Formation and properties of viscoelastic masses made from kafirin by a process of simple coacervation from solution in glacial acetic acid using water

Mohammed S.M. Elhassan, Segun I. Oguntoyinbo, Janet Taylor, John R.N. Taylor*

Institute for Food, Nutrition and Well-being and Department of Food Science, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa

* Corresponding author

Tel.: +27 12 4204296; + Fax: +27 12 4202839

Email address: john.taylor@up.ac.za (J.R.N. Taylor)

Running title: Viscoelastic masses made from kafirin

Highlights

- Kafirin viscoelastic masses can be formed by coacervation from glacial acetic acid.
- Viscoelastic mass formation was achieved by rapid addition of water with low shear.
- Viscoelastic masses could be formed from kafirins differing in their subclasses.
- Glacial acetic acid greatly increased kafirin α-helical conformation.
- Viscoelastic masses maintained their functional properties when stored at 10 °C.

Abstract

Stable viscoelastic masses have been formed from kafirin in a mainly aqueous system. Kafirin was dissolved in glacial acetic acid (GAA) and simple coacervation was performed by rapid addition of 15^{o}C water under low shear. Kafirin precipitated out as a network of hydrated fibrils which could be hand-kneaded into a viscoelastic mass. These could be formed from a very wide range of kafirins, including those where β- or γ-subclass expression was suppressed. Kafirin composition influenced the appearance of the masses but did not fundamentally affect stress-relaxation behaviour. Fresh kafirin masses exhibited similar elasticity and viscous flow balance to gluten. They maintained functionality when stored for several days at 10^{o}C but their elastic component increased. FTIR showed that when kafirin was dissolved in GAA its α-helical conformation increased substantially. Dissociation of the kafirin molecules in GAA, assuming a α-helical conformation may have enhanced water binding, enabling viscoelastic mass formation.

Keywords: dough, elasticity, high protein digestibility, kafirin, stress-relaxation

1. Introduction

Climate change is one of the biggest challenges the world is currently encountering with the greatest impacts being seen in sub-Saharan Africa (FAO, 2014) where agriculture is largely directly dependent on rainfall. Sorghum is one of the most drought-tolerant cereal crops and is highly suited for cultivation in the semi-arid regions (Hadebe, Modi, & Mabhaudhi, 2016). It would be advantageous if sorghum flour could be used as the sole functional component make wheat flour-like bread and other dough-based products, but as yet this has not been possible (Taylor, Taylor, Campanella & Hamaker, 2016).

The primary reason is that unlike wheat gluten, kafirin, the sorghum prolamin protein, does not by itself form a viscoelastic mass when manipulated in water, a fundamental requirement for making leavened wheat-like bread. Several reasons have been proposed for kafirin's lack of functionality in doughs. Kafirin is more hydrophobic than gluten and kafirins can be cross-linked together by disulphide bonding involving the cysteine-rich β- and γ-kafirin subclasses, resulting in the entrapment of the kafirins in their protein bodies (Taylor et al., 2016). As a consequence they are not functional in bread making. Additionally, research suggests that a β-sheet conformation, as exists in glutenin, is critical for prolamins such as zein (the maize homologue of kafirin) to exhibit viscoelastic functionality (Erickson, Campanella & Hamaker, 2012). In their native state zein and kafirin are predominantly in the α-helical conformation (Taylor et al., 2016).

Oom, Pettersson, Taylor & Stading (2008) showed that by plasticizing isolated kafirin (which comprised α- and γ-kafirin subclasses) with oleic acid in a 2:1 proportion a type of viscoelastic mass was formed. However, this viscoelastic mass was more strictly speaking a "resin" as defined by Lai & Padua (2007) since the plasticizer was a lipid. This kafirin resin exhibited similar extensional viscosity and strain hardening properties as oleic acid

plasticized gluten and zein resins but became stiffer much more rapidly. Schober, Bean, Tilley, Smith & Ioerger (2011) found that isolated kafirin (which was comprised predominantly of α-kafirin) could aggregate into a cohesive mass in water at elevated temperature (55^{o}C), but it had very poor functional properties and became hard very rapidly, similar to the findings of Oom et al. (2008).

Goodall, Campanella, Ejeta & Hamaker (2012) investigated the dough making properties conventionally bred high protein digestibility-high lysine (HDHL) sorghum mutants which have modified kafirin expression. They showed that composites of HDHL sorghum flour with wheat flour had improved viscoelastic dough properties compared to normal sorghum-wheat flour composites and similar to 100% wheat flour dough. The authors attributed the improved dough functional properties of these HDHL mutants to their kafirin being "freed" from the protein bodies as a result the γ-kafirin being located in the interior of the protein bodies instead of the periphery in normal sorghum (Oria, Hamaker, Axtell & Huang, 2000), exposing more α-kafirin subclass which is less hydrophobic. Elhassan, Emmambux, Hays, Peterson & Taylor (2015) furthermore found that sorghum mutants expressing both waxy (high amylopectin) and HD traits had improved flour and dough properties associated with bread making quality, compared to normal and normal starch-HD lines.

Chemical treatment with organic acids has been found to improve the functionality of zein. Sly, Taylor & Taylor (2014) showed commercial zein (predominantly α-zein) viscoelastic masses (referred to by the authors as a dough) prepared with dilute acetic acid and lactic acid had greatly improved properties to the extent that zein plus starch or rice flour could form gas-holding doughs as measured by alveography, with properties approaching that of wheat flour dough. Furthermore, King, Taylor & Taylor (2016) showed that a viscoelastic mass (referred to by the authors as a dough) could also be formed from "total" zein

(comprising (α-, β- γ- and δ-zein) by a process of first dissolving it glacial acetic acid and then casting it into a film.

Notwithstanding these developments, neither process could form a viscoelastic mass with kafirin (Sly, 2014: King, 2016). Earlier, however, Taylor, Taylor, Belton, & Minnaar (2009a) and Taylor and Taylor (2010) showed that by dissolving kafirin in glacial acetic acid and adding water in a controlled manner, kafirin precipitated out of solution as organized structures, a process of simple coacervation. Different types of structures such as microparticles and scaffolds were formed, depending on the exact conditions of the coacervation process.

Based on ongoing research into the kafirin coacervation process, this study applied the process under low shear conditions and as shown here a stable, viscoelastic masses can be formed from kafirin. In view of the observations that flours of HD sorghum mutants have improved dough properties compared to normal sorghum, the study investigated the formation of viscoelastic masses and their properties from kafirins isolated from conventionally bred HDHL (normal starch and waxy types) and transgenic HD sorghums with modified kafirin expression.

2. Materials and Methods

2.1. Sorghum grain

For kafirin extraction, whole grain white tan-plant (non-tannin) sorghum grain of two different origins was used:

1) Three conventionally bred HD lines which also expressed the waxy trait (designated WHD1, WHD2 and WHD2), one HD line with normal starch (designated NHD), two waxy-normal protein digestibility lines (designated WN1

and WN2) and two normal starch-normal protein digestibility lines (designated NN1 and NN2). The lines were developed from crosses between parental lines RTx2907 (waxy) and P850029 (HDHL) by Texas A&M University and grown at Halfway, Texas in a controlled field trial. The HDHL line has been identified has having decreased γ-kafirin content, significant down-regulation of 25 kDa α-kafirin, up-regulation of 22 kDa α-kafirin and down-regulation of some β-kafirin subclasses (Benmoussa, Chandrashekar, Ejeta & Hamaker, 2015).

2) Two transgenic high protein digestibility-high lysine (TG-HD) lines (designated TG-HD1 and TG-HD2) and their normal protein digestibility null controls (designated NC1 and NC2). The lines were produced through the Africa Biofortified Sorghum consortium by DuPont Pioneer in an approved controlled field trial at Johnston, Iowa. The TG-HD sorghum lines have suppressed expression of several kafirin subclasses by means of RNAi (RNA interference) technology. TG-HD1 and TG-HD2 were 75% pure with respect to the ABS032 gene construct which suppresses synthesis of α-kafirin A1 and α-kafirin B1and B2 (the 19 and 22 kDa α-kafirin subclasses, respectively), γ-kafirin 1 and 2, and δ-kafirin 2 (Da Silva, Jung, Zhao, Glassman, Grootboom, Mehlo, O'Kennedy, Taylor, & Taylor (2011a).

2.2. Kafirin extraction

Total kafirin was extracted from the sorghum grain essentially as described by Taylor, Taylor, Dutton, & De Kock (2005). Clean, whole grain from each sorghum line (220 g) was milled using an air-cooled laboratory hammer mill fitted with a 500 µm opening screen. The milled grain was extracted with 70% (w/w) ethanol plus 0.35% (w/w) glacial acetic acid and 0.5% (w/w) sodium metabisulphite at 70˚C with vigorous stirring for 1 hour. The supernatant

was collected after centrifugation at 1000 x g at 25°C for 5 minutes. The alcohol was allowed to evaporate from the solute and the precipitated protein washed with cold distilled water (<10°C). The recovered protein was separated by filtration and air dried at 25°C.

