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Wheat glutenin polymers 1. structure, assembly and properties

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This paper is dedicated to our mentor, friend and colleague Dr. Donald D. Kasarda (1933–2021).

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ABSTRACT

The importance of wheat glutenin polymers in determining the processing quality of wheat is generally accepted. Similarly, genetic and molecular studies have provided detailed information on the sequences of the glutenin subunits and identified associations between individual subunits and either good or poor quality for bread-making. However, our knowledge of the polymers themselves, including their molecular masses, structures and pathways of synthesis and assembly, remains incomplete and is largely based on studies carried out between 20 and 50 years ago. The current paper therefore reviews this knowledge and identifies priorities for future research which is required to facilitate the use of modern molecular tools to develop improved types of wheat for future requirements.

2. Introduction

Although many aspects of wheat seed quality remain unresolved, there is general agreement on the importance of glutenin polymers in determining the structure and functional properties of dough and, in particular, in determining dough strength. Similarly, there is general agreement on the importance of the high molecular weight subunits in determining the structure and properties of these polymers. These conclusions are largely based on studies carried out in the last century, which were regarded by the researchers as "work in progress" rather than conclusive. Nevertheless, they have led to concepts and structural models which have become accepted as dogma, being widely and uncritically referenced in more recent studies.

In order to understand our current knowledge it is helpful to briefly review the historical development of the research. The authors entered the field at the end of the 1970s, which was essentially the end of a first phase of research. This was led by physical chemists, particularly at the USDA Regional Research Centres at Peoria and Albany and focused on the development of methods to solubilise and characterise gluten proteins using approaches based on polymer chemistry. Wheat protein research was subsequently revolutionised by the development of sensitive high-resolution analytical systems, electrophoresis and HPLC, followed by molecular genetics. Hence, research after 1980 was largely focused on biochemistry and molecular biology and ground-breaking studies from before this time are rarely read, despite their relevance to our current understanding.

We therefore critically review our current knowledge of glutenin polymer structure and properties, focusing particularly on the early (C20) studies which "established" the currently accepted concepts, but also referring to relevant recent studies. Our aim is to distinguish between scientific facts and conjecture and identify knowledge gaps for future research. Although we briefly introduce the glutenin subunits, details of their role in polymer formation are discussed in the accompanying article (Lafiandra and Shewry, 2022).

2. Glutenin subunits

The glutenin fraction represents about half of the total gluten proteins and consists of polymers stabilised by inter-chain disulphide bonds. Reduction of these disulphide bonds using reducing agents such as 2-mercaptoethanol and dithiothreitol releases the individual glutenin subunits which can be readily separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). This system separates two groups of protein subunits with masses of between about 60,000 and 100,000 and between about 30,000 and 50,000. These are called the high molecular weight subunits (HMW-GS) and low molecular weight subunits (LMW-GS) subunits of glutenin, respectively, and account for about 30–35% and 65–70% of the total fraction, respectively (Wieser, 2000).

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2.1. HMW subunits

The HMW subunits form a clearly defined group, with three to five individual proteins being present in commercial cultivars of bread wheat. They have been extensively studied and reviewed in detail (Shewry et al., 2003b). We will therefore only provide a brief summary here.

1. The HMW subunits are encoded by six genes in bread wheat, two each at the *Glu-1* loci on the long arms of chromosomes 1A (*Glu-A1*), 1B (*Glu-B1*) and 1D (*Glu-D1*).
2. The two genes at each *Glu-1* locus encode related proteins which differ in their characteristics including masses: these are called x-type (masses about 80,000–100,000) and y-type (masses about 60,000–80,000) subunits. Because the two genes are tightly linked recombination is rare and the x-type and y-type subunits are usually inherited as "allelic pairs".
3. The HMW subunits are highly polymorphic, with "allelic variants" being identified by differences in mobility on SDS-PAGE.
4. The variation in subunit number (3, 4 or 5) observed in commercial cultivars results from the fact that not all HMW subunit genes are expressed (due to mutations silencing the different *Glu-1* genes). Hence, all commercial genotypes express 1Dx, 1Dy and 1Bx subunits while some also express a 1Ax and/or 1By subunit. 1Ay subunits are only rarely expressed in bread wheat but do occur in other wheat species.
5. Further mutations resulting in the silencing of all individual *Glu-1* genes can be identified in genotypes in gene banks (e.g. Nap Hal) or in populations developed by mutagenesis. This allows the mutations to be combined to construct sets of "near-isogenic lines", allowing the effects of the loss or substitution of individual subunits or pairs of subunits to be compared in a similar genetic background.
6. The genetic variation in the number and properties of the HMW subunits is associated with effects on dough strength: this results from quantitative effects (more expressed subunits resulting in more HMW subunit protein) and qualitative effects (associated with allelic differences in expressed subunits) (Payne, 1987).
7. The association of the HMW-GS with processing quality has led to the determination of the full amino acid sequences of many subunits and analysis of their structures and properties. These studies have focused on the numbers and distributions of cysteine residues (as sites for cross-linking) and the formation of elastomeric structures and are discussed below.

2.2. LMW subunits

The LMW-GS are the major group of glutenin subunits, accounting for 65–70% of the total glutenin fraction (Wieser, 2000). They are also more diverse than the HMW-GS and their associations with grain quality less well-understood.

SDS-PAGE of the reduced glutenin subunits separates three groups of bands which were initially called B-, C- and D-type glutenin subunits (the HMW-GS being A-type). The B-type LMW-GS are most abundant and comprise a discrete group of proteins which are related to the α-gliadins and γ-gliadins. Their masses vary between 31,000 and 42,000 and all have eight cysteine residues (although their distributions within the proteins vary, as discussed by Shewry et al. (2009a)). By contrast, the C-type and D-type LMW-GS are essentially forms of α/γ-gliadins and ω-gliadins, respectively, in which mutations have led to the presence of odd numbers of cysteine residues (Masci et al., 1999, 2002). This odd number of cysteines means that at least one cysteine is able to form interchain bonds. This contrasts with typical gliadins which have either six or eight cysteines which form three or four intrachain disulphide bonds in α-gliadins and γ-gliadins, respectively, and no cysteine residues in ω-gliadins.

