °C) to remove any residual sodium acetate salt. Protein (N x 6.25) was determined by Dumas nitrogen combustion method (AACC International, 2000) Method 46-30. The extraction procedure was repeated without 1% (w/w) sodium metabisulphite solution to extract kafirin containing high proportions of α- and β-kafirins but relatively low γ-kafirin.

4.1.3.4 Protein preparation purity and yield

The percentage protein yield was determined using the formula below:

$$\text{Percentage protein yield} = \frac{\text{Weight of protein recovered (g)}}{\text{maximum amount of protein present in grain sorghum (g)}} \times 100$$

Weight of protein recovered

$$= \frac{\text{Weight of protein powder recovered (g as is basis)x percentage protein purity}}{100}$$

4.1.3.5 Methods investigated to attempt to form kafirin cohesive mass

4.1.3.5.1 Formation of kafirin cohesive mass using the coacervation from solution in glacial acetic acid method

Dry kafirin preparation (0.20 g) was dissolved glacial acetic acid (0.5 ml) in a covered glass beaker and heated slowly with constant stirring at 50°C for 5 min using a magnetic stirrer hot plate fitted with a temperature probe. Distilled water (1 ml) was then rapidly added (approx. 5 s) into the solution without stirring to form aggregated fibrils (approx. 0.30 g) of final temperature of 25 ± 0.5°C, which were kneaded by hand (wearing rubber gloves) into a cohesive mass (approx. 0.18 g) prior to analyses.

4.1.3.5.2 Kafirin preparation hydrated in acetic acid

Kafirin powder (0.20 g) was weighed and poured into a 50 ml beaker. Acetic acid (1.5 ml, 33% v/v) was pipetted into the 50 ml beaker containing the kafirin and a magnetic stirrer bar and heated slowly at 50°C for 5 min using a magnetic stirrer hot plate fitted with a temperature probe. With this method, a gel was formed, which was studied using a Nikon stereo light microscope (Nikon SMZ 800, Tokyo, Japan).

4.1.3.5.3 Kafirin preparation hydrated in water

The kafirin powder (0.2 g) was weighed into an Eppendorf tube and 1.5 ml distilled water was also pipetted into a separate Eppendorf tube. The Eppendorf tubes were heated in a water bath at 50^{o}C for 1 min. The pre-warmed distilled water was added to the pre-warmed kafirin powder and then vortex for 1 min. The Eppendorf tube was then allowed to cool to ambient temperature before centrifuging at 3000 rpm for 5 min. The supernatant was decanted and the residue was studied using a Nikon stereo light microscope.

4.1.3.6 Electrophoresis of kafirin preparations

4.1.3.6.1 SDS-PAGE

Kafirin preparations isolated with different extraction solvents were characterised by SDS-PAGE under reducing and non-reducing conditions, while kafirin cohesive masses were characterised under non-reducing conditions. An X Cell Sure Lock^{TM} Mini-Cell electrophoresis (Invitrogen Life Technologies, Carlsbad, CA) system was used with 15 well 1 mm thick, pre-prepared Invitrogen NuPAGE 4-12% Bis-Tris gradient gels. Invitrogen Mark12™ unstained standard (2.5-66.3 kDa) was used. The protein loading was ≈20 µg. Particular care was taken to completely solubilize the kafirin cohesive masses in the sample buffer (non-reducing buffer). This was done by further drying the cohesive masses in a fume hood for 72 h. The dry kafirin cohesive masses were ground into fine powders using a ceramic mortal and pestle before solubilizing in a non-reducing sample buffer. The powders in the non-reducing sample buffer were mixed by vortexing vigorously until the powders were completely dissolved. Gels were stained with Coosmassie® Brilliant Blue R250 overnight, destained and then scanned on a flatbed scanner.

4.1.3.6.2 2D-Electrophoresis

Two-dimensional gel electrophoresis was used to further separate kafirin protein subclasses by their isoelectric points (isoelectric focusing) as well as molecular size. Protein (5 mg) was mixed with 500 µl IPG rehydration buffer (60 ųl Zoom$^{@}$ carrier Ampholites + 3 ml DeStreak Rehydration solution) (G-Biosciences, St. Louis, MO). The solution was diluted further to a final concentration of 2 µg protein/µl. Isoelectric focussing (IEF) was performed first using the ZOOM® IPG System according to the manufacturer's instruction (Invitrogen, 2012). An immobilized pH gradient (IGP) strip [pH 3-10 non-linear (NL)] was rehydrated with ≈280 µg

protein loading using the ZOOM® IPGRunner™ cassette. The strip was equilibrated for 1 h during which time the protein sample was absorbed by the gel. IEF was performed using a ZOOM® IGPRunner™ Mini-Cell Chamber at 0.05 mA for each strip and 0.1 Watt/strip, 200 volts for 20 min, 450 volts for 15 min, 750 volts for 15 min and then 2000 volts for 45 min. The strip was then equilibrated using the equilibrium tray for 15 min in 9 ml NUPAGE® diluted sample buffer (LSD) with 1 ml sample reducing agent. Iodoacetamide (232 mg) was dissolved in 10 ml 1X NUPAGE® diluted sample buffer (LSD) to make the alkylating solution. The alkylating solution was then poured onto the strip in the equilibration tray and allowed to equilibrate for 15 min. The strip was then fixed it into the well of a NUPAGE® Novex 4-12% Bis-Tris ZOOM® gel. Agarose solution (0.5%) of approx. 400 µl was added before the SDS-PAGE was performed for the second dimension. Gels were stained with Coosmassie® Brilliant Blue R250 overnight, destained and then scanned on a flatbed scanner.

4.1.3.7 Fourier Transform Infrared spectroscopy (FTIR)

The FTIR was performed on both the dry kafirin preparations and their cohesive masses that were formed when the kafirin preparations were coacervated by distilled water addition from a solution of the protein in glacial acetic acid. The kafirin preparations and the cohesive masses were scanned using a VERTEX 70v FT-IR spectrophotometer (Bruker Optik GmbH, Ettlingen, Germany), with a zinc selenide crystal using 32 sample scan time and 4 cm^{-1} resolution (Anyango et al., 2013). The Attenuated Total Reflectance (ATR) mode in the wavenumber range of 400 to 4000 cm^{-1} was used. The angle of incidence for the ATR was 45^{o}C. The analysis was repeated four times. The convoluted spectra were de-convoluted by Fourier self-deconvolution (FSD) using Lorentzian filter with a resolution enhancement factor of 2 and a 6 cm^{-1} bandwidth. The wavenumbers (cm^{-1}) of α-helical conformation for all samples was 1650 ± 2 and for β-sheet conformation was 1620 ± 2.

The relative proportions of α-helical and β-sheet conformations as well as α/β ratio in the Amide I region were calculated using the following formulas:

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

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

$$\alpha/\beta\ \text{ratio} = \frac{Abs\ \alpha\text{−helix peak} - \text{Abs baseline}}{\text{Abs}\ \beta\text{−sheet peak} - \text{Abs baseline}}$$

4.1.3.8 Stereomicroscopy

Kafirin cohesive mass of approximate size 3 mm x 3 mm and 1 mm thick was placed on a glass slide, stretched and then released before imaging with a magnification of 130x. This test was repeated with kafirin hydrated in water or 33% (v/v) acetic acid to determine their morphology (see sections 4.1.3.5.2 and 4.1.3.5.3).

4.1.3.9 Statistical analyses

The IBM SPSS software version 20 (SPSS, Chicago, IL) was used to analyze the data. The data were subjected to one-way analysis of variance (ANOVA) and the means were separated using Fisher's Least Significance Difference test (LSD). Each experiment was repeated two times.

4.1.4 Results and discussion

4.1.4.1 Protein preparation purity and yield

Glacial acetic acid after sodium metabisulphite presoak was probably the most effective extractant for kafirin. This might be due to the fact that glacial acetic acid is comparatively more hydrophobic with lower dielectric constant than aqueous ethanol (Taylor et al., 2005b). The authors hypothesized that the low dielectric constant of glacial acetic acid enables it to dissolve highly hydrophobic proteins such as kafirin. The kafirin preparations extracted with glacial acetic acid were higher in yield compared to aqueous ethanol (Table 4.1.1). Glacial acetic acid yield was 57 % with respect to the amount of protein in the sorghum, while aqueous ethanol accounted for ≈44 %, whereas kafirin accounts for 50-60 % total protein (Taylor and Schussler, 1986) in sorghum. Sodium hydroxide that was mainly used to adjust the pH to 5 to facilitate kafirin precipitation after extraction might have also contributed to the high extraction of kafirin by probably deamidating glutamine residues, thereby reducing glutamine-glutamine interactions and introducing repulsion through charged glutamate residues (Gao et al., 2005). The kafirin preparations extracted with glacial acetic acid were higher in purity compared to aqueous ethanol (Table 4.1.1). This agrees with the findings of Taylor et al. (2005b) who reported that glacial acetic acid is comparatively more hydrophobic with lower dielectric constant than aqueous ethanol.

The quantity of protein extracted when the reducing agent (sodium metabisulphite) was included in both extractants was double than that obtained without the reducing agent (Table

4.1.1). This is in agreement with the work of Da Silva and Taylor (2005) who found that the inclusion of sodium metabisulphite in the extraction solvent resulted in the extraction of more kafirin as well as of different kafirin subclasses that were initially insoluble in the solvent due to being bonded into large polypeptide polymers in the sorghum grain. El-Nour et al. (1998) also reported an increase in the quantity of extractable sorghum kafirin when 2-mercaptoethanol was included in the extraction solvent. Also, the purity of the kafirin preparations increased when the reducing agent was included in both extractants (Table 4.1.1). When sodium metabisulphite is dissolved in water, it releases sulphur (iv) oxide, which in turn produces dihydrogentrioxosulphate (iv) acid (sulphurous acid) that interacts with cysteine to produce S-sulphocysteine residues (Boundy et al., 1967). This cleaves the disulphide bonds between polypeptide chains (Boundy et al. 1967). The presence of S-sulphocysteine residues prevents the reformation of disulphide bonds, causing partial disruption of sorghum protein matrix (Taylor et al., 2005b). Gao et al. (2005) also found that sodium metabisulphite cleaves the disulphide bond, which then results in the formation of cysteic acid and dehydroalanine. The authors stated further that the formation of cysteic acid and dehydroalanine may reduce the amount of cysteine available for disulphide bonding reformation after the extraction. The impurities in some of the isolated kafirins could have comprised starch, lipids and phelnolics.

Table 4.1. 1 Effects of different extraction solvents on the purity and yield of kafirin preparations

Kafirin preparations	Protein extracted (g protein/100 g sorghum flour) (db)	Protein preparation purity (g protein/100 g preparation)	Protein preparation yield (% of total protein in sorghum flour)
Kafirin extracted with 70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid	$5.67^{b} \pm 0.67$ (117)	$69.2^{c} \pm 2.0$ (24)	$43.8^{b} \pm 1.4$ (170)
Kafirin extracted with 70% ethanol + 0.35% acetic acid	$2.61^{a} \pm 0.15$	$55.8^{a} \pm 0.3$	$16.2^{a} \pm 0.1$
Kafirin extracted with glacial acetic acid after 1% sodium metabisulphite pre-soak	$6.37^{b} \pm 0.54$ (90)	$80.1^{d} \pm 0.7$ (22)	$57.0^{c} \pm 0.6$ (132)
Kafirin extracted with glacial acetic acid	$3.36^{a} \pm 0.16$	$65.6^{b} \pm 0.1$	$24.6^{a} \pm 0.2$

Values are the means ± standard deviations. n=3. Values followed by different superscript letters within a column are significantly different ($p < 0.05$). Protein content of the sorghum flour was 9.55 ± 0.09 g protein / 100 g sorghum flour (db). db = dry basis. Figures in parentheses indicate percentage increase with reducing agent

4.1.4.2 Electrophoresis

SDS-PAGE showed that the band patterns of kafirin extracted with various solvents were typical of kafirin as reported by El Nour et al. (1998). All the kafirin preparations contained all the kafirin subclasses but exhibited different band intensities. Oligomers (> ~62 kDa), monomers such as α-kafirin (23-25 kDa), β-kafirin (12, 16, 18, 20 kDa) and γ-kafirin (~27 kDa, 51 kDa) were identified under non-reducing conditions (Figure 4.1.1.B). This suggests that the kafirin oligomers crosslinked by disulphide bonds have been reduced to monomers when subjected to SDS-PAGE under reducing conditions. All the kafirin preparations had relatively identical faint bands in the 51 kDa region under reducing conditions (Figure 4.1.1.A). This band was designated as γ-kafirin (Sullivan et al., 2018). The polypeptide bands of relative molecular weight ~25 kDa were identified as α_1-kafirin monomers, while bands of apparent molecular weight ~23 kDa were presumed to be α_2-kafirin monomers.

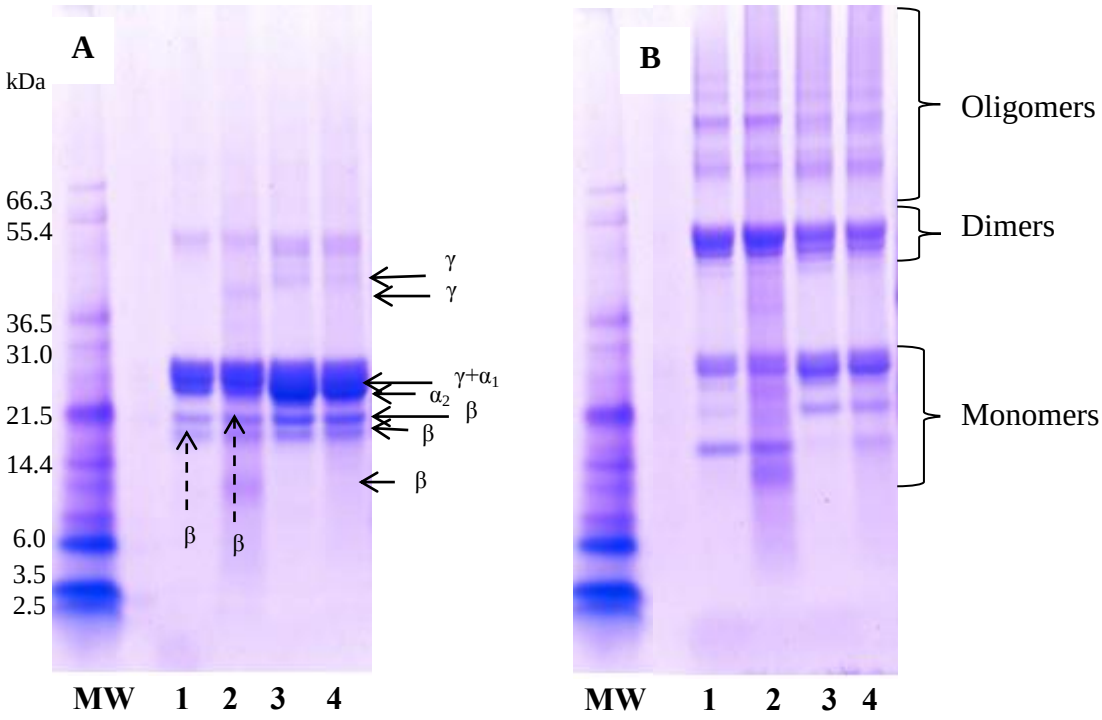

Figure 4.1. 1 SDS-PAGE of kafirin preparations extracted with different solvents. A: SDS-PAGE under reducing conditions; B: SDS-PAGE under non-reducing conditions; lanes: MW: Molecular weight standards; lane 1: kafirin extracted with 70 % ethanol + 0.35 % acetic acid; lane 2: kafirin extracted with glacial acetic acid; lane 3: kafirin extracted with 70 % ethanol + 0.5 % sodium metabisulphite + 0.35 % acetic acid; lane 4: kafirin extracted with glacial acetic acid after 1 % sodium metabisulphite pre-soak

Slight differences were identified in the kafirin preparations extracted with aqueous ethanol. The differences were in the monomeric region (lanes 1 and 3) (Figure 4.1.1.A). Faint bands of relative molecular weights ~18 kDa and 20 kDa (indicated by dotted arrows), which were designated as β-kafirins, were detected in the kafirin preparation extracted without reducing agent (lanes 1 and 2). However, darker bands of similar relative molecular weights ~18 kDa and ~20 kDa (indicated by solid arrows) were detected in the kafirin preparation extracted with aqueous ethanol with reducing agent (lane 3). By reference to the work of El Nour et al. (1998), these bands can be identified as β-kafirins. This indicated that the amount of β-kafirin extracted with aqueous ethanol in the presence of a reducing agent was greater than when the reducing agent was absent. Gao et al. (2005) reported that sodium metabisulphite cleaves the disulphide crosslinking, resulting in an increase in in the proportion of kafirin monomers, as found (Figure 4.1.1.A). Da Silva and Taylor (2005) also found that the inclusion of sodium metabisulphite in the extraction solvent resulted in the extraction of more different kafirin subclasses present as monomers. The faint broad band of apparent molecular weight ~27 kDa in kafirin preparation extracted with ethanol (lane 1) could possibly be a combination of γ-kafirin and α_1-kafirin monomer. A similar finding was reported by Da Silva et al. (2011b) who observed that γ-kafirin was not clearly separated from α-kafirin monomers. The faint bands of β- and γ-kafirins present in the kafirin preparation extracted with ethanol (lane 1) could be due to the inclusion of 0.35% acetic acid. The acetic acid could result in mild deamidation of glutamine residues to glutamate or glutamic acid, leading to the exposure of the hydrophobic regions to solvation (Zhang et al., 2011), making more kafirin subclasses soluble. The broad intense band of molecular weight ~27kDa detected in kafirin preparations extracted with ethanol in the presence of a reducing agent (lane 3) was probably a combination of γ-kafirin and α_1-kafirin (Figure 4.1.1.A). An intense band of relative molecular weight ~23 kDa identified as α_2-kafirin was also detected in kafirin preparations extracted with ethanol in the presence of a reducing agent (lane 3), with reference to the work of Da Silva and Taylor (2004). A band of similar molecular weight ~23 kDa but with reduced intensity (indicated by dotted arrow) was also detected in the kafirin preparations extracted with ethanol (lane 1).

The kafirin preparation extracted with glacial acetic acid without reducing agent showed a faint band of apparent molecular weight ~12 kDa (Figure 4.1.1.A, lane 2), which was absent in other kafirin preparations. This band can be designated as β-kafirin by reference to the

work of Nunes et al. (2005). This indicates the presence of low amounts of β-kafirin. However, the more intense bands of relative molecular weights ~18 kDa and 20 kDa that were detected in the kafirin preparation extracted with glacial acetic acid after a metabisulphite pre-soak (Figure 4.1.1.A, lane 4) showed the presence of a higher proportion of β-kafirin. The use of glacial acetic acid for the extraction of kafirin is a novel method that was developed by Taylor et al. (2005b). As stated above, glacial acetic acid was found to be effective because of its low dielectric constant, which enabled it to dissolve highly hydrophobic proteins such as kafirin. A broad band of relative molecular weight ~27kDa was detected in kafirin preparation extracted with glacial acetic acid in the presence or absence of a reducing agent (Figure 4.1.1.A, lanes 3 and 4). This broad band could possibly be a combination of γ-kafirin and α_1-kafirin monomer. Wang et al. (2009) observed the presence of low amounts of γ-kafirins (~28 kDa) when glacial acetic acid was used for kafirin extraction. A darker band of apparent relative molecular weight ~23 kDa identified as α_2-kafirin was detected in the kafirin preparation from glacial acetic acid after the sorghum flour was pre-soaked in reducing agent (Figure 4.1.1.A, lane 4). Faint bands of apparent molecular weight ~41 kDa designated as γ-kafirin were identified in the kafirin preparation extracted with glacial acetic acid, kafirin preparation extracted with ethanol in the presence of a reducing agent and kafirin preparation extracted with glacial acetic acid after the sorghum flour was pre-soaked in reducing agent. This was similar to the minor γ-kafirin (approximately 46 kDa) described by Anyango et al. (2013). However, this band was absent in kafirin extracted with aqueous ethanol in the absence of a reducing agent.

Considering the presumption that the broad band of molecular weight ~27kDa contained more than one type of kafirin sub-class, a 2-D PAGE was performed in order to identify the individual monomers within this molecular range (Figure 4.1.2). The kafirin extracted with aqueous ethanol displayed bands of apparent molecular weights ~27 kDa (γ-kafirin) and ~20 kDa (β-kafirin) within the acidic region (Figure 4.1.2.A). The kafirin preparation extracted with ethanol in the presence of a reducing agent displayed more bands that spread from the acidic region to the basic region (Figure 4.1.2.C). It displayed clear bands of molecular weights of about ~25 kDa (α_1-kafirin) and ~20 kDa (β-kafirin) as well as faint band of ~27 kDa (γ-kafirin). The kafirin preparation extracted with glacial acetic acid displayed more bands of molecular weights ~23 kDa (α_2-kafirin), ~18 kDa (β-kafirin) and ~27 kDa (γ-kafirin) within the basic region (Figure 4.1.2.B), while kafirin preparation extracted with

glacial acetic acid after the sorghum flour was pre-soaked in reducing agent displayed more bands of molecular weights approximately 23 kDa (α_2-kafirin) ~25 kDa (α_1-kafirin) and 18 kDa (β-kafirin) as well as very faint band approximately 26.6 kDa (γ-kafirin) within the basic region (Figure 4.1.2.D).