2.3. Kafirin viscoelastic mass preparation

The method was based on the kafirin microparticle preparation technique of Taylor et al. (2009a) and Taylor and Taylor (2010). Glacial acetic acid (5 ml) was pipetted into a 50 ml beaker containing a magnetic stirrer bar. One gram dry total kafirin preparation was placed in the beaker with constant stirring. The mixture was heated slowly with continuous stirring to 50°C (within 5 min). The beaker was covered during heating. When the kafirin had dissolved in the glacial acetic acid the stirring was stopped and the beaker was removed from stirrer hotplate. Distilled water (20 ml) at 15°C was added rapidly (5 ±1 sec) to the kafirin solution without stirring using a 20 ml plastic syringe (without needle). This temperature and volume of cool water was selected as it resulted in a final temperature of the acetic acid in the beaker of 25.0 ± 0.5^{o}C, very close to the ambient temperature (approx. 24^{o}C), but substantially below the glass transition temperature (T_g) of water hydrated kafirin of around 40^{o}C (Schober et al., 2011). The magnetic stirrer bar was then removed. The magnetic stirrer bar was then removed. On the rapid addition of the cool water, a soft net of aggregated kafirin formed (Figure 1S), which was collected with a spatula by gentle stirring. This was kneaded for 20 ±2 sec into a cohesive mass by hand (wearing rubber gloves) with squeezing out of the free liquid in the cohesive mass (approx. 2.5 g), resulting in a final kafirin mass of approx. 2.1 g. The brief hand kneading did not raise the temperature of the kafirin dough mass.

2.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to determine which kafirin subclasses were present in the different kafirin preparations. SDS-PAGE was performed under reducing conditions using 4-12% Novex NuPAGE® polyacrylamide gradient gels (8 x 8 cm x1.0 mm thick with 15 wells) (Invitrogen. Carlsbad, CA). Invitrogen Mark12 Unstained Standard was used. Samples were loaded to 10 µg constant protein. Staining was with Coomassie Brilliant Blue R-250. After de-staining, the gels were photographed by scanning on a flatbed scanner.

2.5. Stereomicroscopy

The morphology of the kafirin masses was recorded by stereomicroscopy using a Nikon Stereo SMZ 800 light microscope (Tokyo, Japan) fitted with a Nikon DXM 1200 digital camera. Each piece (5 x 3 x 1.5 mm) was stretched by hand, placed onto a glass slide and then released.

2.6. Confocal Laser Scanning Microscopy (CLSM)

CLSM was used to investigate the internal structure of the kafirin masses. Each piece (5 x 3 x 1.5 mm) was stretched onto a glass slide. They were imaged using a Zeiss 510 META confocal laser scanning microscope (Jena, Germany) fitted with a Plan-Neofluar 10 × 0.3 objective at an excitation wavelength of 405 nm with natural fluorescence (Sly et al., 2014).

2.7. Scanning Electron Microscopy (SEM)

For further investigation of kafirin viscoelastic mass microstructure, ultra high resolution field emission scanning electron microscopy was performed using a JEOL 6000F FEGSEM (JEOL, Tokyo, Japan). Balls of the kafirin cohesive mass of approx. 5 mm diam. were prepared by rolling a piece clockwise 3 times between the palms of the hands. The balls

were mounted on aluminium stubs using carbon glue and dried at 4°C for three days before being sputter coated with gold and viewed.

2.8. Stress-relaxation of kafirin viscoelastic masses

Kafirin viscoelastic masses were prepared as described in 2.3. The pieces (0.5 g) then quickly pressed (within 30 sec) into a longitudinal split, cylindrical flexible plastic mould (4 mm long x 5 mm internal diam.) to obtain a mass of uniform shape and size with excess kafirin material being removed from the ends of the mould using a spatula. The cylinder was then immediately transferred onto the base plate of a Shimadzu Scientific Instruments EZ-Test texture analyser (Kyoto, Japan), fitted with a 10 mm cylindrical probe for analysis. A single compression test was carried out with a test speed of 0.5 mm/s, distance 1 mm and a relaxation time of 100 s. The stress-relaxation properties of vital wheat gluten dough hydrated with distilled water (52% moisture dry powder basis) were determined for comparison. Tests were repeated at 5, 10 and 15 min on the same pieces.

The kafirin masses were then stored in a cold store at 10°C in sealed ziplock type plastic bags for 2 days for gluten and 8 or 16 days for kafirin and the tests repeated. The temperature of 10^{o}C was chosen as previous work had reported that kafirin masses became stiff over time at an ambient temperature 22^{o}C (Oom et al., 2008). The refrigeration temperature of 4^{o}C was not chosen as water has it maximum density at this temperature and it was feared this could influence the kafirin molecular conformation. Different storage times were used for logistical reasons and determine how long the kafirin viscoelastic masses would maintain their rheological properties. F Max (the maximum force at compression), Ft (the force at the time from F Max at which fresh gluten had relaxed to 38.6% of its maximum

force (11.6 seconds) and SR (% stress recovery at 11.6 seconds from F Max) were measured according to Singh, Rockall, Martin, Chung & Lookhart, 2006).

2.9. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was performed on dry and glacial acetic acid wetted kafirin preparations and coacervated kafirin viscoelastic masses to determine any changes in secondary structure. Samples were scanned using a Bruker Optik Vertex 70v FTIR spectrophotometer (Ettlingen, Germany) using 32 scans, 8 cm^{-1} bandwidth, and an interval of 1 cm^{-1} in the Attenuated Total Reflectance (ATR) mode (Anyango, Taylor & Taylor, 2013). The wavenumber range of 400−4000 cm^{-1} was used. The FTIR spectra were normalized and Fourier-deconvoluted using Lorentzian filter with a resolution enhancement factor of 2 and 6 cm^{-1} bandwidth.

2.10. Statistical analysis

Each experiment was repeated at least twice. Data were analysed by one-way analysis of variance (ANOVA). Significant differences among the means were determined by Fisher's Least Significance Difference test ($p < 0.05$ using IBM SPSS Statistics 22 (SPSS, Chicago, IL).

3. Results and discussion

3.1. SDS-PAGE characterization of the kafirins

SDS-PAGE of the kafirins extracted from the sorghum lines indicated that both the transgenic high protein digestibility lines (TG-HD1 and 2) were largely missing a band of apparent molecular weight ~23 kDa (black solid arrows) (Figure 1). It has previously been identified that this protein band is absent in these TG-HD lines (Da Silva, Taylor & Taylor, 2011b). The band represents the cysteine-rich γ-kafirin subclass and its absence is related to their high digestibility (Da Silva et al., 2011a). In contrast, the conventionally bred waxy high protein digestibility lines (WHD 1, 2 and 3) were largely missing a band of apparent molecular weight ~18.5 kDa (black dashed arrows), which with reference to the work of Elkhalifa, Georget, Barker & Belton (2009) can be identified as a β-kafirin, which is also cysteine-rich. This β-kafirin band was present in the kafirins extracted from all the other sorghum lines including the conventionally bred non-waxy high protein digestibility line (NHD1) and the transgenic high protein digestibility lines. The absence of a β-kafirin band is consistent with the observed down-regulation of some β-kafirin subclasses in these HD sorghum lines (Benmoussa et al., 2015).

3.2. Kafirin viscoelastic mass formation and structure

Visual observation and stereomicroscopy (Figure 2A) revealed that viscoelastic masses were successfully made from all the kafirin preparations, irrespective of their subclass composition. As mentioned under 2.3, upon addition of water the kafirin aggregated out of solution in the form of a fibrous net (Figure 1S). The net was comprised of hydrated fibrils. This material could simply be kneaded into a ball. The kafirin balls retained their flexibility for at least one hour at ambient temperature. By application of this coacervation process, kafirin alone, i.e. not as protein composite with gluten, could form a stable viscoelastic mass in a predominantly aqueous medium.

