Functionality of the storage proteins in gluten-free cereals and pseudocereals in dough systems

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Abbreviations: CLSM, confocal laser scanning microscopy; G', shear storage modulus; G", shear loss modulus; HMW-GS, high molecular weight-glutenin subunits; SEM, scanning electron microscopy; SAOS, small amplitude oscillatory test; T_g, glass transition temperature

ABSTRACT

The dough functionality of the storage proteins in "gluten-free" grains has been studied for almost 25 years. Zein, maize prolamin, when isolated as α-zein can form a wheat gluten-like visco-elastic dough when mixed with water above its glass transition temperature. There is good evidence that its dough-forming properties are related to a change in protein conformation from α-helix to β-sheet and association of the molecules into fibrils. Stabilisation of β-sheet structure and visco-elasticity can be enhanced by inclusion of a co-protein. No other isolated cereal or pseudocereal storage protein has been shown to form a visco-elastic dough. Many treatments have been applied to improve "gluten-free" storage protein functionality, including acid/base, deamidation, cross-linking by oxidising agents and transglutaminase, proteolysis, disulphide bond reduction and high pressure treatment. Such treatments have some limited positive benefits on batter-type dough functionality, but none is universally effective and the effects seem to be dependent on the composition and structure of the particular storage protein. Research into mutants where prolamin synthesis is altered appears to be promising in terms of improved dough functionality and scientific understanding. Research into how treatments affect the functionality and structure of isolated storage proteins from "gluten-free" grains other than maize is required.

1. Introduction

Excellent progress has been made in the development of technology to produce gluten-free breads and other dough-based products with the aid of hydrocolloids and gums to improve dough visco-elasticity and gas-holding (Anton et al, 2008; Sciarini et al., 2010). An alternative approach involving improving the visco-elasticity and gas-holding properties of the storage proteins of gluten-free cereals has also been the subject of considerable research since the early 1990s (Lawton, 1992), but progress has been much slower (Erickson et al., 2012).

This approach of using the storage proteins of gluten-free cereals to support the creation of a stable, expanded leavened dough is nevertheless highly desirable. Many gluten-free bread products have poor nutritional quality in terms of proteins, micronutrients and dietary fibre due to them consisting primarily of purified carbohydrates (Matos and Rosell, 2015). Gluten-free dough-based products are generally also disproportionately costly (Singh and Whelan, 2011). Further, there is also a need for non-wheat and low-wheat (as opposed to gluten-free) bread. This is particularly the case in the developing countries of Asia and Africa where there is a huge increase in demand for bread and other Western-type foods, due to continuing high population growth and rapid urbanisation (Pingali, 2007). Cultivation of wheat and barley, which are temperate cereals, is not generally economically viable in these countries which lie in the tropics and semi-arid sub-tropics. Scientific developments in non-wheat dough systems, which parallel those that have taken place in brewing where cereals such as sorghum are now used extensively (Taylor et al., 2013b), would be highly beneficial to both persons who are intolerant to gluten and consumers in developing countries.

This review will focus on research being undertaken to improve the functionality of the storage proteins of maize, sorghum, the millets, oats, rice and the pseudocereals (buckwheat, amaranth and quinoa) in dough systems. Firstly, the composition and structural chemistry of these proteins in

relation to those of wheat glutenin with respect to visco-elasticity will be briefly examined. Next, research into the dough functionality of the "gluten-free" storage proteins as isolated proteins will be reviewed. Then, the major section will review research into improvement of their dough functionality through chemical and physical modifications. After which, improvement through genetic modification of the proteins will be examined. Lastly, possible directions regarding practical application of the findings in bread-making and ongoing research will be considered.

2. Composition and structure of the storage proteins of non-wheat cereals and pseudocereals

To mimic the functional properties of gluten in non-gluten dough systems it is useful to understand how the storage proteins of non-wheat cereals and pseudocereals differ from wheat gluten in composition and structure. The functionality of gluten in wheat dough systems is complex, as recently reviewed by Juhász et al. (2015). As is well-known, gluten comprises monomeric gliadins which are responsible for dough viscosity and extensibility and polymeric glutenins, which critically are responsible for elasticity and strengthening the dough. In particular, the high molecular weight glutenin subunits (HMW-GS) are important in determining gluten elasticity (Shewry et al., 2002).

There are several valuable reviews concerning non-wheat grain storage proteins, including Shewry and Halford (2002), Lawton (2002) and Belton et al. (2006). Table 1 compares the composition and structure of the wheat high molecular weight glutenins with those of the prolamins of the tropical C4 cereals: maize sorghum, pearl millet and teff. The prolamins of maize and sorghum, zein and kafirin, respectively, like the wheat prolamins, are composed of a number of sub-classes (Shull et al., 1991). The polypeptide monomers are, however, all much smaller in size than the wheat HMW-GS, but like the HMW-GS they polymerise through disulphide cross-linking, due to the high cysteine content of the β- and γ- sub-classes. The secondary structures of zein and kafirin are predominately α-helical and tightly folded into a hairpin or rod-like structure , rather than consisting of more open

Table 1
Composition and structures of maize, sorghum, pearl millet and teff prolamins in comparison with wheat high molecular weight glutenin subunits

Species	Storage proteins	% of total prolamin fraction	Polymerisation	Subunit molecular weight (kDa)	Major amino acid composition	Structural model and secondary structure	Hydrophobicity	Refs
Wheat	HMW glutenin subunits	6-12	Macro polymers-linked end to end by disulphide bonding	65-90	Glutamine, proline, glycine cysteine	Extensive repeat sequences based on proline-rich and glutamine rich motifs Rod shaped, α- helical at N- and C- terminal domains, central region of loose spirally repeated β-turns	Less hydrophobic than either kafirin or zein (Belton et al., 2006)	Shewry and Tatham (1990)
Maize	α-zein	80	Limited polymerisation through disulphide bonding with other β- and γ-zein	19 and 22	Glutamine, leucine, cysteine, proline	40-60% α-helix (Argos et al., 1982; Tatham et al., 1993, Forato et al., 2004, Cabra et al., 2005) 7-19.5% β-sheet (Cabra et al., 2005). Classic Argos model based on 9 anti-parallel α-helices within a distorted cylinder (Argos et al., 1982). Modified models based on extended hairpin, rod or ribbon-like structures 17 x 4.5 x 1.2 nm, with clearly defined hydrophilic and hydrophobic domains (Matsushima et al., 1997; Bugs et al., 2004; Forato et al., 2004; Momany et al., 2006)	More hydrophobic than gluten but less hydrophobic than kafirin, has some hydrophilic properties (Belton et al., 2006)	Shewry and Tatham (1990) Colman and Larkins (1999)

	Protein		Polymerisation		Amino acids	Structure	Hydrophobicity	References
	β-zein	7-8	Polymerise through disulphide bonding	14 and 16	Glutamine, alanine, methionine, leucine, cysteine	Mainly β-sheet, β-turn and random coil (Shewry and Tatham, 1990). No structural model.		
	γ-zein	9-12	Polymerise through disulphide bonding	28	Proline, glutamine, leucine, histidine, cysteine	Only γ-zein contains repeated amino acid sequence, hexapeptide repeat of Pro-Pro-Pro-Val-His-Leu. 19-32% α-helix, 11-32% β- sheet (Wu et al., 1983). No structural model.		
	δ- zein			14 and 21	Methionine rich			
Sorghum	α-kafirin	80	Polymerise through disulphide bonding with other β- and γ-kafirin	23-25	Glutamine, leucine, alanine, proline, cysteine	Total kafirin (containing all prolamin sub-units) similar secondary structure contents to α- zein, 40%-60% α- helix (Belton et al., 2006)

No structural models for kafirin or any of its sub-classes but thought to be similar to zein

Only γ-kafirin contains repeated amino acid sequence, hexapeptide repeat of Pro-Pro-Pro-Val-His-Leu | More hydrophobic than gluten and zein, has some hydrophilic properties but is less hydrophilic than zein or gluten (Belton et al., 2006) | Shull et al. (1991) Watterson et al. (1993) Belton et al. (2006) |
	β- kafirin	7-8	Polymerise through disulphide bonding	16-20	Proline, glutamine, alanine, leucine, methionine, cysteine			
	γ- kafirin	9-12	Polymerise through disulphide bonding	28	Proline, glutamine, histidine, cysteine			
	δ- kafirin				Methionine rich			

