Modification of zein dough functionality using kafirin as a coprotein

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ABSTRACT

Kafirin, sorghum prolamin, was investigated as a coprotein for zein as visco-elastic masses and in starch-based model doughs. Regular kafirin and kafirins from waxy and high protein digestibility (HD) sorghum crosses were studied. HPLC revealed that waxy-HD kafirin was of smaller molecular size and low in β-kafirin. It also had greater surface hydrophobicity. Kafirin addition to zein increased visco-elastic mass elasticity up to ≈50% stress-recovery, similar to wheat gluten. Waxy-HD kafirin gave the highest elasticity, possibly due to its hydrophobicity. Kafirin inclusion at 2:8 parts zein increased the tensile strength of model doughs. Maximum strength was, however, only 60% that of gluten-based dough. Kafirin from regular sorghum gave the highest strength, possibly because of greater disulphide-bonded polymerisation. Confocal laser scanning microscopy showed that zein-kafirin copolymers formed fairly linear fibrils in stretched doughs, indicating excellent compatibility between the proteins. Future research should establish how kafirin-zein copolymer performs in non-wheat flour products.

Abbreviations:

CLSM, Confocal laser scanning microscopy; F Max, Maximum force at compression; Ft, 36.8% of maximum force at compression; HD, High protein digestibility; NN, Non-waxy-normal digestibility; RK, Regular kafirin; RP, Reversed phase; SDS, Sodium dodecyl sulphate; SE, Size exclusion; SEM, Scanning electron microscopy; SR, Stress elastic recovery; TZ, Total zein; WHD, waxy, high protein digestibility; WND, waxy, normal protein digestibility; Z, Commercial zein

1. Introduction

The finding by Lawton (1992) that zein, the maize prolamin storage protein, can exhibit wheat gluten-like visco-elastic functionality opened up the potential for the use of zein in dough-based products such as gluten-free and non-wheat bread and pasta (Jeong et al., 2017; Khuzwayo et al., 2020). However, progress towards this objective has been very slow. There are several reasons for this, notably the relatively high temperature required for hydrated zein to behave as a visco-elastic polymer due to its high glass transition temperature (Madeka and Kokini, 1996), that zein is more hydrophobic than gluten (Belton et al., 2006) and because commercial zein, which is essentially just α-zein, has considerably higher extensibility compared to gluten (King et al., 2016). In view of this, its functionality in dough systems has been likened to that of gliadin (Fevziolglu et al., 2012).

Due to the unique properties of zein with regard to visco-elastic functionality, substantial research has been conducted to understand the mechanisms responsible and to improve the quality of zein visco-elastic dough and baked food products (Bean et al., 2021; Zhang et al., 2021). One focus of these research efforts has been the role of protein secondary structure and comparisons between protein structure in zein visco-elastic dough and dough made from wheat gluten. Mejia et al. (2007) found that content of β-sheet structures present in zein increased when mixed at 35ºC in a similar fashion as wheat gluten did during dough mixing. However, when zein was allowed to cool, β-sheet content decreased, which did not occur in gluten. The authors hypothesized that stabilizing the β-sheet content of zein dough through addition of low levels of a second protein would enhance zein dough properties. Later research demonstrated that both small amounts high molecular weight glutenin and casein (Mejia et al., 2012), referred to as coproteins, could in fact stabilise the secondary structure of zein predominantly in the β-sheet conformation. The authors proposed that the coprotein-stabilized β-sheet structure favoured the formation of hydrogen bonds, which are required to maintain the structure of the polymers during their relaxation phase after being subject to mechanical stress. However, more recent work concerning electrospinning of zein with casein, whey protein and rice protein as coproteins encountered practical problems with incorporating these proteins, which were related to their different solubilities compared to zein (Federici et al., 2020). Also, rice starch was recently found to

provide greater improvements in zein visco-elastic properties than low levels of casein, sodium caseinate, gliadin and glutenin added as coproteins (Tandazo et al., 2021), which may reflect the difficulties noted by Federici et al. (2020).

In view of potential adverse reactions to gluten and dairy proteins in bakery products intended for immunocompromised consumers and the practical problems associated with incorporation of such proteins, a potentially better alternative as coproteins, especially in gluten-free products, would be to use prolamin proteins from other cereals that are only distantly related to wheat. Kafirin, the sorghum prolamin, has a very similar structure and composition to zein (Belton et al., 2006). It has been shown, however, that visco-elastic masses (polymeric prolamin protein masses prepared in low concentrations of acetic acid) prepared from kafirin were firmer and far less extensible from those from total zein (which comprises α-, β-, γ-zein and probably also δ-zein) (Oguntoyinbo et al., 2018). Notably, the kafirin masses had similar elasticity to vital wheat gluten masses Hence, it was hypothesised that kafirin could act as a coprotein to improve the functionality of zein-based doughs, which could potentially enable the production of better quality gluten-free type dough-based foods. Thus, this work investigated the effect of various kafirins as coproteins on the functionality of total zein-based visco-elastic masses and commercial zein-based model doughs.

2. Experimental

2.1 Experimental strategy

The study comprised two components:

- Isolation of kafirins from regular, waxy and high-protein digestibility sorghum genotypes, preparation of visco-elastic masses from the kafirins. To help understand their effects as coproteins, the kafirin masses were characterised by reversed phase- and size exclusion-HPLC and in terms of surface hydrophobicity.
- The effects of the kafirins on the functionality of zein-based dough systems were analysed in terms of the stress-recovery characteristics of zein visco-elastic masses made with kafirin masses as a co-protein when compared to zein alone, and the tensile properties and internal structure of model doughs made from blends of the kafirins and zein in different proportions plus maize starch. Wheat bread white flour and vital wheat gluten were included as standards.

2.2 Grain materials and prolamin preparations

Three white tan-plant sorghum lines derived from crosses between lines RTx2907 (waxy, normal protein digestibility) (Miller et al., 1996) and P850029 (non-waxy, high protein digestibility) (Weaver et al., 1998) by Texas A&M Agrilife Research, College Station, TX, USA were studied: non-waxy, normal protein digestibility (designated as NN); waxy, normal protein digestibility (designated as WND); waxy, high protein digestibility (designated as WHD) (Mezgebe et al., 2020). Total kafirin (comprising α-, β- and γ- kafirins) was isolated from the milled grains using 70% (w/w) ethanol plus 0.35% (w/w) glacial acetic acid and 0.5% (w/w) sodium metabisulphite, as described (Elhassan et al., 2018). The isolated kafirins were all defatted with hexane and ground into powders. In addition, grain from the white tan-plant sorghum hybrid PANNAR PEX 602/606 (non-waxy, normal protein digestibility) was used to prepare total kafirin , as described above and is designated as regular kafirin (RK).
Likewise, refined white maize meal (Pride Milling, Centurion, South Africa) was used to extract total zein (designated as TZ) (comprising α-, β- and γ-zeins) as described above for kafirin.