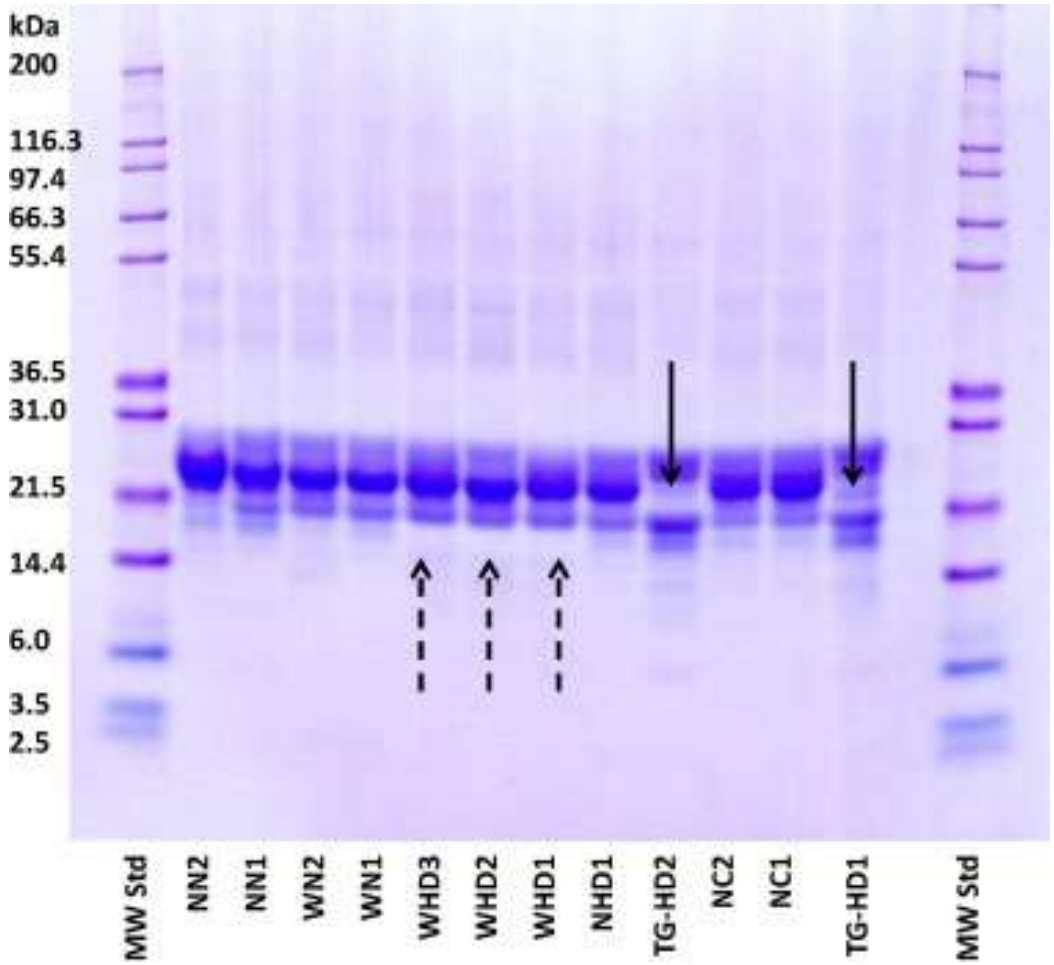


Fig. 1. SDS-PAGE under reducing conditions of the kafirins extracted from the different sorghum lines. NN1, NN2, WN1, WN2 – Conventionally bred normal starch- and waxy-normal protein digestibility lines; NHD1, WHD1, WHD2, WHD3 – Conventionally bred normal starch- and waxy-high digestibility lines; N1, N2, TG-HD1, TG-HD2 – Null controls and transgenic high protein digestibility lines.

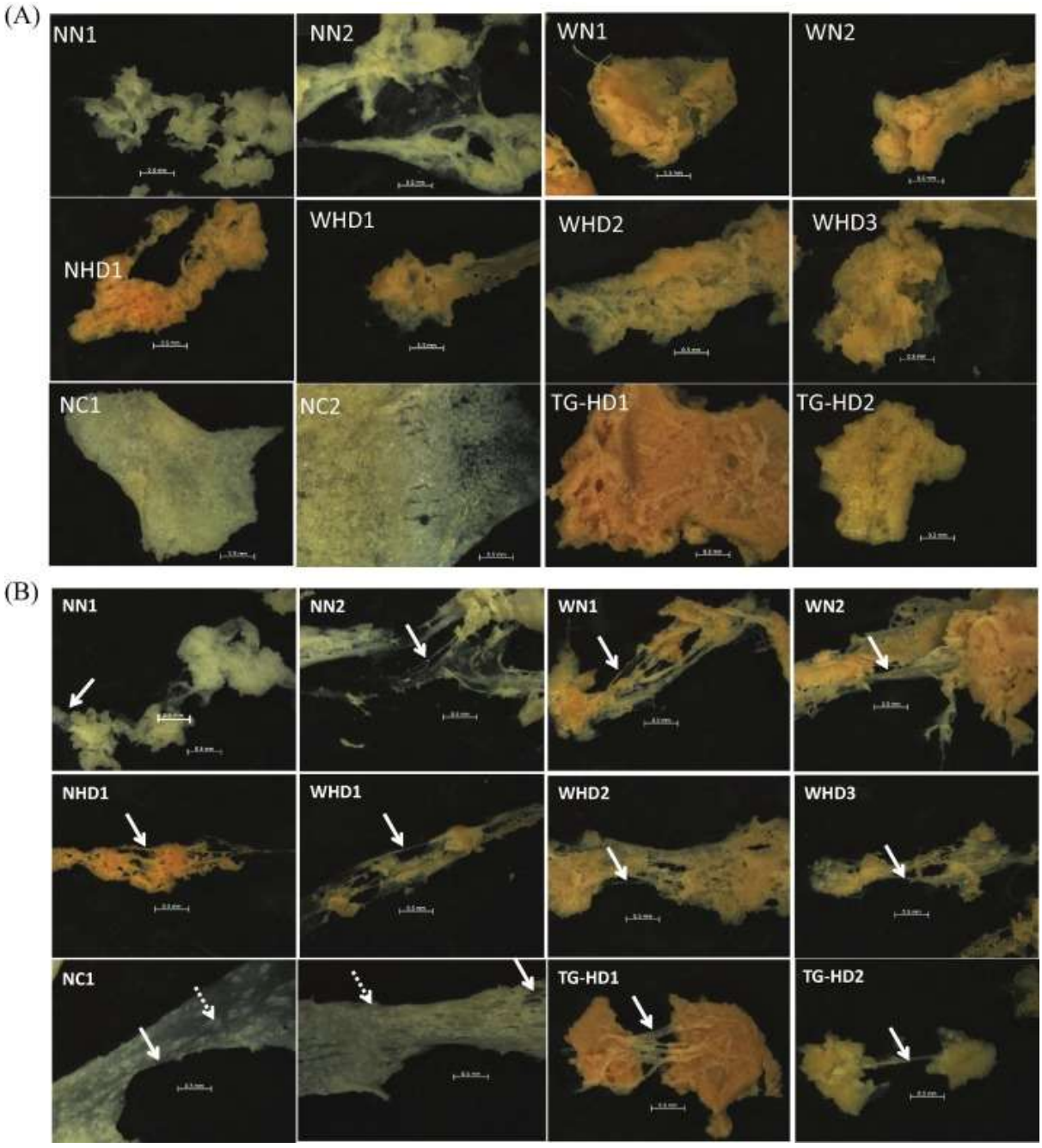


Fig. 2. Stereomicroscopy of the kafirin masses. NN1, NN2, WN1, WN2 – Conventionally bred normal starch- and waxy-normal protein digestibility lines; NHD1, WHD1, WHD2, WHD3 – Conventionally bred normal starch- and waxy-high digestibility lines; N1, N2, TG-HD1, TG-HD2 – Null controls and transgenic high protein digestibility lines. Solid arrows indicate fibrils, dotted arrows indicate kafirin inclusions. Bar = 0.5 mm.

When stretched, all the kafirin masses exhibited fibrils where they had extended under tension (indicated by white arrows) (Figure 2B). Strand formation as long been recognised as an important functional characteristic of wheat glutenin (Orth, Dronzek, & Bushuk, 1973). Furthermore, in the case of zein, the formation of the protein into strands (fibrils/fibres) has been proposed as a critical step in dough formation (Schober, Moreau, Bean & Boyle, 2010). There were some differences in the appearance of the stretched kafirin viscoelastic masses from the different sorghum types. Most notably, NC1 and NC2 (the TG null controls), prepared from kafirin containing all the subclasses, formed sheets containing spherical inclusions (indicated by indicated arrows), which presumably comprised aggregates of kafirin molecules.

The microstructure of stretched kafirin masses observed by CLSM (Figure 3) revealed the presence of the fibrils internally within the masses showing that fibrils were not just present on the surface. Zein viscoelastic masses formed in the presence of dilute acetic and lactic acids have also shown fibrils on the dough interior (Sly et al., 2014). The fibrils could in most cases be seen to be comprised of chains of small particles (dotted arrows). With zein, King et al. (2016) observed such particulate fibrils when a viscoelastic mass was prepared from "total" zein containing all the subclasses, in contrast to the fibrils in dough masses from commercial zein (predominantly α-zein) which were more uniform. With kafirin, similar chains of particles were observed during the formation of cast films from kafirin microparticles using acetic acid (Taylor, Taylor, Belton & Minnaar, 2009b). An analogy was drawn between these chains and the aggregative behaviour of wheat glutenin to form glutenin macropolymer particles (Don, Lichtendonk, Plijter & Hamer, 2003).

In addition, some of the kafirin viscoelastic masses, notably those from the normal sorghums (NN1 and NN2), contained large lumps (approx. 100 μm across) of kafirin (dashed