The B-type LMW-GS are encoded by genes present at the orthologous *Glu-3* loci on the short arms of the homoeologous group 1 chromosomes in bread and durum wheats, closely linked to the *Gli-1* loci encoding gliadins.

3. Glutenin polymers

3.1. Molecular mass range of glutenin polymers

The widely quoted suggestion that glutenin polymers are the largest proteins in nature is almost certainly not true, this record probably being held by covalently cross-linked connective tissues in animals (collagen and elastin). Furthermore, although it is clear that the largest glutenin polymers may be very large, their maximum size and size distribution remain unclear. This uncertainty results from technical limitations to polymer solubilisation and analysis.

Firstly, glutenin polymers are not soluble in the aqueous media which are usually used to extract and characterise proteins. This may relate to two features. Firstly, they interact strongly with other glutenin polymers and gliadins by non-covalent forces, notably hydrogen bonds (as discussed below), and these forces need to be disrupted to separate the individual polymers. Hence, a range of chaotropic solvent systems have been developed to disrupt these forces. However, even under these conditions the individual glutenin polymers and gliadins may still aggregate.

Secondly, the separation of large polymers requires the use of chromatographic media with large pore sizes, such as agarose or controlled pore glass as opposed to acrylamide or cross-linked dextrans. Alternative systems which are not based on the use of chromatographic media, such as ultracentrifugation and asymmetric flow field-flow fractionation (AFFFF), have therefore also been used. However, in all cases the systems must be compatible with the solvents used for solubilisation (which usually contain chaotropic agents and/or detergents).

Finally, calculations of masses will be affected by the assumptions made for calibration and calculation. Gel filtration columns are often calibrated using carbohydrate polymers and protein polymers will almost certainly differ in their shapes and properties. Similarly, calculations of mass based on hydrodynamic properties require assumptions to be made about polymer shape (compact or extended).

Table 1 summarises the results from a range of approaches carried out over 60 years. None can be regarded as definitive, having limitations relating to the methods used to extract and solubilise the samples, the separation ranges of media, the standards used for calibration and the assumptions required for calculations.

The most widely used approach over the past 20 years has been AFFFF, which has given masses up to 10^8 even when the samples were prepared using sonication (Table 1). However, lower polymer masses, up to 2×10^6, were reported when AFFFF was carried out in ethanol: water as a mild chaotropic solvent (Morel at el., 2020). Furthermore, the "polymer" fraction soluble in ethanol:water also contained ω-gliadins, indicating that some non-covalent interactions were not disrupted, and larger "assemblies" (above 2×10^6) even richer in ω-gliadins were also present. This suggests that the higher molecular masses reported by other workers, including by AFFFF of fractions dissolved in SDS, may have included non-covalently bonded gliadins and glutenin polymers as well as covalently bonded polymers.

It should be noted that several of the studies listed in Table 1 used sonication in SDS solution for extraction. Sonication may result in shearing of large polymers, and this also applies to systems in which the proportions of large polymers separated by size-exclusion chromatography are used to predict grain quality (Singh et al., 1990). These systems require strict standardisation of the sonication conditions to ensure that the degree of shearing, and hence the size distribution of the polymers extracted, is reproducible (Morel et al., 2002). Large gluten polymers may also be sheared by vigorous stirring and hence this should be avoided if possible. For example, Wellner et al. (2005) prepared wheat protein bodies by flotation after chopping developing grain to

Table 1
Determination of the masses of glutenin polymers, with details of methods used.