Commercial zein (designated as Z) (essentially α-zein), extracted from yellow maize, was obtained from Sigma-Aldrich, Johannesburg, South Africa (Sigma product code Z3625) and was also defatted with hexane.

Vital wheat gluten was kindly donated by Novozymes SA, Sandton, South Africa.

Commercially available white bread wheat flour, 11.4% protein as is basis (Snowflake, Premier, Waterfall City, South Africa) was used in experiments where wheat flour was used as a standard.

2.3 Visco-elastic mass preparation

Visco-elastic masses were prepared from the total zein and kafirin powders individually and from blends of the powders, essentially as described (Elhassan et al., 2018). In brief, the

powders (200 mg) were dissolved in a 500 µL glacial acetic acid at 50°C and then coacervated out of solution in the form of hydrated fibres by rapid addition of 50 mL 25ºC distilled water. The fibres were then kneaded together into a visco-elastic mass between thumb and fingers and subjected to compression testing directly after (see below). For HPLC and surface hydrophobicity analyses (see below) samples of the kafirin visco-elastic masses were freeze dried.

2.4 Model dough preparation

Model doughs were prepared from commercial zein and blends of commercial zein powder plus total zein powder or plus one of the kafirin types, or from vital gluten together with maize starch (Maizena brand, Pioneer Foods, Tygervalley, South Africa). For the tensile properties study (see below), a ratio of 8:2 commercial zein:other prolamin was used throughout, whereas in the microscopy work the effect of different prolamin ratios was investigated. The method was essentially that described by Sly et al. (2014). One g prolamin powder and 4 g maize starch were mixed thoroughly in a centrifuge tube and placed in a water bath (50℃) for 1 h. Three percent (v/v) acetic acid solution (4.4 g), pre-warmed to 50℃, was slowly added into the prolamin-starch (1:4) blend until a smooth dough was formed. The dough was then hand kneaded for 5 min and rested in a 50℃ water bath for 2 min. A second kneading for 1 min was performed directly prior forming into cylinders for the tensile properties analysis.

2.5 Analyses

2.5.1 Protein contents

The protein contents of the kafirin preparations were determined by the Dumas combustion method using a LECO 628 (LECO Corporation, St. Joseph, MI, USA) according to AACC method 46-03.01 (Cereal & Grains Association, 1999) with a nitrogen to protein conversion factor of 6.25.

2.5.2 Reversed phase (RP) HPLC

For RP-HPLC analysis, samples (5 mg) were dissolved in 1 mL of 60% tert-butanol/0.5% sodium acetate/2% β-mercaptoethanol and then alkylated with 4-vinylpyridine as described in Bean et al. (2011) and centrifuged to clarify before injection into the HPLC. HPLC separations were carried out using a 1260 Infinity II HPLC system (Agilent, Santa Clara, CA, USA) equipped with a Poroshell C18 column (Agilent, Santa Clara, CA, USA) with separation conditions as reported in Bean et al. (2011).

2.5.3 Size exclusion (SE) HPLC

For SE-HPLC analysis, samples (5 mg) were first dissolved in 1 mL pH 10 TRIS-borate buffer containing 2% SDS with continual vortexing for 30 min. Samples were then centrifuged and the supernatant removed for analysis. Next, this residue remaining after the first extract was mixed with 1 mL pH 10 TRIS-borate buffer containing 2% β-mercaptoethanol and vortexed for 60 min before being centrifuged and the supernatant removed for analysis. All samples were stabilized by heating at 80°C as described in Ioerger et al. (2020). SE-HPLC was carried out using a 1260 Infinity II HPLC system (Agilent, Santa Clara, CA, USA) with a Yara SEC-3000 column (Phenomenex, Torrance, CA, USA) as described in Ioerger et al. (2020). Standard proteins with known molecular weights were used as approximate molecular weight markers and were separated under the same conditions as the sorghum kafirins. The proteins used were β-galactosidase (116 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa).

2.5.4 Surface hydrophobicity

Surface hydrophobicity of the samples was evaluated using bromophenol blue binding as described in Chelh et al. (2006) with samples weighed to a constant 5 mg of protein.

2.5.5 Visco-elastic mass stress elastic recovery (SR)

This was determined by the method of Singh et al. (2006) as modified by Elhassan et al. (2018). In brief, a single compression test was performed on small uniform cylinders of freshly prepared total zein, total zein-kafirin and vital gluten visco-elastic masses using a texture analyser. The test speed was 0.5 mm/s, compression distance 1 mm, and the total relaxation time was 100 s. The entire test was performed within 3 min. The test was then repeated at 5, 10 and

15 min intervals. The masses were then stored in sealed ziplock-type polyethylene bags at 4^{o}C for 3 and 7 days and the test repeated. Percentage stress elastic recovery (%SR), a measure of the elasticity of the material (Singh et al., 2006), was calculated from the maximum force at compression (F Max) and the force at the time from F Max at which fresh gluten, the standard, had relaxed to 36.8% of its maximum force (Ft), i.e. Ft/F Max x 100.

2.5.6 Model dough tensile properties

Tensile properties of the prolamin-starch model doughs were determined using a Kieffer rig mounted on a texture analyser, as described (King et al., 2016). In brief, freshly prepared doughs were moulded into uniform cylinder. The dough cylinder was placed on the vertical struts of the Keiffer rig, which were 30 mm apart, and held firmly at both ends using the operator's thumb and index finger. It was stretched at a constant rate of 3.3 mm/s over a distance of up to150 mm. The entire test was performed under room temperature conditions (25^{o}C) within 2 min to prevent the doughs from cooling below their glass transition temperature. Various dough tensile properties measured and calculated. Here, only representative stress-strain curves are presented.

2.5.7 Confocal laser scanning microscopy (CLSM)

The internal structures of stretched fresh pieces of the model doughs (≈ 5 mm x 3 mm x 1 mm thick) were examined using a Zeiss 880 CLSM (Oberkochen, Germany) with a Plan-Neofluar 10×0.3 objective, at an excitation wavelength of 488 nm and emission wavelength filters set to 499-695 nm with natural fluorescence (Sly et al., 2014).

2.5.8 Scanning electron microscopy (SEM)

Fresh stretched model doughs (≈ 2 mm thick) were air-dried in a fan-type incubator at room temperature and then gold coated. They were studied using a Zeiss Evo LS15 field emission SEM (Carl Zeiss, Oberkochen, Germany) at an operating voltage of 3.0 kV.