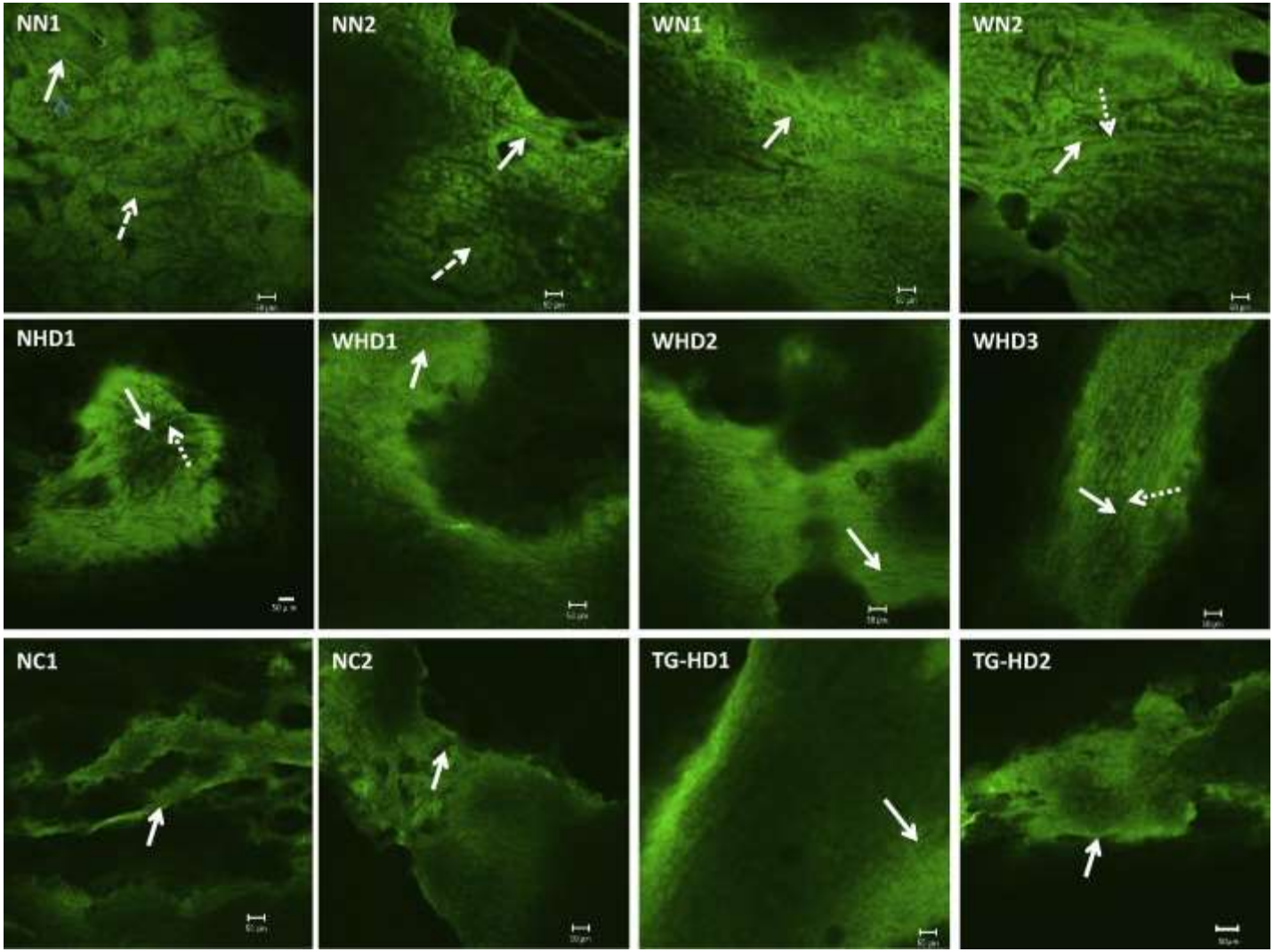


Fig. 3. CLSM of the kafirin masses. NN1, NN2, WN1, WN2 - Conventionally bred normal starch- and waxy-normal protein digestibility lines; NHD1, WHD1, WHD2, WHD3 – Conventionally bred normal starch- and waxy-high digestibility lines; N1, N2, TG-HD1, TG-HD2 – Null controls and transgenic high protein digestibility lines. Solid arrows indicate fibrils, dotted arrows indicate particles in fibrils, dashed arrows indicate lumps of kafirin. Bar = 50 µm.

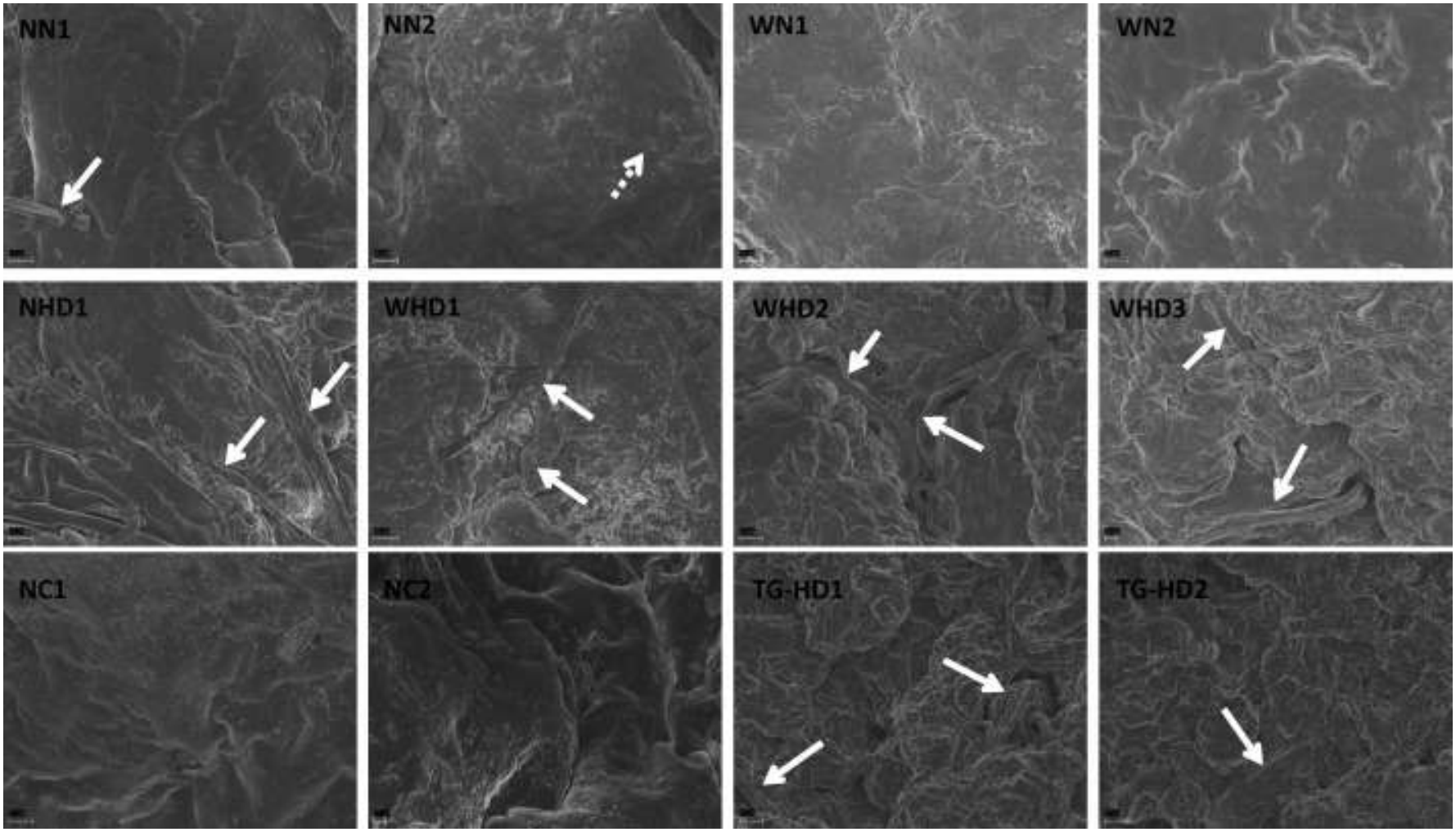


Fig. 4. SEM of the kafirin masses. NN1, NN2, WN1, WN2 – Conventionally bred normal starch- and waxy-normal protein digestibility lines; NHD1, WHD1, WHD2, WHD3 – Conventionally bred normal starch- and waxy-high digestibility lines; N1, N2, TG-HD1, TG-HD – Null controls and transgenic high protein digestibility lines. Solid arrows indicate broad ribbon-like fibrils, dotted arrow indicates fine fibril. Bar = 20 µm.

arrows) which were not in the form of fibrils (Figure 3). These lumps may represent kafirin which had not been properly hydrated during the coacervation process.

SEM of the kafirin viscoelastic masses, which provides a higher resolution image than CLSM but only of the surface structure, revealed clear differences in structure with respect to the kafirins from both the transgenic and conventionally bred high sorghums compared to their controls (Figure 4). The kafirin masses from the TG (TG-HD1 and 2) and conventionally bred HD sorghums (NHD1 and WHD1, 2 and 3) had broad and ribbon-like fibrils, up to 10 μm in diameter (solid arrows). Notably, with the exception of NHD1, all these sorghum lines were either lacking or largely lacking γ- or β-kafirin subclasses. The ribbon-like fibrils were very similar in appearance to the strands formed by isolated wheat glutenin which were observed by Orth et al. (1973) using SEM. In contrast, with the masses made from kafirins from the normal protein digestibility controls (NN1 and 2, WN1 and 2 and NC1 and 2) the fibrils were generally not evident on the surface of the mass, although the occasional fine fibril was observed (dotted arrow).

The rapid addition of water under low shear conditions to a solution of kafirin in glacial acetic acid was observed to be responsible for formation of fibrils and then formation of a viscoelastic mass rather than formation of microparticles as found by Taylor et al. (2009a). Controlled kafirin molecular aggregation and consequent precipitation of protein around a nucleation point is thought to be the mechanism of kafirin microparticle formation (Taylor, Anyango & Taylor, 2013). It is suggested that in this present work kafirin viscoelastic masses formed because the absence of high sheer enabled aggregation of the kafirin molecules into chains and subsequently into a network of hydrated insoluble kafirin fibrils. This is supported by the CLSM work which showed that many of the kafirin fibrils comprised chains of small particles (Figure 3), which were probably the microparticles described by Taylor et al. (2009a). This suggests that the fibrils were formed from strings of

joined microparticles, as observed during kafirin microparticle film formation (Taylor et al. 2009b). This is in keeping with the zein nanoscale self-assembly theory of Wang & Padua (2012) that spheres are the base of all other such protein microphases (nanostructures).

3.3. Rheological properties

The composition of the zein and kafirin in terms of the presence or absence particular subclasses has been proposed to play a key role in their aggregation behaviour (Schober et al., 2011). Using zein, these workers found that a preparation with less than 10% co-extracted cysteine-rich β- plus γ-zein (i.e. >90% α-zein) was needed in order to form a viscoelastic mass, indicating that disulphide bond formation was undesirable in dough formation.