Pearl millet	Pennisetin	40		22 and 20 and 10	Glutamine, alanine, leucine	72% α-helix, 15% β-turns and 9% % β- sheet. Similar to α-zein, but slightly smaller, 14 nm ribbons of folded α-helical segments.		Bugs et al. (2004) Shewry (2002)
			Dimers	49-51				
Teff	Prolamins			22 and 25	Glutamine, proline, valine, leucine, alanine, isoleucine, phenylalanine	Single dominant sequence, high degree of homology with α-prolamins of maize and sorghum		Tatham et al. (1996)

Table 2
Composition of and structures of rice, oats, quinoa, amaranth and buckwheat storage proteins

Species	Storage proteins	%of total storage protein fraction	Polymerisation, structural model and secondary structure	Subunit molecular weight (kDa)	Major amino acid composition	Refs
Rice	Glutelin	66-78	Not soluble in dilute salt solutions so classically defined as glutelins but actually belong to 11-12 S storage globulins family, related to legumin type globulins		Rich in glutamic acid/glutamine, aspartic acid and asparagine, glycine and alanine, poor in tryptophan, methionine and histidine	Shewry and Halford (2002) Wen and Luthe (1985)
	α-		Polymerise by disulphide bonding and hydrophobic interactions to form very large macromolecular complexes (64-500 kD). Insoluble in water due to high levels of hydrophobic, hydrogen and disulphide bonding	30-39	Basic polypeptides	Utsumi (1992) Shewry and Halford (2002) Juliano (2007)
	β-			19-25	Acidic polypeptides	
Oats	12S globulin	50-80	Structurally similar to 11S globulins of legumes. Oligomeric protein quaternary structure similar to legumins, exist as hexamers			Klose and Arendt (2012) Shewry and Halford (2002)

	12Sα-		α- and β-subclasses are disulphide bonded in native globulin, forming a units of 54 kDa which make up the hexamers	33	Acidic polypeptide, rich in aspartic and glutamic acids	Shotwell et al. (1990) Klose and Arendt (2012) Shewry and Halford (2002) Burgess et al. (1983)
	12Sβ			23	Basic polypeptide, rich in histidine, arginine and lysine	Shewry and Halford (2002) Burgess et al. (1983)
Quinoa	11S globulins (chenopodin)					
	A		Disulphide bonded acidic polypeptides. Similar to other acidic subunits of other 11S seed globulins	32-39	High levels aspartate/asparagine, , arginine, serine, leucine and glycine	Brinegar and Goundan (1993)
	B		Disulphide bonded basic polypeptides. Similar to other basic subunits of other 11S seed globulins.	22-23		
Amaranth						
	11S globulins (amarantin)		Like other 11S legumin. High in β-sheet (71%) (β-barrel), β-turn (15-22%) and α-helix (3-15%)		All amino acids present, no particularly high levels of individual amino acids	Segura-Nieto et al. (1992) Marcone and Yada (1998) Barba de la Rosa et al. (1996) Tandang-Silvas et al. (2012)
	Acidic chain			35		
	Basic chain			24		
Buckwheat	13S globulins	33	Typical globulin structure, similar to legumins, consists six sub-units comprising of large acid (32-43 kD) and small basic (20-23 kD) chains, associated by disulphide bonds	280	High in aspartate/asparagine, glutamate/glutamine and glycine	Radović et al. (1996)

spirals of β-turns like HMW-GS (Belton et al., 2006). Also, both zein and kafirin are considerably more hydrophobic than gluten.. Presumably as a consequence of their greater hydrophobicity and different secondary structure, zein and kafirin have a higher glass transition temperature (T_g) than gluten (Taylor et al., 2013a). The prolamins of pearl millet, pennisetin, whilst less studied, are considered to be similar to α-zein in structure (Bugs et al., 2004).

Table 2 summarises the properties of the storage proteins of rice and oats, and of the pseudocereals amaranth, quinoa and buckwheat. The major storage proteins of rice and oats are globulins, similar to those of legumes, and account for some 70-80% of the endosperm storage proteins (Shewry and Halford, 2002). Both are related to the 11-12S legumin type globulins. The rice glutelins comprise acidic and basic polypeptide chains linked by a single disulphide bond (Shotwell et al., 1990) and share similarities with HMW glutenins (Shewry and Halford, 2002). The oat globulins, like the legumins, form hexameric structures. The major storage proteins of pseudocereals are also similar to the legume proteins. They contain 2S albumin and 11S globulin storage proteins, with 7S globulins present in buckwheat and amaranth. Those of amaranth have predominantly β-sheet structure with β-barrel conformation (Tandang-Silvas et al., 2012). The 11S type globulins of oats, rice and the pseudocereals polymerise by disulphide bonding.

It is clear that whilst the composition and structure of these storage proteins share some similarities with glutenin, in particular the extensive disulphide bonded polymerisation of zein and kafirin, there are important differences in terms of amino acid composition, sequence and secondary, tertiary and quaternary structure.

3. Dough forming properties of non-wheat storage proteins

3.1 Isolation in protein bodies

Zein, kafirin and pennisetin prolamins, presumably as a result of their relative hydrophobicity and disulphide bond cross-linking (Shewry, 2002; Belton et al., 2006), are isolated in protein bodies in the starchy endosperm cells of the mature grain (Adams et al., 1976). Likewise, the rice prolamins are isolated in Type I protein bodies (Saito et al., 2012) and the glutelins are isolated in Type II protein bodies (Yamagata et al., 1982) and in oats the globulin and prolamin storage proteins are co-located in the same protein bodies (reviewed by Shewry and Halford, 2002). The albumin and globulin storage proteins of the pseudocereals, amaranth (Coimbra and Salema, 1994), buckwheat (Elpidina et al., 1990) and quinoa (Prego et al., 1998) are also isolated in protein bodies. The localisation of storage proteins in discrete protein bodies in these "gluten-free" grains is unlike the situation in wheat where the glutenin and gliadin proteins form a continuous matrix around the starch granules within the cells of the mature starchy endosperm (reviewed by Shewry and Halford, 2002).

3.2 Dough formation

For zein, kafirin, pennisetin and the rice storage proteins to be functional in doughs, it is presumably necessary for the protein bodies to be disrupted during dough mixing and the proteins freed. However, disruption of the protein bodies has only been observed to happen in maize under conditions when high mechanical energy (specific mechanical energy of ≥100 kJ/kg) was applied using extrusion cooking (Batterman-Azcona et al., 1999) or roller flaking (Batterman-Azcona and Hamaker, 1998). Transmission electron microscopy indicated that the freed α-zein may have formed fibrils (Batterman-Azcona et al., 1999). With oats and pseudocereals, the storage proteins are presumably readily freed from the protein bodies during dough-making due to their aqueous soluble nature (Schoenlechner et al., 2008).

Lawton (1992) in seminal research showed that commercial zein, which is essentially only α-zein (Lawton, 2002; Oom et al., 2008), formed a visco-elastic wheat flour-like dough when mixed with maize starch and the inclusion of dibutyl tartrate (as a plasticizer) at 25°C and above, and at 35°C in the absence of dibutyl tartrate. A visco-elastic dough could not be formed below 25°C and visco-elasticity was lost if the doughs were cooled below 25°C. These temperatures were shown to relate closely to the T_g of zein as a function of moisture content. It was further observed by scanning electron microscopy (SEM) that zein had formed an extensive network of fibres (fibrils). The author concluded that the visco-elasticity of zein was governed by its T_g and that fibre formation was apparently responsible for the visco-elasticity of the zein-starch doughs. Such zein-starch doughs can also expand and hold gas (Sly et al., 2014; Berta et al., 2015). Dough viscosity has been found to be the major factor affecting gas bubble structure formation (Berta et al., 2015).