2.5.9 Statistical analysis

All the tabulated data were subjected to one-way analysis of variance by the Fisher’s least significant difference (LSD) test using the XLSTAT program (Addinsoft, New York). Means

were separated at the 95% probably level, $p < 0.05$. Details of replication of experiments and numbers of samples analysed are given the Table footnotes.

3. Results and discussion

In this work, kafirins extracted from several sorghum types including high protein digestibility (HD)-type sorghum were studied. This was done because Goodall et al. (2012) found that flours from HD-type sorghums produced composite doughs with wheat flour that had higher maximum resistance to extension and greater time to dough breakage compared to composites with regular sorghum flour.

3.1 Characterisation of the kafirin visco-elastic masses

RP-HPLC of the kafirin visco-elastic masses from the regular, NN and WHD sorghums showed that they had broadly similar profiles, comprising mostly peaks eluting from 9.5-12.5 minutes, representing the α-kafirin polypeptides (Fig. 1). The content of γ-kafirin (peaks eluting at 4.5-6 min was similar in the three kafirins (Table 1). However, the peak eluting at just less than 9 min was of significantly smaller area ($p<0.05$) for the closely related NN and WHD lines than from the regular sorghum, with the high protein digestibility (WHD) line having the smallest peak area. Kafirin subclasses in the RP-HPLC separations were identified from previous work (Bean et al., 2000, 2011; Cremer et al., 2014) with additional research using extracts of a genetically derived β-kafirin null variant and purified β-kafirin (Ioerger and Bean, *unpublished data*) This is also supported by SDS-PAGE data from Elhassan et al. (2018) that showed that the kafirins from three WHD lines were essentially missing a band of $\approx M_r$ 18.5 kDa, similar to that stated for β-kafirin by Shull et al. (1991).

SE-HPLC was performed to estimate the proportion of polymeric and oligomeric kafirins in the kafirins from the sorghum types. It revealed that the fraction solubilised with SDS solution (i.e. under non-reducing conditions) from WHD had a significantly higher content ($p<0.05$) of oligomers, M_r >116 kDa compared to the RK and NN kafirins (Fig. 2, Table 1). The pellets remaining were extracted with β-mercaptoethanol (i.e. under reducing conditions) to hydrolyse inter- and intra-molecular disulphide bonds in order to the break the kafirin polymers into their

monomeric polypeptides so that they solubilised and could be analysed. SE-HPLC revealed that this kafirin fraction was much smaller ($p<0.05$) in the WHD kafirin compared to that in the NN and RK kafirins. The much higher ratio of the non-reducing faction total peak area to reducing peak area for the WHD kafirin (5.73:1) compared to the RK and NN kafirins (1:83:1 and 2.05:1, respectively) (Table 1) indicates the WHD kafirins were much less polymerised than the NN and RK kafirins. This can be attributed to a lower proportion of β-kafirin in the WHD kafirin as β-kafirin is considered to act as the chain length extender when kafirin polymerises by disulphide bonding (El Nour et al., 1998).

In dough formation, the surface hydrophobicity of proteins is important with respect to them interacting together (Jazaeri et al., 2015). Moreover, hydrophobic interactions have specifically been proposed to be involved in the formation of zein visco-elastic materials (Smith et al., 2014). Table 1 shows that the WHD kafirin had significantly higher ($p<0.05$) surface hydrophobicity than the RK and NN kafirins. This could be related to its lower β-kafirin content. Gamma-kafirin, the most hydrophobic of the kafirin classes (Duodu et al., 2003), has the least negative free energy of hydration as calculated from its amino acid sequence, whereas β-kafirin has the most negative (Table 1).

3.2 Visco-elastic mass functionality

Table 2 shows that the fresh (Day 0, 1st compression) visco-elastic masses prepared from total zein, the various kafirins and their 1:1 ratio composites all had substantially lower percentage stress-recoveries (%SR), than the wheat gluten. Of these, WND kafirin had the highest %SR (22.7%) and total zein and the total zein-NN kafirin composite had the lowest (≈ 8%), showing that they all had lower elastic recovery at 10.5 s compared to the gluten. It should be noted that the %SRs of the kafirins from the waxy, high protein digestibility sorghum crosses were substantially lower than the previous work on these lines by this group (Elhassan et al., 2018). This difference is because in the work of Elhassan et al. (2018) the acetic acid concentration in the visco-elastic masses was 25%, whereas it was only 1% in the present work. Oguntoyinbo al. (2018) found that with kafirin masses there was a progressive reduction in %SR with decreasing acetic acid concentration. The values reported here are similar to those reported by Oguntoyinbo et al. (2018) who investigated the effect of acetic acid concentration. The

reduction in SR was attributed to decreased plasticization of the kafirin and also to the increase in pH of the masses due to the lower acid concentration.

With repeated compression testing on Day 0, there were significant increases ($p<0.05$) in the %SRs of all the kafirins and the total zein-kafirin composites but there was no increase with the total zein alone (Table 2). The increases with the WND kafirin and WHD kafirin were such that the %SR of WND was the same as the gluten, which exhibited little change in %SR with repeated compression, and that of WHD was significantly higher ($p<0.05$). After storage at 4^{o}C for up to 7 days, there were further substantial increases in %SR in all the viscoelastic masses, with the exception of gluten, total zein and WND kafirin. The increases with the total zein-WND kafirin and total zein-WHD kafirin composites were very large, to the extent that after 7 days storage their %SRs were significantly higher ($p<0.05$) than the gluten, as was also the WHD kafirin These had %SRs of 47.5%, 43.7% and 52.7%, respectively.

The increases in %SR with the kafirins on their own and as composites with the total zein and absence of an increase with total zein on its own when the visco-elastic masses were subjected to repeated compression and storage (Table 2) indicate the development of a functional protein "dough". In the case of gluten, this takes place by the molecules assuming principally a β-sheet secondary structure and interacting together by disulphide bonding and non-covalent interactions, especially hydrogen bonding (Belton, 2005). With the kafirins, disulphide bonding has been proposed to also be involved its elastic functionality (Oguntoyinbo et al., 2018) as kafirin has a higher number of cysteine residues than zein. The 19 kDa and 22 kDa α-type kafirins, the major prolamin class, each contain two cysteine residues, whereas their zein homologues contain only one cysteine residue (Belton et al., 2006). Furthermore, kafirin has been found to have a degree of disulphide-bonded polymerization than zein (Emmambux and Taylor, 2009). However, as there were significant differences in SR between the different kafirins on their own and when composited with total zein, there were clearly other contributing factors. Notably, the total zein-WHD kafirin composite and WHD kafirin on its own had the highest %SRs despite the fact that the WHD kafirin was clearly less polymerized than normal protein digestibility sorghum kafirin (Table 1).