In this present work, the properties of the kafirin masses were initially evaluated by hand stretching (Figure 2B). Nevertheless, it was evident that the masses from all the different kafirin preparations exhibited the critical dough rheological property of elasticity, irrespective of the presence of absence of particular cysteine-rich β- and γ-kafirins. The masses retained pliability and elasticity even after storage in a sealed ziplock-type bag at 10°C. This is probably as a result of the presence of acetic acid acting as a plasticizer (Sly et al., 2014).

Quantitative data were then obtained by stress-relaxation testing (Table 1). As observed visually, all the kafirin masses exhibited elasticity, regardless of their composition. With fresh masses (analysed at day 0), kafirin masses from sorghum lines WN1 and 2, NHD1 and WHD1, 2 and 3 had slightly lower or similar stress recovery to gluten (mean 43% recovery), indicating that they had a similar balance of viscous flow and elasticity to gluten. In contrast, NN1 and 2, NC1 and 2 and TG-HD1 and 2 exhibited significantly higher stress recovery ($p < 0.05$) than gluten, indicating that they were more elastic. NC1 and 2 and

Table 1
Stress-relaxation of gluten and kafirin from different sorghum lines on day 0 and after storage at 10°C for either 2, 8 or 16 days.

Sorghum line	**Repeated stress-relaxation testing**	**F Max (N)**		**Ft (N)**		**%SR**	
Gluten Standard		Day 0	After 2 days	Day 0	After 2 days	Day 0	After 2 days
	1st test	0.828 ± 0.216	0.724 ± 0.159	0.305 ± 0.080	0.233 ± 0.043	36.8 ± 0.0	32.4 ± 2.3
	Repeat 5 min	1.931 ± 0.239	1.844 ± 0.216	0.826 ± 0.093	0.668 ± 0.123	42.8 ± 0.9	36.1 ± 4.0
	Repeat 10 min	2.980 ± 0.031	2.990 ± 0.162	1.348 ± 0.223	1.167 ± 0.177	45.0 ± 3.1	38.9 ± 4.4
	Repeat 15 min	3.538 ± 0.373	3.747 ± 0.105	1.695 ± 0.200	2.110 ± 0.022	47.9 ± 3.0	42.2 ± 4.7
Mean						**43.1B[a]a ± 4.7**	**37.4Aa[b] ± 4.2**
NN1		Day 0	After 8 days	Day 0	After 8 days	Day 0	After 8 days
	1st test	0.041 ± 0.003	0.704 ± 0.052	0.025 ± 0.001	0.547 ± 0.030	59.8 ± 2.4	77.7 ± 1.4
	Repeat 5 min	0.069 ± 0.013	0.760 ± 0.026	0.042 ± 0.004	0.580 ± 0.033	60.7 ± 6.1	76.3 ± 1.7
	Repeat 10 min	0.095 ± 0.004	0.842 ± 0.012	0.058 ± 0.010	0.631 ± 0.006	61.6 ± 12.7	75.0 ± 0.4
	Repeat 15 min	0.135 ± 0.028	0.878 ± 0.013	0.083 ± 0.001	0.650 ± 0.012	62.9 ± 11.9	74.0 ± 0.2
Mean						**61.3Ca ± 1.3**	**75.8Fb ± 1.6**
NN2		Day 0	After 8 days	Day 0	After 8 days	Day 0	After 8 days
	1st test	0.037 ± 0.010	0.628 ± 0.004	0.028 ± 0.006	0.518 ± 0.003	74.7 ± 2.8	82.5 ± 1.0
	Repeat 5 min	0.076 ± 0.010	0.671 ± 0.035	0.053 ± 0.007	0.538 ± 0.025	69.7 ± 0.2	80.2 ± 0.5
	Repeat 10 min	0.098 ± 0.015	0.712 ± 0.048	0.067 ± 0.013	0.568 ± 0.054	67.9 ± 3.4	79.7 ± 2.2
	Repeat 15 min	0.117 ± 0.015	0.757 ± 0.039	0.085 ± 0.012	0.598 ± 0.045	72.5 ± 1.1	78.9 ± 1.8
Mean						**71.2Da ± 3.0**	**80.3Gb ± 1.5**
WN1		Day 0	After 8 days	Day 0	After 8 days	Day 0	After 8 days
	1st test	0.046 ± 0.002	0.939 ± 0.018	0.015 ± 0.001	0.496 ± 0.003	32.9 ± 1.6	52.9± 1.3
	Repeat 5 min	0.075 ± 0.006	1.065 ± 0.054	0.022 ± 0.005	0.533 ± 0.039	28.7 ± 4.2	50.0 ± 1.1
	Repeat 10 min	0.096 ± 0.014	1.134 ± 0.028	0.038 ± 0.012	0.577 ± 0.030	38.6 ± 6.8	50.8 ± 1.4
	Repeat 15 min	0.143 ± 0.59	1.224 ± 0.025	0.051 ± 0.023	0.626 ± 0.030	35.5 ± 1.2	51.2 ± 3.5
Mean						**33.9Aa ± 4.2**	**51.2Bb ± 1.2**

WN2		Day 0	After 8 days	Day 0	After 8 days	Day 0	After 8 days
	1st test	0.059 ± 0.007	0.847 ± 0.157	0.015 ± 0.002	0.515 ± 0.150	24.5 ± 0.7	60.2 ± 6.5
	Repeat 5 min	0.105 ± 0.001	1.116 ± 0.021	0.032 ± 0.001	0.646 ± 0.151	30.5 ± 0.9	57.7 ± 12.4
	Repeat 10 min	0.140 ± 0.006	1.256 ± 0.046	0.050 ± 0.007	0.696 ± 0.140	36.0 ± 6.7	52.3 ± 9.1
	Repeat 15 min	0.218 ± 0.020	1.379 ± 0.063	0.075 ± 0.014	0.774 ± 0.094	34.3 ± 3.4	56.3 ±9.4
Mean						**31.3Aa ± 5.1**	**56.6Cb ± 3.3**
NHD1		Day 0	After 8 days	Day 0	After 8 days	Day 0	After 8 days
	1st test	0.081 ± 0.022	3.069 ± 0.069	0.022 ± 0.002	2.186 ± 0.142	27.4 ± 4.8	71.2 ± 3.0
	Repeat 5 min	0.250 ± 0.013	3.679 ± 0.025	0.094 ± 0.013	2.527 ± 0.047	37.3 ± 3.4	68.7 ± 0.8
	Repeat 10 min	0.308 ± 0.009	3.858 ± 0.070	0.117 ± 0.012	2.589 ± 0.098	37.9 ± 5.0	67.1 ± 1.3
	Repeat 15 min	0.460 ± 0.040	3.958 ± 0.122	0.184 ± 0.005	2.749 ± 0.028	40.1 ± 4.6	69.5 ± 1.5
Mean						**35.7Aa ± 5.6**	**69.1Eb ± 1.7**
WHD1		Day 0	After 8 days	Day 0	After 8 days	Day 0	After 8 days
	1st test	0.069 ± 0.000	0.791 ± 0.025	0.032 ± 0.001	0.554 ± 0.035	45.7 ± 1.0	69.9 ± 2.1
	Repeat 5 min	0.119 ± 0.021	0.938 ± 0.073	0.046 ± 0.006	0.644 ± 0.028	39.0 ± 2.0	68.8 ± 2.32.4
	Repeat 10 min	0.178 ± 0.023	1.108 ± 0.035	0.072 ± 0.003	0.750 ± 0.060	40.8 ± 3.8	67.6 ± 3.3
	Repeat 15 min	0.224 ± 0.014	1.209 ± 0.070	0.100 ± 0.002	0.833 ± 0.091	44.5 ± 3.6	68.8 ± 3.6
Mean						**45.2Ba ± 3.1**	**68.8Eb ± 0.9**
WHD2		Day 0	After 16 days	Day 0	After 16 days	Day 0	After 16 days
	1st test	0.057 ± 0.001	1.002 ± 0.008	0.018 ± 0.000	0.632 ± 0.007	31.9 ± 0.4	63.1 ± 1.2
	Repeat 5 min	0.104 ± 0.003	1.134 ± 0.030	0.040 ± 0.005	0.713 ± 0.016	38.1 ± 5.8	62.8 ± 0.2
	Repeat 10 min	0.145 ± 0.004	1.185 ± 0.048	0.055 ± 0.006	0.743 ± 0.016	38.0 ± 3.0	62.7 ± 1.2
	Repeat 15 min	0.173 ± 0.007	1.220 ± 0.008	0.071 ± 0.008	0.770 ± 0.016	40.7 ± 2.8	63.1 ± 1.8
Mean						**37.2ABa ± 3.7**	**62.9Db ± 0.2**
WHD3		Day 0	After 16 days	Day 0	After 16 days	Day 0	After 16 days
	1st test	0.034 ±0.006	0.185 ± 0.011	0.008 ± 0.003	0.161 ± 0.004	23.5 ± 4.0	87.3 ± 2.7
	Repeat 5 min	0.061 ± 0.004	0.207 ± 0.002	0.021 ± 0.004	0.173 ± 0.004	33.8 ± 3.9	83.8 ± 1.2
	Repeat 10 min	0.070 ± 0.007	0.227 ± 0.011	0.023 ± 0.001	0.195 ± 0.011	32.4 ± 4.3	85.7 ± 0.4
	Repeat 15 min	0.091 ± 0.009	0.245 ± 0.008	0.031 ± 0.001	0.194 ± 0.025	33.8 ± 2.7	78.9 ± 7.3
Mean						**30.9Aa ± 5.0**	**83.9Hb ± 3.6**