SDS-PAGE under non-reducing conditions was also performed to determine whether kafirin cohesive masses formation was due to disulphide bonding. The band patterns were typical of kafirin (Figure 4.1.3), as reported by El Nour et al. (1998). All the kafirin preparations subjected to different treatments contained all the kafirin subclasses but with different intensities. The predominant bands identified in all the kafirin preparations hydrated in 1.5 ml distilled water or hydrated in 33% (v/v) acetic acid and in the kafirin cohesive masses (formed through coacervation by distilled water from a solution of the protein in glacial acetic acid) comprised oligomers (> 65 kDa), monomers such as α-kafirin (23-25 kDa), β-kafirin (~12, 16, 18, 20 kDa) and γ-kafirin (~27 kDa, 51kDa). No additional or more intense trimer or dimer bands were detected in the kafirin cohesive masses regardless of the extraction solvents. All the band patterns were similar to those of all the kafirin preparations under non-reducing conditions (Figure 4.1.3) i.e. SDS-PAGE did not show any further disulphide cross-linking. Cohesive mass formation may have been as a result of dissolution of the kafirin molecules in glacial acetic acid, enabling them to better interact with water molecules (Elhassan et al., 2018). Similarly, King et al. (2016) suggested that dissolution of zein molecules in acetic acid could enhance water binding, enabling zein dough formation

209

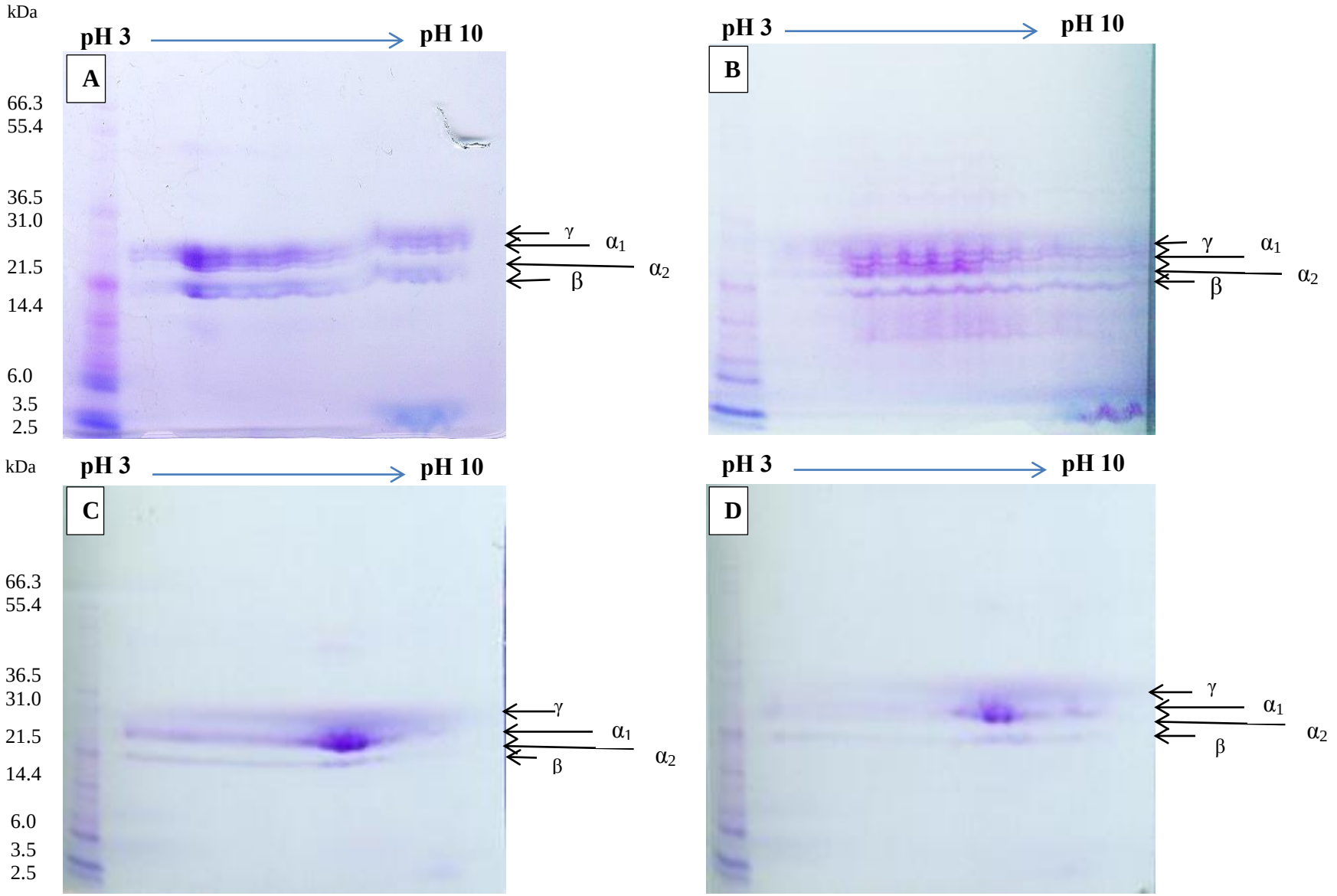

Figure 4.1. 2 2D-PAGE band pattern of kafirin preparations extracted with different solvents. A: kafirin extracted with 70 % ethanol + 0.35 % acetic acid, B: kafirin extracted with glacial acetic acid, C: kafirin extracted with 70 % ethanol + 0.5 % sodium metabisulphite + 0.35 % acetic acid, D: kafirin extracted with glacial acetic acid after 1 % sodium metabisulphite pre-soak

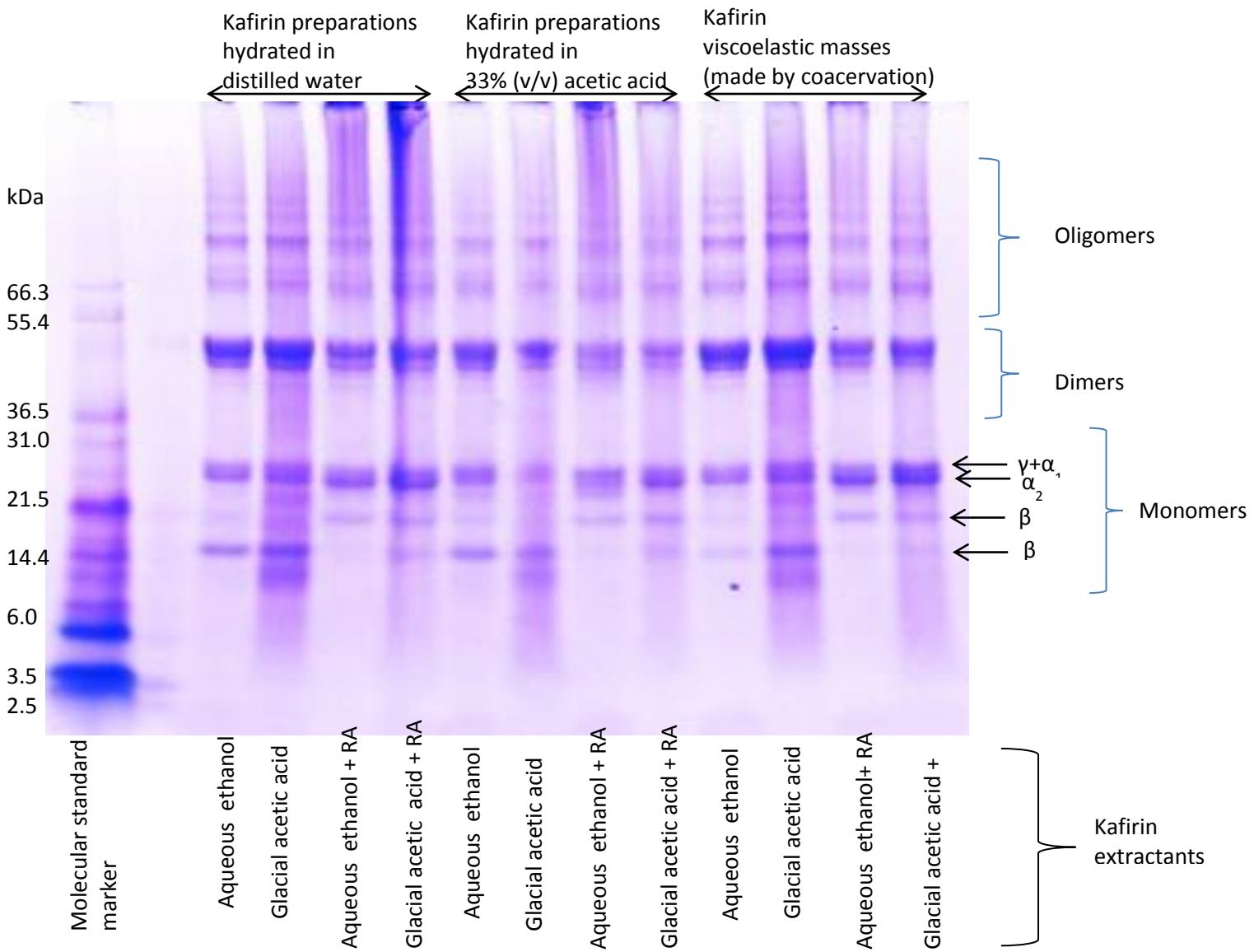

Figure 4.1. 3 SDS-PAGE of kafirin preparations isolated with different solvents subjected to different treatments under non-reducing conditions, RA-reducing agent (sodium metabisulphite)

4.1.4.3 Secondary structure of kafirin preparations and cohesive masses

Two main regions (Amide I and Amide II) were identified in the deconvoluted FTIR spectra of kafirin preparations and cohesive masses from the frequency range of 1700-1400 cm^{-1} (Figure 4.1.4) The frequency range from approx. 1700-1600 cm^{-1} and 1575-1475 cm^{-1} has been identified for Amide I and Amide II, respectively for both kafirin and zein (Duodu et al., 2001). Only the amide I is usually considered for analysing the secondary structure of protein in cohesive systems as well as in solution because the frequencies of vibration of each secondary structural component of these proteins have been found to correlate with the frequencies of the Amide I bands (Kong and Yu, 2007). The amide II is considered less reliable for protein secondary structure determination because it is more sensitive to hydration, protein-solvent interactions (Wellner et al., 1996). Different deconvoluted frequencies have been assigned approximately to secondary structure of protein at the Amide I region (Kong and Yu, 2007). These include 1624-1642 cm^{-1} (β-sheet), 1691-1699 (β-sheet), 1640-1648 cm^{-1} (random coil), 1654-1658 cm^{-1} (α-helix), 1663 (3_{10} Helix) and 1665-1690 cm^{-1} (β-turns).

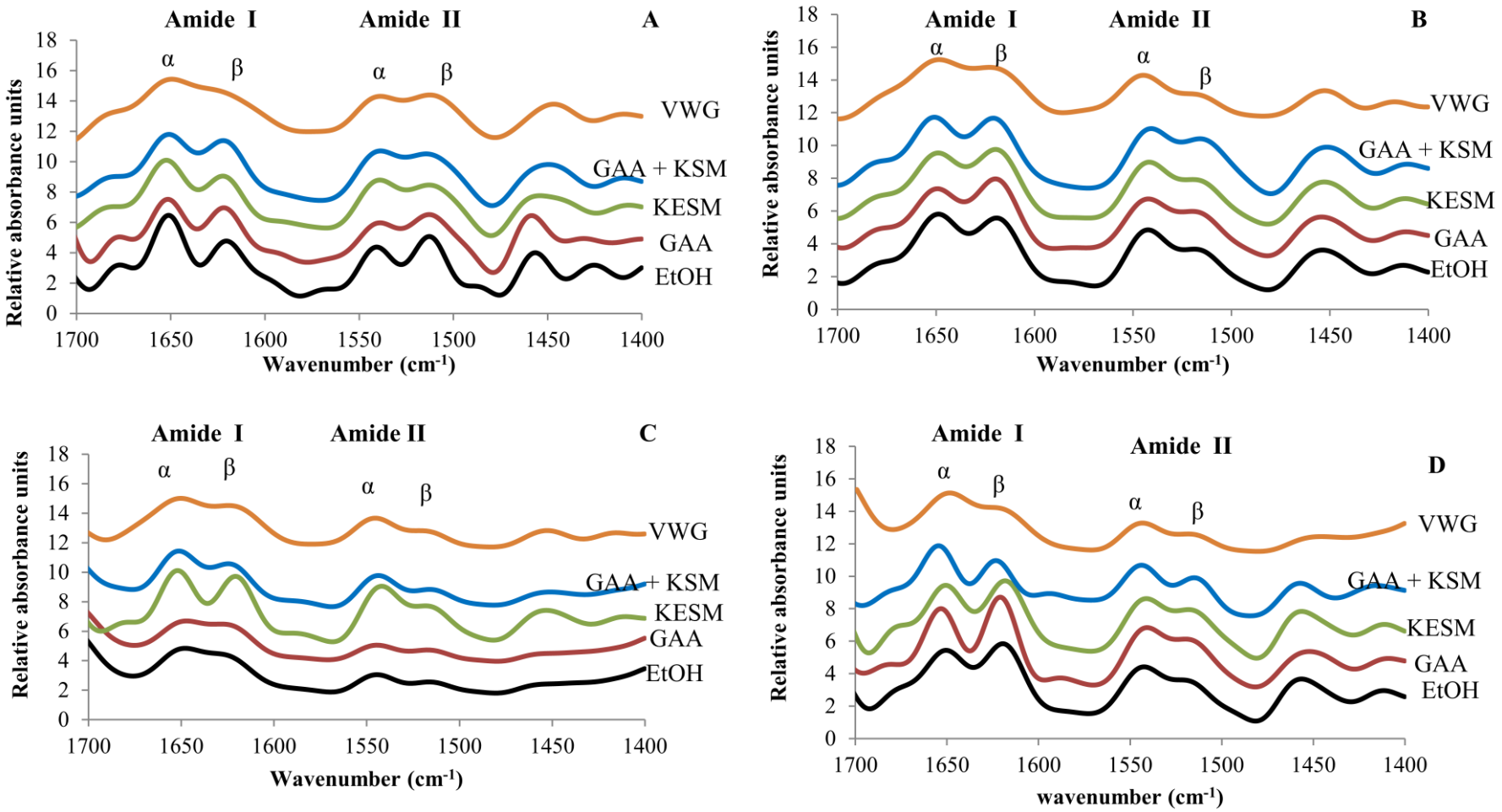

Figure 4.1. 4 FTIR of kafirin preparations and cohesive masses. A: Dry kafirin preparations, B: Kafirin preparations hydrated in water, C: Kafirin preparations hydrated in 33% (v/v) acetic acid, D: kafirin viscoelastic masses. EtOH: kafirin extracted with 70 % ethanol + 0.35 % acetic acid; GAA: kafirin extracted with glacial acetic acid; KESM: kafirin extracted with 70 % ethanol, 0.35 % acetic acid and 0.5 % sodium metabisulphite; GAA + KMS: kafirin extracted with glacial acetic acid with 1% sodium metabisulphite presoak; VWG: vital wheat gluten

There were no clear differences in peak pattern among the different kafirin preparations regardless of their compositions (Figure 4.1.4). The relative proportions of α-helices were higher than the β-sheet conformations in all the dry kafirin preparations. Duodu et al. (2001) and Belton et al. (2006) reported that kafirin in its native state contains more α-helices than the β-sheets. Belton et al. (2006) stated that kafirin is approximately 60% α-helical in its native state. Interestingly, the vital wheat gluten powder also exhibited a high proportion of α-helix (Table 4.1.2). This finding was not expected because β-sheet and random conformations are the major secondary structures in an unhydrated gluten (Wellner et al., 1996), and these β-sheet and random conformations are transformed into β-turn when gluten is hydrated (Bock and Damodaran, 2012). The vital wheat gluten (VWG) spectra differ because the VWG, which was used as the standard, was also subjected to the treatments as the kafirin was subjected to, for comparison. The α/β ratios of the dry kafirin preparations extracted with glacial acetic acid with or without reducing agent were similar (1.11:1) but slightly lower compared to the α/β ratios of the dry kafirin preparations extracted with aqueous ethanol based solvents (1.22-1.44:1). The lower α/β ratio exhibited by the dry kafirin preparations extracted with glacial acetic acid can be attributed to the unfolding of the hydrophobic protein polypeptides (α-helical secondary structure) into a more open β-sheet conformation. Li et al. (2012) showed that acetic acid was a good solvent for zein and kafirin. This allowed the molecules to unfold with a resultant better protonation. Taylor et al. (2009) also reported that the ratio of α-helical to intermolecular β-sheet conformation of kafirin microparticles decreased when the acetic acid concentration in the extraction solvent was increased. The α/β ratios of the dry kafirin preparations extracted with aqueous ethanol with or without the reducing agent were not significantly different ($p > 0.05$). This finding suggests that the inclusion of sodium metabisulphite in the extractants did not have any significant effect on the secondary structure.

Table 4.1. 2 Effects of different types of extractants and solvents on the secondary structure of kafirin measured by FTIR as indicated by differences in the Amide I region

	Solvents							
	Dry kafirin preparation		Kafirin preparation hydrated in water		Kafirin preparation hydrated in 33% (v/v) acetic acid		Kafirin cohesive mass	
Kafirin preparation	**α/β ratio**	**Relative α-helical conformation (%)**	**α/β ratio**	**Relative α-helical conformation (%)**	**α/β ratio**	**Relative α-helical conformation (%)**	**α/β ratio**	**Relative α-helical conformation (%)**
Vital gluten powder	$1.24^{aAB} \pm 0.11$	56.3	$1.17^{cdA} \pm 0.12$	51.7	$1.20^{bAB} \pm 0.10$	54.1	$1.74^{bcC} \pm 0.50$	58.5
Kafirin extracted with 70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid	$1.22^{abB} \pm 0.19$	55.4	$0.95^{bA} \pm 0.02$	48.5	$1.12^{abAB} \pm 0.14$	52.6	$0.93^{aA} \pm 0.06$	48.0
Kafirin extracted with 70% ethanol + 0.35% acetic acid	$1.44^{bC} \pm 0.05$	61.3	$1.06^{cAB} \pm 0.04$	51.6	$1.15^{abB} \pm 0.12$	54.1	$0.90^{aA} \pm 0.08$	47.2
Kafirin extracted with glacial acetic acid after 1% sodium metabisulphite presoak	$1.11^{aAB} \pm 0.02$	53.0	$1.00^{bcA} \pm 0.08$	49.9	$1.28^{bC} \pm 0.27$	56.6	$1.23^{bC} \pm 0.10$	56.3
Kafirin extracted with glacial acetic acid	$1.11^{aB} \pm 0.04$	54.5	$0.86^{aA} \pm 0.18$	45.9	$1.06^{abAB} \pm 0.02$	51.8	$0.84^{aA} \pm 0.05$	45.4

Mean values followed by different lower case letters in a column or upper case superscript letters in a row are significantly different ($p < 0.05$). n=4
Wavenumber (cm-1) of α-helix conformation for all samples was 1650 ± 2 and for β-sheet conformation was 1620 ± 2.

There was a decrease in the proportion of α-helices in all the kafirin powders when hydrated in warm water. This was also observed by Sly et al. (2014) with zein. These authors found that the relative proportion of the α-helical conformation decreased from 60.3% to 51.2% when zein powder was hydrated in warm water. There was also slight increase but not significant ($p > 0.05$) in the β-sheet conformation of the vital wheat gluten when hydrated with water. This result is in agreement with the theory that the intermolecular β-sheet content of gluten protein increases upon hydration with water (Popineau et al., 1994; Belton, 1999). The α-helical conformations of all the kafirin preparations hydrated in 33% (v/v) acetic acid slightly increased compared to the kafirin preparations hydrated in water (Table 4.1.2). Sly et al. (2014) found that the relative proportion of α-helical conformation of zein dough increased as the concentration of organic acids increased compared to zein mixed with water. The increase was attributed to deamination. In this study, the proportion of α-helical conformation reduced slightly when dry the kafirin powders extracted with ethanol with or without reducing agent were hydrated in 33% acetic acid. There was also a slight reduction of helical conformation in the kafirin extracted with glacial acetic acid. This can be attributed to the partial protein protonation. However, the α/β ratio increased substantially when the kafirin extracted with glacial acetic acid plus reducing agent was hydrated in 33% acetic acid solution. This again can be attributed to partial re-folding of the protein molecules.

When kafirin preparations extracted from aqueous ethanol with or without reducing agent were coacervated by distilled water addition from a solution of the protein in glacial acetic acid, the cohesive masses formed exhibited a relatively higher β/α ratio (i.e. lower α/β ratio compared to the kafirin preparations, regardless of their different compositions (Table 4.1.2). This change in secondary structure can be attributed to protonation of kafirin protein surface. Glutamine-rich bridges may have been formed via hydrogen bonding (Wang and Padua, 2012). Again, similarly, when kafirin isolated from glacial acetic acid without reducing agent was coacervated, the cohesive mass formed exhibited a greater proportion of β-sheet conformation (Table 4.1.2). However, the secondary structure of the cohesive mass formed from kafirin extracted with glacial acetic acid with reducing agent exhibited a higher proportion of α-helical conformation, similar to that of vital wheat gluten.

4.1.4.4 Appearance of the hydrated kafirin and cohesive masses

Stereomicroscopy showed that cohesive masses were formed when all the kafirin preparations, regardless of their extraction solvent, were coacervated by distilled water addition from a solution of the protein in glacial acetic acid by using rapid water addition with no stirring (without high shear) (Figure 4.1.5). This was unlike the situation where microparticles were formed by coacervation of a solution of kafirin in glacial acetic acid with addition of water with stirring (Taylor et al., 2009). The aggregation of the kafirin molecules into a network of fibrils was probably facilitated by the absence of high shear. Schober et al. (2011) found that a mainly α-kafirin isolate aggregated into a cohesive substance when it was dissolved in warm water in the presence of reducing agent. However, the kafirin mass immediately became stiff and lost its extensibility on cooling. The coacervation process resulted in improved functionality of kafirin cohesive mass compared to the kafirin cohesive substance obtained by Schober et al. (2011). The elastic masses in this study were formed at ambient temperature (25^{o}C), which was well below the glass transition temperature of hydrated kafirin (~41^{o}C) (Schober et al., 2011).

The cohesive mass formed with kafirin extracted with aqueous ethanol without reducing agent, with α/β ratio of 0.90:1 and low levels of β- and γ-kafirins, was cohesive with narrow fibrils (solid arrow). The narrow fibrils were similar to the cohesive mass formed from kafirin extracted with aqueous ethanol with the reducing agent, which also had α/β ratio of 0.90:1 but high levels of β- and γ-kafirin. This indicates that the morphology of fibrils formed may not depend on the proportions of the secondary structure. Schober et al. (2010) proposed that prolamin fibril formation is a critical step in dough formation. These fibrils correspond to the fibrils in both waxy and non-waxy normal protein digestibility sorghum lines observed by Elhassan et al. (2018). As stated, the glacial acetic acid used to dissolve the kafirin preparations might have allowed the kafirin molecules to unfold for better protonation. The formation of kafirin cohesive masses can in part be due to hydrogen bonding between water and protein molecules, as suggested by Elhassan et al. (2018).