Oom et al. (2008) showed that kafirin (comprising α- and γ-kafirin) plus starch in water mixtures would not form visco-elastic doughs, even at the elevated temperature of 55°C and with addition of lactic acid as a plasticizer. However, a "dough" could be formed with kafirin by plasticizing kafirin (which had been hydrated in water) into a resin using oleic acid in a 2:1 ratio (kafirin:oleic acid). At 22°C, kafirin and commercial zein-oleic acid resins showed similar extensional viscosity and strain hardening as a gluten resin. However, the kafirin resin, unlike the gluten and zein resins, rapidly became stiff (Fig. 1). This was attributed to higher levels of disulphide bonding in the kafirin. In slight contrast, Schober et al. (2011) found that while relatively pure α-zein isolated with 70% ethanol readily aggregated into a visco-elastic dough at 40°C, kafirin isolated using 83% isopropanol (apparently relatively pure α-kafirin) could aggregate into cohesive mass in water at 55°C, but kafirin isolated using the more hydrophilic 70% ethanol did not aggregate. However, the aggregated kafirin mass had very poor dough properties as it immediately became hard. The inclusion of 5% mercaptoethanol (as reducing agent) improved its properties somewhat, as the mass remained
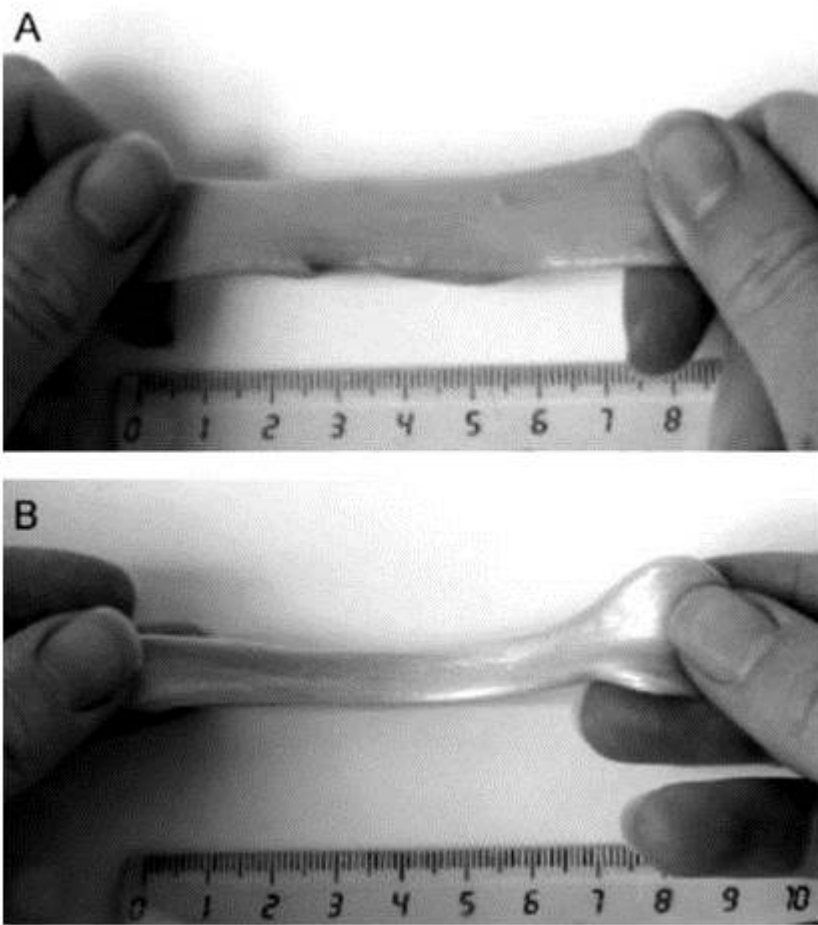
Fig. 1. Extensibility of (A) kafirin and (B) α-zein resin doughs showing the stiffer nature of kafirin dough compared to α-zein dough (Oom et al., 2008). Reproduced with the permission of the copyright holder.

slightly extensible for a few minutes. The effect was attributed to breaking of disulphide bonds. The authors, on the basis of hydrophobicity of the various zein and kafirin sub-classes and the above described findings, suggested that hydrophobic interactions were the key to visco-elastic functionality of these proteins, and that disulphide bonds are undesirable. Smith et al. (2014) investigated the impact of various salts in the Hofmeister series on the formation of zein visco-elastic material (dough). It was found that NaI and NaSCN made the material softer and more extensible, while NaCl and Na_2SO_4 impacted negatively or even disrupted formation of the material, adding further support to the concept that zein visco-elastic properties result from non-covalent interactions.

Despite the fact that the storage proteins from all the gluten-free grains have been isolated and studied, there is essentially no evidence that any of them, apart from α-zein, will form a gluten-like visco-elastic dough when mixed with water. Although, Avanza et al. (2005), for example,showed that amaranth protein produced an elastic gel when an aqueous protein dispersion was first heated to 70-95°C to denature the protein and then cooled.

In view of this, work to determine the molecular basis of α-zein dough functionality will be discussed. This has focused on changes in protein secondary structure and has been explained in detail by Erickson et al. (2012).

3.3 Influence of secondary structure on dough functionality

Mejia et al. (2007) found that when commercial zein (α-zein) was mixed into a dough at 35°C, above its T_g in water, there was a substantial increase in β-sheet structure and concomitant decrease in α-helical structure. This rearrangement was similar, but not identical, to that observed with wheat gluten where with extension there is an increase in β-sheet content and a concomitant decrease in β-turn content (Belton, 2005). However, when the temperature was reduced to 25°C, the

proportion of β-sheet structure in zein decreased substantially, which did not happen with gluten.

Also, upon removal of shear stress, there was a rapid decrease in the proportion of β-sheet

structure, which also did not happen with gluten. This led to Erickson et al. (2012) to theorise that

the visco-elastic properties of zein were related to the formation of a β-sheet rich secondary

structure, similar to that which is believed to be involved in the elasticity of the wheat HMW

glutenin polymers (Belton, 2005). As noted, the major storage protein of amaranth, amarantin, an

11S globulin also has mainly β-sheet structure (Tandang-Silvas et al., 2012) and it might be expected

to have visco-elastic properties, which is not the case. However, since the structure in mainly

intramolecular (not intermolecular) with β-barrel domains and is very stable, it has been suggested

that this could not contribute to the required protein-protein interactions (Taylor et al., 2014).

Figure 2 shows the model developed by Wang and Padua (2012) to explain how zein (α-zein) self-

assembles into nanostructures during film formation through a change from α-helical to antiparallel

β-sheet secondary structure. What has been proposed by Erickson et al. (2012) for when zein is

mixed with water into a dough clearly parallels this. In the Wang and Padua model the antiparallel

β-sheets form what they refer to as a "ribbon". Lai and Padua (1997) showed that films prepared

from zein-oleic resins appeared to be made up of fibres (fibrils) , which presumably are bundles of

ribbons. The ribbons were also observed to form entanglements. Entanglements of fibrils have also

been observed when kafirin was coacervated out of solution into microparticle-type materials

(Taylor et al., 2009). These entanglements resembled an expanded foam (Fig. 3). Several

researchers have observed fibrils in zein doughs by SEM and confocal laser scanning microscopy

(CLSM) and suggested that fibril formation is a critical step in zein visco-elastic dough formation

(Lawton, 1992; Schober et al., 2010; Sly et al., 2014).

Erickson et al. (2012) on the basis of their ongoing research also proposed that the inclusion of a

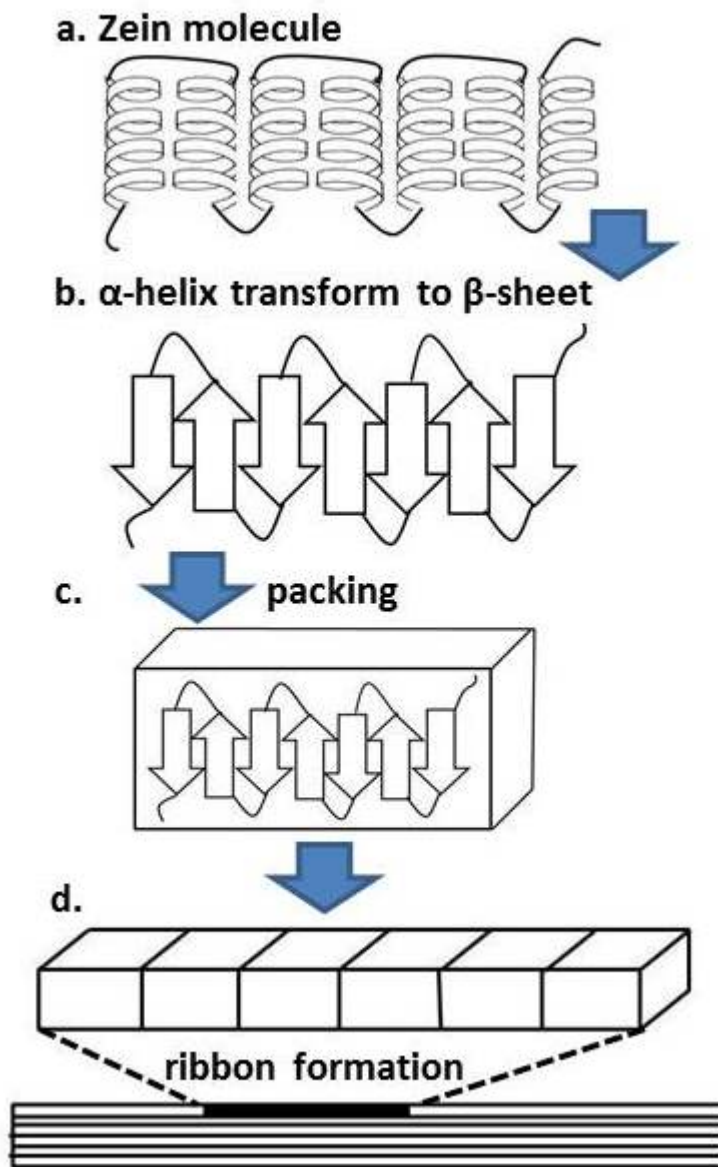small proportion of another protein in the dough such as HMW glutenin or even casein, which they