NC1		Day 0	After 16 days	Day 0	After 16 days	Day 0	After 16 days
	1st test	0.036 ± 0.002	0.330[3] ± 0.006	0.027 ± 0.001	0.272 ± 0.005	74.7 ± 2.5	82.3 ± 2.9
	Repeat 5 min	0.069 ± 0.012	0.362 ± 0.006	0.054 ± 0.011	0.298 ± 0.000	77.9 ± 1.8	82.4 ± 1.5
	Repeat 10 min	0.082 ± 0.003	0.375 ± 0.007	0.065 ± 0.006	0.313 ± 0.023	79.2 ± 4.2	83.4 ± 4.5
	Repeat 15 min	0.103 ± 0.008	0.435 ± 0.024	0.077 ± 0.005	0.368 ± 0.048	74.3 ± 1.3	84.4 ± 6.4
Mean						**76.5DEa ± 2.4**	**83.1GHa ± 1.0**
NC2		Day 0	After16 days	Day 0	After 16 days	Day 0	After 16 days
	1st test	0.038 ± 0.008	0.271 ± 0.032	0.024 ± 0.006	0.231 ± 0.046	63.8 ± 1.9	84.8 ± 7.0
	Repeat 5 min	0.071 ± 0.008	0.333 ± 0.004	0.056 ± 0.008	0.271 ± 0.017	78.6 ± 2.4	81.5 ± 4.2
	Repeat 10 min	0.089 ± 0.006	0.366 ± 0.023	0.074 ± 0.009	0.298 ± 0.023	82.9 ± 4.4	81.2 ± 1.4
	Repeat 15 min	0.106 ± 0.006	0.386 ± 0.001	0.088 ± 0.006	0.309 ± 0.002	83.0 ± 0.9	79.9 ± 0.3
Mean						**77.1DEa ± 9.1**	**81.9GHa ± 2.1**
TG-HD1		Day 0	After 8 days	Day 0	After 8 days	Day 0	After 8 days
	1st test	0.071 ± 0.007	0.394 ± 0.001	0.059 ± 0.006	0.315 ± 0.010	82.4 ± 0.8	80.0 ± 2.4
	Repeat 5 min	0.087 ± 0.009	0.438 ± 0.005	0.071 ± 0.009	0.354 ± 0.009	81.4 ± 0.1	80.8 ± 3.0
	Repeat 10 min	0.114 ± 0.001	0.456 ± 0.006	0.093 ± 0.004	0.368 ± 0.011	81.5 ± 3.6	80.6 ± 1.3
	Repeat 15 min	0.120 ± 0.002	0.483 ± 0.009	0.095 ± 0.004	0.394 ± 0.005	79.5 ± 2.1	81.6 ± 0.5
Mean						**81.2Ea ± 1.2**	**80.8Ga ± 0.7**
TG-HD2		Day 0	After 16 days	Day 0	After 16 days	Day 0	After 16 days
	1st test	0.073 ± 0.005	0.553[3] ± 0.028	0.054 ± 0.005	0.456 ± 0.026	73.7 ± 1.8	82.0 ± 0.5
	Repeat 5 min	0.107 ± 0.001	0.665 ± 0.009	0.074 ± 0.004	0.542 ± 0.018	69.1 ± 3.1	81.5 ± 1.5
	Repeat 10 min	0.123 ± 0.004	0.705 ± 0.011	0.091 ± 0.003	0.582 ± 0.035	74.4 ± 4.5	82.5 ± 3.7
	Repeat 15 min	0.155 ± 0.006	0.746 ± 0.024	0.115 ± 0.003	0.616 ± 0.053	74.2 ± 0.9	82.4 ± 4.5
Mean						**72.9Da ± 2.5**	**82.1GHb ± 0.5**

F Max. = Maximum force
Ft = Force at time at which fresh gluten had relaxed to 38.6% of its maximum force (11.6 seconds after maximum force)
SR = % stress recovery at 11.6 seconds from F Max
[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 ($p<0.05$)

presumably NN1 and 2 masses contained the γ-kafirin subclass, whereas TG-HD1 and 2 did not. This implies that the presence or reduced expression of this γ-kafirin does not greatly influence kafirin elastic behaviour. With masses that had been stored, all the kafirin masses had significantly higher stress recovery than gluten, i.e. they showed greater elastic behaviour with TG-HD1 (deficient in γ-kafirin) and WHD1 (deficient in β-kafirin). This indicates that the presence or reduced expression of either of these cysteine-rich kafirin subclasses although it affects the appearance of the masses (Figure 2), does not greatly influence elastic behaviour. However, the reason for the increase in elasticity of the kafirin masses during storage is needs to be established.

All the stored kafirin masses exhibited a high degree of elastic behaviour, similar to that observed with gelatine gel (Singh et al., 2006), a food material with characteristic high elasticity. This is contrast to masses made from commercial zein (predominantly α-zein) which show predominantly viscous flow properties, especially when prepared in dilute organic acids (Sly et al., 2014). The rheological behaviour the kafirin masses was, however, more similar to that of total zein (comprising α-, β-, δ- and γ-zein) masses (King et al., 2016), which was also prepared by first dissolving the protein in glacial acid.

All the kafirin masses became progressively firmer with repeated stress-relaxation testing, like the gluten (Table 1). However, all the kafirin masses became firmer on storage for either 8 or 16 days (showing a several-fold increase in F Max and Ft), apparently unlike the gluten which recovered its initial softness when stored for 2 days (similar F max and Ft at 2 days storage as at day 0). Notwithstanding them becoming firmer on storage, the kafirin masses maintained or increased their elastic behaviour, as stated above.

Thus, the visual differences between the masses made from kafirins with different compositions (Figure 2) were not reflected in their stress-relaxation behaviour (Table 1). However, a grouping was evident. Those from the normal sorghum varieties, which had

kafirin with all the subclasses (NN1 and 2) and the TG without γ-kafirin but with β-kafirin (TG-HD1 and 2) and their null controls with all kafirin subclasses (NC1 and 2) showed high elasticity initially, with stress recovery values in the range 61.6-81.2% (Table 1). In contrast, the waxy-normal protein digestibility (WN1 and 2) and the waxy-high protein digestibility (NHD1) and the waxy-high protein digestibility (WHD1, 2 and 3) exhibited much lower elasticity initially, with stress recovery values in the range 30.9-45.2%. On storage, however, the percent stress recovery of these sorghums increased considerably to 51.2-83.9%. This difference in elastic behaviour between these groups is in part a result of their different genetic background. The transgenic sorghums and their null controls were from a different line P898012 x Macia (Da Silva et al., 2011a) than the conventionally bred high protein digestibility sorghum and their controls (RTx2907 x P850029).

3.4. Kafirin and kafirin mass secondary structure

As shown, viscoelastic masses can be produced from kafirin by dissolving the protein in glacial acetic acid before coacervating it from solution using water. It was expected that this treatment would result in conformational changes in the proteins.

FTIR Amide I region of the dry kafirin preparations did not show any substantial differences in band pattern between the different kafirin preparations regardless of their composition in terms of kafirin subclasses (Figure 2S A). The WHD2 (deficient in β-kafirin) and WN1 (containing all kafirin subclasses) kafirin preparations had the highest α-helical content of 74.2 and 67.3%, respectively (Table 2). With the exception of these two kafirin preparations, the range of relative α-helical conformation for the preparations was 48.4-69.1%. This is general agreement with recent FTIR data on the secondary structure of dry kafirin where it was found that the most predominant bands originated from α-helical structures and that by calculation there was 49% α-helical structure with much smaller

Table 2

Calculated ratio of α-helical to β-sheet conformation and relative α-helical conformation of the different kafirin preparations in dry form, kafirin wetted with glacial acetic acid and in viscoelastic mass form.

Sorghum line	Dry kafirin preparation		Dry kafirin preparation wetted with glacial acetic acid		Kafirin viscoelastic mass	
	α/β ratio	Relative α-helical conformation (%)	α/β ratio	Relative α-helical conformation (%)	α/β ratio	Relative α-helical conformation (%)
NN1	1.63^{a} ±0.10	61.9	2.51^{b} ±0.05	71.5	1.41^{a} ±0.04	58.5
NN2	1.57^{b} ±0.10	61.0	2.27^{c} ±0.01	69.4	1.30^{a} ±0.04	56.4
WN1	2.07^{b} ±0.15	67.3	2.27^{b}±0.01	69.4	1.36^{a} ±0.01	57.6
WN1	1.06^{a} ±0.01	51.6	2.16^{c}±0.01	68.4	1.10^{b} ±0.01	52.3
NHD1	1.14^{a} ±0.01	53.3	2.49^{c} ±0.01	71.4	1.26^{b} ±0.01	54.2
WHD1	1.45^{a} ±0.02	59.2	2.42^{b} ±0.03	70.7	1.27^{a} ±0.11	55.9
WHD2	2.89^{b} ±0.22	74.2	3.01^{b} ±0.09	75.1	1.37^{a} ±0.05	57.7
WHD3	1.45^{a} ±0.05	59.2	2.31^{b} ±0.01	69.8	1.47^{a} ±0.05	59.5
NC1	1.34^{b} ±0.02	57.3	2.67^{c} ±0.07	72.8	1.11^{a} ±0.06	52.6
NC2	1.17^{a} ±0.01	53.9	2.82^{b} ±0.16	73.8	1.18^{a} ±0.01	54.2
TG-HD1	1.27^{a} ±0.04	55.9	3.02^{b} ±0.11	75.1	1.13^{a} ±0.01	53.0
TG-HD2	0.94^{a} ±0.01	48.4	2.55^{c} ±0.06	71.8	1.27^{b} ± 0.06	55.9