Here, the greater surface hydrophobicity of WHD kafirin (Table 1), which itself may be due to its low content of β-kafirin, may have played a role. Smith et al. (2014) working with commercial zein found that treatment of the zein with β-mercaptoethanol had little impact on its

ability to form a viscoelastic material, indicating that the degree of polypeptide polymerisation was not directly involved in its visco-elasticity. On the basis of the effects of chaotropic and kosmotropic salt solutions on the zein's hydrophobicity, Smith et al (2014) proposed that non-covalent interactions within and between the zein molecules play a role in their arrangement to form a material with visco-elasticity properties and suggested that interactions between external facing hydrophobic regions of the zein could be involved. The greater surface hydrophobicity of WHD may have impacted on protein interaction and functionality.

3.3 Model dough functionality

On the basis that compositing kafirins with total zein resulted in visco-elastic masses with much higher elastic recovery than zein alone, their effects in a model gluten-free type dough system made with maize starch were studied. Here, commercial zein was used because of its wide availability and in order to more directly compare the findings with published studies. The zein and kafirin model doughs were made with a 3% solution of acetic acid as preliminary work revealed that its inclusion greatly improved dough cohesiveness, as previously reported (Sly et al., 2014). It is recognised that hydrating the doughs with dilute acetic acid would render them unfit for consumption due to the unpleasant flavour. In this present work, dilute acetic acid was used to demonstrate proof of concept. However, the same effect can be obtained with dilute lactic acid (Sly et al., 2014) at a concentration similar to that in sourdoughs. The improved dough cohesiveness brought about by dilute acetic acid or lactic acid is probably due to the zein molecules being protonated by the acidic solution (Li et al., 2012), forming a conjugate acid. A 1:4 ratio of prolamin to maize starch was used to make the model doughs because with vital gluten, this ratio resulted in a similar tensile functionality compared to the wheat flour dough standard (Fig. 3).

The maximum stress of the zein-kafirin composite model doughs were all considerably higher than that of commercial zein on its own or when composited with total zein (Fig. 3). In principle this finding is the same as that of Meija et al. (2012) using other proteins as coproteins. These authors studied high molecular weight gluten as coprotein with commercial zein at a 1:9 ratio in a prolamin-maize starch model dough. At 23^{o}C (i.e. similar to the assay temperature in this present work), the gluten greatly increased the firmness of the dough, although the dough

firmness was still considerably less than of the gluten-starch standard. Although it was stated that the inclusion of the gluten slowed dough relaxation time to that resembling the gluten dough, it is not clear whether the elasticity of the dough was affected as data on this was not presented. In this present work, the doughs in descending order of maximum strength were zein-RK kafirin, zein-NN kafirin, zein-WND kafirin, zein-WHD kafirin, zein-total zein and zein alone (Fig. 3). This suggests that that there was not a relationship between the strength of model doughs and the elasticity of their visco-elastic masses as the WND kafirin composite had a much lower %SR than the NN kafirin composite (Table 2). It is notable that the zein-kafirin composite doughs made with the more highly polymerized kafirins (RK and NN) (Table 1) had the highest stress, while the less polymerized WHD kafirin composite had rather lower stress and the total zein composite and zein alone had the lowest stress. This implies that the dough strength was related to the size of the disulphide bonded polymers, as is generally considered to be the case with gluten (Ortolan and Steel, 2017). Also of note was the fact that the stress-strain curve linear slopes of the kafirin composite model doughs were all higher than those of the zein doughs, indicating that the kafirin composites were stiffer. This is also likely to be a consequence of their greater polymerization. Meija et al. (2012) showed that at 23^{o}C, gluten inclusion with zein increased dough stiffness and damping to the same levels as the gluten dough during dough relaxation, indicating that the co-polymer dough had much greater capacity to store energy. . Similar effects on zein elasticity have also been observed if it is thermally treated to bring about controlled heat induced cross-linking and structural rearrangement (Frederici et al., 2021).

However, in the present work the overall tensile functionality of the zein-kafirin model doughs was still markedly inferior to that of the gluten model dough. The maximum stress value for zein-RK kafirin dough, which was the highest, was only 60% of that of the gluten model dough. The relatively low strength of the zein-kafirin composites was in part a consequence of making the doughs in 3% acetic acid (Sly et al., 2014), but this was necessitated by the need to give these doughs cohesiveness. Notwithstanding this, their extensibility at maximum stress was only about 40% of that the gluten model dough.

3.4 Model dough microstructure

The microstructures of stretched model doughs were studied using CLSM and SEM (Fig. 4) to gain insight into the reasons for their differing rheological properties. With CLSM, the protein autofluoresced and in all the doughs (wheat flour, wheat gluten, zein and zein-kafirin) existed as continuous matrix (white arrows) completely enveloping the starch granule (seen as dark tiny points as they did not autofluoresce) (Fig. 4A). However, when viewed by SEM the protein appeared as very thin strands (Fig. 4B). This was due to the fact that the doughs analysed by CLSM were fresh (i.e. normally hydrated), whereas all the water had been removed from the doughs viewed by SEM during sample preparation. As a result, with SEM the protein matrix had shrunk dramatically, whereas the starch granules were barely affected. Thus, the images obtained by CLSM are a more accurate representation of the actual starch-protein arrangement in the doughs.

As revealed by CLSM, the microstructures of the wheat bread flour dough (Fig 4Aa) and the gluten-maize starch model dough (Fig. 4Ab) were similar in that the gluten protein matrix was amorphous (dotted white arrows). However, when viewed by SEM (Fig. 4B), the gluten in the gluten-maize starch model dough exhibited distinct fine strands, which were not visible in wheat bread flour dough. The reason that the two gluten-based doughs appeared to be different by SEM was probably because with dehydration of the wheat flour dough the matrix protein shrank onto the starch granules to form a film, whereas with the gluten-maize starch model dough the gluten did not adhere to the granules and formed strands. The adhesion of the protein to the granules in the wheat flour dough was probably due to the presence of amphoteric molecules in wheat flour (Gan et al., 1995), which would be absent in commercial gluten. In contrast, CLSM revealed that the starch-granule containing protein matrix in the zein and zein-kafirin composite doughs was predominantly in the form of distinct fibrils (Fig. 4Ac-n) of $\approx$ 20 μm diameter, which were aligned together into much larger fibres that were up to 500 μm across. This fibril-fibre structure was previous reported in zein visco-elastic masses (Sly et al., 2014). The fact that zein and kafirin form such a structure is probably related to the fact that they are more hydrophobic than gluten (Belton et al., 2006; Duodu et al., 2003). As a result, the protein molecules strongly interacted together to form fibrils, which adhered together into fibres, rather than existing as more amorphous strands as with the gluten in wheat dough (Jekle and Becker, 2015).