Fig. 2. Model for zein β-sheet ribbon formation according to Wang and Padua (2012). Adapted and redrawn.
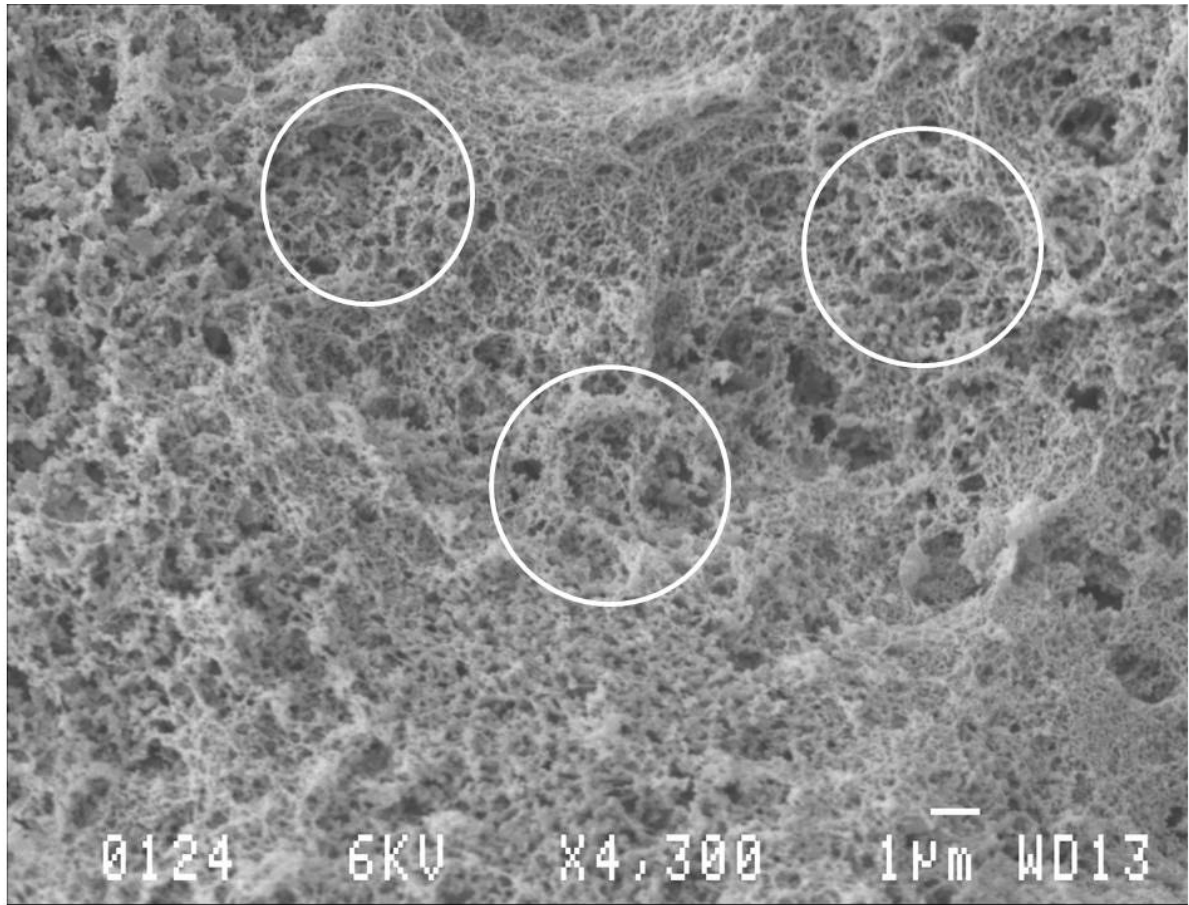
Fig. 3. SEM of entanglements of fibrils of kafirin coacervated from solution. Circles show foam-like structures.

refer to as a "co-protein" is crucial to the stabilisation of the zein β-sheet structure, through the formation of fibrous β-sheet-rich networks. Marco and Rosell (2008a,b) showed that addition of various proteins (pea, soy, egg albumen and whey) to rice flour significantly changed the storage and viscous moduli of the doughs, possible evidence of a co-protein effect.

4. The co-protein concept

4.1 Comparison of wheat gluten and maize zein

For zein, as discussed, a structure with a viscoelastic behaviour can be obtained above its glass transition temperature at appropriate moisture levels (Lawton, 1992; Mejia et al., 2007). Yet, the structure is lost immediately after removal of the stress applied during mixing. At Purdue University, researchers pursued the hypothesis that the maize endosperm proteins are simply missing additional elastic HMW-GS that is in wheat, and that addition of a minor amount of this or another similar protein would confer viscoelasticity to the mixture. Mejia et al. (2012) showed that addition of such a co-protein, namely HMW-GS or casein (as a non-wheat protein), stabilized the viscoelasticity of the hydrated, heated (to 35$^{\mathrm{o}}$C), and mixed maize zein, as well as held stable the β-sheet content after mixing had stopped. Furthermore, they hypothesized that both zein and gliadin are affected both in structure and function by their respective co-proteins. Understanding the properties of gliadin and zein systems with addition of the co-protein or plasticizers has provided a basic knowledge on how to functionalize zein and other zein-type cereal prolamins that might be used for the development of gluten-free systems (Erickson et al., 2012). The gliadin-zein hypothesis has been supported by a rheological and physicochemical study of the effect of HMW-GS addition to gliadin and zein composites (Fevzioglu et al., 2012), with an attempt made to relate structural and rheological data.

4.2 Rheological data

Small Amplitude Oscillatory Shear Tests (SAOS)

The SAOS technique is ideal to characterize the structural properties of viscoelastic materials (Morrison, 2001). Typical data are shear storage modulus (G´) and shear loss modulus (G´´). G´ is defined as the component of stress in phase with the strain and provides an indication of dough elasticity. On the other hand, G´´ is related to the viscous nature of the material and represents the energy lost, and is as the component of stress that is 90° out of phase with the strain (Campanella, 2011). Another material function used to describe the viscoelastic character of a material is the phase angle (δ). A lower phase angle value indicates a dough with higher elasticity. In materials with an amorphous nature such has dough, it provides a better indication of the dough elasticity than the moduli defined above (Fevzioglu et al., 2012).