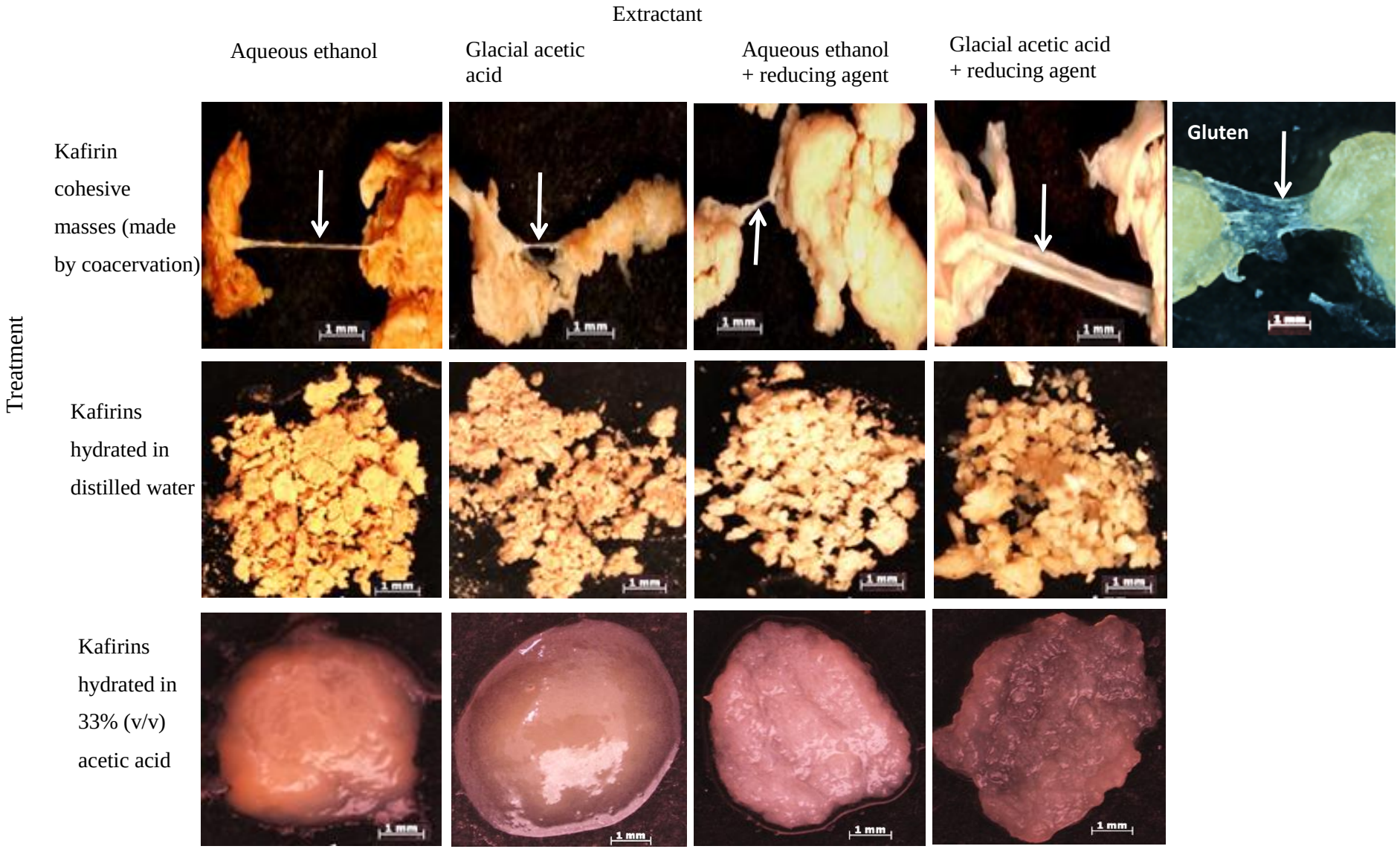

Figure 4.1. 5 Effect of kafirin preparations on the morphology of kafirin cohesive mass by stereomicroscopy. Solid arrows indicate fibrils

All the kafirin preparations isolated with aqueous ethanol or glacial acetic acid with or without reducing agent formed viscoelastic masses regardless of the solvent used for the kafirin extraction or the different compositions of the extraction solvents. Stereomicroscopy indicated that the cohesive masses formed from kafirin extracted with aqueous ethanol with reducing agent (comprising high level of α- and β- and γ-kafirins) or without reducing agent (comprising high proportion of α-kafirin but low levels of β- and γ-kafirins) and glacial acetic acid without reducing agent (comprising high levels of α- and β-kafirins but low level of γ-kafirin) had similar narrow fibrils. However, the cohesive mass formed from glacial acetic acid with reducing agent (high levels of α- and β-kafirins but low level of γ-kafirin) had broad and ribbon-like fibrils, which was similar in appearance to wheat glutenin (Orth et al., 1973) and α-zein dough (Schober et al., 2011). Schober et al. (2011) found that functional zein comprised mainly α-zeins, while non-functional zein contained high levels of β- and γ-zeins. All functional zeins comprised very low levels of β- and γ-zeins.

The kafirin preparations hydrated in warm distilled water did not result in cohesive mass formation but particles (Figure 4.1.5), while the kafirin preparations hydrated in 33% (v/v) acetic acid solution without coacervation process resulted into gels (Figure 4.1.5). This can be due to the fact that the solvents were not sufficiently hydrophobic.

4.1.5 Conclusions

Kafirin preparations can form cohesive masses by simple coacervation process regardless of the solvents used for the kafirin extraction or difference in their secondary structure. The dissolution of the kafirin molecules in glacial acetic acid may enable them to better interact with water molecules, facilitating cohesive mass formation. The formation of kafirin cohesive masses does not appear to be as a result of additional disulphide cross-linking or polymerization. Hydration of kafirin preparations in distilled water or 33% (v/v) acetic acid does not result in the formation of kafirin cohesive mass.

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4.2 THE EFFECT OF KAFIRIN AND ZEIN COMPOSITION ON ASPECTS OF THE RHEOLOGICAL PROPERTIES OF KAFIRIN AND ZEIN VISCOELASTIC MASSES

4.2.1 Abstract

Kafirin and zein viscoelastic masses were subjected to uniaxial stress-relaxation and dynamic rheological analysis to determine the effects of kafirin and zein sub-class composition on their rheological properties. Viscoelastic masses were formed through coacervation with water from solution in glacial acetic acid. Several different kafirins and zeins were investigated: kafirins extracted with different solvents, high α-kafirin, high α-zein, zein (containing α-, β-, γ-zeins) kafirin minus γ-kafirin, high kafirin minus γ-kafirin, kafirins from high protein digestibility and waxy sorghum lines and their controls. Stable viscoelastic masses could be formed from all kafirins and zeins through coacervation, apparently regardless of their composition. This is probably because the dissolution of kafirin or zein in glacial acetic acid enabled their protonation and complete solvation. Kafirin viscoelastic masses had a higher elastic component than the zein viscoelastic masses, which exhibited greater viscous flow characteristics, even after storage below the glass transition temperature (T_g), probably because of kafirin's greater propensity to disulphide bond. In fact, kafirin had a proportionally greater elastic component than gluten. Most of the kafirins, like gluten, could maintain their elastic recovery when stored at 4^oC for up 16 days. Kafirin extracted with aqueous ethanol without reducing agent had a loss tangent closest to gluten and therefore had a similar ratio of loss and storage moduli. The absence of a reducing agent presumably prevented intermolecular disulphide bond breakage, which probably resulted in a stronger viscoelastic mass. This study reveals that viscoelastic masses can be formed from kafirin and zein through coacervation from solution in glacial acetic acid, apparently regardless of the composition of the kafirin and zein.

4.2.2 Introduction

A major problem with the sorghum kafirin prolamin protein is that it does not by itself exhibit viscoelastic properties when hydrated in water, unlike wheat gluten (Taylor et al., 2016b). This accounts for why sorghum is not generally used for making leavened bread. In fact, very few proteins except for isolated maize zein and carob germ protein have been reported to have similar viscoelastic properties as wheat gluten (Lawton, 1992; Smith et al. 2010).

In gluten-free bread-making research, it is common practice to investigate not only doughs, but isolated proteins. It has been predicted that conventionally bred (non-GM) waxy (W) (high amylopectin) and high protein digestibility (HD) sorghum lines developed by Texas A&M University (Jampala et al., 2012) as well as transgenic high protein digestibility-high lysine (TG-HD) lines, which have suppressed expression of kafirin subclasses, including α-kafirin A1 and α-kafirin B1 and B2, γ-kafirin 1 and 2, and δ-kafirin 2 (Goodall et al., 2012; Da Silva et al., 2011a.b), could have improved dough quality, compared to normal sorghum flour (Elhassan et al., 2017). The conventionally bred (HD) lines have decreased γ-kafirin content, decreased 25 kDa α-kafirin, increased 22 kDa and decreased β-kafirin sub-classes (Benmoussa et al., 2015).

However, there is paucity of knowledge on the rheological behaviour of kafirin viscoelastic doughs (also referred to as viscoelastic masses). The progress made with the formation of kafirin masses through coacervation by Elhassan et al. (2018) has stimulated research into the rheological properties of the kafirin masses. Both zein and kafirin have been considered to exhibit similar functional properties (Taylor et al., 2013). Kafirin and zein have similar amino acid compositions (Correa de Souza et al., 2015), and are largely homologous (De Rose et al., 1989; Belton et al., 2006). Thus, there is need to research into how the composition of kafirins and zeins with respect to their subclass composition affect the rheological properties of their viscoelastic masses. Therefore, the objective of this study was to determine the effect of kafirin and zein composition on the rheological properties of kafirin and zein viscoelastic masses.

4.2.3 Materials and Methods

4.2.3.1 Sorghum lines for kafirin extraction

Several different types of sorghums were used to extract kafirin:
A mixture of two very similar white tan-plant, non-tannin sorghum cultivars PANNAR PEX 202 and PEX 606.

Conventionally bred waxy and HD lines (coded WHD1, WHD2 and WHD3), one HD line with normal (non-waxy) starch (coded NHD). The sorghum lines were obtained from crosses between parental lines RTx2907 (waxy) and P850029 (HD and high lysine) by Texas A&M University and grown at Halfway, Texas, USA in a controlled field trial. The HDHL line has decreased γ-kafirin content, decreased 25 kDa α-kafirin, increased 22 kDa and decreased β-kafirin sub-classes (Benmoussa et al., 2015). There has been no quantification of the different subclasses present in the HDHL lines published in literature. Only qualitative data has been published (Benmoussa et al., 2015).

Transgenic high protein digestibility-high lysine (TG-HD) lines with suppressed expression of several kafirin subclasses by means of RNAi technology and their normal protein digestibility null controls. The lines were developed by DuPont Pioneer through the Africa Biofortified Sorghum consortium and multiplied in an approved controlled field trial at Johnston, Iowa, USA.

4.2.3.2 Prolamin preparations

Commercial zein (Sigma Z3625) (essentially α-zein) - Purchased from Sigma-Aldrich, Johannesburg, South Africa.

Kafirin preparations extracted using 70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid, 70% ethanol + 0.35% acetic acid, glacial acetic acid after 1% sodium metabisulphite pre-soak, and glacial acetic acid were isolated using the various extractants as described in Chapter 4.1

Kafirin was extracted from decorticated sorghum grain PANNAR PEX 202/606. Also, kafirins were extracted from various W and HD lines and their controls using 70% (w/w) ethanol containing sodium metabisulphite and acetic acid as described by Emmambux and Taylor (2003).

Total zein (containing α-, β-, γ- and δ-zein) - Extracted from whole grain white maize (cultivar unknown) by the same method.

Kafirin minus γ-kafirin (kafirin minus-γ) - Extracted from the kafirin preparation from PANNAR PEX 202/606 using sodium lactate (0.05 M) containing 2% (v/v) 2-mercaptoethanol (Evans et al., 1987; Taylor et al., 2007).

High α-prolamin preparations were prepared from the defatted prolamins, as described by Schober et al. (2011). The prolamin preparations were extracted with 70% (w/w) aqueous ethanol at 50^{o}C for 1 h, with intermittent shaking. The preparations were then centrifuged at 9050 x *g* at 25^{o}C for 10 min to recover the supernatant and the residue. The supernatant was decanted and the residue was further washed with 50 g 70% (w/w) aqueous ethanol at 50^{o}C and centrifuged. The two separate supernatants recovered from each centrifugation process were combined, placed in open stainless trays and the ethanol was evaporated off in a fume hood at 25^{o}C. Excess cold, distilled water at 10^{o}C was added to the residue to precipitate the protein. The precipitated protein was recovered by filtration under vacuum and air-dried over-night at 25^{o}C. The preparations obtained were designated high α-prolamin. The weights of the high α-prolamin preparations were recorded and their protein content determined. The amount of α-prolamin present in the high α-prolamin preparations was obtained by dividing the total protein recovered by the starting amount of protein, expressed as a percentage. Protein preparations were characterised by 2-D electrophoresis (Taylor et al., 2018). Protein (N x 6.25) was determined by a Dumas nitrogen combustion method, AACC International standard method 46-30 (American Association of Cereal Chemists International, 2000).

4.2.3.3 Preparation of viscoelastic masses

Kafirin viscoelastic masses were prepared using the kafirin microparticle preparation technique of Taylor et al. (2009). Dry kafirin preparation (0.20 g) was dissolved glacial acetic acid (1 ml) and heated slowly with constant stirring at 50^{o}C for 5 minutes. The beaker was

covered with aluminium foil during heating. Distilled water (2 ml) at 24^{o}C was then rapidly added (5 ± 1 s) into the solution without stirring to form fibril aggregates of final temperature 25 ± 0.5^{o}C which were kneaded into viscoelastic masses with fingers for 20 ± 2 s. The final acetic acid concentration was 33% (v/v). Once formed, the resulting hydrated solids or viscoelastic masses were stored in polyethylene ziplock-type plastic bags. Microbiological spoilage of the viscoelastic masses was prevented during storage by the low pH (pH 2.3-3.5) of the viscoelastic masses and by low storage temperature storage (4°C). The moisture content of the viscoelastic mass was approximately 48% when formed. Moisture loss was prevented by storage of the viscoelastic masses in sealed ziplock-type plastic bags. The material was viscoelastic in nature, since when it was manually compressed with the fingers it largely returned to its original form on release. The stress-relaxation test confirmed and quantified the degree of elastic recovery.

4.2.3.4 Stress-relaxation test (large deformation test)

Directly after preparation, the kafirin viscoelastic masses were pressed for 30 s into a longitudinal split, cylindrical silicone plastic mould (4 mm long x 5 mm internal diam.) to obtain a viscoelastic mass piece of uniform shape and size. The viscoelastic mass piece was then immediately transferred onto the base plate of a Shimadzu EZ-Test texture analyser (Kyoto, Japan), fitted with a 10 mm cylindrical probe for analysis. A single compression test was performed on the viscoelastic mass with a test speed of 30 mm/min (0.5 mm/s), distance 1 mm, test strain of 25% and a relaxation time of 100 s at 0, 5, 10 and 15 min intervals. The tests were repeated after the kafirin viscoelastic masses were stored in sealed ziplock-type polythylene bags at 4^{o}C for 2, 8 and 16 days. This test was also carried out on vital wheat gluten (kindly provided by Novozymes SA, Johannesburg, South Africa) (0.2 g) hydrated with distilled water (0.12 g). FMax (the maximum force at compression), Ft (the force at which fresh gluten viscoelastic mass had relaxed to 36.8% of its maximum force (11.6 s after FMax) and SR (percent stress-recovery at 11.6 s after FMax) were measured as described by Singh et al. (2006).

4.2.3.5 Dynamic rheological analysis (small deformation test)

A Physica MCR 101 Rheometer fitted with Rheoplus software (Anton Paar, Osfildern, Germany) was used. Directly after preparation, the kafirin viscoelastic masses were moulded

into a cylindrical disc of 25 mm diam. over 4 min and transferred onto the Rheometer parallel base plate geometry fitted with 25 mm diameter probe and 2 mm gap between the top and the bottom plates. Paraffin oil was applied at the edges of the viscoelastic masses to prevent them from drying out. Strain-sweep analysis was performed to establish the linear viscoelastic range of the masses. The strain measured ranged from 0.01 to 100 % at a constant frequency of 6.3 rad/s (1 Hz) at 25^{o}C (Elhassan et al., 2017). Frequency sweep analysis was performed within the linear viscoelastic region over an angular frequency (ω) range of 0.1-100 rad/s at constant 0.1% strain at 25^{o}C. Vital wheat gluten was again used as a standard. The vital wheat gluten viscoelastic mass was prepared by vortexing vital wheat gluten (1 g) and distilled water (0.6 g) in a test tube for 1 min.

4.2.3.6 Statistical analyses

The data were subjected to one-way analysis of variance (ANOVA) at a confidence level of p = 0.05 and the means were separated using the Fisher's Least Significance Difference test (LSD) using IBM SPSS software version 20 (SPSS, Chicago, IL). Each experiment was repeated two times

4.2.4 Results and discussion

4.2.4.1 Stress-relaxation and dynamic rheological analysis of kafirin viscoelastic masses

4.2.4.1.1 Kafirin viscoelastic masses from kafirin extracted with different extraction solvents

Regardless of the type of extraction solvents used, all the kafirin preparations formed viscoelastic masses. However, the kafirin viscoelastic masses were 10 times softer (lower FMax and Ft) when compared to gluten, even after storage (Table 4.2.1). Significantly, all the kafirin preparations produced viscoelastic masses with a much higher relative elastic component (as measured by percent stress-recovery) (71-89%) than gluten (43%) at day 0. All the kafirin viscoelastic masses, regardless of the method of preparation, like the gluten, maintained their initial elasticity when stored for 2 days. The viscoelastic mass prepared from the kafirin preparation isolated with aqueous ethanol plus the reducing agent (KESM) became slightly harder with repeated relaxation testing time at day 0 to day 16. However, it was softer

than gluten viscoelastic mass. Its percent stress-recovery (80.2-82.9%) was very high but remained the same even after storage for 2 days, thereafter its elasticity then decreased on day 8 or 16 with a concomitant increase in viscous flow component. The decrease is possibly due to progressive loss of bound water or residual acetic acid molecules. The residual acetic acid probably acted as a plasticiser, as suggested by Sly et al. (2014), since the kafirin preparations were coacervated in glacial acetic acid during viscoelastic mass formation.

Table 4.2. 1 Stress-relaxation of kafirin viscoelastic masses from kafirins extracted with different solvents and after storage for 2, 8 and 16 days at 4°C

Viscoelastic mass type and extractant	Fmax (N)				Ft (N)				Stress-recovery (%)			
	Day 0	Day 2	Day 8	Day 16	Day 0	Day 2	Day 8	Day 16	Day 0	Day 2	Day 8	Day 16
Gluten standard	2.319B[a]a[b] (1.197)	2.326Ba (1.324)	3.956Bab (1.251)	5.793Bb (1.002)	1.044Ba (0.608)	1.045Ba (0.806)	1.184Ba (0.382)	2.268Bb (0.611)	43.1A[a]b[b] (4.7)	37.9Ab (3.4)	29.7Aa (0.4)	38.6Ab (4.2)
Kafirin extracted with 70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid	0.251Aa (0.052)	0.297Aa (0.063)	0.475Ab (0.039)	0.635Ac (0.071)	0.207Aa (0.036)	0.237Aa (0.053)	0.339Ab (0.027)	0.475Ac (0.047)	82.9Cc (3.1)	80.2Cc (2.4)	71.3Ba (0.6)	74.9Bb (1.1)
Kafirin extracted with 70% ethanol + 0.35% acetic acid	0.138Aa (0.057)	0.301Ab (0.050)	0.374Ab (0.041)	0.467Ac (0.055)	0.097Aa (0.036)	0.222Ab (0.036)	0.281Ac (0.025	0.348Ad (0.039)	71.4Ba (4.6)	73.7Ba (0.6)	75.2Ca (2.1)	74.4Ba (0.8)
Kafirin extracted with glacial acetic acid after 1% sodium metabisulphite pre-soak	0.029Aa (0.002)	0.123Ab (0.016)	0.258Ac (0.023)	0.387Ad (0.072)	0.025Aa (0.001)	0.098Ab (0.009)	0.201Ac (0.012)	0.296Ad (0.046)	84.1Ca (0.9)	79.2Ca (4.7)	78.2Da (2.6)	77.0Ba (2.7)
Kafirin extracted with glacial acetic acid	0.050Aa (0.010)	0.142Ab (0.017)	0.220Ac (0.021)	0.431Ad (0.075)	0.040Aa (0.011)	0.118Ab (0.015)	0.178Ac (0.016)	0.324Ad (0.050)	78.9BCa (11.3)	82.6Ca (1.0)	80.9Ea (1.5)	75.4Ba (2.0)

FMax. =Maximum force
Ft = Force at which fresh gluten dough had relaxed to 36.8 % of its maximum force 11.6 s after FMax
Stress-relaxation (%) = % stress-recovery at 11.6 seconds after FMax as calculated by Singh et al. (2006)
[a]Effect of different extraction solvents – Mean values with different upper case letter in a column differ significantly from each other ($p < 0.05$)
[b]Effect of storage – Mean values with different lower case letter in a row differ significantly from each other ($p < 0.05$)
The Ft value used (11.6 seconds) was the average of 5 closely agreeing independent experiments.

The FMax of the kafirin viscoelastic mass made from kafirin extracted with aqueous ethanol plus a reducing agent increased from 0.251 N on day 0 to 0.635 N on day 16 (Table 4.2.1). The FMax of the kafirin viscoelastic mass made from kafirin extracted with aqueous ethanol without a reducing agent increased from 0.138 N on day 0 to 0.467 N on day 16 (Table 4.2.1). Both preparations were significantly softer ($p < 0.05$) than gluten on each of the days tested. Although, the kafirin viscoelastic mass maintained its elasticity from day 0 to day 16, its percent stress-recovery (71.4-74.4%) was still not as high as that of the kafirin viscoelastic mass prepared with aqueous ethanol plus reducing agent as extractant (71.3-82.9%). Viscoelastic masses made from kafirin extracted with glacial acetic acid both with and without a reducing agent had lower FMax values than those of kafirin extracted with aqueous ethanol, either with or without reducing agent (Table 4.2.1). As discussed in Chapter 4.3, it appears that when kafirin is dissolved in glacial acetic acid, there is an irreversible change happening at the molecular level which enables the kafirin to bind water strongly. As a consequence, it would be expected that kafirin extracted with glacial acetic acid would have the ability to bind more water than kafirin extracted with aqueous ethanol. This additional water would act as a plasticiser resulting in a softer viscoelastic mass than kafirin extracted from aqueous ethanol.