Fraction analysed	Method of separation	Solvent for separation	Method of mass determination	mass range	comments	Reference
Freeze-dried gluten dissolved in aluminium lactate/lactic acid at pH3.5, glutenin precipitated by addition of sodium hydroxide to pH4.6	Sedimentation equilibrium ultracentrifigation	4M guanidine thiocyanate	Calculated from sedimentation data	Up to 3 x 10^6	Also smaller components which may have included gliadins.	Jones et al., 1961.
Flour extracted with AUC* (total gluten proteins)	Gel filtration on Sepharose 2B and 4B	5.5M guanidinium chloride	Based on fractionation range of column	100,000 to over 20 x 10^6	Use of chaotropic acids ensured almost complete solubility. Calculated masses affected by column calibration.	Huebner and Wall, 1976.
TGlutenin extracted with 0.1M acetic acid and separated on Sephadex G200. Void volume peak taken as Ac-OH glutenin.	Gel filtration on Sepharose CL-4B	AGC*	Based on fractionation range of column	200,000 to over 10 x 10^6	Unlikely to contain largest and smallest polymers. Calculated masses affected by column calibration.	Rao and Nigam, 1988
Glutenin extracted with 0.5% SDS, pH7.0 and precipitated with 70% ethanol	Gel filtration on Toyopearl HW-75F	0.2% SDS	Based on void volume of column and calibration with dextrans	Up to over 10 x 10^6,		Danno et al., 1990.
Fractions extracted with progressively increasing concentration of HCl and dissolved in0.25% SDS pH6.8 with sonication	AFFFF	0.1% SDS, pH6.8.	From hydrodynamic diameter	In the range 440,00 (extended form) to 1.1 x 10^6 (spherical form)	Sonication may shear large polymers. Calculated masses may be affected by SDS. Sonication may shear large	Wahlund et al., 1996
Sequential extraction of flour with 0.05M acetic acid (gliadins and extractable glutenins) followed by 0.001MHCl or 0.001M HCl + sonication or 0.05M acetic acid + sonication	AFFFF	0.05M acetic acid + 0.002% FL-70 (surfactant)	Based on Stokes diameters calculated based on calibration with protein of mass range 12,500 to 450,000	Consistent with "from less than 200,000 into the millions"	polymers. Calculations based on calibration with smaller proteins.	Stevenson and Preston, 1996.
Flour extracted 0.05M acetic acid then sonicated with 0.05M acetic acid	AFFFF	0.05M acetic acid + 0.002% FL-70 (surfactant)	Multiangle laser light scattering	Major peak 225,000 to 300,000; values up to 10 x 10^6	Sonication may shear large polymers. Biased towards larger polymer species due to light scattering calculations.	Stevenson et al., 2003.
Monomeric proteins removed and then sonicated with 2% SDS in Na phosphate buffer pH6.9.	AFFFF	2% SDS in Na phosphate buffer pH6.9.	Photon correlation spectroscopy	Two particle populations, molecular weights estimated as from 1 to 6.7 x 10^6 and from 11 to 34 x 10^6 in 6 cultivars	Particles may represent aggregated polymers. Smaller particles underestimated as obscured by larger particles.	Sutton, 1996
Glutenin extracted with 2% SDS at pH8.5 and precipitated with acetone.	Agarose gel electrophoresis	0.1% SDS	Dynamic light scattering	500,000 to over 5x10^6	Only fractions containing large polymers analysed. Masses will depend on assumptions of protein shape	Egorov et al., 1998
Glutenin dissolved by stirring with 0.25% SDS at pH 6.8 for 3-7 days at 4°C	AFFFF	0.25% SDS	Multiangle laser light scattering	2-3 x 10^7	Sonication resulted in lower mass of 0.4-0.6 x 10^7	Arfvidsson et al., 2004
Polymeric proteins extracted with 0.1M phosphate buffer, pH6.9, containing 2% SDS with sonication	AFFFF	0.1M phosphate buffer, pH6.9, containing 0.1% SDS	Multiangle laser light scattering	Up to 10^8	Sonication may have resulted in some shearing.	Lemelin et al., 2005
Commercial gluten samples dissolved by sonication in 0.05M acetic acid	AFFFF	0.05M acetic acid	Multiangle laser light scattering	8 x 10^6 to 3.5 x 10^8	Cannot rule out effects of production methods on polymers. Sonication would result in some shearing.	Pitkanen et al., 2014
Glutenin fraction suspended in 50:50 ethanol:water	AFFFF	50:50 ethanol: water	Multiangle laser light scattering	10^5 to 2 x 10^6	Ethanol:water is a mild chaotropic solvent	Morel et al., 2020

AFFFF, asymmetrical Flow Field-Flow Fractionation; AUC: 0.1N Acetic acid, 3M urea, 0.01N cetyltrimethyl ammonium bromide; AGC: 0.1M acetic acid, 2,25M guanidinium chloride, 0.01M cetyltrimethyl ammonium bromide.

avoid effects of mechanical work input on polymer interactions.

It can therefore be concluded that the size of the covalently-stabilised glutenin polymers extends up to at least 1 to 2 × 10^6, with at least some reports of higher masses resulting from strongly bound gliadins and interactions between glutenin polymers which were not dissociated by the solvents used for extraction and separation.

3.2. Analysis of polymer size classes show relationships between mass, subunit composition and solubility

Although individual glutenin polymers have not been purified and characterised it is clear that they vary in size and subunit composition, and that this variation relates to solubility.

The most convincing evidence for this comes from analyses of two fractions. The "classical gliadin" fraction extracted with 70% (v/v) aqueous ethanol contains substantial amounts of oligomeric and/or polymeric components which can be separated by gel filtration chromatography in denaturing solvents: these have been called high molecular weight gliadin and aggregated gliadin. SDS-PAGE of reduced subunits and partial amino acid sequencing shows that this polymeric fraction is highly enriched in B-type and C-type LMW subunits, with only traces of HMW subunits (Bietz and Wall, 1980; Shewry et al., 1983). Whereas Bietz and Wall (1980) suggested that this fraction comprises mainly oligomers of mass 100,00–150,000, Shewry et al. (1983) showed that they were excluded from a Sephacryl S300 column with a fraction range for globular proteins of 1.5×10^6. However, the true mass has not been established.

By contrast, a number of studies have shown that high molecular weight polymers are enriched in HMW subunits of glutenin. For example, high molecular mass fractions prepared by gel filtration chromatography after solubilisation using chaotropic agents such as AUC (0.1N acetic acid, 3M urea, 0.01N cetyltrimethyl ammonium bromide) (Field et al., 1983) or sodium stearate (Hamauzu et al., 1979) or by SE-HPLC after sonication in SDS buffer (Larroque et al., 1997). However, as noted above, it must be borne in mind that the use of sonication to solubilise polymers for separation may result in some shearing.

Hence, it is clear that the glutenin subunit composition varies with polymer mass and that the HMW-GS are concentrated in large polymers. However further studies are required to quantify these differences, and in particular to avoid the shearing of polymers during extraction.

3.3. Genetic variation in polymer size and composition

Several observations indicate that genetic variation in glutenin composition results in effects on polymer size and structure.

Firstly, the proportion of high molecular mass polymers varies between genotypes and is associated with differences in processing quality (see, for example, Singh et al., 1990). Similarly, the relationship between allelic variation in HMW subunit composition and dough strength is well-established (Payne, 1987) and HMW-GS are known to be enriched in high molecular mass polymers, the proportions of which are also correlated with dough strength (reviewed by Shewry et al., 2003b). These observations therefore imply that allelic variation in HMW-GS affects polymer amount and size.

Although this hypothesis is widely accepted there is limited direct evidence or information about the mechanism. The most convincing evidence comes from comparisons of near-isogenic lines which differ in alleles at the *Glu-1* loci (PopineauCornec et al., 1994; Naeem and MacRitchie, 2005). The subunit pairs compared in these experiments differed in cysteine content, with the "good quality" subunit 1Dx5 having an additional cysteine residue compared with 1Dx2 and the "poor quality" 1Bx20 having two less cysteine residues (due to amino acid substitutions) than 1Bx7 (Shewry et al., 2003a,b). Analysis of polymers from 16 cultivars by AFFFF also showed strong genetic effects on the size distribution of polymers with the number average molecular weight being higher in cultivars with the good quality subunits 1Dx5+1Dy10 than those with the poor quality subunits 1Dx2+1Dy12 (Lemelin et al., 2005).