Based on their phase angle values, two different rheological behaviours were observed between pure proteins and composites containing the "co-protein" HMW-GS (Fig. 4). Doughs with high phase angle values, like zein and gliadin and also HMW-GS alone, were characterized as materials with a low elasticity (Fevzioglu et al., 2012). Conversely,doughs having low phase angle values, which included gluten and composites of gliadin with 20 and 10% HMW-GS and zein with 10 and 5% HMW-GS, had high elasticity. As expected, the dough prepared with gluten showed the lowest phase angle value, suggesting it has a more elastic behaviour than the other materials. In particular, with zein, addition of a small amount of HMW-GS (5 and 10%) caused a substantial change in its viscoelastic properties with phase angle values close to gluten. The finding that 5% HMW-GS co-protein incorporation was nearly equal to 10% HMW-GS supported the view that the low amount of co-protein addition fundamentally changes to the structure and function of the larger "parent" prolamin protein.
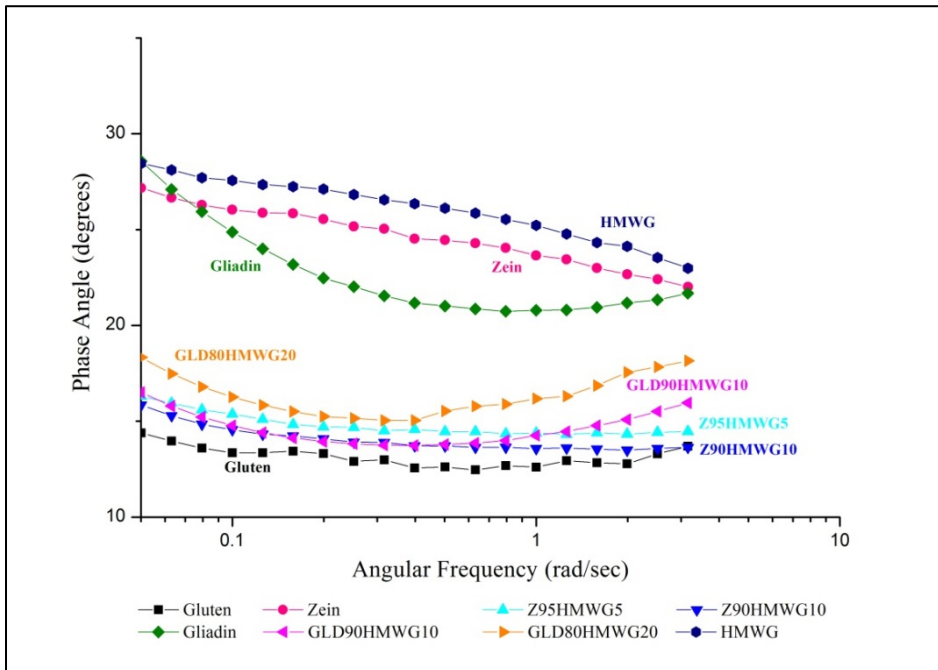
Fig. 4. Changes in phase angle values of zein dough systems with HMW-GS addition (adapted from Fevzioglu et. al., 2012). GLD90HMW-GS10 composite with 90% gliadin and 10% HMW-GS, GLD80HMW-GS20 composite with 80% gliadin and 20% HMW-GS, Z90HMW-GS10 composite with 90% zein and 10% HMW-GS and Z95HMW-GS5 composite with 95% zein and 5% HMW-GS. All these percentages are based on the total amount of protein added to the sample.

Large deformation rheological properties by lubricated squeezing flow test

Rheological measurements using large strains provide a better understanding of baking performance because the dough undergoes deformations/strains that are of the same order than those experienced during baking (Dobraszczyk and Morgenstern., 2003; Sliwinski et al., 2004; Dobraszczyk and Salmanowicz, 2008). The type of deformations to which doughs are subjected during the baking process of dough are biaxial extension and uniaxial elongation (Sliwinski et al., 2004). Thus, it is important to characterize the rheological properties of these composites. Lubricated squeezing flow viscometry is a rheological technique that applies biaxial deformation to a material (Campanella and Peleg, 2002)

This technique was employed to test the hypothesis that the addition of a co-protein (i.e. HMW-GS) enhances the viscoelastic properties of the gliadin and zein (Fevzioglu et al., 2012). Extensional viscosity that measures the resistance of the dough to be stretched was also used to assess the effect of the co-protein. Results showed that, at constant extensional rate, extensional viscosity of the gluten dough was higher than for the other dough types. It also showed significant strain hardening, a rheological property that is associated to good baking performance of dough (van Vliet, 2008). Extensional viscosities of all dough samples, except the HMW-GS dough, exhibited strain hardening (Fevzioglu et al., 2012).. The effect of the co-protein addition was observed by increase in extensional viscosities of zein and gliadin doughs. Notably, the impact of HMW-GS addition was more drastic on zein than on gliadin. Comparisons at higher strain rates showed that composites containing zein in the presence of the co-protein exhibited higher extensional viscosities. Overall, the rheological data gave good support to the concept of using a co-protein to improve the visco-elastic property of zein and hence other non-wheat cereal prolamins such as kafirin and pennisetin .

5. Chemical and physical modification

A number of chemical (especially enzymic) and physical treatments have been applied to non-wheat proteins and their flours in order to modify their protein functional properties, with the aim of forming cohesive and elastic doughs. A weakness of much of this research is that the studies have generally involved flours, as opposed to isolated proteins. This approach is understandable since wheat flour, not isolated gluten, is normally used to make doughs and breads. Also, since essentially only zein is available commercially, the proteins have to be isolated and this can be technically difficult and may alter their properties. However, working with flours does make the understanding of how these "gluten-free" proteins function in dough systems difficult. Hence, in this review, research work into modification of isolated proteins will be discussed first where information is available, followed by studies involving modification of gluten-free flours.

5.1 Acid/base treatments

Acidification is widely used in wheat dough systems to modify dough rheology by increasing water-holding capacity with the aim of increasing shelf-life and also to improve product aroma profile (Houben et al., 2010). Acidification is achieved by sourdough fermentation or by acid addition or by a combination of both. These treatments have also been applied to gluten-free dough systems in attempts to improve the textural, nutritional and shelf-life properties of gluten-free breads.

Strong acid or alkali (0.5-2 M HCl and NaOH) addition, with or without heat, to isolated Z19 α-zein caused deamidation of the zein and partial unfolding of the protein resulting in improved emulsifying properties (Cabra et al., 2007). However, such severe conditions do not reflect what would happen in a food system. Zhang et al. (2011) examined the effects of milder acid and base treatments (0.0005-0.002 HCl and NaOH) on commercial zein at 20℃, below zein's T_g in water. They found structural changes occurred under both acidic and basic conditions (pH 2.7 and 12.5)

compared to near neutral conditions. These changes involved a decrease in α-helix, β-sheet and β-turns and were attributed to mild deamidation of glutamine residues, since there was no evidence of fragmentation or polymerisation of zein molecules by SDS-PAGE. Deamidation of glutamine by acid or base results in the formation of glutamate (Zhang et al., 2011), which has an additional third pKa of 4.07. The conformational changes in the zein resulted in decreased viscosity, elastic modulus and viscous modulus.

Mixing zein into a dough using weak organic acids such as lactic acid and acetic acid, as produced in sourdough fermentations, was found to result in highly extensible but weak doughs (Sly et al., 2014). Increasing the acid concentrations from 0.7% to 5.4% caused a progressive and substantial decrease in dough strength but with retention of dough cohesion. These changes in dough functional properties were thought to be due to the uniformity of the dough and linear orientation of zein fibrils as shown by CLSM. Secondary structural changes above the T_g of zein in water showed that with increasing acid concentration there was an increase in proportion of α-helix when compared to zein mixed with water. However, in all cases the proportion β-sheet structure was still high. When zein was mixed with maize starch or rice flour in the presence of the dilute acids (pH<4.0) cohesive doughs were formed. These doughs could hold air and inflate a small dough bubble by alveograph, although small tears in the bubble rapidly formed. CLSM of the stretched zein-rice flour doughs showed linearly oriented continuous fibrils, which enveloped the flour particles (Fig. 5).