The stress-recovery of the viscoelastic mass prepared from kafirin extracted with glacial acetic acid without the reducing agent remained the same with repeated relaxation testing time from day 0 to day 16 (Table 4.2.1). The repeated stress-relaxation testing was done on the same sample. This viscoelastic mass also maintained its elasticity on storage for 16 days. It was also more elastic than the kafirin viscoelastic mass prepared from kafirin extracted with aqueous ethanol without reducing agent, except at day 16. It was, however, softer than the kafirin viscoelastic masses prepared from kafirin extracted with aqueous ethanol with or without the reducing agent.

The kafirin viscoelastic mass from kafirin extracted with glacial acetic acid plus reducing agent maintained its elasticity throughout. Its elasticity at day 0 and 2 (79.2-84.1%) was similar to the elasticity of kafirin viscoelastic mass prepared from kafirin extracted with glacial acetic acid without the reducing agent (78.9-82.6%) and viscoelastic mass prepared with kafirin preparation extracted with aqueous ethanol plus reducing agent (80.2-82.9%).

Notably, all the kafirin viscoelastic masses prepared from kafirins extracted with the various solvents maintained their elastic behaviour even after storage for 16 days at 4^{o}C. This temperature was far below the glass transition temperature (40-44^{o}C) of hydrated kafirin (Schober et al., 2011). The elasticity of the kafirin viscoelastic masses could be due to the formation of hydrogen bond between the kafirin molecules and water molecules during the coacervation of kafirin from solution in glacial acetic acid by addition of water. Elhassan et al. (2018) proposed that essentially complete dissolution of the kafirin molecules in glacial acetic acid might have enabled them to better interact with water molecules, allowing viscoelastic masses formation.

The frequency sweep rheological test provides information about changes in the viscoelastic properties of the polymer network at different observation times (Ortolan et al., 2017). The responses of the kafirin viscoelastic masses to increasing frequency were monitored at a constant amplitude and temperature. Like gluten, there was an increase in the storage modulus (G') of all the kafirin viscoelastic masses, with increasing frequency, irrespective of solvent used to extract the kafirin (Figure 4.2.1).

With the exception of the viscoelastic mass prepared from kafirin extracted with 70% ethanol plus acetic acid but without reducing agent, they were all very soft relative to gluten. This is in agreement with the FMax and Ft data from the stress-relaxation testing (Table 4.2.1). Although SDS-PAGE (section 4.1.3.2) showed that the viscoelastic mass from kafirin extracted with aqueous ethanol without reducing agent was less polymerised, as indicated by its relatively low content of the cysteine-rich β- and γ-kafirins, it exhibited more resistance to deformation than all other kafirin viscoelastic masses (Figure 4.2.1). An explanation for the greater firmness of the mass from kafirin extracted with 70% ethanol plus acetic acid but without reducing agent could be that the protein was probably only incompleted hydrated. Li et al. (2012) observed that α-zein behaved like a swollen colloid (not a solution) in aqueous ethanol.

All the kafirin viscoelastic masses from kafirin extracted with either glacial acetic acid or aqueous ethanol with or without reducing agent showed a high elastic modulus (Figure 4.2.1), regardless of their composition and the proportions of β-sheet conformation (sections 4.1.3.2

and 4.1.3.3). This is in agreement with the stress-recovery behaviour of the viscoelastic masses when they were subjected to large deformation (Table 4.2.1).

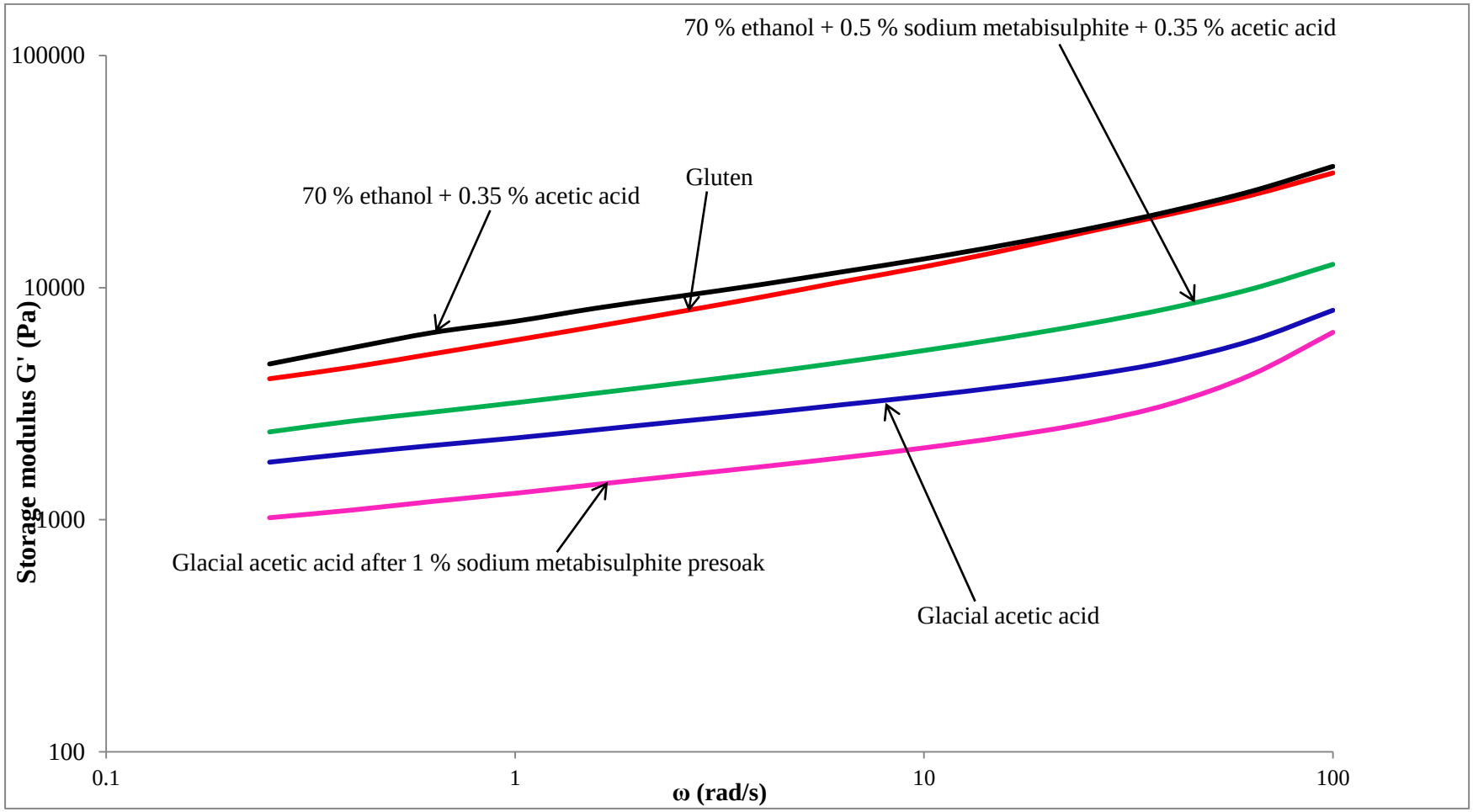

Figure 4.2. 1 Frequency sweep curve for storage (G') modulus of kafirin viscoelastic masses from kafirins extracted with different solvents.

Loss tangent (tan δ) plots give insight in the relative contribution of the elastic and viscous moduli to the viscoelastic behaviour of a material (Steffe, 1992), in this case, of the kafirin viscoelastic masses. The loss tangent data confirmed that all the kafirin viscoelastic masses formed from kafirin extracted with different solvents were predominantly elastic regardless of their compositions (Table 4.2.2). According to Hooke's law, the loss tangent of an ideal or perfect elastic material is equal to zero. The shear stress is proportional to the relative deformation (shear strain). This implies that the lower the value of loss tangent, the higher the elasticity of the viscoelastic mass. Stathopoulos et al. (2008) reported that a higher loss tangent is an indication of greater degree of viscous behaviour, while a lower value indicates higher degree of elasticity. Hence, the kafirin viscoelastic masses had higher relative elastic behaviour than the gluten, as indicated by the stress relaxation data (Table 4.2.1). Interestingly, strong wheat flour dough of good bread making quality has a low loss tangent (tan δ), while dough from weak flour of poor bread making quality has high tan δ (Miller and Hoseney, 1999). Notably, in this present study, the viscoelastic mass from kafirin preparation extracted with aqueous ethanol without reducing agent had a tan δ value closest to gluten (Table 4.2.2). It therefore appears to have similar viscoelastic behaviour to gluten, whereas other kafirin viscoelastic masses were softer but proportionally more elastic than gluten. The use of glacial acetic acid, which would more completely solvate the prolamin (Li et al., 2012) and/or inclusion of reducing agent to break inter-chain disulphide bonds probably allowed better water binding through hydrogen bonding and also plasticisation, resulting in weaker viscoelastic masses.

Table 4.2. 2 Dynamic rheological analysis (small deformation) frequency-sweep test at 1 rad/s of kafirin viscoelastic masses from kafirins extracted with different solvents

Viscoelastic mass type and extractant	Loss tangent tan (δ)
Gluten standard	$0.522^{e} \pm 0.014$
Kafirin extracted with 70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid	$0.313^{c} \pm 0.010$
Kafirin extracted with 70% ethanol + 0.35% acetic acid	$0.423^{d} \pm 0.002$
Kafirin extracted with glacial acetic acid after 1% sodium metabisulphite pre-soak	$0.281^{b} \pm 0.001$
Kafirin extracted with glacial acetic acid	$0.258^{a} \pm 0.001$

Mean values with different lower case letter in a column differ significantly from each other ($p < 0.05$)

At low frequency (1 rad/s), the kafirin viscoelastic masses from kafirins extracted with glacial acetic acid plus reducing agent, which was rich in β-kafirin but low in γ-kafirin or without reducing agent which had less β- and γ-kafirins (section 4.1.3.2) were more elastic than all other kafirin viscoelastic masses (Figure 4.2.1). However, the presence of more β-kafirin and α1-kafirin in the kafirin viscoelastic mass from kafirin extracted with glacial acetic acid plus reducing agent might have resulted in more disulphide bond mediated polymerisation, which perhaps made it more cohesive. The kafirin viscoelastic mass from kafirin extracted with glacial acetic acid plus reducing agent had a secondary structure with a high α/β conformation ratio (1.23:1) (section 4.1.3.3), unlike the other kafirin viscoelastic masses with greater proportion of β-sheet secondary structure. This indicates that kafirin secondary structure may not have any significant effect on the elasticity of kafirin viscoelastic masses.

4.2.4.1.2 Kafirin viscoelastic masses from high protein digestibility sorghum lines

The presence or absence of particular subclasses in kafirin has been proposed to determine its aggregate formation behaviour (Schober et al., 2011). These authors also showed that zein that was predominantly α-zein with less than 10% β+γ zeins was required to form a stable viscoelastic material, which indicated that disulphide bond formation was undesirable in the formation of zein viscoelastic masses.

However, in this present study, regardless of the composition of the different kafirin preparations, in terms of either presence or essentially absence of cysteine-rich β- and γ-kafirins (Elhassan et al., 2018), all the viscoelastic masses exhibited elasticity even after storage at 4^{o}C for up to 16 days (Table 4.2.3). However, like the gluten standard, all the kafirin viscoelastic masses increasingly became firmer on storage for either 8 or 16 days, with a many-fold increase in FMax and Ft.

Table 4.2. 3 Stress-relaxation of kafirins from waxy and high protein digestibility sorghum lines on day 0 and after storage for either 8 or 16 days at 4^{o}C

Sorghum line	FMax (N)		Ft (N)		Stress-recovery (%)	
	Day 0	**After16 days**	**Day 0**	**After 16 days**	**Day 0**	**After 16 days**
Gluten standard	2.319 ± 1.197	5.793 ± 1.002	1.044 ± 0.608	2.268 ± 0.611	43.1B^{a}b^{b} ± 4.7	38.6Ab ± 4.2
KESM	0.251 ± 0.052	0.635 ± 0.071	0.207 ± 0.036	0.475 ± 0.047	82.9Eb± 3.1	74.9Da ± 1.1
WHD2	0.120 ± 0.051	1.135 ± 0.095	0.046 ± 0.023	0.715 ± 0.060	37.2Aa ± 3.7	62.9Bb ± 0.2
WHD3	0.064 ± 0.024	0.216 ± 0.026	0.021 ± 0.010	0.181 ± 0.017	30.9Aa ± 5.0	83.9EFb ± 3.6
TG-HD	0.115 ± 0.034	0.667 ± 0.083	0.084 ± 0.026	0.549 ± 0.069	72.9Ca ± 2.5	82.1Eb ± 0.5
Null control	0.076 ± 0.029	0.339 ± 0.050	0.061 ± 0.028	0.277 ± 0.035	77.1CDa ± 9.1	81.9Ea ± 2.1
	Day 0	**After 8 days**	**Day 0**	**After 8 days**	**Day 0**	**After 8 days**
WHD1	0.148 ± 0.068	1.012 ± 0.185	0.063 ± 0.030	0.695 ± 0.122	45.2Ba ± 3.1	68.8Cb ± 0.9

FMax. = Maximum force. Ft = Force at time at which fresh gluten had relaxed to 38.6% of its maximum force (11.6 s after maximum force). Stress-relaxation (%) = % stress-recovery at 11.6 s from FMax. as calculated by Singh et al. (2006)
[a]Effect of protein type – Mean values with different upper case letter in a column differ significantly from each other ($p < 0.05$).
[b]Effect of storage – Mean values with different lower case letter in a row differ significantly from each other ($p < 0.05$).
Kafirin viscoelastic masses from waxy high protein digestibility lines (WHD1, WHD2, WHD3), transgenic high protein digestibility-high lysine line (TG-HD), its Null control, KESM = kafirin extracted with 70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid.
The Ft value used (11.6 seconds) was the average of 5 closely agreeing independent experiments.

Freshly prepared kafirin viscoelastic masses (analysed at day 0) from NHD, WHD1, WHD2 and WHD3 had similar or slightly lower relative elasticity compared to gluten (43%). However, KESM, TG-HD as well as its null control exhibited a relatively higher elastic component than gluten as they exhibited significantly higher stress-recovery ($p < 0.05$). KESM and null control kafirin viscoelastic masses contained all kafirin subclasses, whereas it was absent in TG-HD (Da Silva et al., 2011b; Elhassan et al., 2018). Hence, the complete or reduced expression of γ-kafirin did not greatly affect the kafirin elastic behaviour. All the kafirin viscoelastic masses that had been stored for either 8 or 16 days exhibited significantly higher stress-recovery compared to gluten. This implies that they exhibited greater relative elastic behaviour than gluten on storage. However, more research is needed to understand the rationale behind increase in elasticity of kafirin viscoelastic masses on storage. The high degree of elastic behaviour possessed by all the kafirin viscoelastic masses stored for either 8 or 16 days was similar to that of gelatine gel (Singh et al., 2006), a highly elastic food material. However, commercial zein (predominantly α-zein) viscoelastic mass has been found to exhibit highly viscous flow behaviour, especially when formed with dilute organic acids (Sly et al., 2014).

The stress-recovery values of the kafirin masses with different subclass composition show some groupings. The kafirin masses from the normal sorghum varieties, containing all the subclasses (KESM) and the TG-HD with β-kafirin but without γ-kafirin and its null control exhibited high elasticity at day 0, with stress-recovery values of 72.9-82.9% (Table 4.2.3). In contrast, the initial stress-recovery values of the waxy-high protein digestibility (WHD1, WHD2, WHD3) and the non-waxy-high protein digestibility (NHD) at day 0 ranged from 30.9-45.2%, which showed that they exhibited much lower elasticity. However, the stress-recovery of these kafirin masses increased significantly on storage to 62.9-83.9%. The differences between the groups appear to be somewhat related to their differing genetic backgrounds.

The storage modulus (G') of kafirin viscoelastic masses from the transgenic sorghum line (TG-HD), which was deficient in γ-kafirin (Elhassan et al., 2018), was higher than the G' of its null control (containing γ-kafirin) (Figure 4.2.2). Hence, the presence or reduced expression of γ-kafirin did not significantly affect or influence the elastic behaviour of kafirin viscoelastic mass. A high G' value is indicate of a strong and elastic material (Falade et al.,

2014; Ortolan et al., 2017). However, the G' values of TG-HD and its null control were still higher than that of gluten. This implies that they had a greater elastic component than gluten. These results were also similar to the significantly higher stress-relaxation exhibited by both the TG-HD and its null control compared to gluten (Table 4.2.3).

The loss tangent values also showed that both TG-HD and its null control had a higher elastic component than gluten (Table 4.2.4) as they were significantly lower ($p < 0.05$). The loss tangent values also revealed that kafirin viscoelastic masses from the waxy-high protein digestibility sorghum lines deficient in β-kafirin (WHD1, WHD2, WHD3) and the non-waxy high protein digestibility sorghum line (NHD) exhibited low elastic behaviour, similar to the low stress-recovery measured by the large deformation test (Table 4.2.3). However, they had higher G' and G" than gluten (Figure 4.2.3). This further emphasises that the kafirin viscoelastic masses possess a higher proportion of the elastic rheological component compared to gluten.

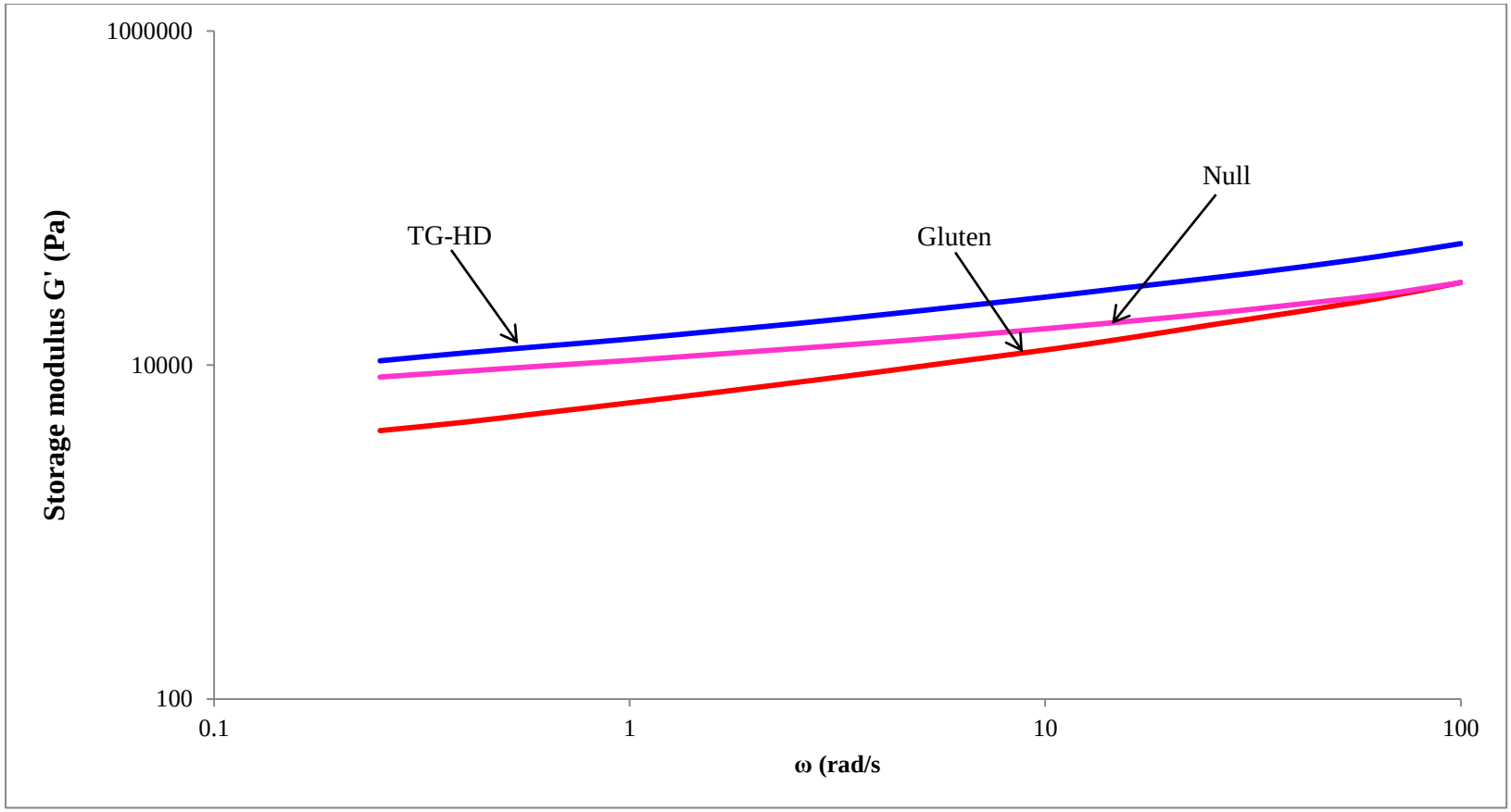

Figure 4.2.2 Frequency sweep curve for storage (G') modulus of kafirin viscoelastic masses from transgenic high protein digestibility-high lysine line (TG-HD) and its null control (Null)