Whereas the effects of subunits 1Dx5 and 1Bx7 on polymer size could be attributed to differences in cross-linking. this mechanism will not apply to differences between other allelic pairs of subunits which do not differ in cysteine content.

3.4. How are glutenin polymers stabilised: covalent and non-covalent interactions

It is generally accepted that glutenin polymers are stabilised by interchain disulphide bonds. This conclusion was initially based on observing the effects of reducing agents but has since been demonstrated by the identification of interchain disulphide bonds between glutenin subunit. Thus, HMW-GS:HMW-GS, LMW-GS:LMW-GS and HMW-GS:LMW-GS bonds have been identified by digestion with proteases followed by the isolation and characterisation of peptides containing disulphide bonds (as discussed below). Although low levels of other types of covalent cross-links have been suggested, notably dityrosine bonds (Tilley et al., 2001), these may only be formed under extreme conditions during processing and there is no evidence for their formation *in planta*.

In addition, glutenin polymers associate with each other and with gliadins by non-covalent forces. Although hydrophobic and electrostatic interactions have been suggested, there is little doubt that the most important non-covalent forces are hydrogen bonds. There is a range of evidence for this including the effects on dough rheology of gluten esterification and of mixing in the presence of chaotropic agents (such as dilute urea), under nitrogen and with deuterium oxide instead of water. It is also consistent with the behaviour of dough and gluten on heating, which will "melt" hydrogen bonds but strengthen hydrophobic interactions. Readers are referred to the review of Belton (2005) and a lively correspondence in this journal (MacRitchie, 2007; Belton, 2007; van Vliet and Hamer, 2007) for a more detailed discussion of the relationship between the structures and interactions of glutenin polymers and the mechanical properties of gluten.

All gluten proteins are rich in glutamine residues, resulting in a "hydrophilic" amino acid composition. The fact that they are not soluble in water, as would be predicted from their compositions, is due to the formation of hydrogen bonds with glutamine residues in the same or other gluten proteins, rather than with water (as discussed by Shewry et al., 2003b). This formation of hydrogen bonds may be facilitated by the fact that the glutamine residues are regularly spaced in repeated peptide motifs. These regularly spaced bonds have been suggested to form "glutamine zips", which are similar to those formed by protein deposits in neurodegenerative diseases of humans (Perutz et al., 1994).

4. Models of glutenin structure

A number of "models" of the covalent structure of glutenin polymers have been proposed. We will therefore re-examine these in the light of our current knowledge.

Direct evidence for polymer structure comes from biochemical analyses to identify interchain disulphide bonds (discussed above) and the presence of oligomeric and dimeric components (discussed below) and this information must be included in any models. In addition, models must account for our knowledge of polymer mass and composition, notably the ratio of HMW:LMW subunits in total glutenin (about 1:4) and the variation in this ratio in relation to mass. Models also need to account for the biophysical properties of glutenin, and for the effects of various factors such as temperature and chaotropic agents on these. However, it is important to stress that even the most sophisticated models are not "established structures", but hypotheses which should be testable by experimentation.

The first serious attempt to develop a structural model for gluten was the "Linear Glutenin Hypothesis" of Ewart (1977). He rejected the widely held assumption that glutenin formed a cross-linked rubber-like structure because this structure could not explain a number of properties, notably viscous flow and work hardening. He therefore suggested that glutenin consisted of linear chains (concatenations) of glutenin molecules joined by disulphide bonds. He also proposed that work hardening results from the build-up of secondary forces, which we would now consider to be mainly hydrogen bonds, between these

molecules, rather than the entanglement of chain (knots).

Bietz and Wall (1980) discriminated between polymers formed by HMW and LMW subunits. They suggested that the HMW subunits formed linear chains as proposed by Ewart, but that the LMW subunits formed oligomers of mass 100,000 to 150,000. They further suggested that the oligomers and individual LMW subunits could be linked to the HMW subunit concatenates by disulphide bonds. A similar but more detailed model was proposed by Graveland et al. (1985). This was based on analyses of glutenin fractions prepared by precipitation/redissolution and considered the roles of individual HMW subunits.

These models have led to a generally accepted view that the HMW subunits form the "backbone" of glutenin with LMW subunits forming side branches (see, for example, Wrigley, 1996; Shewry et al., 2003b; Wieser, 2007) (Fig. 1). The central role of the HMW subunits is based on the identification of head-to-tail disulphide bonds by mass spectroscopy of peptides from protease digestion of unreduced glutenin, and the identification of x-y dimers released by partial reduction of glutenin (although disulphide exchange could be catalysed by the reducing agents used) (these studies are reviewed by Shewry and Tatham, 1997).

We know much less about the organisation of LMW subunits in glutenin polymers. A number of disulphide bonds involving LMW subunits (B, C and D-type) have been mapped, including several which appear to be interchain bonds between B-type and C-type (α/γ-gliadin related) LMW subunits and HMW subunits (Lutz et al., 2012; Schmid et al., 2017). The C-type and D-type LMW subunits usually contain only single cysteine residues available for the formation of interchain bonds. Hence, they will either be attached to HMW subunits as single branches or block the further extension of chains of LMW subunits (ie. act as chain terminators). Consistent with this, Venzel et al. (2014) showed that these proteins are enriched in polymers which are extracted in SDS without sonication.

Our current knowledge can therefore be summarised as follows:

1. HMW subunits may form head-to-tail concatenates which form the backbone of large glutenin polymers.
2. Individual LMW subunits and/or LMW subunit oligomers and polymers may be attached to this backbone by interchain disulphide bonds
3. Soluble polymers are enriched in chain-terminating C-type and D-type LMW subunits.
4. Smaller polymers and oligomers comprising only LMW subunits also occur.