When isolated oat globulins were treated with NaOH to give a pH 9.7 at 25, 37 and 55ºC, insoluble aggregates formed at the lower temperatures and soluble aggregates at 55ºC (Ma et al., 1990). Free sulphydryl content was found to decrease progressively with time at all temperatures and was attributed to disulphide bond formation. Surface hydrophobicity increased at 25ºC but decreased at 37ºC. The changes were suggested to be due to protein conformational changes and partial denaturation of the proteins due to proteolysis by proteases co-extracted with the proteins.
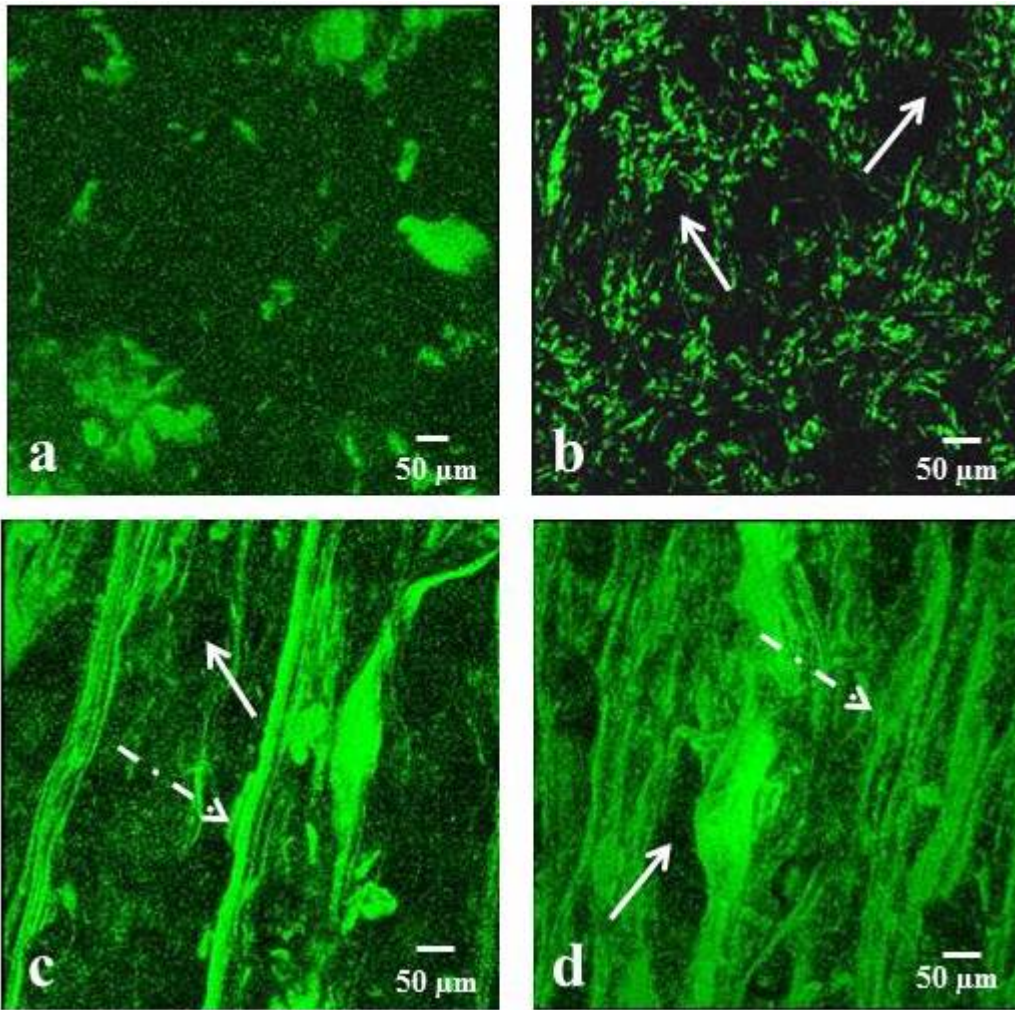
Fig. 5. CLSM of stretched zein-rice flour doughs (Sly et al., 2014). Doughs prepared with: a. distilled water at room temp.; b. distilled water at 40oC; c. 1.3% lactic acid at 40oC; d. 5.4% lactic acid at 40oC. Dashed arrows indicate oriented zein fibrils; solid arrows indicate rice flour particles. Reproduced with the permission of the copyright holder.

When isolated amaranth proteins were exposed to extremes of pH, they also underwent conformational changes and partial or total denaturation (Abugoch et al., 2010). Surface hydrophobicity was found to be lower at acid pH than at alkaline pH. When amaranth flour doughs were acidified with lactic acid to a pH 4.4, the doughs were firmer and but not substantially more elastic than when no acid was present (Houben et al., 2010). Increasing the amount of acid used increased dough firmness further. It appeared that the added protons increased the binding capacity of the proteins in the dough.

5.2 Oxidative cross-linking

Oxidising enzymes such as glucose oxidase and laccase (diphenol oxidase) are used in wheat dough systems to increase dough strength and stability and hence increase bread volume and crumb softness and structure (Goesaert et al., 2005). In gluten-free research, such enzymes have been applied to oats to generate covalent linkages and positively influence the bread-making properties of flours (Flander et al., 2008, 2011; Renzetti et al., 2010). Tyrosinase, which specifically oxidises tyrosine to benzoquinone, cross-linked isolated oat globulins but not oat albumins or prolamins (Flander et al., 2011). The microstructure of tyrosinase cross-linked oat globulins showed aggregated globulins, which resulted in increased oat dough hardness. High levels of enzyme addition resulted in dough softening, attributed to xylanase and β-glucanase side activities in the tyrosinase preparation. This softening was insufficient to counteract the protein cross-linking caused by the tyrosinase, since the oat bread was still harder than the control. Tyrosinase did not affect the extensibility of the oat dough.

Renzetti et al. (2010) found that laccase increased loaf volume and decreased crumb hardness and chewiness of oat breads. The laccase affected oat dough rheology by increasing elasticity, which led to increased stability of batter films during expansion of gas cells. This prevented premature gas cell

rupture. The ability of the batter to deform more easily probably led to improved bread properties. In addition, small protein aggregates in laccase bread crumb led to better bread-making performance. Improvements of dough rheology were also thought to be due β-glucanase side activities. In contrast, Flander et al. (2008) found that laccase treatment increased oat bread firmness. The authors found an increase the amount of middle-sized soluble proteins, (63-65 kDa) with a concomitant decrease in the number of smaller soluble proteins and no effect on oat globulins. They thought that the oat dough had sufficient free ferulic acid for laccase to form phenoxy radicals leading to disulphide bonds between proteins and low molecular weight thiols, resulting in an increase in oat bread firmness. Both research groups measured a reduction in the number of sulphydryl groups (Flander et al., 2008; Renzetti et al., 2010).

Glucose oxidase catalyses the oxidation of glucose to form gluconic acid and hydrogen peroxide. The hydrogen peroxide promotes inter- and intra-molecular disulphide cross-linking in wheat bread systems (Primo-Martin et al., 2003). When applied to gluten-free systems glucose oxidase treatment had different effects, depending on the type of gluten-free cereal (Renzetti et al., 2009a). Maize and sorghum breads showed improved loaf volumes and reduced collapsing of the loaf top, which was attributed to protein polymerisation, aggregated protein structures and enhanced batter elasticity. Positive effects on dough rheology, loaf volume and bread texture were found when rice flour was treated with glucose oxidase in the presence of hydoxypropyl methycellulose as a result of cross-linking of the proteins (Gujral and Rosell, 2004a). When oat flour was treated with glucose oxidase extensive protein polymerisation resulted in large globular protein aggregates as viewed by CLSM (Renzetti et al., 2010). These large protein aggregates were thought to cause detrimental effects on oat bread texture in the form of an increase in crumb hardness. However, the enzyme had no significant effect on the quality of buckwheat or teff breads (Renzetti et al., 2009a).

5.3 Transglutaminase mediated cross-linking and deamidation

Transglutaminase catalyses the formation of ε-(γ-glutamyl)lysine cross-links in proteins (Ohtsuka et al., 2000) but can also deamidate glutamine in lysine-poor proteins such as the cereal prolamins (Larré et al., 1993). It has been widely investigated in gluten-free bread-making but often in combination with other treatments such as protein and hydrocolloid addition (Gujral and Rosell, 2004b; Moore et al., 2006; Marco and Rosell, 2008 a,b), which makes it difficult to establish whether the observed effects on bread quality are due to protein modification or the additives or a combination.

Concerning research on where transglutaminase was used as a sole treatment, buckwheat and brown rice batters showed significant increases in pseudoplastic behaviour and improved baking performance in terms of crumb structure and chewiness but not loaf volume, at high levels of enzyme addition, which were attributed to protein polymerisation (Renzetti el al., 2008a). Increases in crumb hardness, chewiness and decreased specific volumes, at high enzyme addition, were attributed to protein cross-linking. Oat and sorghum batters also showed increased pseudoplastic behaviour but this did not result in improved baking performance or textural properties. Maize batters showed a decrease in elastic properties and improved baking performance in terms of increased specific loaf volume, decreased crumb hardness and chewiness. The authors speculated that due to maize flour's low protein content and low lysine content, deamidation reactions were more prevalent than protein cross-linking. The deamidation reactions could have been responsible for the decrease in viscosity, facilitating expansion of the batter. Using CLSM, the authors observed formation of protein complexes due to transglutaminase action in the case of buckwheat and brown rice batters but no differences could be seen for sorghum, oat, teff or maize batters. This was probably because both glutamine and lysine are needed for transglutaminase cross-linking, and lysine was limiting. Huang et al. (2010) found increased visco-elasticity and enhanced thermal stability of oat doughs treated with transglutaminase, which they attributed to protein cross-linking.