Means with different superscript letters within a row are significantly different ($p < 0.05$). n=3
Wavenumber ($cm^{-1)}$ of α-helix for all samples was 1650 ±2 and for β-sheet was 1620 ±2.

components of β-turn and β-sheet structures (Xiao, Li, Li, Gonzalez, Xia & Huang, 2015). When a small quantity of glacial acetic acid was added to these dry kafirin preparations a cohesive substance was formed in all cases. The proportion of α-helical conformation was increased ($p < 0.05$) very substantially with a concomitant reduction in β-sheet conformation in all the lines except for WHD2 and WN1, i.e. the lines which had a high α-helical conformation in the dry form (Figure 2S B). The α/β conformation ratio of these cohesive substances in glacial acetic acid was high, in the range 2.16-3.02:1 (Table 2). However, the proportion of α-helical conformation in all the coacervated viscoelastic masses was much lower than in the cohesive substances and was generally similar to that of the dry kafirin preparations, i.e. still predominantly α-helical (Figure 2S C). On coacervation, the proportion of α-helical conformation of the WHD2 and WN1 masses was similar to all the others (Table 2). For the masses, the α/β conformation ratio was in the range 1.11-1.47:1, which is in the same range of as for dry kafirin microparticles produced by coacervation from a kafirin solution in glacial acetic acid of 1.3:1 (Taylor et al., 2009a) and 1.2:1 (Anyango et al., 2013) as similarly measured by FTIR.

Findings on the role of protein secondary structure in zein dough functionality are apparently somewhat contradictory. On the basis research into zein dough behaviour under shear stress when on its own and in combination with co-proteins, it has been proposed that a β-sheet conformation is critical for zein viscoelastic functionality (Erickson et al., 2012). However, in agreement with this present work, Sly et al. (2014) found that viscoelastic masses made from commercial zein and prepared in dilute organic acids had a predominantly α-helical conformation and that its proportion increased with acid concentration. King et al. (2016) also found that total zein viscoelastic mass in the presence of dilute acetic acid was predominantly α-helical in conformation. These authors additionally found that total zein in distilled water exhibited a predominantly β-sheet conformation but did not form a

viscoelastic masses, indicating that this conformation does not guarantee zein dough formation.

In the present work with kafirin, it is suggested that dissolving the protein in glacial acetic acid resulted in disassociation of the molecules from each other and enabled them to assume a majorly ordered α-helical structure. It is proposed that in the coacervation process when water was added rapidly with low shear, the existence of separated kafirin molecules with an ordered structure resulted in the formation of fibrils. These could then be kneaded into a mass which had viscoelastic properties. This is analogous to the "loop and train" model for glutenin viscoelasticity proposed by Belton (1999).

4. Conclusions

Stable viscoelastic masses can be formed from kafirin when it is dissolved in glacial acetic acid and the protein precipitated by rapid coacervation with cool water under low shear. It is proposed that dissociation of the kafirin molecules and them assuming a majorly α-helical conformation enables the molecules to bind with water to form a viscoelastic masses. The presence or reduced expression of the cysteine-rich β- and γ-kafirin subclass in the kafirin influences dough appearance but does not fundamentally affect stress-relaxation behaviour. This indicates that the degree of kafirin polymerization does not affect its ability to form a viscoelastic mass. The kafirin masses can maintain functionality for at least 16 days when stored at 10^{o}C, well below the T_g of water hydrated kafirin, probably due to plasticisation by the presence of acetic acid.

Acknowledgements

The Howard G. Buffett Foundation through the Norman E. Borlaug Institute for International Agriculture of Texas A&M University. The Bill and Melinda Gates Grand Challenges 9, Africa Biofortified Sorghum Project through a sub-grant from the Africa Harvest Biotechnology Foundation International. The University of Pretoria for a Research Support Bursary for MSM Elhassan. Alan Hall and Antoinette Buys of the Laboratory for Microscopy and Microanalysis for assistance with the microscopy.

References

Anyango , J. O., Taylor, J. R .N., & Taylor, J. (2013). Role of γ-kafirin in the formation and organization of kafirin microstructures. *Journal of Agricultural and Food Chemistry, 61,* 10757-10765.

Belton, P .S. (1999). Mini review: On the elasticity of wheat gluten. *Journal of Cereal Science, 29,* 103–107.

Benmoussa, M., Chandrashekar, A., Ejeta, G., & Hamaker, B. R. (2015). Cellular response to the high protein digestibility/high-lysine (*hdhl*) sorghum mutation. *Plant Science, 241,* 70-77.

Da Silva, L. S., Jung, R., Zhao, Z., Glassman, K., Grootboom, A. W., Mehlo, L., O'Kennedy, M. M., Taylor, J., & Taylor, J. R. N. (2011a). Effect of suppressing the synthesis of different kafirin sub-classes on grain endosperm texture, protein body structure and protein nutritional quality in improved sorghum lines. *Journal of Cereal Science, 54,* 160-167.

Da Silva, L. S., Taylor, J., & Taylor, J. R. N. (2011b). Transgenic sorghum with altered kafirin synthesis: kafirin solubility, polymerization, and protein digestion. *Journal of Agricultural and Food Chemistry, 59,* 9265–9270.

Don, C., Lichtendonk, W., Plijter, J. J., Hamer, R.J. (2003). Gluten macropolymer: a gel formed by glutenin particles. *Journal of Cereal Science, 37*, 1–7.

Elhassan, M. S., Emmambux, M. N., Hays, D.B., Peterson, G. C., & Taylor, J. R. N. (2015). Novel biofortified sorghum lines with combined waxy (high amylopectin) starch and high protein digestibility traits: Effects on endosperm and flour properties. *Journal of Cereal Science*, *65*, 132-139.

Elkhalifa, A. E. O., Georget, D. M., Barker, S. A., & Belton, P. S. (2009). Study of the physical properties of kafirin during the fabrication of tablets for pharmaceutical applications. *Journal of Cereal Science,* 50, 159-165.

Erickson, D. P., Campanella, O. H., & Hamaker, B. R. (2012). Functionalizing maize zein in viscoelastic dough systems through fibrous, β-sheet-rich protein networks: An alternative, physicochemical approach to gluten-free breadmaking. *Trends in Food Science & Technology*, *24*, 74-81.

Hadebe, S. T., Modi, A. T., & Mabhaudhi, T. (2016). Drought tolerance and water use of cereal crops: A focus on sorghum as a food security crop in sub-Saharan Africa. *Journal of Agronomy and Crop Science*, DOI: 10.1111/jac.12191.

FAO. (2014). FAO Statistical Yearbook 2014 Africa Food and Agriculture. www.fao.org/ Accessed 01/11/16.

Goodall, M. A., Campanella, O. H., Ejeta, G., & Hamaker, B. R. (2012). Grain of high digestible, high lysine (HDHL) sorghum contains kafirins which enhance the protein network of composite dough and bread. *Journal of Cereal Science*, 56, 352-357.

King, B. L. (2016. Formation of a viscoelastic dough from total zein (α-, β- and γ-zein) isolated from white maize. MSc Dissertation, University of Pretoria, Pretoria.

King, B. L., Taylor, J., & Taylor, J. R. N. (2016). Formation of a viscoelastic dough from isolated total zein (α-, β- and γ-zein) using a glacial acetic acid treatment. *Journal of Cereal Science, 71*, 250-257.

Lai, H.-M., & Padua, G. W. (1997). Properties and microstructure of plasticized zein films. *Cereal Chemistry 74*, 771-775.

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

Oria, M. P., Hamaker, B. R., Axtell, J. D., & Huang, C. P. (2000). A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. *Proceedings of the National Academy of Sciences, USA, 97*, 5065-5070.

Orth, R. A., Dronzek, B. L., & Bushuk, W. (1973). Studies of glutenin. IV. Microscopic structure and its relations to breadmaking quality. *Cereal Chemistry, 50*, 688-696.

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

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

Singh, H., Rockall, A., Martin, C. R., Chung, O. K., & Lookhart, G. L. (2006). The analysis of stress relaxation data of some viscoelastic foods using a texture analyzer. *Journal of Textural Studies 37*, 383-392.

Sly, A. C. (2014). Improvement of zein dough characteristics using dilute organic acids. MSc Dissertation, University of Pretoria, Pretoria.

Sly, A. C., Taylor, J., & Taylor, J. R. N. (2014). Improvement of zein dough characteristics using dilute organic acids. *Journal of Cereal Science, 60,* 157-163.

Taylor, J., & Taylor, J. R. N. (2010). Patent Cooperation Treaty Application 2010. WO 2010/041203 A1 Process for Producing Protein Microparticles.

Taylor, J., Anyango, A. O., & Taylor, J. R. N. (2013). Developments in the science of zein, kafirin and gluten protein bio-plastic materials. *Cereal Chemistry, 90,* 344-357.

Taylor, J., Taylor, J. R. N., Dutton, M .F., & De Kock, S. (2005). Glacial acetic acid - A novel food-compatible solvent for kafirin. *Cereal Chemistry, 82,* 485-487.