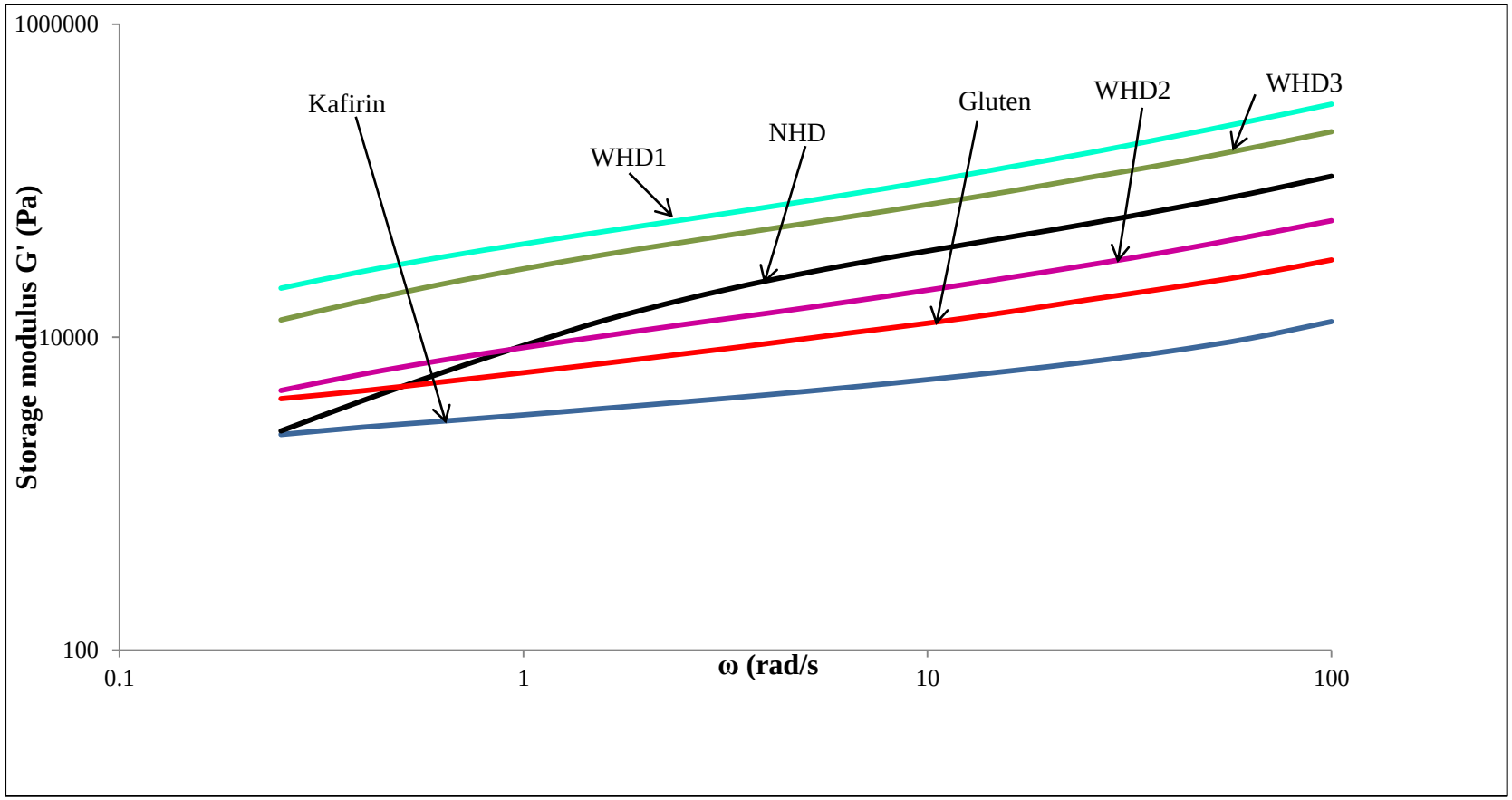

Figure 4.2. 3 Frequency sweep curve for storage (G') modulus of kafirin viscoelastic masses from waxy high protein digestibility lines (WHD1, WHD2, WHD3), non-waxy high protein digestibility line (NHD) and kafirin extracted with 70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid

Table 4.2. 4 Dynamic rheological analysis (small deformation) frequency-sweep test at 1 rad/s of kafirin viscoelastic masses from waxy and high protein digestibility sorghum lines

Sorghum line	Loss tangent tan (δ)
Gluten standard	$0.522^{b} \pm 0.014$
KESM	$0.313^{a} \pm 0.010$
NHD	$1.390^{d} \pm 0.099$
WHD1	$0.706^{bc} \pm 0.024$
WHD2	$0.793^{c} \pm 0.042$
WHD3	$0.782^{c} \pm 0.030$
TG-HD	$0.333^{a} \pm 0.019$
Null control	$0.279^{a} \pm 0.035$

Mean values with different lower case letter in a column differ significantly from each other ($p < 0.05$).

Waxy high protein digestibility lines (WHD1, WHD2, WHD3), non-waxy high protein digestibility line (NHD), transgenic high protein digestibility-high lysine line (TG-HD), its null control, KESM = kafirin extracted with 70% ethanol + 0.5% sodium metabisulphite + 0.35% acetic acid.

4.2.4.1.3 Stress-relaxation of kafirin viscoelastic masses from kafirin and zein of different subclasses

Regardless of their subclass composition, both the zein and kafirin viscoelastic masses were very soft in comparison with gluten and remained soft at day 0 (i.e. not stored), even after repeated compression (Table 4.2.5). This was unlike gluten viscoelastic masses that became firmer, with repeated compression at day 0. Notably, it was not possible to measure the stress-recovery of any of the zein viscoelastic masses immediately after preparation due to their extreme softness (gel-like). Thus, the viscoelastic masses were "rested" (held) for 4 h at 4^{o}C, after which they became firm (solid-like) enough to be subjected to stress-relaxation test. The increase in firmness might have been due to the formation of intermolecular hydrogen bonding. Unlike the zein masses that had lower stress-recovery, all the kafirin viscoelastic masses exhibited higher stress-recovery, regardless of their compositions. The effect of prolamin composition on the strength or firmness of the viscoelastic masses was noticeable after storage for 16 days. The various kafirin and zein were ranked in order from softness to firmness: kafirin (comprising all the subclasses) (0.6 N), zein (1.5 N), high α-kafirin (2.2 N), high α-zein (7.5 N), kafirin minus-γ (14.9 N), commercial zein (25.2 N) and high α-kafirin minus-γ (34.0 N). Both kafirin (comprising all the subclasses) and high α-kafirin viscoelastic masses maintained their elasticity (78.3-76.9%, and 83.4-59.1% stress-recovery, respectively) even after storage for 16 days. However, the degree to which the high α-kafirin viscoelastic mass maintained its elasticity was less compared to the kafirin viscoelastic mass. This can be attributed to the lower levels of γ-kafirin present in the high α-kafirin preparation. The kafirin minus-γ and high α-kafirin minus-γ (32.3% and 32.1% stress-recovery, respectively) did not maintain their high elasticity on storage. This indicates that the presence of γ-kafirin (rich in disulphide bond forming cysteine residues) is essential for the maintenance of elasticity of the viscoelastic mass during storage. This finding differs slightly from that of Elhassan et al. (2018) that the presence or absence of either γ- or β-kafirin sub-classes did not affect stress-relaxation behaviour. This difference could be attributed to the removal of γ-kafirin subclass from kafirin in this present study without any compensatory synthesis of other kafirin subclass, whereas Elhassan et al. (2018) used high protein digestibility lines where the suppression of γ-kafirin was compensated by synthesis of other kafirin subclasses (Da Silva et al., 2011b). In slight contrast, all zein viscoelastic masses exhibited similar viscous flow behaviour on storage, irrespective of the presence or absence

of the γ-subclass. This may be related to the lower degree of crosslinking in zein compared to kafirin (Emmambux and Taylor, 2009).

Table 4.2. 5 Stress-relaxation behaviour of viscoelastic masses from kafirin and zein of different sub-class composition after repeated compression on day 0 and after storage for 2 and 16 days at 4°C

Viscoelastic mass type	FMax (N)			Ft (N)			Stress-recovery (%)		
	Day 0	**Day 2**	**Day 16**	**Day 0**	**Day 2**	**Day 16**	**Day 0**	**Day 2**	**Day 16**
Gluten standard	3.538 (0.373)	3.747 (0.105)	6.874 (0.108)	1.695 (0.200)	2.110 (0.022)	2.976 (0.125)	47.9Ca (3.0)	42.2Ca (4.7)	43.3Da (2.5)
Kafirin (total kafirin containing all sub-classes)	0.073 (0.008)	0.290 (0.013)	0.585 (0.011)	0.057 (0.010)	0.248 (0.013)	0.450 (0.023)	78.3Ea (5.2)	85.7Fb (0.4)	76.9Fa (3.3)
High α-kafirin	0.091 (0.002)	0.204 (0.006)	2.191 (0.014)	0.076 (0.002)	0.185 (0.003)	1.295 (0.021)	83.4Fb (0.4)	90.7Fb (3.9)	59.1Ea (0.6)
Kafirin minus-γ	0.223 (0.007)	1.048 (0.001)	14.856 (0.026)	0.146 (0.011)	0.621 (0.034)	4.796 (0.067)	65.4Db (3.0)	59.3Eb (3.3)	32.3Ca (0.5)
High α-kafirin minus-γ	0.510 (0.017)	4.061 (0.008)	33.970 (2.588)	0.340 (0.018)	2.135 (0.107)	10.967 (2.349)	66.5Dc (1.3)	52.6Db (2.5)	32.1Ca (4.5)
Zein (total zein, α-, β- and γ-zeins)	0.054 (0.004)	0.063 (0.005)	1.530 (0.072)	0.009 (0.001)	0.012 (0.002)	0.404 (0.030)	16.8Ba (1.5)	18.6Ba (4.9)	26.4Bb (0.7)
High α-zein	0.080 (0.015)	0.111 (0.001)	7.523 (0.241)	0.003 (0.001)	0.003 (0.001)	2.349 (0.111)	3.1Aa (0.3)	2.3Aa (0.7)	31.2Cb (0.5)
Commercial zein (essential α-zein)	0.070 (0.011)	0.200 (0.008)	25.195 (0.474)	0.003 (0.001)	0.007 (0.001)	5.498 (0.856)	3.5Aa (0.4)	3.5Aa (0.6)	21.8Ab (3.0)

FMax. =Maximum force, Ft = Force at which fresh gluten dough had relaxed to 36.8 % of its maximum force 11.6 s after FMax
SR = % stress-recovery at 11.6 seconds after FMax as calculated by Singh et al. (2006). The Ft value used (11.6 seconds) was the average of 5 closely agreeing independent experiments. [a]Effect of different extraction solvents – Mean values with different upper case letter in a column differ significantly from each other ($p < 0.05$). [b]Effect of storage – Mean values with different lower case letter in a row differ significantly from each other ($p < 0.05$)

4.2.5 Conclusions

This study reveals that viscoelastic masses can be formed from kafirin and zein through coacervation from glacial acetic acid, apparently regardless of the composition of the kafirin and zein. Kafirin extracted with aqueous ethanol without reducing agent has a loss tangent closest to gluten when subjected to small deformation rheological analysis and therefore exhibits more similar viscoelastic properties to gluten. Kafirin and zein exhibit different rheological properties. Measurement of large deformations shows that kafirin exhibit greater elastic recovery than zein, whereas zein exhibits more viscous flow properties under the measurement conditions used. In fact, kafirin has a proportionally greater elastic recovery than gluten. Furthermore, all the kafirin viscoelastic masses prepared from kafirin regardless of the solvent used for their isolation can maintain their initial elastic recovery when stored at 4^{o}C far below the kafirin T_g, regardless of their compositions. Maintenance of elasticity of kafirin when stored appears to require the presence of γ-kafirin.

4.2.6 References

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4.3 KAFIRIN AND ZEIN FORMATION: EFFECTS OF ACETIC ACID AND PROTEIN CONCENTRATIONS

4.3.1 Abstract

Kafirin and zein could be used in making wheat-free leavened dough-based products if their functionality can be modified to more closely resemble gluten. Recently, stable viscoelastic masses were produced from isolated kafirin and total zein by dissolution of the prolamins in glacial acetic acid, followed by simple coacervation with rapid water addition. The methodology was, however, not compatible with food systems, as the final acid concentration was too high (33%). This work revealed that coacervation with reduction in the final acetic acid concentration down to 0.1% still enabled formation of kafirin and zein viscoelastic masses, with functionality retained on storage at 4°C for an extended period; indicating an irreversible molecular change with dissolution in glacial acetic acid. Kafirin masses were much firmer than zein masses but both were softer than gluten. However, kafirin displayed a similarly elastic high component to gluten, whereas zein exhibited more viscous flow characteristics than elastic when compared to kafirin. This was probably due to the presence of more disulphide bonds in kafirin than zein. A model to explain this behaviour is proposed. Regarding the effect of prolamin concentration in glacial acetic acid, a minimum, between 5 and 10% was necessary for viscoelastic mass formation at low final acetic acid concentration (5%).

4.3.2 Introduction

Improvement in the functional properties of non-wheat prolamin proteins such as kafirin and zein has potential to enable them to be used in making wheat-free leavened dough-based products such as bread. Successful application of this approach to bread making would be highly beneficial especially in countries in the semi-arid regions of Africa and Asia where sorghum and maize are widely grown, reducing the need for expensive wheat imports. However, since the first report of the formation of model doughs made from commercial zein and starch, which had wheat flour dough-like viscoelastic behaviour (Lawton, 1992), progress has been slow. Most work has been carried out on commercial zein, which comprises mainly the α-zein subclass, although it is commercially available, it is highly variable between batches (Selling et al., 2005).

Recently, four factors that influence the formation of doughs, (sometimes referred to as viscoelastic masses) from non-wheat prolamin proteins have been identified (Taylor et al., 2018). They are: prolamin composition in terms of prolamin subclasses, secondary structure, glass transition temperature (T_g) and the relative hydrophobicity of the prolamin. With regard to subclass composition, the presence of the γ-subclass and its propensity for disulphide crosslinking is thought to impact negatively on viscoelastic mass formation (Schober et al., 2011; King et al., 2016). However, when kafirin and zein viscoelastic masses were formed by coacervation from glacial acetic acid, it was found that the γ-subclass was necessary to retain softness on storage with kafirin and zein and was important for the retention of elastic recovery of kafirin (Chapter 4.2).

Some workers have proposed that the presence of β-sheet formation is necessary for viscoelastic mass functionality (Erickson et al., 2012) and that viscoelastic masses can only be formed above the prolamin's hydrated T_g (Lawton, 1992). The inclusion of additional proteins (co-proteins), which can help stabilise a β-sheet formation of zein has been suggested as a way to create a commercial zein dough, which is functionally similar to a wheat dough (Mejia et al., 2007, 2012). In contrast, Taylor et al. (2018) using different acid treatments and a final temperature below the prolamin's hydrated T_g, found that the FTIR spectra of kafirin and zein viscoelastic masses were different from each other and largely independent of subclass composition. All were predominately α-helical in conformation but the proportion of α-helical to β-sheet varied, dependant on treatment. The proportion of α-

helical conformation increased with increased acid concentration (Taylor et al., 2018; Elhassan et al., 2018). This was attributed to changes in solvent polarity and was in agreement with the findings of Xiao et al. (2015) working with kafirin. The authors found that when kafirin was dissolved in 65% (v/v) isopropanol, 60% (v/v) tert-butanol, and equeous ethanol solvents, the relative α-helical conformation of kafirin increased with the decrease of solvent polarity. Also, the degree of hydrophobicity of the prolamin affects its ability to hydrate and retain water (Taylor and Belton, 2002). This would affect the prolamin's ability to remain hydrated during viscoelastic mass formation. Furthermore, work by Smith et al. (2014) showed that the addition of salts affected α-zein's surface hydrophobicity, which in turn affected its ability to form viscoelastic masses.

What is also becoming clear from this research is that kafirin and zein, which have been considered to have very similar functional properties (Taylor et al., 2013), are in fact very different in terms of their ability to form viscoelastic masses under certain specific conditions (Taylor et al., 2018). Zein viscoelastic masses demonstrated predominantly viscous flow characteristics, whereas kafirin masses were more elastic.

Up until recently, attempts to form stable viscoelastic masses from kafirin using aqueous systems had not been successful. The masses rapidly lost functionality (Schober et al., 2011). This is attributable to kafirin's higher hydrophobicity and greater propensity to polymerise through disulphide crosslinking (El Nour et al., 1998; Duodu et al., 2003; Belton et al., 2006; Emmambux and Taylor, 2009). Smith (2012) found that steeping of sorghum flour for an extended period ahead of extracting kafirin enabled the formation of a 'resin', which was extensible. Formation of this 'resin' was attributed to partial hydrolysis of kafirins by endogenous sorghum enzymes or by bacterial enzymes produced during steeping. An alternative explanation may be that this was due to the production of lactic acid by action of endogenous, lactic acid bacteria and would be analogous to the effect of dilute organic acid treatments (Sly et al., 2014; King et al., 2016). Recently, however, stable viscoelastic masses have been successfully produced from both isolated kafirin and total zein (zein containing the full complement of subclasses present in the maize grain) by dissolution of the prolamins (28.6%) in glacial acetic acid, followed by simple coacervation with rapid water addition to a final acid concentration of 33% (Elhassan et al., 2018; Taylor et al., 2018). The authors attributed the formation of stable viscoelastic masses to the glacial acetic acid enabling

complete solvation, protonation, and partial unfolding of the prolamins, which were thought to be present in solution mainly as monomers. These changes were thought to enable fibril and viscoelastic mass formation on water addition.

Whilst interesting, the methodology of kafirin viscoelastic mass formation is far from applicable in a food system due to the high final acetic acid concentration (33%). Hence, in order to further develop the coacervation-type process, it was necessary to determine whether kafirin and total zein viscoelastic mass formation and mass functionality was affected by the final concentration of acetic acid, and whether mass formation and functionality was dependent on the concentration of protein present. Additionally, the work resulted in greater understanding of the intrinsic differences in viscoelastic mass formation and functionality between kafirin and zein and deeper insight into how the coacervation process enables kafirin and total zein viscoelastic mass formation.

4.3.3 Materials and Methods

4.3.3.1 Materials

Kafirin (comprising all the subclasses) and zein (i.e. total zein comprising α-, β-, δ- and γ-zein), (Taylor et al., 2018) were extracted with 70% aqueous ethanol (w/w) containing 0.5% sodium metabisulphite (w/w) and 0.35% acetic acid (w/w), according to the method described by Emmambux and Taylor (2003). Decorticated sorghum from a tan-plant, non-tannin white sorghum (cultivars PANNAR PEX 202/206) and milled whole grain white maize were used for extraction, respectively. The prolamins were air dried at ambient temperature (25^{o}C). Protein content was determined (N x 6.25) by a Dumas combustion method, according to AACCI standard method 46–30 (American Association of Cereal Chemists International, 2000).

4.3.3.2 Viscoelastic mass formation

Kafirin and zein viscoelastic masses were prepared by the coacervation method described by Elhassan et al. (2018). In brief, the prolamins were dissolved in glacial acetic acid at 50°C and then coacervated out of solution in the form of fibrils by rapid addition of cold (15°C) distilled water. The fibrils formed were then kneaded into a viscoelastic mass using the

fingers. The final temperature of the masses was approx. 25°C, which is below the glass transition temperature (T_g) of kafirin (approx. 40°C) (Schober et al., 2011) and similar to that of hydrated commercial zein (T_g at high water content close to room temperature (25°C) (Lawton, 1992). The resulting hydrated solids or viscoelastic masses were stored in ziplock-type bags at 4°C between testing periods.

To determine the effects of final acetic concentration at a constant protein content of 28.6%, on viscoelastic mass formation and properties, different levels of water addition were investigated to obtain final acetic acid concentrations ranging from 20% to 0.1% (w/w).

To determine the effect of varying the protein concentration on viscoelastic mass formation, kafirin or zein were dissolved in glacial acetic acid at protein concentrations of 28.6%, 15, 10 and 5% (w/w), as described above. Water was then added to a final constant acid concentration of 5% (w/w). The 5% final acetic acid was selected because the stress-recovery of freshly prepared kafirin and zein viscoelastic masses remained constant at acetic acid concentrations below 5% and the masses exhibited formation of uniform, broad fibrils, which has been identified as an important functional characteristic of wheat glutenin (Orth et al., 1973). When fibril aggregates were formed, they were manipulated by hand into a cohesive mass, which was then analysed. Vital wheat gluten was also formed into a viscoelastic mass as described in Chapter 4.2 and used for comparison.

4.3.3.3 Analyses

4.3.3.3.1 Microscopy

The resulting structures formed were examined by stereomicroscopy (Nikon SMZ 800, Tokyo, Japan) and using Ultra high resolution field emission scanning electron microscope (SEM) (JEOL 6000F FEGSEM, Tokyo, Japan). Additionally, the structures formed at the different protein contents were examined using a Zeiss 510 META Confocal Laser Scanning Microscope (CLSM) (Jena, Germany) coupled with a Plan-Neofluar 10 x 0.3 objective, at an excitation and emission wavelengths of 405 nm and 425 nm, respectively, with natural fluorescence (Taylor et al., 2018). Preparation techniques used were as described.

4.3.3.3.2 Rheology of viscoelastic masses

Rheological properties of the viscoelastic masses were determined using the methods described in Chapter 4.2 on day 0 and then after storage in polyethylene ziplock type bags at 4°C on day 2, day 8 and day 16. FMax (the maximum compression force), Ft (the force at the time from FMax at which fresh gluten had relaxed to 36.8% of its maximum force (11.6 seconds) and Stress-recovery (percentage stress-recovery at 11.6 seconds from FMax) were measured as described by Singh et al. (2006).

4.3.4 Results and discussion

4.3.4.1 Microscopy of masses formed at different final acetic concentrations

Kafirin could form cohesive viscoelastic masses by coacervation over the full range of final acetic acid concentrations, decreasing from 20% down to 0.1% (w/w) (1 g/l, 1.7 mM) (Figure 4.3.1). This very low acetic acid concentration can be considered as food compatible as it is within the range of acetic acid concentrations found in sourdough breads, 0.7 g/l (1.1 mM) to 7.0 g/l (11.7 mM) (Pontonio et al., 2017).