A particular limitation of all these models is that the role of the LMW subunits is not adequately explained. Because they account for about 65–70% of total glutenin they must form extensive side-branches to the HMW subunit concatenates and/or a substantial population of separate oligomers and polymers.

5. How does glutenin structure determine gluten elasticity?

It is likely that several mechanisms contribute to gluten elasticity.

Firstly, it has been suggested, based on a range of biophysical studies and predictions, that the central repetitive domain of the HMW subunits forms a loose β-spiral structure, based on interspersed β-reverse turns and poly-proline II structure (reviewed by Shewry et al., 2003b, 2009a). This structure may look like a loose spring and is likely to resemble a spring in exhibiting a level of intrinsic activity. Indeed, elastic properties have been demonstrated at the single molecule level by mechanical stretching of synthetic peptides based on consensus repeat motifs using atomic force microscopy (Haward et al., 2011). The increased rigidity which was observed when the stretching was carried out in D_20 compared to H_20 also indicated a role of intrapeptide hydrogen bonding.

Belton also suggested that the formation of localised arrays of hydrogen bonds between adjacent gluten proteins (glutamine zips) contributes to gluten elasticity (Belton, 1999; 2005, 2007; Shewry et al., 2003b). Belton suggested that dry gluten is disordered but that regular hydrogen bonded structures are formed on hydration by orientation of the β-turns in adjacent β-spirals to form structures resembling "interchain" β-sheet. However, further hydration results in the replacement of some of the interchain hydrogen bonds with hydrogen bonds with water, resulting in an equilibrium between aligned regions (trains) and loop regions. This structure is suggested to contribute to elasticity as mechanical deformation will lead to disruption, initially extending the loops but eventually also separating the train regions. When the force is released the equilibrium balance between loops and trains will be restored. Evidence for these interactions comes from the application of mechanical deformation to "gluten" prepared from protein bodies (and therefore not previously subjected to mechanical treatments) which showed a progressive increase in β-sheet structure (Wellner et al., 2005).

The precise sequences of amino acids within the repetitive domains of the HMW subunits would be expected to affect the ability to form hydrogen bonds within and between β-spirals, as opposed to hydrogen bonds to water, and this has been demonstrated by the effects of amino acid substitutions on the solubility of synthetic peptides (Wellner et al., 2006). Tatham et al. (2001) studied the elastomeric mechanism by stress-strain analysis of HMW subunits after cross-linking with γ-radiation. This indicated that elastic recoil may be associated, at least in part, with extensive hydrogen bonding within and between subunits and that entropic and energetic mechanisms contribute to the observed elasticity

The importance of hydrogen bonding by the repetitive domains of the HMW subunits implies that subunits with longer repetitive domains should contribute to more elastic gluten. This has been proposed by several workers and HMW subunits with unusually high masses identified. For example, HMW subunit 1Ax2.1* is likely to be derived from HMW subunit 1Ax2* by the insertion of about 100 amino acids in the central repetitive domain (Tahir et al., 1996). However, this hypothesis

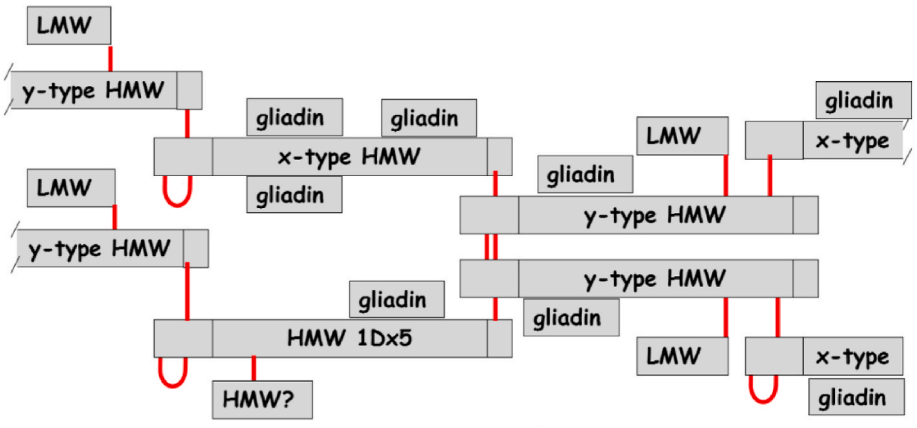

Fig. 1. Model to explain the role of the HMW subunits of glutenin in determining the structure of wheat glutenin polymers, based on the identification of interchain disulphide bonds (shown as vertical lines). A single inter-chain bond has been identified in the N-terminal domain of x-type subunits. HMW subunit 1Dx5 is also proposed to form an addition cross-link to another HMW subunit based on the effects of over-expression on gluten rheology. The HMW subunit polymers also form disulphide bonds with LMW subunits while gliadins interact by non-covalent forces. Based on Shewry et al. (2003a) which gives a more detailed explanation.

has not been proved by experimental studies.

Finally, there is no doubt that inter-chain disuphide bonds are essential in stabilising glutenin polymers, with reduction leading to the release of the component subunits and the complete loss of elasticity. However, the extent to which disulphide bonds actually contribute to the elastic mechanism, as opposed to stabilising the polymers, is not clear. The application of mechanical stress will deform the disulphide bonds leading to the re-establishment of the structure when the stress is released. In addition to limiting the length of "trains" stabilised by hydrogen bonds (as discussed above), the number and spacing of the disulphide bonds will affect the stiffness of the polymers (ie their ability to be extended). Effects on polymer cross-linking have therefore been proposed to be responsible for differences in quality associated with HMW subunits which differ in their numbers and distributions of cysteine residues, such as 1Dx5+1Dy10 v 1Dx2+1Dy12 and 1Bx7+1By9 v 1Bx20x+1By20y (Naeem and MacRitchie, 2005; Shewry et al., 2003a, b).