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

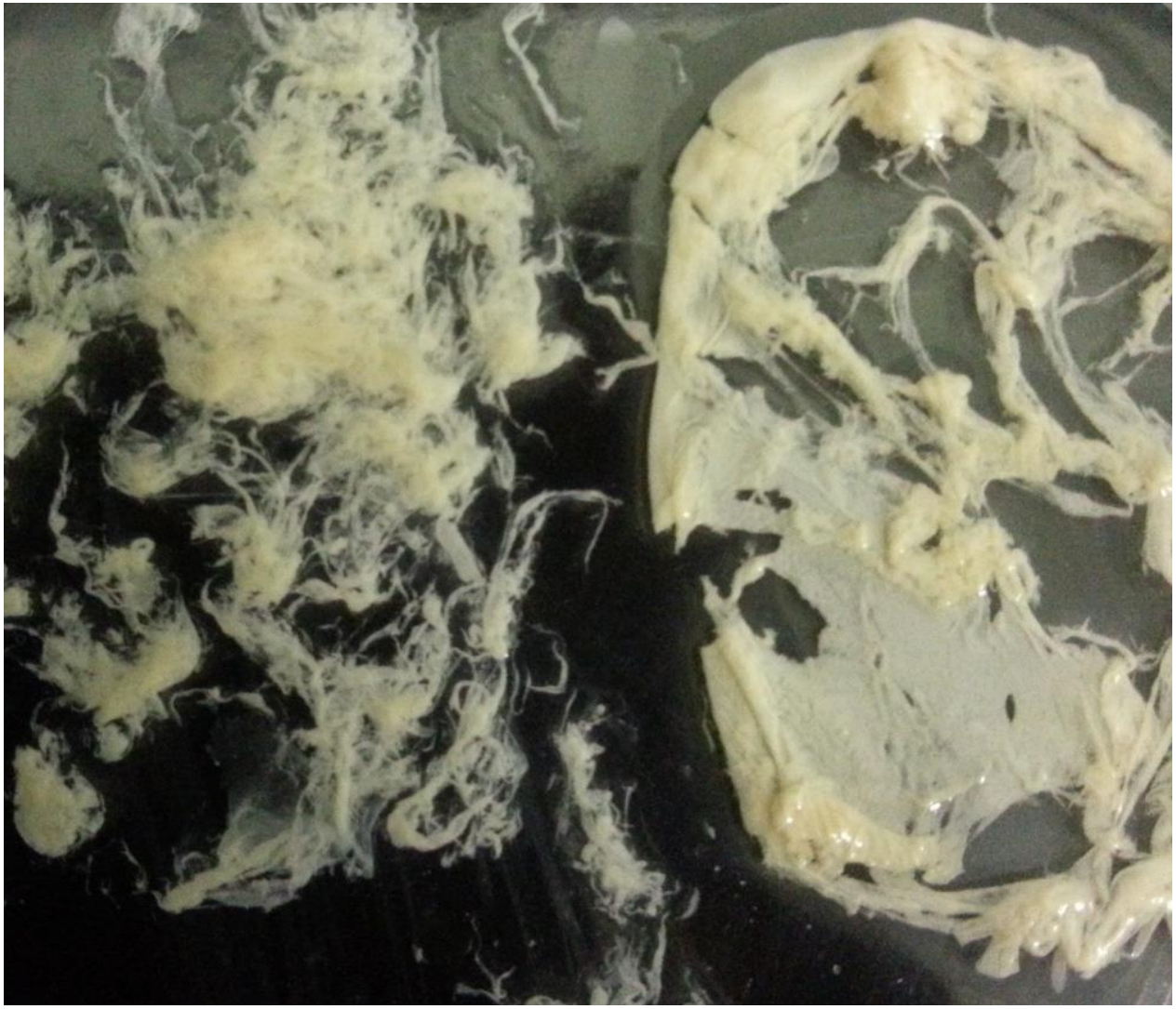
Taylor, J., Taylor, J. R. N., Belton, P. S., & Minnaar, A. (2009b). Preparation of free-standing films from kafirin protein microparticles: mechanism of formation and functional properties. *Journal of Agricultural and Food Chemistry,* 57, 6729-6735.

Taylor, J. R. N., Taylor J., Campanella, O. H., & Hamaker, B. R. (2016). Functionality of the storage proteins in gluten-free cereals and pseudocereals in dough systems. *Journal of Cereal Science,* 67:22-34.

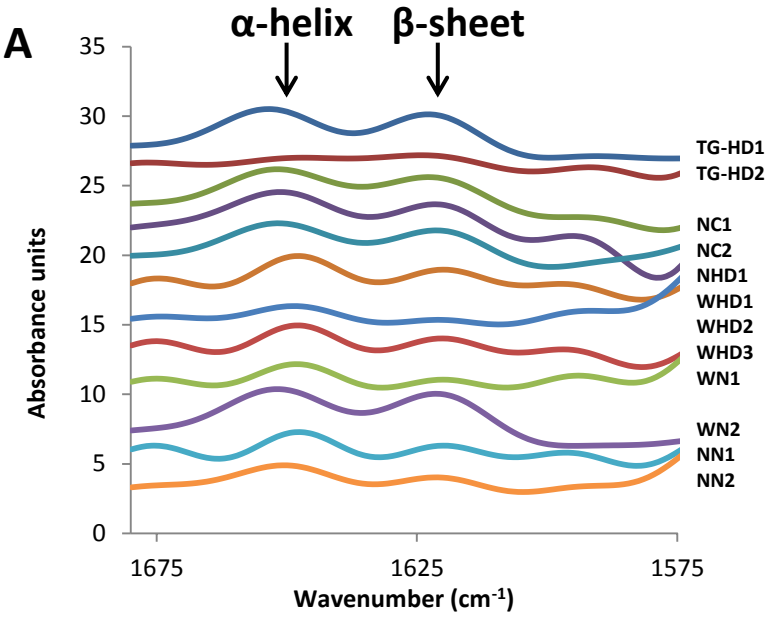
Wang, Y., & Padua, G. W. (2012). Nanoscale characterization of zein self-assembly. *Langmuir, 28,* 2429−2435.

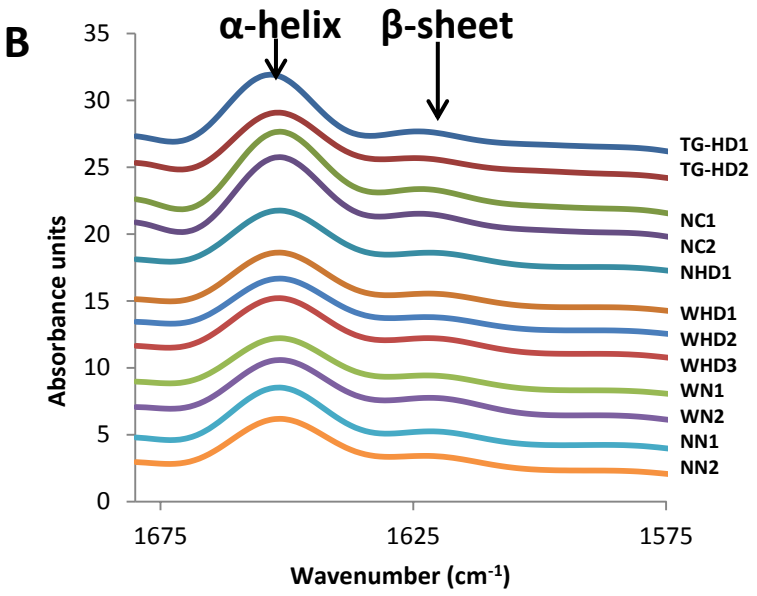
Xi, J., Li, Y., Gonzalez, A. P., Xia, Q., & Huang, Q. (2015). Structure, morphology and assembly behaviour of kafirin. *Journal of Agricultural and Food Chemistry, 63,* 216-224.

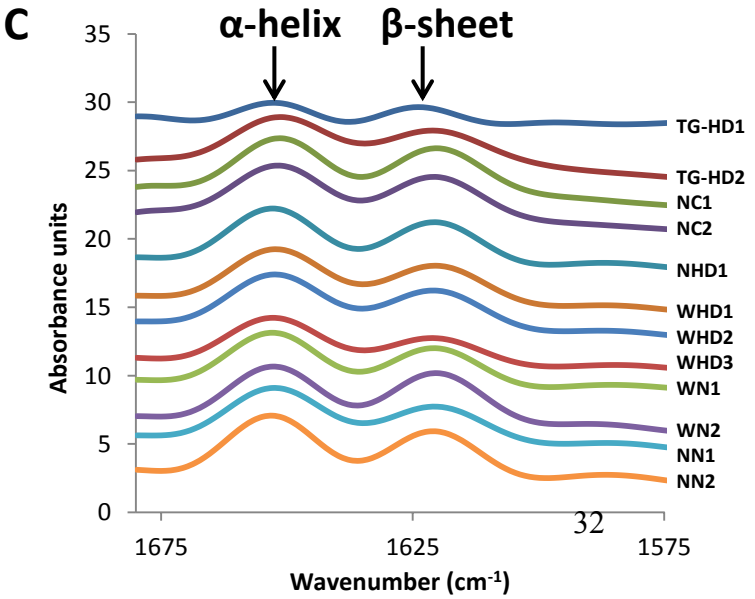
Appendix A. Supplementary data



Supplementary Fig. 1S. Formation of kafirin net of hydrated fibrils.







Supplementary Fig. 2S. FTIR of kafirin preparations and masses. A – Dry kafirin preparations; B – Kafirin preparations wetted with glacial acetic acid; C – Kafirin masses.