Enzyme accessibility to substrate and protein solubility have been identified as playing roles in transglutaminase cross-linking (Coussons et al., 1992; Kang et al., 1994). Thus, Renzetti et al. (2008a) concluded that the improved baking performance of buckwheat flours treated with transglutaminase was due to the high level of glutamine and lysine in the soluble protein fraction of the flour and also potentially the presence of more accessible enzyme binding sites than oat flours.

A detailed study of the effect of transglutaminase activity on protein fractions (2S albumin, 13S and 8S globulin) of buckwheat flours showed that all the fractions were reactive with transglutaminase (Renzetti et al., 2008b). An increase in molecular weight of all the protein fractions and the formation of high molecular weight protein polymers were thought responsible for the improved flour functionality, resulting in improved baking characteristics. A study of the protein fractions of brown rice flour after transglutaminase treatment showed that the α- and β-glutenin subunits were the primary substrates for polymerisation into supramolecular structures (Renzetti et al., 2012). The brown rice globulin and albumin fractions did not aggregate, despite the high lysine content the albumins. Thus, the specificity of transglutaminase for brown rice glutenins was thought to be due to greater accessibility of the enzyme to their glutamine and lysine residues compared with the albumin and globulin fractions. Transglutaminase polymerisation also promoted new and stronger hydrophobic interactions between proteins in brown rice batters. This, along with transglutaminase polymerisation of the glutelin complexes, was thought to be responsible for the improved textural properties of transglutaminase treated brown rice breads (Renzetti et al., 2008a). The authors speculated that deamidation reactions could be responsible for the improved baking performance of maize flour treated with transglutaminase due to a decrease in batter viscosity facilitating expansion of the batter.

5.4 Specific deamidation

Deamidation of isolated α-zein using glutaminase was found to result in conformational changes

(Yong et al., 2004). A slight decrease in both α-helical and antiparallel β-sheet structure was

accompanied by a concomitant increase in β-turn and random coil.). These changes weakened the

ability of zein to aggregate and led to increased solubility and better emulsion properties. Increased

protein solubility was also found when rice glutelin was deamidated when treated with glutaminase

(Liu et al., 2011). Deamidation resulted in the breaking of hydrophobic, hydrogen and some

intermolecular disulphide bonds and changed protein conformation from α-helix to random coil and

β-turns.

5.5 Disulphide bond reduction

In contrast to improving storage protein dough functionality by cross-linking, there have been a few

investigations in the effects of addition of reducing agents to break disulphide bonding. Smith et al.

(2014) working with isolated zein found that the reducing agent mercaptothanol had little effect on

the ability of isolated zein to form a dough, supporting the authors' hypothesis that non-covalent

interactions are involved in zein dough formation. In apparent contrast, Yano (2010) working with

rice batter found that the reducing agent glutathione dramatically improved batter gas-holding and

subsequent loaf volume. Glutathione was found to prevent the formation of disulphide bonded

"macromolecular protein barrier". In contrast, Capuani et al. (2014) found no substantial effect of

glutathione addition on brown (whole grain) rice and buckwheat batters or breads, and attributed

the different findings to type of redox agent used and molecular oxygen concentrations.

5.6 Protein hydrolysis

The positive effects of lactic acid bacteria and sourdough fermentations on bread quality go far

beyond those caused simply by acid production. Concerning protein effects, fermentation results in

activation of endogenous flour proteases (Hüttner and Arendt, 2010), and where the fermentative

bacteria have high reductive activity such as *Weisella cibaria,* increase the amount of free thiol

groups (Capuani et al., 2013). According to Clarke et al. (2002) sourdough fermentation changes the

dough pH slowly, passing through a range of pH optima for various proteases and other enzymes

present in the dough system. This then results in changes to the protein and starch properties of the

dough, which in turn affects the final dough functionality. Various workers have described resultant

protein degradation, detected by CLSM or by capillary electrophoresis, brought about by sourdough

fermentation of brown rice (Capuani et al., 2014), buckwheat (Capuani et al., 2014; Moroni et al.,

2011; Wolter et al., 2014), sorghum (Schober et al., 2007; Wolter et al., 2014), maize, teff, oat and

quinoa (Wolter et al., 2014). On isolation of dough proteins from sourdough fermented sorghum,

Schober et al. (2007) found that proteases degraded proteins that were already soluble at the

beginning of fermentation. The resulting smaller sized peptides (<19 kDa) could no longer cross-link

and so did not aggregate upon baking. According to Elkhalifa et al. (2006), the small peptide

proteolysis products are taken up by the bacteria for growth. Degradation of the kafirin by proteases

has been found to be limited due to its location in protein bodies (Elkhalifa et al., 2006; Schober et

al. (2007). Proteolysis also contributes to bread flavour (Arendt et al., 2007). In the case of maize

sourdoughs, endosperm protein matrix degradation by proteases was thought to allow partial starch

hydrolysis and leaching of amylose, resulting in less elastic doughs which were able to trap and

retain carbon dioxide in a cohesive dough (Falade et al., 2014). Sourdough fermentation of

buckwheat flours degraded the globulin fractions of the flour releasing smaller polypeptides, thus

weakening protein-protein and protein-starch interactions and possibly influencing network

connectivity (Moroni et al., 2011).

Proteases have been used as additives to improve dough and bread quality in rice breads, resulting

in increased loaf volume and softer crumb structure (Hamada et al., 2013; Kawamura-Konishi et al.,

2013; Renzetti and Arendt, 2009b; Hatta et al., 2015) and lower staling rate (Kawamura-Konishi et

al., 2013). The mechanism seems to involve the hydrolysis of natively present disulphide-linked

macromolecular proteins, which form a barrier around the rice starch granules and limit the swelling

of the starch (Renzetti and Arendt, 2009b). Examination of the effects of different types of

proteases showed that metallo, cysteine and serine type proteases were more effective at improving

rice bread quality than aspartyl proteases (Hatta et al., 2015). Limited hydrolytic activity by

proteases on rice storage proteins has been considered sufficient for improving rice bread quality

(Hamada et al., 2013; Kawamura-Konishi et al., 2013). However, Hatta et al. (2015) described an

increase in smaller proteins derived from the degradation of α- and β-glutelin. They considered that

these small proteins formed aggregates through disulphide cross-linking and hydrophobic

interactions on kneading the rice batter after endo-type protein hydrolysis. The protein aggregates

formed fine networks which retained carbon dioxide during fermentation and resulted in increase in

loaf volume and decrease in crumb hardness.

Protease addition to buckwheat, sorghum, maize and teff flours resulted in no significant effects on

loaf volume or crumb hardness for maize and teff (Renzetti and Arendt, 2009a). However, with

sorghum a liquid-like batter was produced. As only slight differences in protein microstructure and

protein profiles were observed, this indicated that limited protein hydrolysis had occurred. When

baked, these batters showed increased loaf volume but reduced crumb cohesiveness. Buckwheat

batters also showed decreased viscosity with protease treatment. Protein degradation to low

molecular weight peptides was observed along with decreased protein continuity when the batters

were examined by CLSM. Baking of buckwheat batters also resulted in increased loaf volumes but

with the presence of a large hole in the centre of the loaf. Oat bread batter quality was found to be

improved with protease addition in terms of batter softness and increased ability of the batter to

deform and increased elasticity, resulting in increased specific volume and decreased crumb harness

and chewiness of breads (Renzetti et al., 2010). These improvements were attributed to extensive

protein hydrolysis by the protease and depolymerisation of β-glucan by β-glucanase side activities in the protease preparation.

5.7 High pressure

Very high pressure treatment (≥200 MPa) has been investigated as a method of improving gluten–free cereal batter functionality. Hüttner et al. (2009) found that the application of hydrostatic pressure (≥300 MPa) to oat batter (flour-water batter) induced the formation of urea-insoluble protein complexes and/or disulphide bonds. The pressure treatment also induced starch gelatinisation. High pressure treatment was also found to induce protein polymerisation with rice and teff batters through thiol group-sulphydryl group interchanges, but this did not occur with buckwheat batter (Vallons et al., 2011), presumably due to the low level of cysteine in buckwheat storage proteins. With sorghum batters, pressures ≤300 MPa weakened the structure, due to protein polymerisation, whereas at pressures >300 MPa batter constancy increased due to pressure induced starch gelatinisation (Vallons et al., 2010). Breads with 2% pressure-treated sorghum showed delayed staling but at 10% addition the breads had low volume and poor quality. Similarly, high pressure treatment (350 MPa) of several different cereal flour batters was found to have negative effects on important quality parameters of wheat plus gluten-free cereal composite breads (Angioloni and Collar, 2012). With oat-wheat composite bread, loaf volume was reduced and with sorghum- and finger millet-wheat composites, whilst there was no adverse effect on loaf volume, the staling kinetics of the breads were more rapid.