Some differences in the prolamin matrix between the stretched zein and the zein-kafirin model doughs and also between the doughs made with different kafirins were evident by CLSM

(Fig. 4). With the commercial zein-maize starch dough, the protein fibrils were clearly linearly aligned in the direction of stretching (Fig. 4Ac). This was also the case with the model doughs made from blends of commercial zein and total zein with increasing proportions of the latter from 9:1 to 7:3 (Fig. 4Ad-g). This indicates that there was very high compatibility between the total zein and commercial zein molecules, which enabled them to align together to form linear fibrils. When kafirins were used as a coprotein, there was also good compatibility between them and commercial zein at a 8:2 zein:kafirin ratio, as indicated by the fact that the prolamin networks were predominantly in the form of linearly orientated fibrils (Fig. 4Ai,l-n). However, with blends of commercial zein and RK there was a progressive loss of fibril linearity in at the higher ratios of 7.5:2.5 and 7:3 as evidenced by CLSM (Fig. 4Aj,k). At these higher ratios of RK kafirin to commercial zein, the prolamin protein matrix appeared to exist as clumps (dashed white arrows) instead of fibrils and fibres. This is possibly indicative of three-dimensional polymerisation between the two types of prolamins. Greater disulphide bonding due to the higher number of cysteine residues in kafirin than in zein (Belton et al., 2006) could be responsible. As stated, such three-dimensional polymerisation may also have been responsible for the fact that the RK kafirin-commercial zein blend model dough also had the highest strength (Fig. 3).

Concerning the model doughs produced from blends of commercial zein with the kafirins from crosses between the waxy and high protein digestibility type sorghums (WND, NN and WHD), the dough made with NN kafirin as coprotein appeared to have the most linearly orientated prolamin fibrils (Fig. 4A), whereas the fibrils in the dough made with WHD kafirin were the least well defined (Fig. 4An). The former may have been due to the NN kafirin having the lowest surface hydrophobicity (Table 1), enabling good interaction between the kafirin and zein molecules. This is analogous to the generally accepted model for glutenin polymer interaction of Belton (1999), the “loop and train” hypothesis. This is based on hydration of the glutenin polymers and hydrogen bonded interaction between the polymers and water molecules, and between the polymers themselves, respectively. The latter may have been a consequence of relatively poor interaction between the WHD kafirin and zein molecules resulting from the high surface hydrophobicity of the WHD kafirin (Table 1).

4. Conclusions

Kafirin can act as an effective coprotein to zein as they have excellent compatibility, unlike several other types of proteins. The inclusion of kafirin increases the elasticity of zein-kafirin visco-elastic masses and the strength of prolamin and maize starch model doughs. Zein-kafirin visco-elastic masses have similar elasticity to gluten, which may due to greater disulphide bonding between the molecules as a consequence there being twice the number of cysteine residues in α-kafirin compared to α-zein. Their model doughs have greater strength than zein doughs, which is possibly because their disulphide bonded polymers are of high molecular size, as is the case with glutenin strength. Different kafirins impart slightly different behaviours to zein-kafirin coprotein materials. Of the types studied, kafirin from high protein digestibility sorghum imparts the highest elasticity to the visco-elastic masses, which may be due to its greater surface hydrophobicity (at least 50% higher than the regular and non-waxy, normal protein digestibility sorghum types), which is likely to be a consequence of its lower content of β-kafirin (at least 40% lower relative to γ-kafirin). Kafirin from regular sorghum imparts the highest strength to the model doughs, possibly because of greater three-dimensional polymerisation through disulphide bonding.

The next step to be answered in future work on kafirin as a coprotein to zein in food doughs is to determine its impact on the quality of products such as pasta and breads made from non-wheat flours.

Declaration of competing interest

The authors declare that they have no competing interests

CRediT authorship contribution statement

M.B. Ncube: Investigation, Writing - original draft, J. Taylor: Supervision, Data curation, S.R. Bean: Formal analysis, Writing – review and editing, B.P. Ioerger: Investigation, Writing – review and editing, J.R.N. Taylor: Conceptualization, Supervision, Writing – review and editing

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CAPTIONS TO FIGURES

Figure 1. RP-HPLC of kafirin visco-elastic masses prepared from kafirins from regular, NN and WHD sorghum types.

NN = Non waxy, Normal protein digestibility; WHD = Waxy, High protein digestibility.

Figure 2. SE-HPLC of kafirin visco-elastic masses prepared from kafirins from regular, NN and WHD sorghum types.

NN = Non waxy, Normal protein digestibility; WHD = Waxy, High protein digestibility.

Figure 3. Stress-strain curves of prolamin-maize starch model doughs.

WBF = Wheat bread flour; G = Gluten, Z-RK = Commercial zein-Regular kafirin; Z-NNK = Commercial zein-Non waxy, normal protein digestibility kafirin; Z-WNDK = Waxy, Normal protein digestibility kafirin; Z-WHDK = Commercial zein-Waxy, High protein digestibility kafirin; Z-TZ = Commercial zein-Total zein; Z = Commercial zein only.

Figure 4.
4A. CLSM of the microstructure of prolamin-maize starch model doughs.

a. Wheat bread flour; b. Gluten; c. Commercial zein only; Commercial zein-Total zein ratios - d. 9:1, e. 8:2, f. 7.5:2.5, g. 7:3; Commercial zein-Regular kafirin ratios - h. 9:1, i. 8:2, j. 7.5:2.5, k.7:3; l. Commercial zein-Waxy, Normal protein digestibility kafirin ratio 8:2; m. Commercial zein-Non waxy, Normal protein digestibility kafirin ratio 8:2; n. Commercial zein-Waxy, High protein digestibility kafirin ratio 8:2. Dotted white arrows = Amorphous gluten matrix, Solid

white arrows = Prolamin protein fibrils; Dashed white arrows = Clumps of prolamin protein; Bar = 100 µm.

Figure 4.

4B. SEM of the microstructure of prolamin-maize starch model doughs.

a. Wheat bread flour; b. Gluten; c. Commercial zein only; Commercial zein-Total zein ratios - d. 9:1, e. 8:2, f. 7.5:2.5, g. 7:3; Commercial zein-Regular kafirin ratios - h. 9:1, i. 8:2, j. 7.5:2.5, k.7:3; l. Commercial zein-Waxy, Normal protein digestibility kafirin ratio 8:2; m. Commercial zein-Non waxy, Normal protein digestibility kafirin ratio 8:2; n. Commercial zein-Waxy, High protein digestibility kafirin ratio 8:2. Solid white arrows = Prolamin protein strands; Black arrows = Starch granules; Bar = 10 µm.