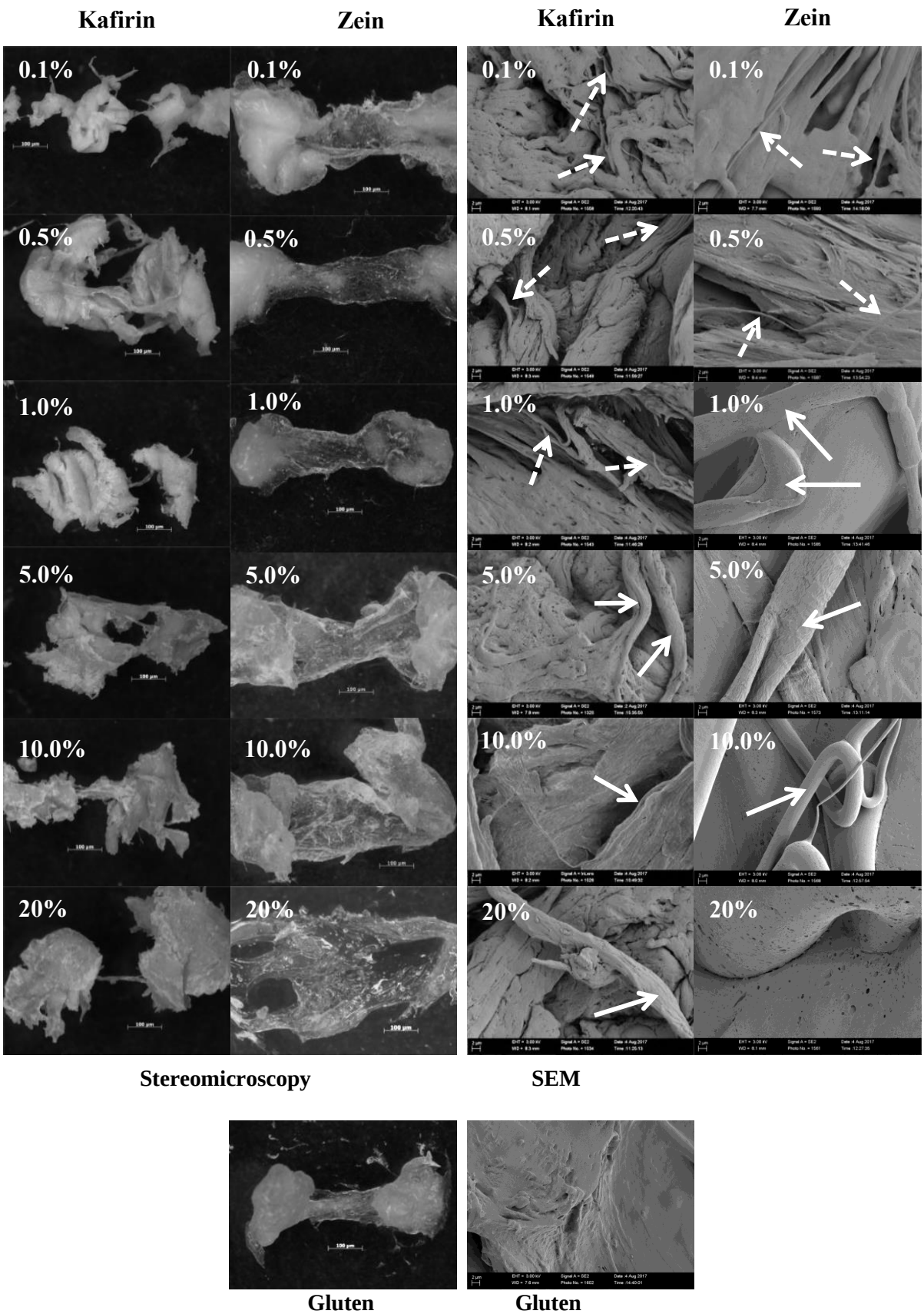

Figure 4.3. 1 Stereomicroscopy (left, bar = 100 μm) and scanning electron microscopy (right, bar = 2 μm) of kafirin and zein cohesive masses formed at different final acetic acid concentrations. Solid arrows indicate thick bands of fibrils, dotted arrows indicate finer fibrils

SEM of kafirin cohesive masses formed at acid concentrations decreasing from 20% to 5% showed evidence of extensive intertwined, broad fibrils, indicated by solid arrows (Figure 4.3.1). At lower final acid concentrations (1%-0.1%), intertwined masses of fibrils were present but the individual fibrils appeared somewhat finer and less cohesive, indicated by dotted arrows. However, under the same conditions zein could only form handleable viscoelastic masses at a final acetic acid concentration of 5% (w/w) or less. With zein, progressive further reduction of the final acid concentration from 5% to 0.1%, produced a more dough-like material, which could be stretched. Final acid concentrations above 5% resulted in a sticky, taffy-like mass of zein, to the extent that stress-relaxation values could not be measured. This material did, however, show clear evidence of fibril formation up to a final acetic acid concentration of 10% when examined by stereomicroscopy and SEM. At a final acid concentration of 10%, the zein taffy appeared dough-like by stereomicroscopy but was still too soft to be handled. At 20% final acetic acid concentration, the zein material resembled a shiny, partially solubilised mass when viewed by stereomicroscopy. This same treatment, when viewed by SEM appeared as a soft mass, with no distinct features. In general, when observed by SEM, zein viscoelastic masses were softer and more fluid-like than their equivalent kafirin cohesive masses at the same final acetic acid concentrations, which was borne-out by rheological analyses. When made with final acetic acid concentrations of 0.5% and 0.1%, the zein cohesive masses resembled the gluten cohesive mass, with many interconnected fibrils. In contrast, only at final acetic acid concentrations of 5% and 10% did the kafirin cohesive masses somewhat resemble gluten.

4.3.4.2 Rheological properties of masses formed at different acetic acid concentrations

Whilst the ability to form fibrils has been identified as a factor critical to dough formation (Lawton, 1992; Schober et al., 2010, 2011), ideally the prolamin fibrils once formed must be able to form a cohesive mass with rheological properties similar to that of gluten in order to be functional in a gluten-free dough system. Previous work has shown that coacervated zein (total zein) viscoelastic masses demonstrated predominantly viscous flow characteristics (Chapter 4.2), whereas coacervated kafirin rheology was more similar to that of gluten, with both viscous flow and elastic recovery properties.

All kafirin and zein viscoelastic masses were softer than gluten, even after repeated compression and after 16 days storage at 4^{o}C, regardless of the final acid concentration of the preparation (Table 4.3.1). For example, the FMax of kafirin viscoelastic mass (20% final acetic acid concentration) at day 0 was 0.6 N increasing to 4.1 N by day 16 storage, whereas the zein viscoelastic mass was too soft to allow measurement at this final acid concentration. Comparative gluten values were 3.5 N on day 0 and 6.9 N after 16 days storage. On any individual day of testing, as the final acetic acid concentration was reduced, the kafirin viscoelastic masses generally retained the same degree of firmness or became slightly softer but zein viscoelastic masses consistently became firmer with decreasing final acetic acid concentration. Within the same acid concentration, both the kafirin and zein viscoelastic masses became firmer with storage. At the lowest final acetic acid concentration, 0.1% the F Max of kafirin viscoelastic mass was 0.3 N at day 0, increasing to 4.2 N after 16 days storage, whereas for zein the F Max was 0.2 N increasing to 2.4 N after 16 days.

Table 4.3. 1 Effect of different final acid acetic concentrations on the stress-relaxation of kafirin and zein viscoelastic masses prepared by coacervation at day 0 and after storage at 4°C for 2, 8 and 16 days. Protein concentration in glacial acetic acetic 28.6%

Final acetic acid conc (%)	Final pH of viscoelastic mass	FMax (N)				Ft (N)				Stress-recovery (%)			
		Day 0	Day 2	Day 8	Day 16	Day 0	Day 2	Day 8	Day 16	Day 0	Day 2	Day 8	Day 16
Gluten													
Not applicable		3.538 (0.0373)	3.747 (0.105)	5.510 (0.518)	6.874 (0.108)	1.695 (0.200)	2.110 (0.022)	1.659 (0.040)	2.976 (0.125)	47.9Db (3.0)	42.2Bb (4.7)	30.2Ba (2.1)	43.3Bb (2.5)
Kafirin													
20	2.3	0.608 (0.081)	2.379 (0.144)	3.378 (0.086)	4.144 (0.134)	0.350 (0.028)	1.701 (0.054)	2.283 (0.054)	2.751 (0.023)	57.8Ea (3.0)	71.6Gc (0.1)	67.6Hb (0.1)	66.4Gb (1.6)
15	2.4	0.656 (0.005)	2.355 (0.076)	3.256 (0.063)	4.177 (0.020)	0.305 (0.015)	1.453 (0.030)	1.938 (0.066)	2.473 (0.140)	46.5Da (2.6)	61.8Fb (3.3)	59.5FGb (3.2)	59.2EFb (3.6)
10	2.5	0.378 (0.051)	1.640 (0.045)	2.420 (0.004)	2.830 (0.026)	0.241 (0.036)	1.015 (0.030)	1.535 (0.051)	1.810 (0.080)	63.6Fa (1.0)	61.9Fa (0.1)	63.4GHa (2.2)	64.0FGa (2.2)
5	2.7	0.412 (0.021)	2.053 (0.021)	2.608 (0.076)	4.896 (0.312)	0.131 (0.009)	1.076 (0.151)	1.405 (0.208)	2.465 (0.153)	31.7BCa (0.6)	52.4DEb (6.8)	53.8DEFb (6.4)	50.4CDb (0.1)
3	2.8	0.231 (0.001)	1.333 (0.049)	2.172 (0.098)	3.702 (0.088)	0.072 (0.004)	0.744 (0.016)	1.298 (0.125)	1.966 (0.321)	31.2BCa (1.7)	55.8EFb (0.9)	59.9FGb (8.5)	53.0DEb (7.4)
2	2.8	0.385 (0.043)	1.755 (0.026)	2.481 (0.175)	3.649 (0.113)	0.108 (0.028)	0.798 (0.145)	1.185 (0.098)	1.770 (0.255)	27.7Ba (4.1)	45.5BCb (8.9)	48.0CDb (7.3)	48.5BCDb (0.9)
1	3.0	0.345 (0.047)	1.537 (0.080)	2.207 (0.238)	3.426 (0.035)	0.104 (0.013)	0.753 (0.081)	1.110 (0.127)	1.643 (0.003)	30.2BCa (0.5)	48.9CDb (2.7)	50.3CDEb (0.3)	48.0BCDb (0.6)
0.5	3.2	0.335 (0.007)	1.260 (0.013)	1.815 (0.004)	3.086 (0.628)	0.115 (0.028)	0.658 (0.059)	1.025 (0.140)	1.492 (0.074)	34.4Ca (9.2)	52.2DEb (5.2)	56.5EFGb (7.6)	49.1BCDb (7.6)
0.2	3.3	0.360 (0.069)	2.171 (0.076)	2.364 (0.033)	3.523 (0.028)	0.107 (0.006)	0.954 (0.001)	1.135 (0.045)	1.589 (0.011)	30.0BCa (4.0)	44.0BCb (1.6)	45.0Cb (1.2)	45.1BCb (0.1)
0.1	3.5	0.302 (0.025)	2.334 (0.122)	3.233 (0.129)	4.207 (0.190)	0.087 (0.021)	1.026 (0.049)	1.733 (0.035)	2.058 (0.263)	28.5Ba (4.4)	44.0BCb (0.2)	53.6DEFc (1.1)	48.8BCDbc (4.1)

Zein													
5	2.5	0.088 (0.007)	0.183 (0.035)	0.370 (0.016)	0.687 (0.069)	0.004 (0.001)	0.039 (0.001)	0.090 (0.029)	0.195 (0.046)	4.5Aa (1.2)	20.3Ab (8.1)	24.3Ab (8.9)	28.8Ab (9.6)
3	2.6	0.159 (0.043)	0.155 (0.001)	0.432 (0.001)	0.589 (0.043)	0.007 (0.006)	0.025 (0.002)	0.098 (0.020)	0.142 (0.020)	3.7Aa (3.0)	15.9Ab (1.4)	22.7Abc (4.6)	24.3Ac (5.1)
2	2.8	0.131 (0.003)	0.172 (0.020)	0.476 (0.040)	0.724 (0.019)	0.012 (0.001)	0.035 (0.008)	0.125 (0.003)	0.195 (0.016)	8.8Aa (0.4)	20.2Ab (2.6)	26.4Ac (1.6)	26.9Ac (1.5)
1	2.9	0.217 (0.087)	0.221 (0.004)	0.546 (0.051)	1.143 (0.178)	0.018 (0.004)	0.048 (0.009)	0.146 (0.023)	0.307 (0.045)	8.4Aa (1.8)	21.5Ab (3.8)	26.7Ac (1.7)	26.9Ac (0.2)
0.5	3.3	0.154 (0.045)	0.317 (0.050)	0.577 (0.024)	1.166 (0.047)	0.011 (0.005)	0.059 (0.012)	0.153 (0.120)	0.317 (0.042)	6.6Aa (1.2)	18.4Ab (0.9)	26.5Ac (3.2)	27.1Ac (2.5)
0.2	3.3	0.173 (0.039)	0.356 (0.058)	0.830 (0.129)	1.671 (0.193)	0.015 (0.007)	0.071 (0.013)	0.180 (0.009)	0.401 (0.030)	8.6Aa (1.5)	19.8Ab (0.6)	22.0Ab (4.5)	24.1Ab (1.0)
0.1	3.4	0.171 (0.019)	0.413 (0.072)	1.106 (0.149)	2.449 (0.343)	0.014 (0.003)	0.072 (0.021)	0.286 (0.030)	0.565 (0.063)	8.4Aa (2.6)	17.2Ab (2.1)	25.9Ac (0.8)	23.1Ac (0.7)

FMax. =Maximum force
Ft = Force at which fresh gluten dough had relaxed to 36.8% of its maximum force 11.6 s after F Max
Stress-relaxation (%) = % stress-recovery at 11.6 seconds after FMax as calculated by Singh et al. (2006)
Mean values with different upper case letter in a column differ significantly from each other ($p < 0.05$)
Mean values within a protein type with different lower case letter in a row differ significantly from each other ($p < 0.05$)
The Ft value used (11.6 seconds) was the average of 5 closely agreeing independent experiments.
n = 2

The increase in mass firmness with decreasing acid concentration found in zein is in agreement with previous work. Sly et al. (2014) working with commercial zein found that increasing concentrations of both dilute acetic acid and lactic acid resulted in softer viscoelastic masses. The authors attributed this to plasticisation by the organic acids. Furthermore, it has been shown that viscoelastic masses made from total zein films cast from glacial acetic acid, that had residual acid washed away, were firmer and less extensible than viscoelastic masses made from similar films where residual acetic acid remained (King et al., 2016). Thus, in this present work it appears that at least for zein, as the final acetic acid concentration decreased its plasticising effect was reduced and the viscoelastic masses became firmer. Similar work has not previously been carried out with kafirin.

Earlier, it was shown that percentage stress-recovery of kafirin viscoelastic masses, at a final acid concentration of 33%, exhibited a very high elastic component, which was retained after 16 days storage (Chapter 4.2). Here, as the final acid concentration decreased from 20% to 0.1%, there was a gradual reduction in the percentage stress-recovery for kafirin, from 57.8% (20% final acid concentration) to 28.5% (0.1% acid) on day 0, and from 66.4% (20% acid) to 48.8% (0.1% acid) on day 16 (Table 4.3.1, Figure 4.3.2). This may also be related to the reduction in concentration in the viscoelastic masses. However, the role of the differing pH of the viscoelastic masses, which increased from pH 2.3 at 20% acetic acid to pH 3.5 at 0.1% acetic acid (Table 4.3.1) cannot be entirely ignored. Zhang et al. (2011) observed that α-zein became slightly firmer when the pH was increased from pH 2.7 to pH 3.3. The kafirin viscoelastic masses formed at lower final acid concentrations had stress-recovery behaviour that was closer to that of gluten. Potentially, manipulation of final acid concentrations could enable kafirin viscoelastic masses with rheological properties similar to gluten to be formed at a food compatible final acid concentration. For kafirin viscoelastic masses produced at all final acid concentrations, the elastic component increased on storage. This may have been due to the formation of additional disulphide linkages on storage. It has been suggested that hydrophobic interactions are responsible for kafirin and zein gluten-like functionality and that disulphide bonding is in fact detrimental to this process (Schober et al., 2011; Smith et al., 2014). However, Chapter 4.2 showed that the γ-subclass, with its propensity to form disulphide linkages, is necessary for retention of viscoelastic mass softness of kafirin and zein and for elastic recovery of kafirin.

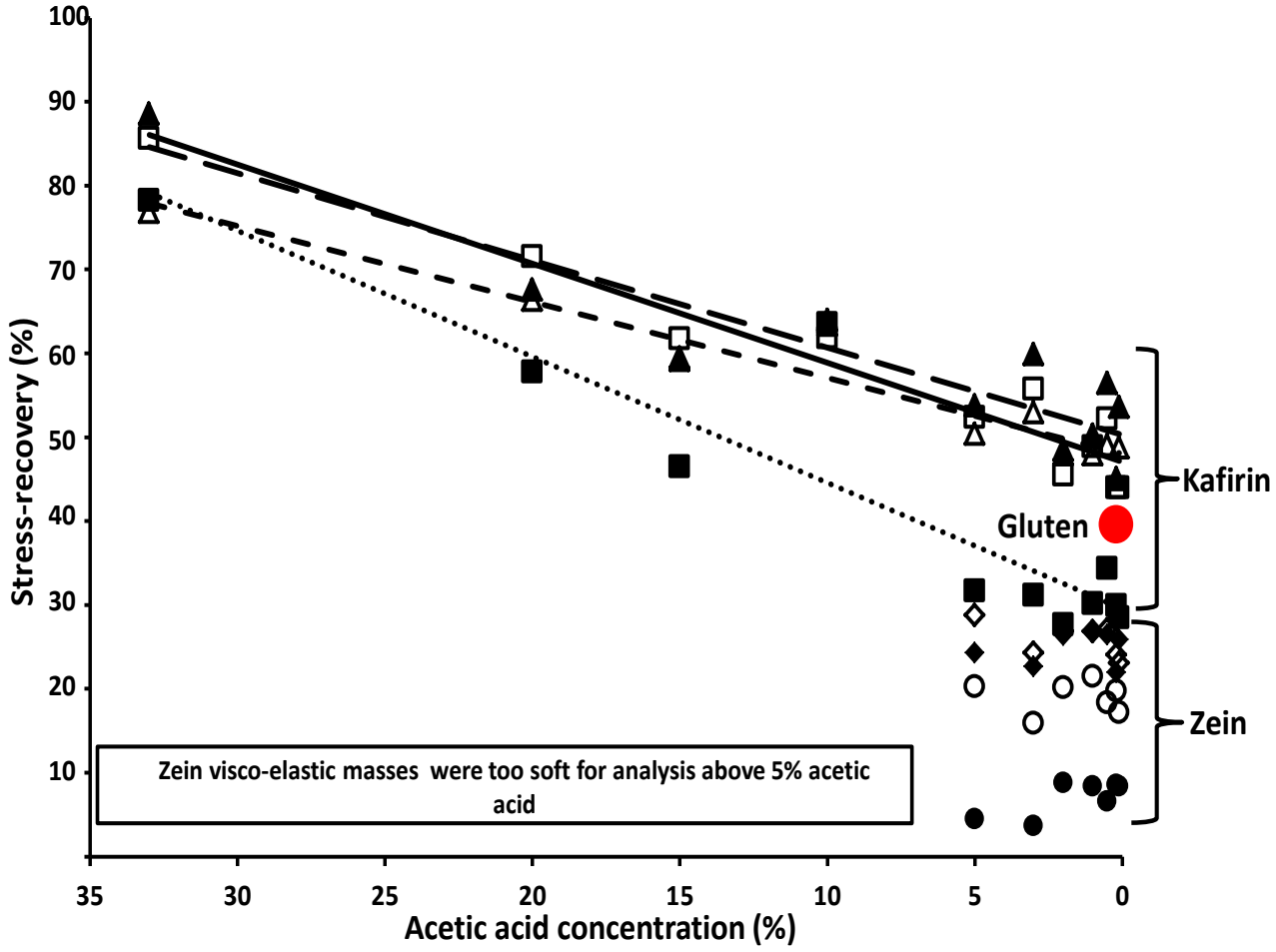

Figure 4.3. 2 Effect of final acetic acid concentration on the stress-recovery behaviour of kafirin and zein viscoelastic masses

Zein behaved differently to kafirin, even at the very low final acid concentrations (5%-0.1%), where viscoelastic masses could be formed. Zein viscoelastic masses were even softer than kafirin viscoelastic masses as described, 0.09 N (5% acid) to 0.17 N (0.1% acid) on Day 0, and remained soft even after 16 days storage (0.69 N (5% acid) - 2.45 N (0.1% acid) (Table 4.3.1). Furthermore, similar to as reported in Chapter 4.2, these zein viscoelastic masses exhibited much greater viscous flow characteristic than the kafirin viscoelastic masses (Figure 4.3.2). However, in contrast to kafirin, reduction in final acid concentration did not affect stress-recovery of the zein viscoelastic masses on Day 0, as these values were not statistically different, ranging from 4.5-8.8%. On storage, stress-recovery increased to between 23.1-28.8%, again with no statistically significant effect of final acid concentration.

Thus, it can be concluded that when kafirin and zein are dissolved in glacial acetic acid, there is an irreversible change occurring at a molecular level. This change, which affects their functionality to the extent, that apart from water, no additional plasticiser is required in for them to exhibit viscoelasticity is probably due to protonation of the prolamins, causing partial unfolding of the prolamin structure, resulting in more solvent accessible areas and allowing hydration on the molecular surface (Li et al., 2012).

4.3.4.3 Effect of protein concentration during coacervation

Since viscoelastic masses could be formed at very low final acetic acid concentration from kafirin and zein solutions in glacial acetic acid of high protein concentration (28.6%), the effect of reducing protein concentration on viscoelastic mass formation at low final acid concentration (5%) was investigated. Viscoelastic masses could be formed from both kafirin and zein with decreasing protein concentrations from 28.6% down to 10%. Figure 4.3.3 shows CLSM and SEM of the effects of coacervation of 15, 10 and 5% solutions of kafirin and zein on the characteristics of the materials produced. Cohesive masses were not clearly produced from 5% protein solutions, although with kafirin there was some indication of fibril formation when observed by CSLM (Figure 4.3.3A). With coacervation of 10 and 15% protein solutions, the kafirin cohesive masses appeared to comprise of more ordered parallel fibrils than the zein masses when viewed by CLSM (Figure 4.3.3A). When viewed at much higher magnification using SEM, the kafirin materials comprised a mass of rough surfaced fibrils (Figure 4.3.3B). In contrast, the zein cohesive masses appeared to comprise a smooth,

more uniform “dough-like” material with some broad fibrils, especially when formed from a 15% protein solution. These differences in appearance were reflected in their stress-relaxation behaviours. Kafirin viscoelastic mass softness on day 0 was similar regardless of the protein concentration used to produce the masses, varying between 0.4 and 0.5 N (Table 4.3.2). After 16 days storage, the kafirin viscoelastic masses had become firmer (4.9-5.8 N) and closer in firmness to the gluten standard (6.9 N). In contrast, the zein viscoelastic masses were softer than both gluten and kafirin viscoelastic masses on day 0 (0.09-0.24 N), increasing to 0.69-2.9 N by day 16.