6. Genetic modification of storage protein expression

High protein digestibility sorghums have been developed using mutation breeding that have altered, folded (invaginated) shaped protein bodies, due to the γ-kafirins being located at the base of the
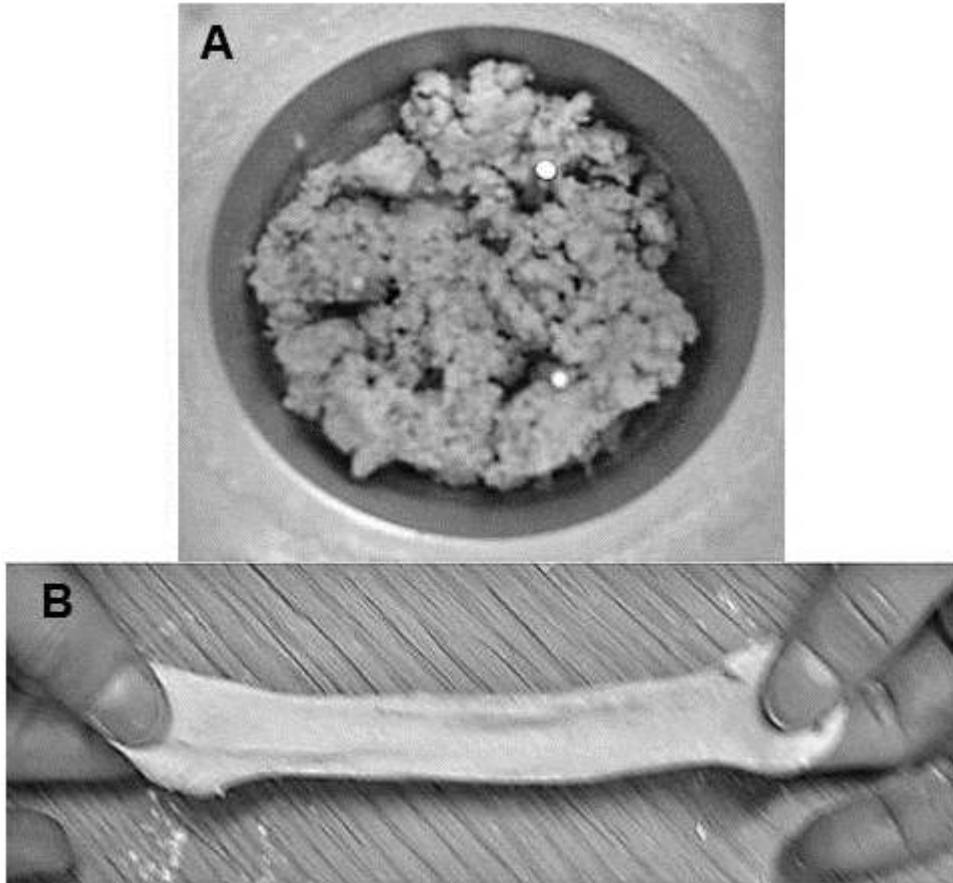
Fig. 6. Normal sorghum (left) and HDHL sorghum (right) flour doughs with 18% vital wheat gluten addition (Goodall et al., 2012). Reproduced with the permission of the copyright holder.

invaginations, rather than around the protein body periphery (Oria et al., 2000). Goodall et al. (2012) showed that composites of high protein digestibility sorghum flour with added vital wheat gluten (18%) formed a visco-elastic dough, whereas normal sorghum flour plus gluten did not (Fig. 6). They also showed that composite flour doughs of wheat and high protein digestibility sorghum had substantially greater resistance to extension than composites made with normal sorghum flour and that their breads were significantly larger. Kumar et al. (2012) concluded that the kafirin was freed from the protein bodies in these high protein digestibility sorghums and hence had the ability to form improved protein networks in wheat flour composites compared to normal sorghum.

Regarding the application of recombinant DNA technology (genetic engineering), the wheat HMW-GS protein Glu-1Dx5 which notably increases dough strength (Blechl et al., 2007), has been expressed in maize (Sangtong et al., 2002). Recently, the expression of the Glu-1Dx5 protein in maize was examined in relation to its interaction with maize prolamins during maize endosperm development (Zhang et al., 2013). Its expression resulted in some distortion of protein body shape. Further, when the expression of γ-zein was down-regulated, protein deformation was enhanced and a non-vitreous phenotype was produced. Apparently, the dough functionality of this phenotype was not investigated.

Down-regulating the expression of the cysteine-rich γ-kafirin in sorghum using recombinant DNA technology with the aim of reducing disulphide bond cross-linking and hence improving protein digestibility has been achieved in different studies. Da Silva et al. (2011a) and Grootboom et al. (2014) showed that co-suppression of the synthesis of α-, γ-, β- and δ-kafirins modified the structure of the protein bodies so that they had invaginations like those of the high protein digestibility mutant (Oria et al., 2000) and that they were less densely packed in the endosperm. These changes resulted in the endosperm structure becoming floury. Da Silva et al. (2011b) showed that these transgenic sorghums also had a much reduced proportion of disulphide bond cross-linked type

kafirin. Dough-based baked products including biscuits and flatbreads of comparable quality to those from normal sorghum could be produced from these transgenic sorghums (Taylor and Taylor, 2011). However, their actual dough properties and leavened bread-making quality were not investigated. Kumar et al. (2012), in general agreement with the findings of Da Silva et al. (2011a) and Grootboom et al. (2014), showed that γ-kafirin suppression alone did not alone alter protein body structure, but attributed this to the silencing of a predicted 29 kDa α-kafirin sub-class (presumably a cysteine-poor protein). With the aim of improving the roti (flatbread) making quality of sorghum, gene cassettes containing a wheat HMW-GS gene driven by the γ-kafirin promoter have been constructed (Mishra et al., 2008). Latterly, similar gene cassettes have been used to express the wheat HMW-GSs Dy10/Dx5 in sorghum (Kumar et al., 2012). However, the impact of expression on wheat HMW-GS on sorghum dough and breadmaking quality has not as yet been determined.

There has been little research into genetic modification of storage proteins in the pseudocereals. Carrazco-Peňa et al. (2013) modified the primary structure of amaranth 11S globulin with the aim of improving its functional properties, by insertion of four continuous methionine residues and expressing the gene in *E. coli*. The modification increased the hydrophobicity of the protein and resulting in it forming medium strength gels.

7. Conclusions

Despite nearly 25 years research into the functionality of "gluten-free" storage proteins in dough systems, only zein been shown to form a wheat gluten-like visco-elastic dough. Further, this is only when the zein has been extracted from the maize in the form of α-zein, the most hydrophilic zein sub-class (Duodu et al., 2003). Clearly, mimicking gluten functionality is very challenging. While the co-protein concept has potential, this technology used alone has been unable to produce a

comparable product to wheat bread. This probably related to the fact that zein is less hydrophilic than the wheat gluten proteins (Duodu et al., 2003; Shewry et al., 2003).

Perhaps, it will take a combination of technologies to produce gluten-free products that have all the attributes of wheat bread. Addition of a functional visco-elastic zein plus an appropriate non-gluten co-protein, together with hydrocolloids to a non-wheat cereal/pseudocereal flour may combine the desirable nutritional, protein-associated textural properties and high loaf volume associated with wheat bread.

With regard to improving the functionality of the storage proteins in oats, rice and the pseudocereals, enzyme treatments such as transglutaminase and proteolysis have some limited positive benefits on the functionality of the batter dough systems, but the effects seem to be grain protein type dependant. Hence, the best practical approach seems to be to use a combination of hydrocolloids and protein treatment to improve bread texture.

Regarding future research directions, the theory that a change in secondary structure from α-helical to β-sheet is key to zein functionality appears to be well founded. However, as indicated, β-sheet structures are themselves highly complex (e.g. intra vs. inter, coils vs. barrels). At present we do not know enough about the nature of the β-structure in maize zein dough and what factors influence this and what prevents formation of doughs with sorghum kafirin, which is structurally very similar. Research into mutants where zein/kafirin synthesis is altered appears to be a promising avenue. Concerning other gluten-free grains, in order to improve our fundamental understanding of the observed effects on their flours of protein-altering treatments , there is a clear need to investigate how these treatments affect the functionality and structure of their isolated proteins.

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