Table 1. RP-HPLC and SEC-HPLC peak areas of the kafirin visco-elastic masses* and their surface hydrophobicity, plus the free energies of hydration of the α-, β- and γ-kafirin classes as calculated from their amino acid sequences

Sorghum type	RP-HPLC			SE-HPLC			Surface hydrophobicity	Kafirin class	Free energy of hydration (kcal/mol)[4, 5]
	Peak area at ≈ 9 min (likely β-kafirin) (mV)	Peak area at 4.5-6 min. (γ-kafirin) (mV)	Ratio of β:γ	Total peak area non-reducing extraction (mV)	Total peak area reducing extraction (mV)	Ratio of non-reducing: reducing	(µg bromophenol blue bound/5 mg protein)		
Regular	821±37[1]C[2]	1217±89A[2]	0.67:1	320484 ±7027A[1]	175103 ±2730B	1.83:1	20.5±0.5[3]B	**α-**	-140.4[4]
Non-waxy, normal protein digestibility (NN)	569±28B	1113±64A	0.51:1	298652 ±23842A	145634 ±6429C	2.05:1	17.4±0.4A	**β-**	-166.5[5]
Waxy, high protein digestibility (WHD)	349±18A	1172±79A	0.30:1	441424 ±7046B	77088 ±2118A	5.73:1	30.8±1.5C	**γ-**	-113.6[4]

*The waxy, normal protein digestibility kafirin was not characterized as SDS-PAGE analysis had shown that it was intermediate in protein profile between the non-waxy, normal protein digestibility and the waxy, high protein digestibility lines (Elhassan et al., 2018) and because the time and cost of the HPLC analyses was a constraint.

[1]Mean and standard deviation of two replicate protein extractions each sample, [2]Mean values with different letters in a column differ significantly from each other ($p < 0.05$), [3]Mean and standard deviation of two analyses per sample, [4]Data from Duodu et al. (2003) which was calculated from the free energies of hydration of the individual amino acids in the α- and γ-kafirin amino acid sequences, [5]Similarly calculated from the β-kafirin amino acid sequence in Chamba et al. (2005)

Table 2. Percentage stress-recovery[1] of total zein, kafirin and zein:kafirin visco-elastic masses at day 0 and after storage at 4°C for 3 and 7 days

Storage time	**Day 0**				**Day 3**	**Day 7**
Prolamin type[2]	**0 min (1st compression)**	**5 min (2nd compression)**	**10 min (3rd compression)**	**15 min (4th compression)**	**(5th compression)**	**(6th compression)**
Wheat gluten standard	36.8±3.5E[3]bc[4]	37.1±2.5Ec	34.6±1.3CDabc	31.2±1.8Da	35.9±0.4CDbc	33.2±1.4BCab
Total zein	7.6±1.8Abc	3.8±0.4Aa	6.7±1.0Ab	6.6±0.1Ab	6.4±0.7Ab	8.6±1.0Ac
NN kafirin	16.6±1.2Ca	18.1±1.1Ba	21.4±3.1Bab	24.3±3.7Cb	37.5±3.9CDd	38.9±2.9Cd
WND kafirin	22.7±2.8Da	26.9±3.4Cab	32.0±4.4Cbc	34.9±3.7Dc	34.3±2.4Cc	29.7±2.4Bbc
WHD kafirin	16.5±1.4Ca	32.9±3.9Db	39.8±4.6Dc	43.5±3.1Ec	54.5±1.0Ed	52.7±1.5Fd
Total zein-NN kafirin	8.0±0.4Aa	7.4±0.2Aa	8.3±2.1Aa	11.9±2.1Bb	28.5±3.4Bc	27.2±2.9Bd
Total zein-WND kafirin	11.5±0.1Ba	16.0±5.0Bb	21.7±5.5Bc	20.9±3.4Cc	38.2±2.1Dd	47.5±1.0Ee
Total zein-WHD kafirin	9.1±0.3Ba	13.8±2.1Bb	19.9±2.6Bc	24.2±2.5Cd	51.5±2.7Ee	43.7±1.0De

[1]% Stress Recovery at 10.5 s after F Max. Mean ± standard deviation, n=2.

[2]NN = Non-waxy, normal protein digestibility, WND = Waxy, normal protein digestibility, WHD = Waxy, high protein digestibility

[3]Effect of prolamin type - Mean values with different uppercase letters in a column differ significantly from each other ($p < 0.05$).

[4]Effect of storage - Mean values with different lower case letter in a row differ significantly from each other ($p < 0.05$).

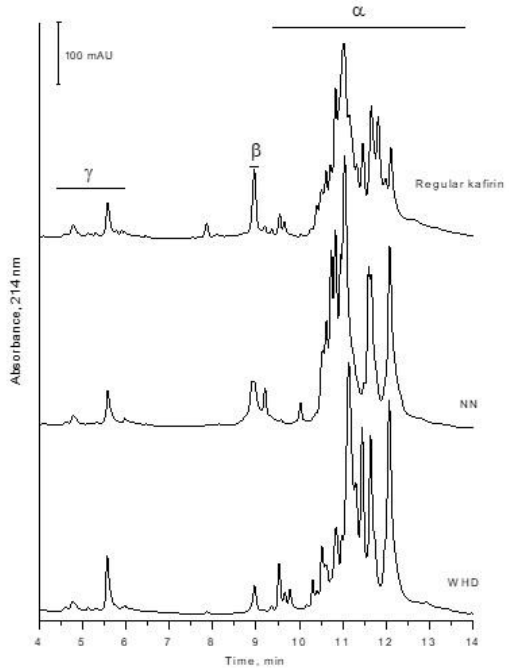
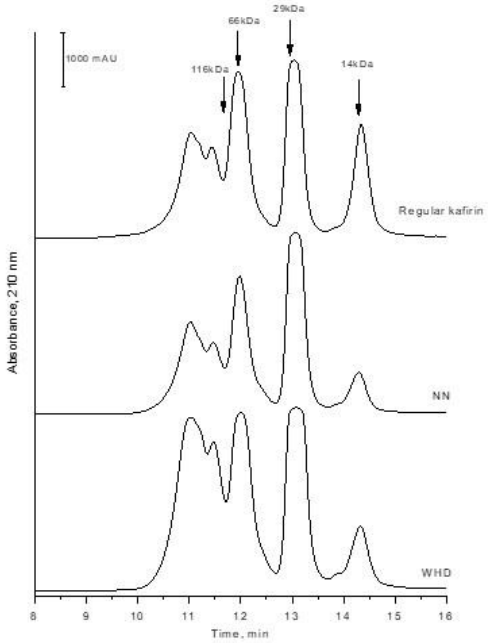