Protein concentration did not affect the stress-recovery of kafirin viscoelastic masses over the range where viscoelastic masses were formed, 28.6 to 10% protein. The stress-recovery values were lower (31.7-38.9%) than gluten (47.9%) on Day 0 regardless of protein concentration, and becoming slightly higher (46.8-56.4%) than gluten (43.3%) after 16 days storage (Table 4.3.2). The stress-recovery of the zein viscoelastic masses was considerably lower (4.5-16.1%) than both kafirin and gluten viscoelastic masses on day 0, increasing to values close to gluten on storage (Figure 4.3.4). The higher stress-recovery of kafirin (23.5-34.2%) compared to zein (4.5-13.8%) can be attributed to greater polymerisation of the kafirin protein compared to zein, as described above. When the effect of protein concentration (28.6, 15, 10 and 5%) in glacial acetic acid on viscoelastic mass formation was determined, viscoelastic masses could only be formed from both kafirin and zein by reducing the protein concentrations from 28.6% down to 10%. Thus, the stress-relaxation test could not be performed below a protein concentration of 10%. The points 0-9 on x-axis were included to reveal the inability of the prolamins to form fibrils when dissolved in glacial acetic acid solution at low prolamin concentrations.

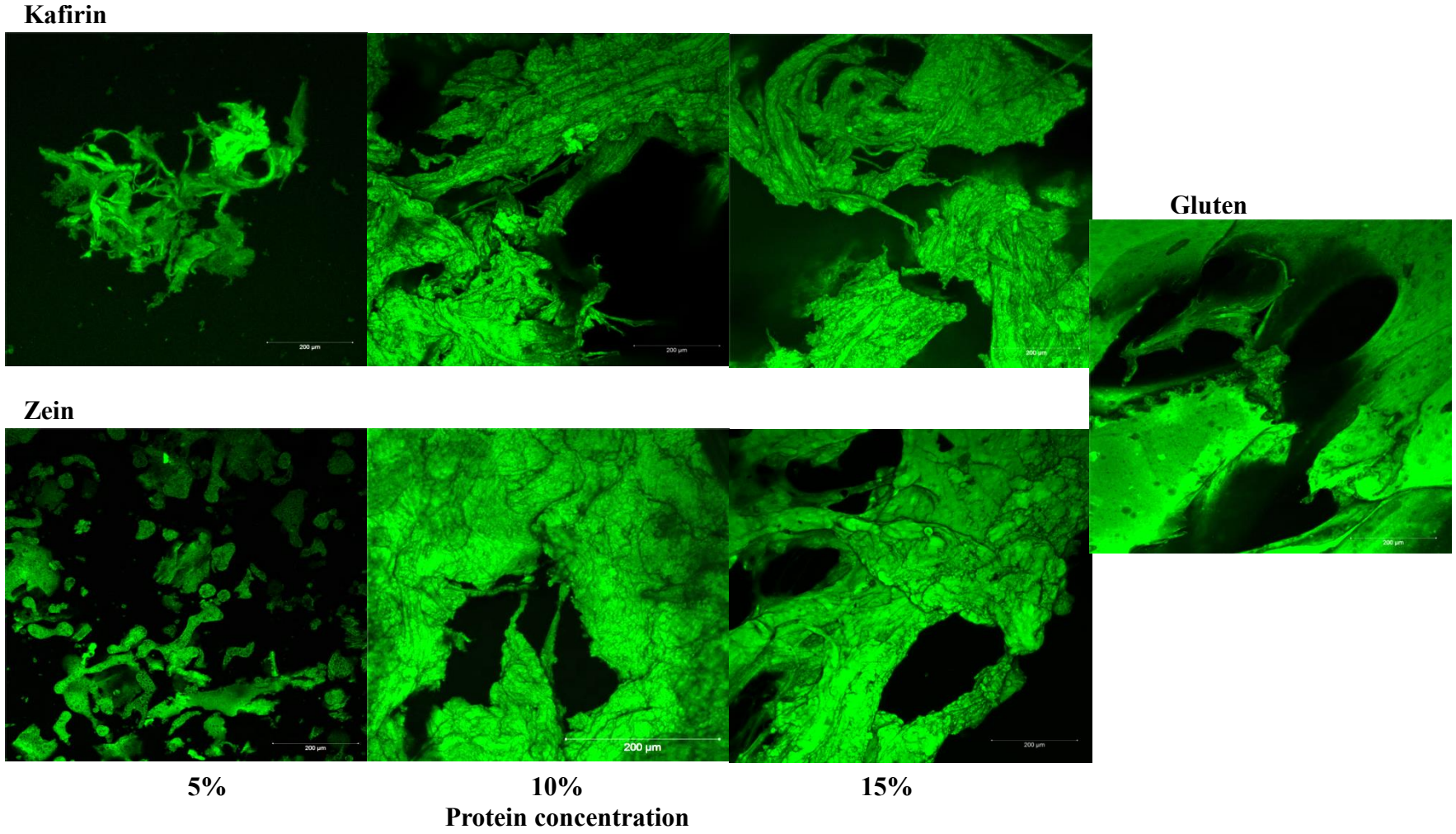

Figure 4.3. 3.A Confocal laser scanning microscopy of kafirin and zein cohesive masses prepared by coacervation at different protein concentrations (bar = 200 µm).

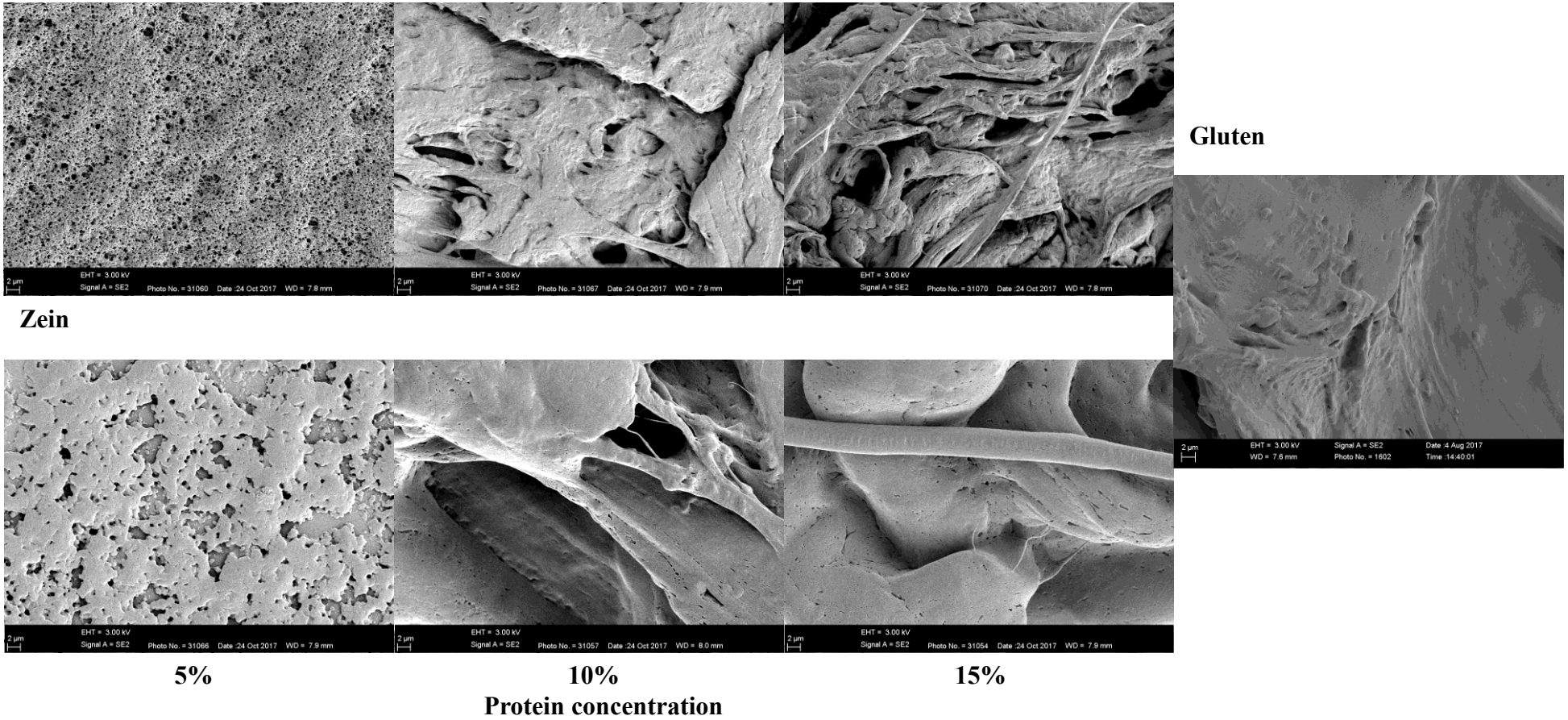

Figure 4.3. 3.B Scanning electron microscopy of kafirin and zein cohesive masses prepared by coacervation at different protein concentrations (bar = 2 μm).

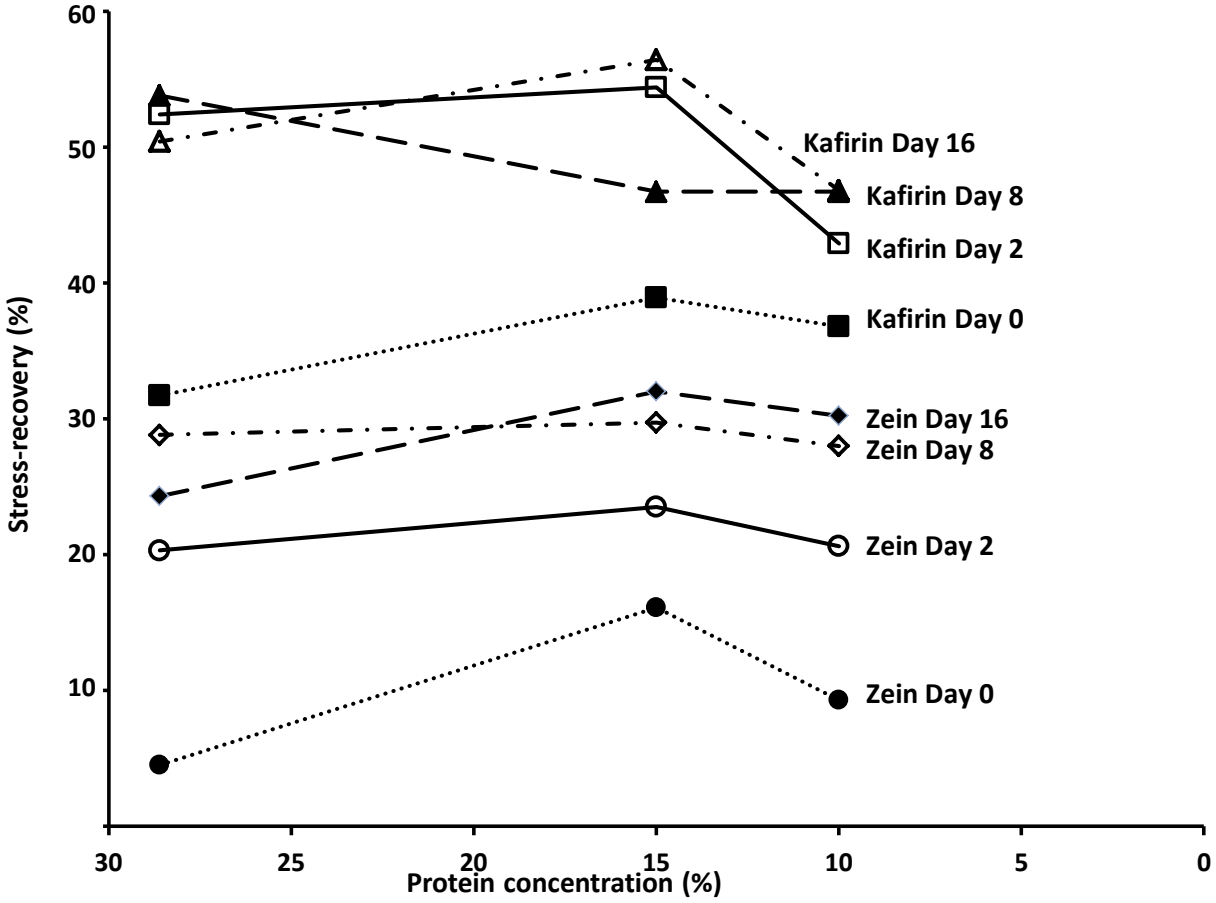

Figure 4.3.4 Effect of final protein concentration in glacial acetic acid on the stress-recovery behaviour of kafirin and zein viscoelastic masses

Table 4.3. 2 Effect of protein concentration in glacial acetic acid during preparation of kafirin and zein viscoelastic masses by coacervation on their stress relaxation at day 0 and after storage at 4°C for 2, 8 and 16 days. Coacervation to a final acetic acid concentration of 5%

Protein	FMax (N)				Ft (N)				Stress-recovery (%)			
Type	Day 0	Day 2	Day 8	Day 16	Day 0	Day 2	Day 8	Day 16	Day 0	Day 2	Day 8	Day 16
Gluten												
	3.538 (0.0373)	3.747 (0.105)	5.510 (0.518)	6.874 (0.108)	1.695 (0.200)	2.110 (0.022)	1.659 (0.040)	2.976 (0.125)	47.9Eb (3.0)	42.2Bb (4.7)	30.2 Ba (2.1)	43.3Bb (2.5)
Kafirin												
28.6%	0.412 (0.021)	2.053 (0.021)	2.608 (0.076)	4.896 (0.312)	0.131 (0.009)	1.076 (0.151)	1.405 (0.208)	2.465 (0.153)	31.7Ca (0.6)	52.4BC b (6.8)	53.8Cb (6.4)	50.4BC (0.1)
15%	0.508 (0.127)	2.420 (0.075)	3.810 (0.111)	5.767 (0.093)	0.197 (0.001)	1.317 (0.037)	1.781 (0.058)	3.253 (0.225)	38.9Da (0.7)	54.4Cc (3.2)	46.7Cb (0.2)	56.4Cc (3.0)
10%	0.482 (0.071)	2.772 (0.284)	3.079 (0.076)	5.103 (0.427)	0.180 (0.054)	1.178 (0.090)	1.797 (0.081)	5.103 (0.427)	36.8CDa (5.9)	42.9Ba (7.6)	46.7Ca (0.2)	46.8Ba (6.5)
Zein												
28.6%	0.088 (0.007)	0.183 (0.035)	0.370 (0.016)	0.687 (0.069)	0.004 (0.001)	0.039 (0.001)	0.090 (0.029)	0.195 (0.046)	4.5Aa (1.2)	20.3Ab b (8.1)	24.3Ab (8.9)	28.8Ab (9.6)
15%	0.184 (0.034)	0.547 (0.021)	1.298 (0.037)	2.438 (0.235)	0.030 (0.010)	0.128 (0.018)	0.415 (0.011)	0.723 (0.051)	16.1Ba (2.4)	23.5Ab (4.2)	32.0ABc (1.8)	29.7Ac (0.9)
10%	0.235 (0.042)	0.623 (0.045)	1.247 (0.052)	2.865 (0.151)	0.023 (0.016)	0.129 (0.021)	0.377 (0.025)	0.808 (0.221)	9.3Aa (2.8)	20.6Ab (4.7)	30.2ABc (0.7)	28.0Abc (6.2)

FMax. =Maximum force

Ft = Force at which fresh gluten dough had relaxed to 36.8 % of its maximum force 11.6 s after FMax

% stress-recovery at 11.6 seconds after FMax as calculated by Singh et al. (2006). The Ft value used (11.6 seconds) was the average of 5 closely agreeing independent experiments. [a]Mean values with different upper case letter in a column differ significantly from each other ($p < 0.05$)

[b]Mean values within a protein type with different lower case letter in a row differ significantly from each other ($p < 0.05$)

n = 2

As indicated above, at a 5% protein concentration in glacial acetic acid, the kafirin and zein did not form fibrils that could be kneaded into a viscoelastic mass on coacervation. In fact, a suspension of non-cohesive, aggregated protein was formed with both kafirin and zein. Hence, stress-relaxation analysis could not be performed on these preparations. When these materials were viewed using SEM, a fine sponge-like matrix was observed for kafirin aggregates, whereas zein aggregates looked like a discontinuous mat of a fused matrix (Figure 4.3.3B). The kafirin aggregates were similar in appearance to kafirin structures formed by coacervation at relatively high protein content (15.2%) and high final acetic acid content (40%) (Taylor et al., 2009). These kafirin structures were prepared with gentle stirring with a magnetic stirrer and were described as a continuous open matrix resembling an expanded foam. In contrast, the zein aggregates from this study more closely resembled kafirin aggregates that had been prepared with low protein content (2%) and low acid content (5.4%) but with the application of very high shear using an Ultra-Turrax blender (Taylor et al., 2009).

The fact that no fibrils were formed by coacervation with water from a glacial acetic acid solution of low prolamin protein concentration, indicates that there is a minimum concentration of protein that is required to enable intermolecular association (probably by hydrogen bonding) between the molecules to form fibrils. The degree of shear applied during water addition must also be low to allow fibril formation since higher shear appears to result in aggregation of the molecules into particles rather than fibrils.

4.3.5 Conclusions

Kafirin and zein viscoelastic masses can be formed by coacervation with water from solutions of the proteins in glacial acetic acid down to very low final acetic acid concentrations, down to 0.1% acetic acid. These low final acetic acid concentrations, since similar to the acid levels of sourdough fermentations (Falade et al., 2014) would permit yeast and chemical leavening of doughs and it is likely that baked products made from them would be sensorially acceptable. Even at this very low final acetic acid concentration, the masses largely retained their functionality when stored at 4°C for an extended period of time. This implies that when kafirin and zein are dissolved in glacial acetic acid, there is an irreversible change occurring at a molecular level. This change affects their functionality, to the extent

that apart from water, no additional plasticiser is required for them to exhibit viscoelasticity. Furthermore, it appears that kafirin and zein are binding water so strongly that they do not become glassy when stored below their glass transition temperatures for several days. Hence, after coacervation from glacial acetic acid kafirin and zein appear to be more hydrophilic in nature and exhibit functionality that is more similar to gluten. However, there are differences in the rheological properties of kafirin and zein viscoelastic masses when formed by this coacervation process. Both kafirin and zein were softer than gluten but kafirin displayed a higher elastic component than zein, which exhibited more viscous flow characteristics. This is probably due to the greater degree of disulphide bonding present in the kafirin viscoelastic masses.

Additionally, this work has shown that there is a minimum concentration of protein in glacial acetic acid, between 5 and 10%, that must be present for fibrils to form from these prolamins, which can be kneaded into viscoelastic masses.

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5 GENERAL DISCUSSION

The general discussion is divided into three parts. The first is a critical review of the methodology applied in this study, in which the weaknesses in the work performed and omissions are appraised. The second part proposes and discusses mechanisms through the means of models to explain the rheological behaviour of kafirin viscoelastic masses of different composition and the differences in behaviour between kafirin and zein masses. The third part proposes future research on development of a composite from both kafirin and zein proteins to obtain a viscoelastic mass with the desired balance of elasticity and viscous flow properties as gluten.

5.1 Methodological considerations

The two very similar tan-plant, non-tannin white sorghum cultivars PANNAR PEX 202 and 606 were decorticated before milling. Bran with the attached germ and minimal starch are removed from the sorghum kernel during decortication. These sorghum grains were decorticated to reduce protein-lipid interactions. This might have contributed to the good recovery and chemical quality of the kafirins due to reduced co-extraction of fat with the kafirin preparations.

SDS-PAGE analysis was performed to determine whether the formation of kafirin viscoelastic masses from kafirin preparations extracted with various solvents was due to disulphide bonding (section 4.1.3.9.2). SDS-PAGE did not show any evidence of induced polymerisation through disulphide bonding. The formation of stable viscoelastic masses is attributed to the glacial acetic acid which would enable complete solvation, protonation, and partial unfolding of the prolamins (Li et al., 2012). The kafirin molecules were proposed to be present in solution at least in part as monomeric polypeptide chains (Elhassan et al., 2018; Taylor et al., 2018), with kafirin dimeric and polymeric polypeptide chains also present. In fact, when prolamins were dissolved in glacial acetic acid, it was evident that an irreversible change occurred at a molecular level. When kafirin and zein are dissolved in glacial acetic acid, it is hypothesised that there is protonation of the prolamins, causing partial unfolding and allowing hydration on the molecular surface, as proposed by Li et al. (2012). This in turn could enable inter- and intramolecular covalent bonding, probably mainly disulphide bond

crosslinking, which would stabilise the hydrated prolamin conformation. This was on account of the fact that apart from water, no additional plasticiser was needed for them to exhibit viscoelasticity. However, the retention of viscoelastic mass softness and elastic recovery on storage requires the presence of γ-kafirin, with its propensity to form disulphide bonding (Chapter 4.2). Additionally, the elastic component of all the kafirin viscoelastic masses produced at different final acetic acid concentrations increased on storage. This could be due to the formation of additional disulphide linkages on storage.

CLSM has been used to investigate the microstructural changes in foods (Choi et al., 2008). CLSM does not only provide an image with better resolution compared to the conventional light or fluorescence microscopy, it has the ability to produce optical sections through a three dimensional specimen (Dürrenberger et al., 2001). CLSM was used to study the microstructures of the kafirin and zein cohesive masses produced at different protein and final acetic acid concentrations. Cohesive masses were clearly produced at 10% and 15% protein solutions. The kafirin cohesive masses appeared to contain more ordered parallel fibrils than the zein cohesive masses. However, when viewed by SEM at much higher magnification, zein masses appeared to be a smooth and more uniform "dough-like" material with some broad fibrils, whereas kafirin masses appeared to comprise a mass of rough surfaced fibrils. This could be attributed to greater hydrophobicity of kafirin compared to zein. However, all the kafirin and zein cohesive masses viewed by SEM were dried in a desiccator before examination. Hence, the images obtained were not necessarily of the native state of the cohesive masses. This was due to the artefacts which could have been formed during the removal of the acetic acid.

The large angle deformation compression test used to evaluate the stress relaxation of kafirin and zein viscoelastic masses revealed that both kafirin and zein, which had been considered to have similar functional properties, were indeed very different. It was found that kafirin viscoelastic masses were predominantly elastic, whereas zein masses had predominantly viscous flow characteristics (Chapter 4.2). Kafirin viscoelastic masses were much firmer compared to zein masses but both were softer relative to gluten, regardless of their subclass composition. The dynamic rheological analysis (small angle deformation) was used to obtain complementary information on the rheological properties of several different kafirins types. However, the dynamic rheological analysis could not be carried out on the high α-prolamin

preparations and the viscoelastic masses formed at different protein and final acetic acid concentrations. This was due to the very small amounts of certain of sorghum lines from which the kafirins were extracted. It was therefore difficult to evaluate the responses of the viscoelastic masses to increasing frequency at constant amplitude and temperature. Inability to perform the dynamic rheological analysis on these prolamins made it difficult to confirm the results obtained by the large angle deformation test.