6. Molecular interactions of glutenin polymers: "glutenin macropolymer"

Even allowing for polymer masses extending to 2×10^6 or greater, it is clear that the gluten network in dough is not a continuous covalently linked polymer but consists of many polymers and monomers interacting by non-covalent forces. There has been substantial debate about the nature of these forces (as discussed above) but there is little doubt that the major non-covalent forces are hydrogen bonds, formed between the regularly repeated glutamine residues in the repetitive domains of the proteins. Such non-covalent interactions may stabilise "glutenin macropolymer".

It has long been known that the proportion of gluten protein that is not extracted by solvents and chaotropic agents (such as dilute acetic acid, 3M urea, 2-chloroethanol and AUC) is positively correlated with breadmaking quality. Early studies also showed that the fraction insoluble in dilute acetic acid become hydrated by the solvent and could be recovered as a "gel protein" fraction after centrifugation (Inamine et al., 1967).

A similar approach was used to prepare and characterise the gel layer recovered after extraction of flour with 1.5% SDS (Graveland et al., 1982). This fraction has since been defined as "glutenin macropolymer" (GMP) and the amount shown to correlate with dough elasticity and breadmaking quality (Dachkevitch and Autran, 1989; Weegels et al., 1996). Hence, it has been widely measured and used to predict quality. Weegels (1994) compared three cultivars, showing that GMP accounted for between 55% and 82% of the glutenin remaining after extraction of flour with 70% ethanol. Weegels et al. (1996) then compared a wider selection of 15 cultivars and lines, showing that GMP accounted for between 2.1 and 4.4% dry wt. in flours containing between 8.3 and 12.8% protein. GMP and total glutenin prepared by Osborne fractionation had similar contents of HMW-GS, about 30% of the total protein present in the fraction. By contrast, the SDS-soluble glutenin fraction contained only 4–6% HMW-GS. However, GMP contained lower proportions of x-type HMW-GS, 70–75% compared to 90–96% total HMW-GS in SDS-soluble glutenins (Weegels et al., 1995).

Mueller et al. (2016) quantified and analysed GMP fractions from two cultivars differing in HMW-GS composition and baking quality. The flours of the good- and poor-quality lines contained 36 and 6 mg/g of dried GMP gel, respectively, comprising starch (56% and 70%), protein (35% and 8%), SDS (5% and 16%) and water (8% and 2%). The protein was 90% glutenin, with LMW-GS: HMW-GS ratios of 1.7 and 1.3 in the good- and poor-quality cultivars, respectively. A comparison of grain samples from plants subjected to heat and drought stress showed similar trends in the concentrations of HMW-GS and GMP, implying (but not proving) a relationship (Zhang et al., 2013).

The amount of GMP prepared from dough changes during processing which has been described as resulting from "de-polymerisation and re-polymerisation" (Weegels et al., 1996). In molecular terms it may result from changes in non-covalent interactions between disulphide-stabilised polymers and may therefore more be correctly described as de-aggregation and re-aggregation (Don et al., 2003) (restricting the use of the term "polymers" to covalently linked molecules). Similarly, using "glutenin macropolymer" to describe covalently stabilised glutenin polymers (see for example, Lindsay and Skerritt, 1999) should be avoided.

Don et al. (2003) also dispersed GMP in 1.5% SDS and used confocal scanning laser microscopy to show the presence of spherical "bodies" of 5–30 μm in diameter. They suggested that these may represent individual protein bodies deposited in the developing grain. This hypothesis was tested by Van Herpen et al. (2008) who compared GMP with protein bodies isolated from developing grain. Although the protein bodies contained high molecular mass polymers (as discussed below) they were much smaller than the particles of GMP with a higher proportion of SDS-soluble protein. They therefore concluded that they differed in their degree of polymerisation (or aggregation). A direct correspondence between protein bodies and GMP is also unlikely as the protein bodies merge during the later stages of grain maturation to form a continuous matrix surrounding the starch granules in the starchy endosperm cells (Tosi, 2012). Hence, GMP is likely to be derived from this matrix rather than individual protein bodies.

As discussed above, the presence of loose assemblies of glutenin polymers and gliadins, notably ω-gliadins, with masses above 2×10^6 has been demonstrated by AFFFF using ethanol:water as a mild chaotropic solvent (Morel et al., 2020). These aggregates are much smaller than the GMP particles described by other authors. The authors suggested that the aggregation resulted from hydrogen bonding between glutamine residues in the proteins (as discussed below) and they may have been released from the larger GMP aggregates by partial disruption of the hydrogen bonds by the chaotropic solvent.

To summarise, GMP is clearly of interest to wheat scientists because of its relationship to grain processing quality. However, it remains ill-defined in respect to its subunit composition, molecular size and stabilising forces. Furthermore, the effects of genotype, environment and the interactions of these factors on its amount, composition and properties require further studies.

7. When, where and how are glutenin polymers assembled?

7.1. Polymer assembly within the cell

Wheat gluten proteins are synthesised on the rough endoplasmic reticulum (ER) with a signal peptide which co-translationally transports the newly synthesised protein into the ER lumen. It is well established for other proteins that folding and disulphide bond formation occur rapidly, assisted by molecular chaperones and catalysed by enzymes such as protein disulphide isomerase and peptidylprolyl cis−trans isomerases (which include cyclophilins) ("folding proteins") (reviewed by Osipova et al., 2012). These early steps in protein folding and assembly should apply to the non-repetitive domains of the α-type gliadins, γ-gliadins and B- and C-type LMW subunits, which appear to have globular conformations which are rich in α-helix and form intrachain disulphide bonds. However, the roles of folding proteins in the folding of the repetitive domains which form extended structures and in the formation of interchain disulphide bonds is not clear.