α
100 mAU
γ
β
Regular kafirin
NN
WHD
Absorbance, 214 nm
4
5
6
7
8
9
10
11
12
13
14
Time, min

Fig. 1

SEC non-reduced



SEC reduced

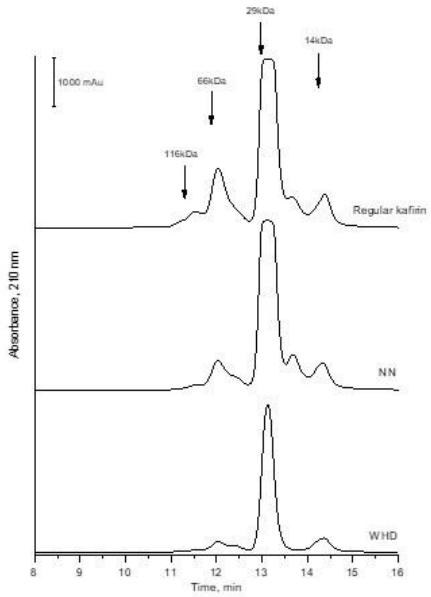


Fig. 2

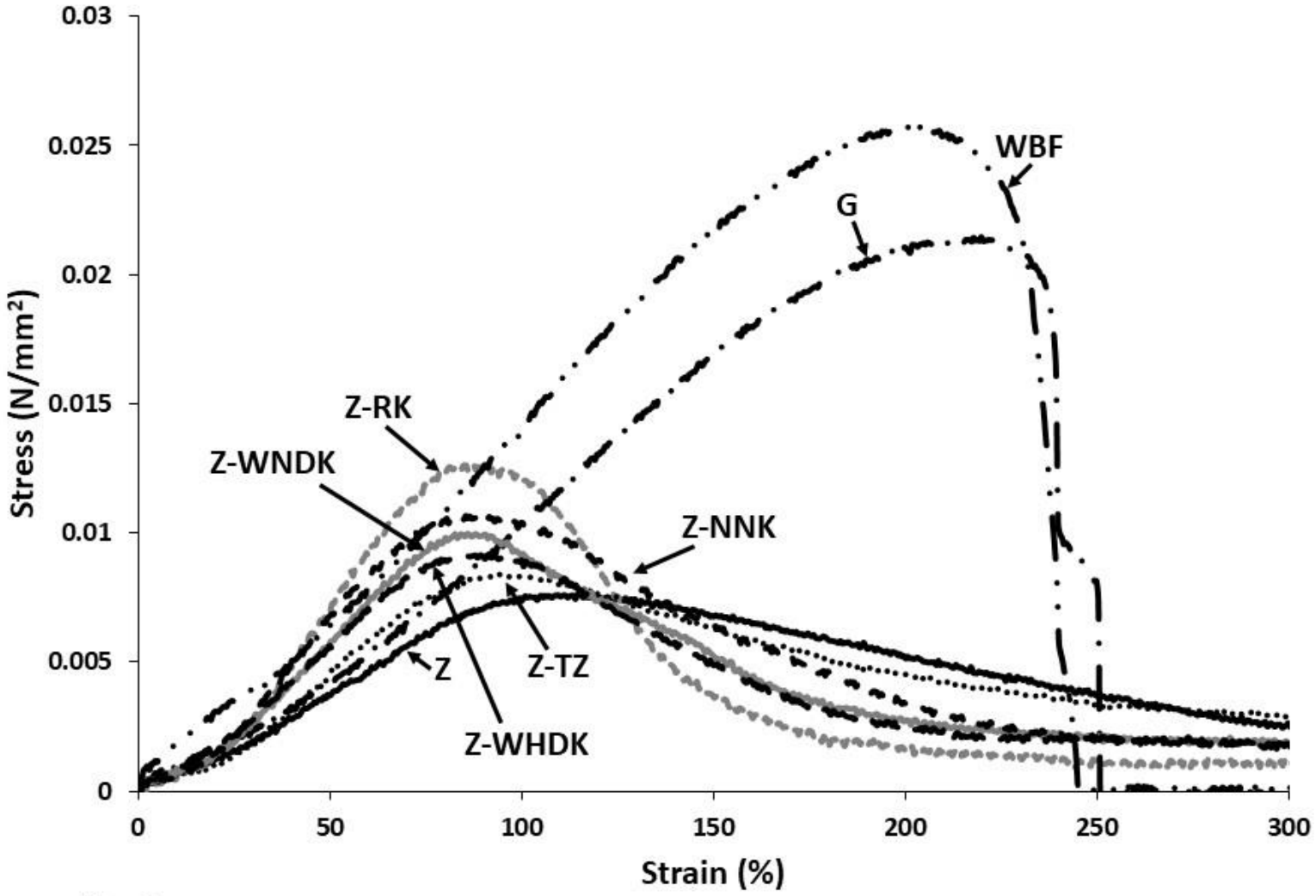

0.03
0.025
0.02
0.015
0.01
0.005
0
Stress (N/mm²)
0
50
100
150
200
250
300
Strain (%)
WBF
G
Z-RK
Z-WNDK
Z-NNK
Z
Z-TZ
Z-WHDK