5.2 Mechanisms to explain the rheological behaviour of kafirin viscoelastic masses of different composition and the differences in behaviour between kafirin and zein masses

Models to explain the stress-recovery behaviours of viscoelastic masses from several different kafirins and zeins types: kafirins extracted with different solvents, high α-prolamin and prolamins minus γ-prolamin, and kafirins from high protein digestibility and waxy lines are proposed (Figures 5.1, 5.2, 5.3, and 5.4). Kafirin (comprising all prolamin subclasses) displayed a similarly high elastic component to gluten, whereas zein (comprising all prolamin subclasses) exhibited more viscous flow properties (sections 4.2.4.1.3 and 4.3.4.2). When the masses are compressed, it is proposed that the force is sufficient to break hydrogen bonds but the strong covalent disulphide bonds will remain intact (Figure 5.1B). During compression, zein masses will deform more than kafirin and more energy will be dissipated, whereas kafirin with its higher number of disulphide bonds will exhibit greater resistance to compression, and more energy will be stored. On removal of the force, the kafirin mass will release the stored energy and recover almost to its original shape, with hydrogen bond reformation (Figure 5.1C). However, zein with its lower number of disulphide bonds, will release insufficient energy to return to its original shape (Figure 5.1C). Callaghan and Gil (1999) found that the effect of shear and extensional deformations on the structure of soft gluten showed that hydrogen bonds between glutamine residues were broken when stress was applied. The hydrogen bonds were reformed after the cessation of stress. Thus, in this study, it is proposed that when both kafirin and zein masses are compressed, the force is sufficient to break bonds between water and protein molecules.

The viscoelastic masses made from all kafirins extracted with different solvents contained all kafirin subclasses and they exhibited high elastic behaviour (section 4.2.4.1.1). The masses

from the high α-kafirins had high proportion of the α-subclass with a concomitant decrease in both β- and γ-subclasses (section 4.2.4.1.3). They also exhibited high elastic recovery (section 4.2.4.1.3). A high proportion of the α-subclass appears to improve the functionality of the viscoelastic masses. They appeared to have similar elastic behaviour as proposed for kafirin viscoelastic mass (comprising all prolamin subclasses) during and after compression (section 4.2.4.1.3), which can be explained in the same way (Figure 5.2). TG-HD kafirin from sorghum and its null control exhibited high elastic recovery (section 4.2.4.1.2). TG-HD kafirin did not contain γ-subclass but there was a compensatory synthesis of other kafirin sub-class. This elastic behaviour could due to the genetic background of the transgenic sorghum. The transgenic sorghum and its null control were from a different line sorghum P898012 x Macia (Da Silva et al., 2011a.b) than the conventionally bred waxy and high protein digestibility sorghum (RTx2907 x P850029)

There would be less disulphide bonding in the α-kafirins minus γ-kafirin due to the absence of γ-kafirin compared to the kafirins with all kafirin sub-classes (Figure 5.3A) (Taylor et al., 2018). Kafirin polymerises through disulphide bond cross-linking due to the presence of cysteine-rich γ- and β-kafirins (El Nour et al., 1998). Thus, the absence of γ-kafirin subclass suggests lower amounts of disulphide cross-linking (El Nour et al., 1998; Belton et al., 2006). Therefore during compression, where the hydrogen bonds in the kafirin masses are broken and the masses tend to dissipate more energy and exhibit lower resistance to force of compression (Figure 5.3B) as compared to kafirin with all the subclasses (Figure 5.1B). The kafirins minus γ-kafirin lost their elasticity on storage and they were more viscous unlike kafirins with all the subclasses that maintained their elasticity on storage. This shows that the γ-kafirin subclass is necessary to retain the elasticity of viscoelastic mass during storage, and it appears to be so required for maintaining the softness of both kafirin and zein viscoelastic masses (Chapter 4.2).

The kafirins from the waxy and high protein digestibility sorghum lines (WHD1, WHD2 and WHD3) that were deficient in β-kafirin subclass exhibited much lower elasticity initially (section 4.2.4.1.2) compared to the kafirin from the transgenic high protein digestibility-high lysine (TG-HD) line without γ-kafirin but with β-kafirin and also compared to its null control. It is proposed that they behaved as illustrated in Figure 5.4. Beta-kafirin has been reported to act as a bridge or a link between the oligomers of α_1- and γ-kafirin (El Nour et al., 1998).

These authors further stated that β-kafirin appears to be one of the necessary components that determine the degree of polymerisation. The presence or absence of β-kafirin appears to influence the ability of kafirin or zein to form viscoelastic masses, more than the γ-kafirin (Taylor et al., 2018).

It is well known that the glutenins are responsible for strengthen and elasticity of the wheat dough, especially the high molecular weight glutenin subunits (HMW-GS). This is due to the glutenin HMW subunits forming hydrogen bonds between the polypeptides, and storing energy (Wiesser, 2007). The monomeric gliadins which are responsible for dough viscosity and extensibility due to the gliadins forming hydrogen bonds between the monomeric polypeptides (Wiesser, 2007). However in this present study, the elasticity of kafirin viscoelastic masses is proposed to depend on the presence of covalent disulphide bonds which allow it to exhibit greater resistance to compression and greater storage of energy, which in turn will allow it to recover almost to its original shape upon removal of compression force. In contrast, due to the lower number of disulphide bonds in zein mass, it will deform more than kafirin during compression and store less energy. On release, insufficient energy is available to return the zein mass to its original shape upon removal of compression force. Hydrogen bonding is proposed to be involved in the extensibility of zein mass in a similar way to gliadin. It is proposed that kafirin is analogous to the HMW glutenins as it exhibits elasticity, and zein, particularly commercial (essentially α-zein) is analogous way to gliadin as it exhibits viscous flow behaviour.

5.3 Potential future work

Future work is required on the formation and functional properties of synthetic gluten-free doughs prepared with kafirin or zein protein plus sorghum or maize starch. This may be more practical to start with, since starch is the main component of bread. Up to 50% water is absorbed by starch and it interacts with protein to form a continuous network during bread making (Goesaert et al., 2005). The methodology could involve viscoelastic mass formation by the simple coacervation process followed by compositing with starch.

If good progress is made with an isolated kafirin-zein-starch composite, then doughs made with the kafirin-zein composite masses plus maize and/or sorghum flour may be studied. Dough sheeting could be used to improve the rheological properties of the dough. During

sheeting, the dough formed is kneaded and rolled into a sheet by compression between two rotating cylinders (Petitot et al., 2009). Sheeting has been found to increase wheat dough elasticity and extensibility (Chakrabarti-Bell et al., 2010). Khuzwayo (2016) also demonstrated that dough sheeting in combination with pre-gelatinized maize flour, addition of maize sourdough and the surfactant DATEM could form a dough that could mimic the viscoelastic nature of wheat flour dough.

Both zein and kafirin (consisting of all the subclasses) are not produced on a commercial scale. However, α-zein is commercially produced. The major problem at present is that there are no economical processes for kafirin extraction. Even the high cost of kafirin production on a laboratory scale has hindered extensive research on kafirin functionality. In the absence of a commercially available kafirin, the full potential of kafirin for making gluten-free bread products may not be fulfilled. However, the percolation-type extraction process for kafirin developed by Muhiwa et al., 2017 using distillers dried grains with solubles (DDGS), a prolamin-rich by-product from the grain bioethanol industry, which required low solvent usage, which gave high protein recovery could be adopted for the extraction of kafirin and zein. This extraction process could enable more but less expensive prolamin production compared to the existing laboratory methods. This would allow for more extensive and in-depth study on the viscoelastic masses.

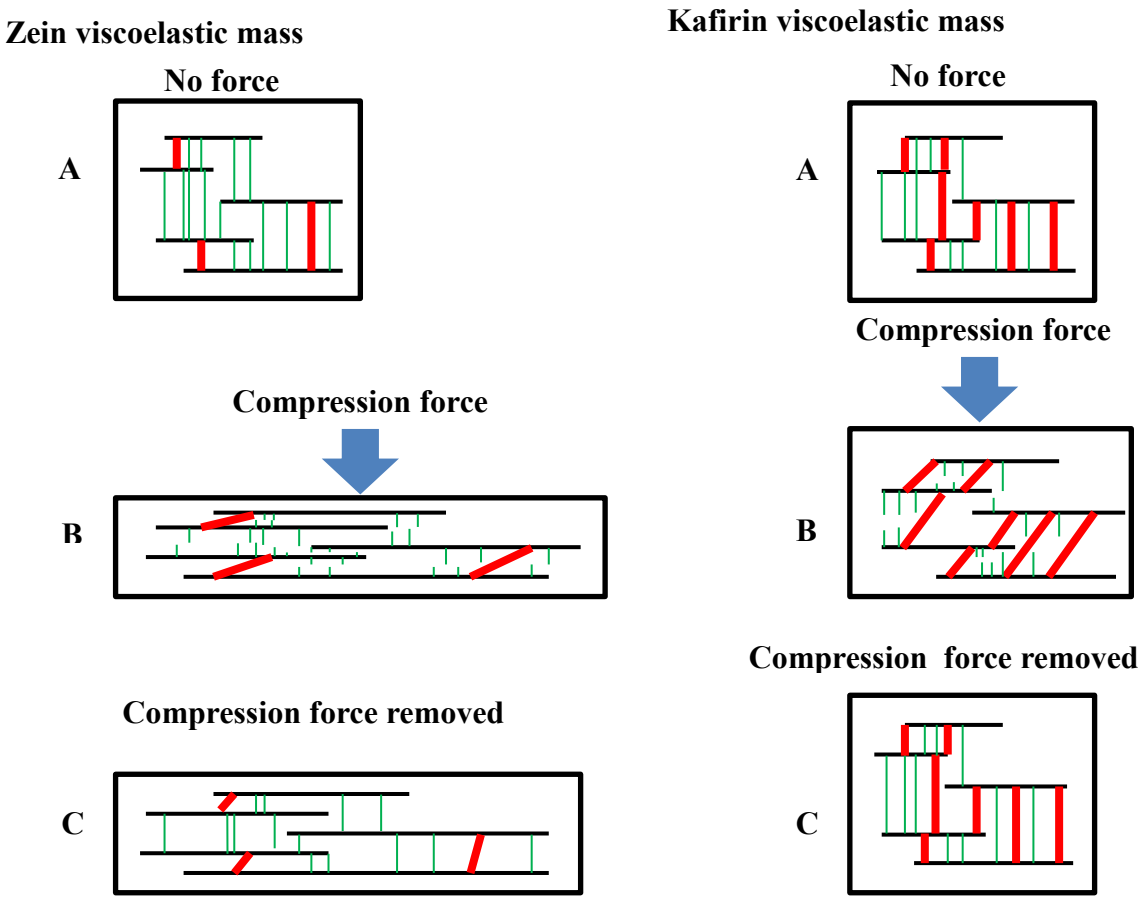

Figure 5. 1 Model to explain the differences in kafirin and zein stress-recovery behaviour when compressed.

Disulphide bonds-Thick red lines, Hydrogen bonds-Thin green lines. A-Viscoelastic masses at rest with no force applied. B-Compression force applied to viscoelastic masses. C-Compression force removed from viscoelastic masses.

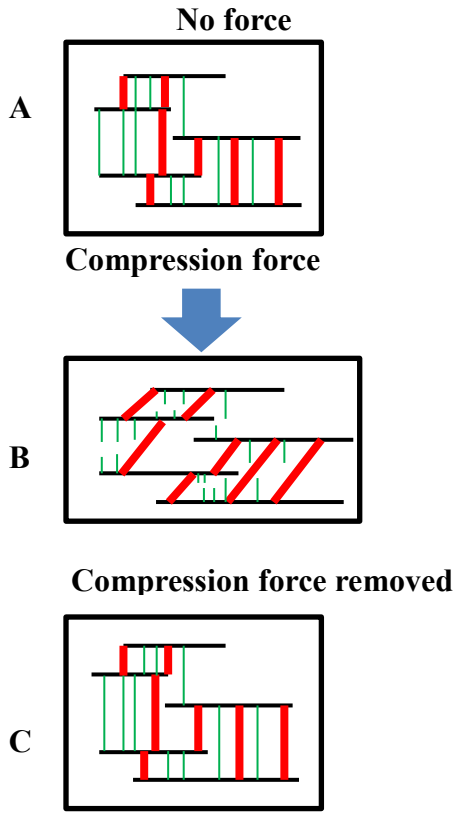

Figure 5. 2 Model to explain the stress-recovery behaviour of all kafirins extracted with different solvents, high α-kaifrn, kafirin fromTG-HD and its null control when compressed.

Disulphide bonds-Thick red lines, Hydrogen bonds-Thin green lines. A-Viscoelastic masses at rest with no force applied. B-Compression force applied to viscoelastic masses. C-Compression forces removed from viscoelastic masses.

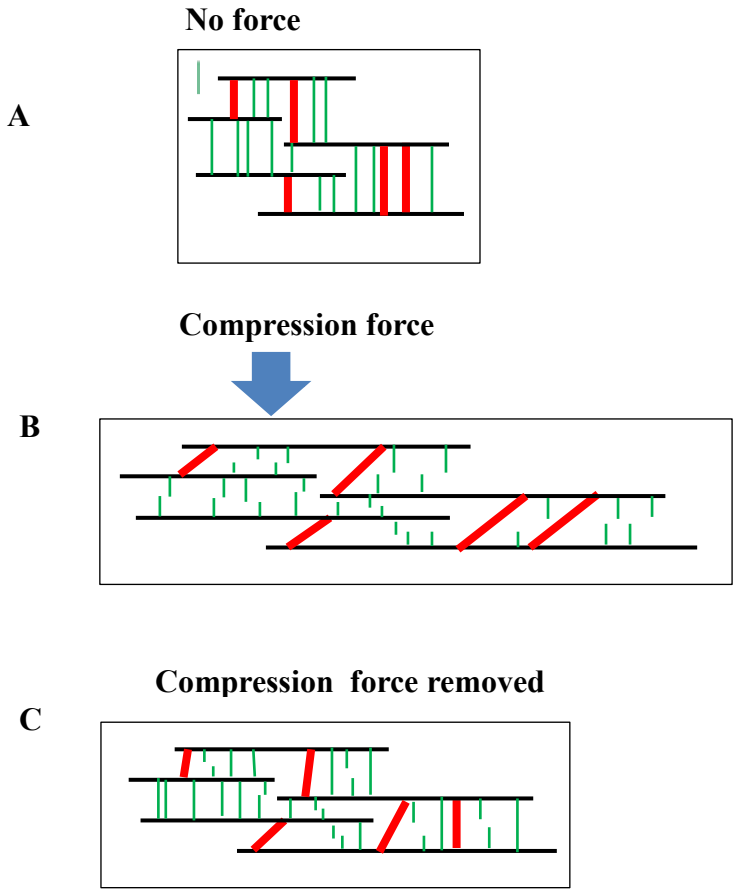

Figure 5. 3 Model to explain the stress-recovery behaviour of the kafirins minus γ-kafirin when compressed.

Disulphide bonds-Thick red lines, Hydrogen bonds-Thin green lines. A-Viscoelastic masses at rest with no force applied. B-Compression force applied to viscoelastic masses. C-Compression force removed from viscoelastic masses.

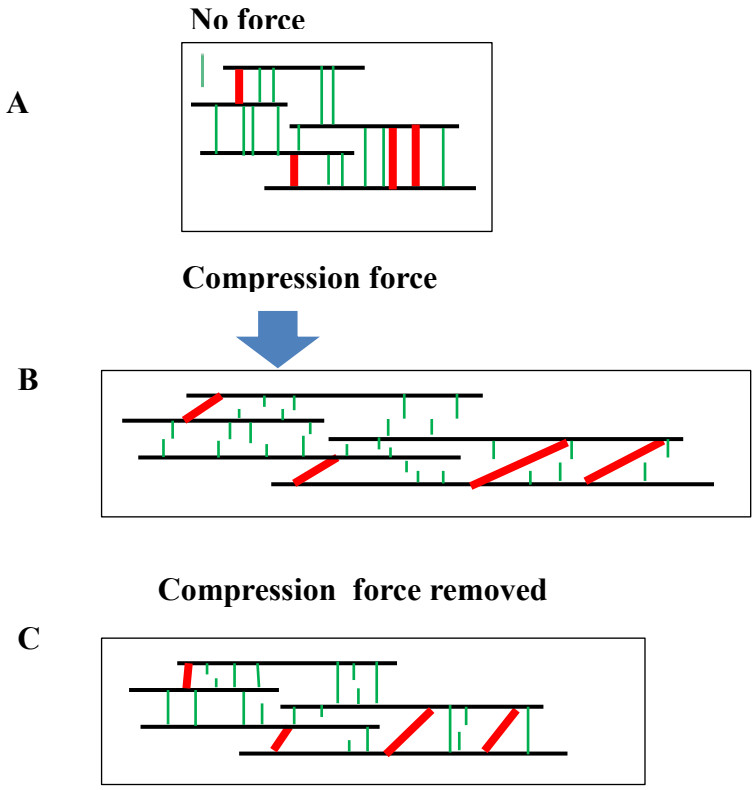

Figure 5. 4 Model to explain the stress-recovery behaviour of kafirins from waxy (W) and high protein digestibility (HD) WHD1, WHD2, WHD3, NHD when compressed.

Disulphide bonds-Thick red lines, Hydrogen bonds-Thin green lines. A-Viscoelastic masses at rest with no force applied. B-Compression force applied to viscoelastic masses. C-Compression force removed from viscoelastic masses.

6 CONCLUSIONS AND RECOMMENDATIONS

Kafirin and zein preparations can form stable viscoelastic masses by dissolution of the prolamins in glacial acetic acid, followed by simple coacervation with rapid water addition; apparently regardless of the subclass composition of kafirin and zein. Various factors such as prolamin composition in terms of prolamin subclasses, the relative hydrophobicity of the prolamins, protein and final acetic acid concentrations have been identified to influence the functionality of kafirin and zein viscoelastic masses.

Like gluten, both kafirin viscoelastic masses containing all the subclasses and kafirin that is high α-kafirin can maintain their initial elasticity when stored. However, the extent to which a high α-kafirin viscoelastic mass can maintain its elasticity during storage is much lower when compared to the kafirin viscoelastic mass containing all the subclasses. This may be due to the lower amount of γ-kafirin in the high α-kafirin preparation. The kafirins minus γ-kafirin can not maintain their elasticity on storage after 2 days, probably due to the absence of γ-kafirin. Maintenance of kafirin elasticity when stored appears to depend on the presence of γ-kafirin.

Manipulation of the final acetic acid concentrations can enable the formation of kafirin viscoelastic masses with a similar balance of elasticity and viscous flow rheological properties to wheat gluten at levels which are food compatible, i.e. very low final acetic acid concentration. In fact, coacervation with reduction in the final acetic acid concentration down to 0.1% can still enable formation of kafirin and zein viscoelastic masses, with functionality retained on storage at 4^{o}C for an extended period. However, a minimum of prolamin concentration in glacial acetic acid between 5 and 10% is essential for viscoelastic mass formation at low final acetic acid concentration (5%). This is because no fibrils may be formed by coacervation with water from a glacial acetic acid solution of lower prolamin concentration. There is an irreversible change i.e. change in the secondary structure accompanied by breakage of non-covalent bonds that occurs at a molecular level in kafirin and zein when they are dissolved in glacial acetic acid, such that apart from water, no additional plasticiser is needed for them to exhibit viscoelasticity.

Kafirin and zein viscoelastic masses differ in their elastic and viscous flow properties. Kafirin is more elastic, while zein exhibits predominantly viscous flow characteristics, even after storage, probably because kafirin is more hydrophobic than zein.

Models to explain the stress-recovery behaviours of viscoelastic masses from several different kafirin and zeins types are proposed. When the masses are compressed, kafirin viscoelastic mass (comprising all prolamin subclasses) with its higher number of disulphide bonds will exhibit greater resistance to compression, and more energy will be stored, whereas zein mass (comprising all prolamin subclasses) with its lower number of disulphide bonds will deform more than kafirin and more energy will be dssipitated. On removal of the force, the kafirin mass will release the stored energy and recover to its original shape, with hydrogen bond reformation. However, zein will release insufficient energy to return to its original shape. During compression, the α-kafirins minus γ-kafirin tend to dissipate more energy and exhibit lower resistance to force of compression when compared to kafirin with all the subclasses. This is because the α-kafirins minus γ-kafirin probably has less disulphide bonding due to the absence of γ-kafirin. Thus, the elasticity of kafirin viscoelastic masses is proposed to depend on the presence of covalent disulphide bonds, whereas hydrogen bonding is involved in the extensibility of zein viscoelastic masses in the same way as gliadin. Kafirin behaves in an analogous way to the HMW glutenins and zein, particularly commercial (essentially alpha-zein) behaves in an analogous way to gliadin.

Kafirin viscoelastic mass formation by coacervation from solution in glacial acetic acid can not be considered as an economical method of dough formation. Therefore, more economical methods can be developed with similar action for making gluten-free bread products.

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8 PUBLICATIONS AND PRESENTATIONS BASED ON THIS RESEARCH

Elhassan, M.S.M., Oguntoyinbo, S.I., Taylor, J. and Taylor, J.R.N., 2018. Formation and properties of viscoelastic masses made from kafirin by a process of simple coacervation from solution in glacial acetic acid using water. Food Chem. 239, 333-342.

Oguntoyinbo, S.I., Taylor, J. and Taylor, J.R.N., 2017. Improvement in the functionality of kafirin dough through chemical modification. Oral presentation in the New Voice Symposium of the Association of Cereal Science and Technology Southern Africa (CST-SA), Pretoria. South Africa, May, 2017.

Oguntoyinbo, S.I., Taylor, J. and Taylor, J.R.N., 2017. Properties of viscoelastic masses prepared from kafirin (sorghum prolamin protein) isolated with different solvents. Oral presentation in 22nd Biennial International SAAFoST Congress and Exhibition, Cape Town, South Africa, September, 2017.

Oguntoyinbo, S.I., Taylor, J. and Taylor, J.R.N., 2018. Comparative functional properties of kafirin and zein viscoelastic "doughs" formed by simple coacervation at different acetic acid and protein concentrations. Oral presentation at the International Sorghum Conference "Sorghum in the 21st Century", Cape Town, South Africa, April, 2018.

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