The normal trafficking pathway for proteins transported into the ER lumen is to the Golgi apparatus where they are sorted based on targeting sequences and transported via vesicles either to the vacuole or the plasmalemma and apoplast (outside the cell). Most of gluten proteins in wheat appear to be transported to the vacuole, where they aggregate to form protein bodies. However, some gluten proteins are not transported to the Golgi apparatus but remain within the lumen of the ER where they aggregate to form a second population of protein bodies of ER origin. Although the segregation of gluten protein types into the two

populations of protein bodies is not precise, it is clear that most of the gliadins are transported to the vacuoles while the ER-derived protein bodies are enriched in HMW-GS (reviewed by Tosi, 2012). No specific signals for transport or retention have been identified in gluten proteins and it is therefore possible that the segregation results from differences in their propensity to aggregate. The newly synthesis and folded gliadin monomers may be soluble in the ER and remain as monomers until their concentration is sufficiently high to aggregate, which may occur in the Golgi or Golgi-derived vesicles. By contrast, the formation of interchain disulphide bonds between the newly synthesised HMW subunits may result in polymerisation, aggregation and insolubility within the ER.

The two populations of protein bodies may continue to grow during the grain filling period but ultimately merge during grain desiccation to form a continuous matrix surrounding the starch granules. Further assembly and rearrangements of glutenin polymers may occur in the protein bodies and matrix during grain maturation, perhaps catalysed by low molecular weight red-ox reagents (reduced/oxidised glutathione and ascorbic acid/dehydroascorbic acid) and catalysed by thiol oxidoreductase enzymes. These processes are reviewed by Osipova et al. (2012) and discussed by Branlard et al. (2020). .

This model, which is summarised in Fig. 2, provides a framework for discussion but clearly does not provide a full account of glutenin polymer assembly. In particular, it does not provide an adequate explanation for the following.

1. The assembly of the LMW-GS into oligomers and polymers.

LMW-GS account for about 65–70% of glutenin and 30–35% of the total gluten proteins and are known to form oligomers and small polymers comprising only LMW subunits as well as contribute to larger polymers which are considered to be based on a backbone of HMW subunits. We therefore need to know where the oligomers/small polymers comprising LMW subunits are assembled and deposited. Furthermore, are some of these oligomers/small polymers subsequently incorporated into HMW polymers and, if so, where and how does this occur? Bietz and Wall (1980) suggested that they are assembled in the vacuolar protein bodies and subsequently become incorporated into HMW polymers when the protein bodies merge but there is no experimental evidence for this and it is possible that some LMW-GS are incorporated directly into HMW polymers in the ER, rather than forming "intermediate oligomers".

2. The mechanism and specificity of formation of interchain disulphide bonds.

The pattern of interchain disulphide bonds is considered to be specific, rather than random. However, we do not have enough direct evidence to be certain that this is the case, due to the technical challenges of mapping disulphide bonds in the glutenin polymers.

We therefore need to know the extent to which the formation of disulphide bonds is regular, as opposed to random, and how this specificity is determined (bearing in mind the presence of two cellular pathways and decreasing solubility of the growing polymers). Assuming that at least some interchain disulphide bonds are initially formed in the ER, are further bonds formed in the protein bodies and matrix as the polymers grow and to what extent are the disulphide bonds rearranged during grain maturation? Finally, what are the roles of folding proteins and redox systems (redox pairs and enzymes) in the formation and rearrangement of disulphide bonds in the different cellular compartments where glutenin polymers are assembled and deposited?

Answers to some of these questions are being provided by studies of glutenin polymer formation in developing grain.

7.2. Developmental pathway and environmental impacts on polymer assembly during grain development

A number of studies have shown that the proportion of HMW polymers increases during grain development, particularly during the later stages of grain filling and desiccation (Gupta et al., 1996; Carceller and Aussenac, 1999, 2001; Shewry et al., 2009b; Naeem et al., 2012; Ferreira et al., 2012). This is illustrated in Fig. 3, which shows that the proportion of the F1 peak separated by SE-HPLC, (which comprises high molecular weight glutenin polymers) increased particularly during grain desiccation (42 days post anthesis to maturity) while the total protein content remained constant.

The mechanisms determining this late polymerisation are not known, but it is clearly inversely correlated with the water content of the tissue (Carceller and Aussenac, 1999; Naeem et al., 2012). Naeem et al.

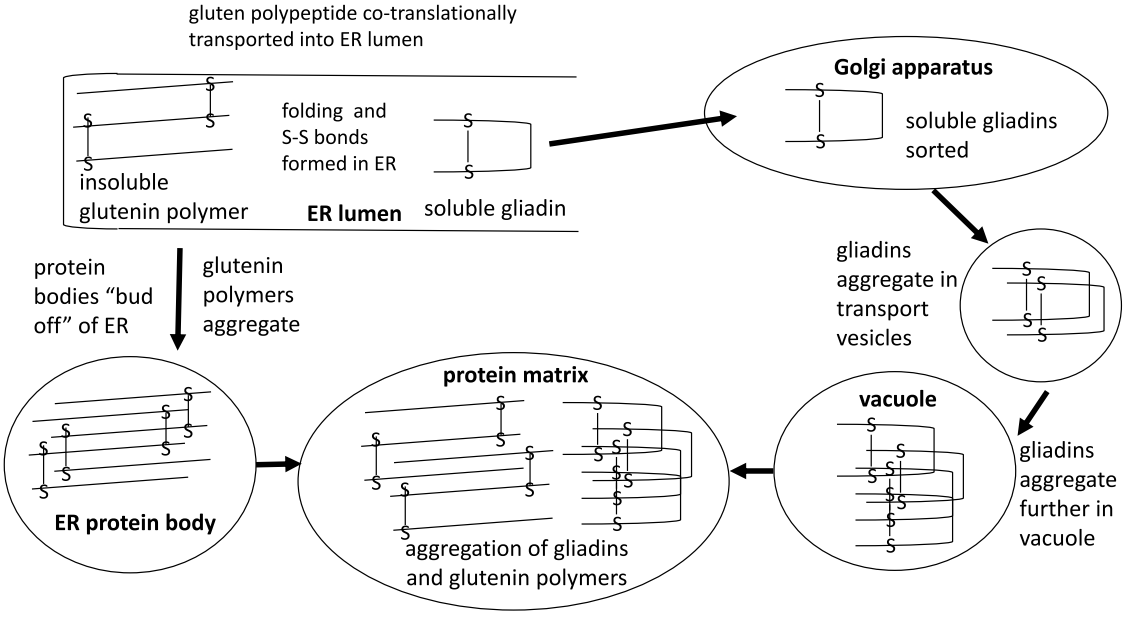

Fig. 2. Schematic summary of the possible pathway of glutenin polymer assembly and aggregation in the developing wheat grain.