Fig. 3

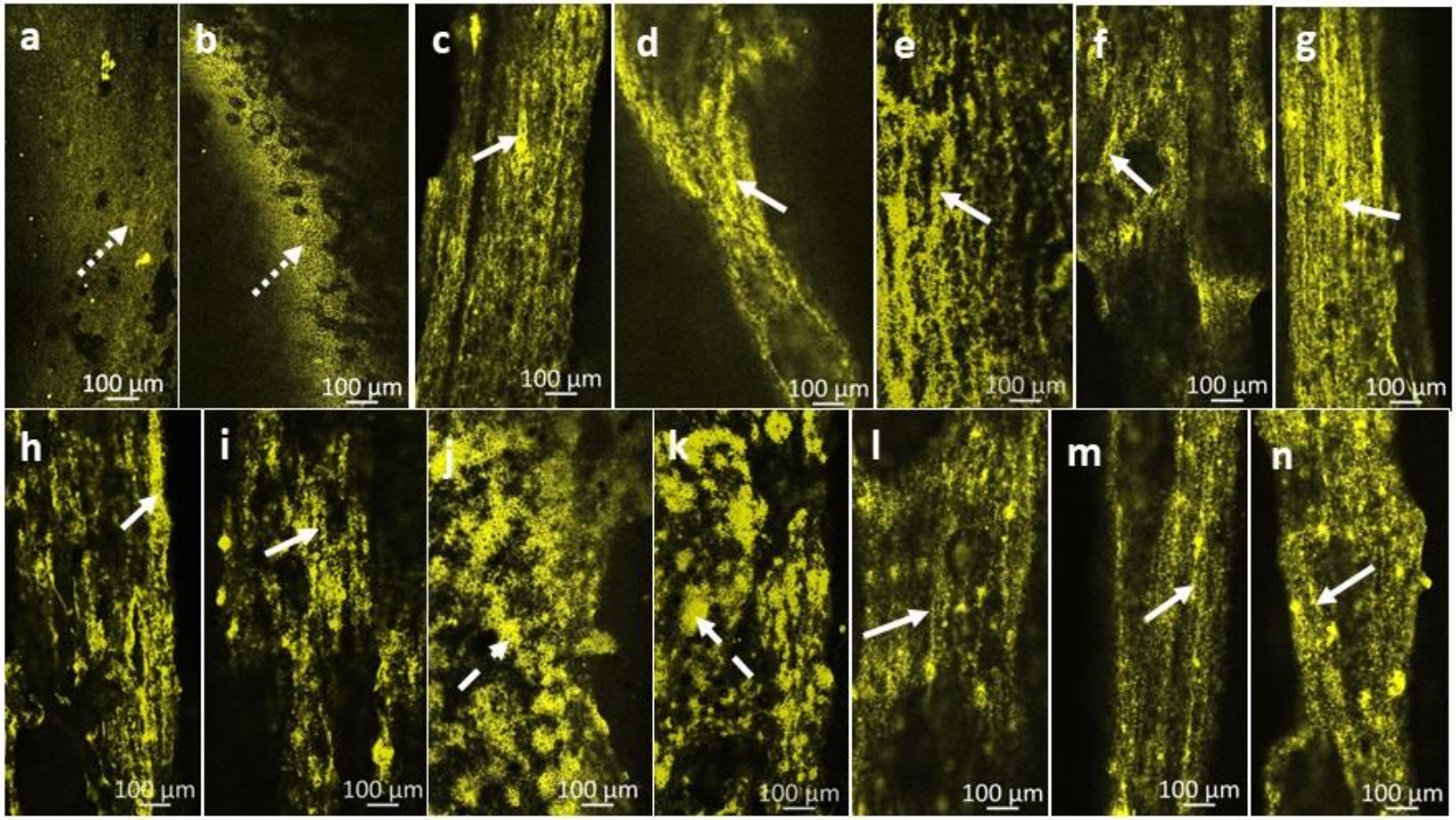


Fig. 4A

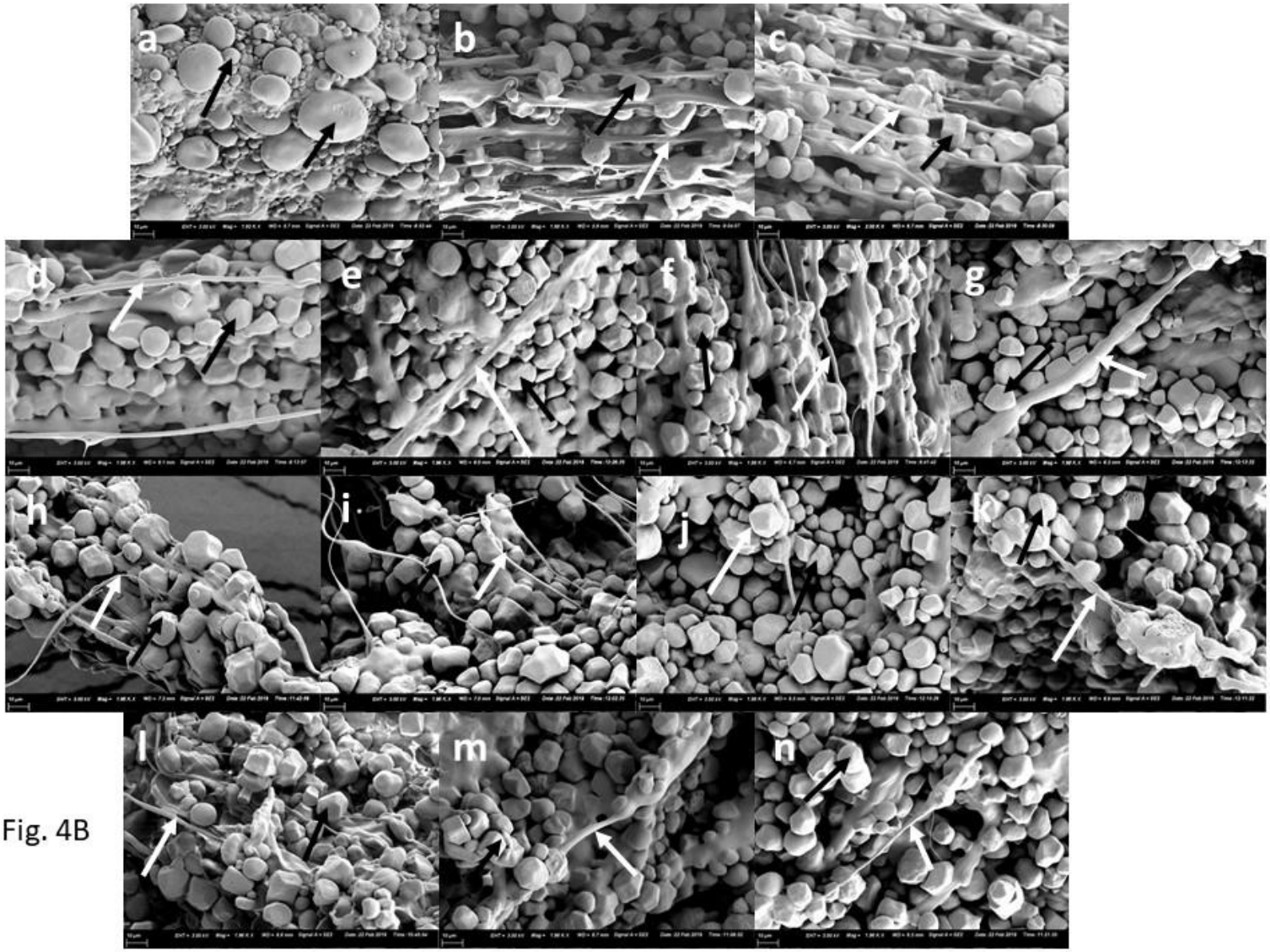


Fig. 4B