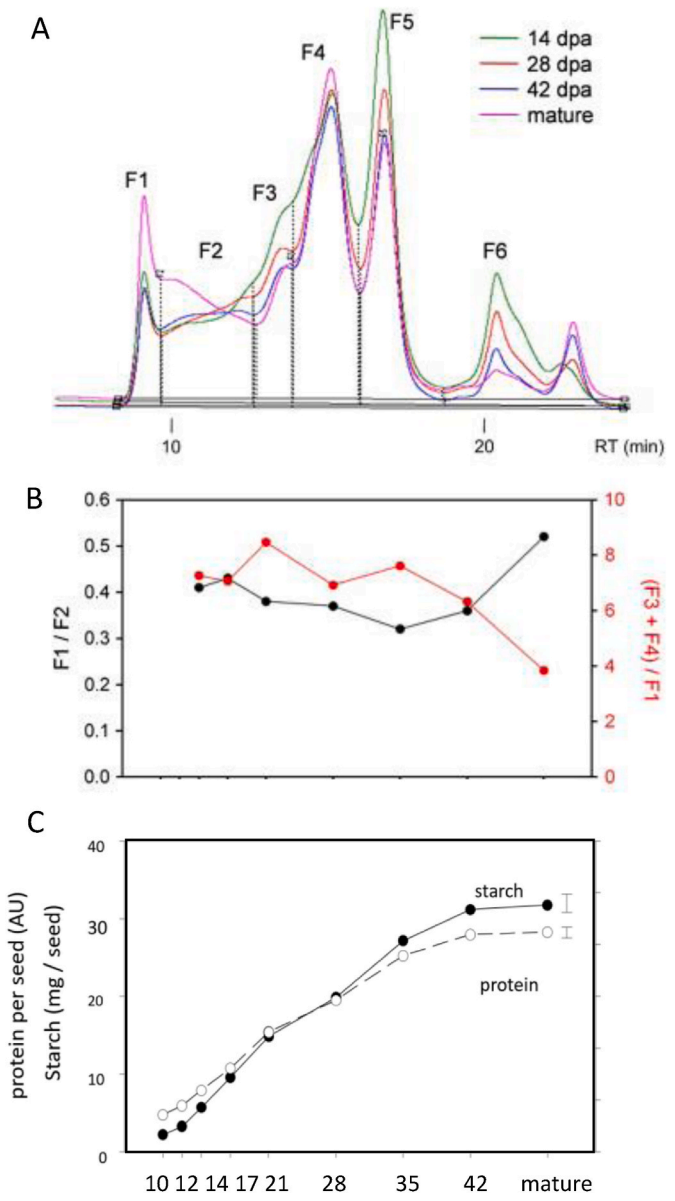

Fig. 3. Polymer accumulation in developing grain. A, SE-HPLC profiles; B, ratios of peaks F1/F2 and F3+F4/F1 calculated from the profiles; C, accumulation of starch and protein. 10, 12, 14, 17, 21, 28, 35 and 42 are days post-anthesis. AU, arbitrary units (from gel scanning). Taken from Shewry et al. (2009b) with permission.

(2012) suggest that it "may be explained by a higher concentration of SH groups as reactants in the formation of S–S bonds in the polymerisation reaction" while Rhazi et al. (2003) suggest a two-stage model in which HMW polymers assembled during grain filling undergo a second level aggregation during maturation, or due to desiccation earlier in development. They suggest that this occurs "by entanglement, stabilised by hydrogen bonding and additional disulphide bridges" and leads to the formation of unextractable polymeric protein (UPP). Ferreira et al. (2012) showed that free glutenin subunits disappeared and the size distribution of glutenin polymers increased during grain development and that this was accompanied by a decrease in cellular redox potential. They also suggested that protein-bound glutathione acted as a protective mechanism against irreversible thiol oxidation rather than limiting glutenin polymer assembly. Ferreira et al. (2014) also studied polymer assembly using MALDI-mass spectrometry. They showed that HMW-GS 1Bx20 was present as monomers and in small oligomers with free cysteine residues early in grain development and that complete disulphide bond formation was only achieved late in development.

More recently, Koga et al. (2017, 2020) compared natural desiccation and artificial drying and determining the effects of environmental factors. They suggest that the process involves the activation of enzymes involved in folding and disulphide bond formation, as reviewed by Osipova et al. (2012).

Two detailed recent studies have used AFFFF to characterise glutenin polymers in sets of cultivars grown under a range of environmental conditions: these were 130 cultivars grown in 6 locations (Ausennac et al., 2020) and 192 cultivars grown in 11 locations (Branlard et al., 2020). Their analyses confirmed that LMW-GS and HMW-GS alleles had effects on glutenin polymer size, but showed that these effects were much smaller than the effects of the growing conditions. In fact, the ratio of variances due to environmental and genetic factors (σ 2 E/σ 2 G) was 16.88 for polymeric protein content and 11.56 for the weight average molecular mass of polymers (Ausennac et al., 2020). Similarly, Branlard et al. (2020) reported that protein content, grain hardness and glutenin diversity together accounted for about 20% of the phenotypic variation in gluten polymer molecular mass, which increased to 28.3% when rainfall was included and 60.5% when rainfall and temperature during grain development were included in the regression. They suggested that these effects are mediated by the redox status of non-protein free thiol compounds, such as glutathione which forms polymeric protein-bound glutathione conjugates and limits polymer formation.

These studies show that glutenin polymerisation is incomplete when the proteins are initially deposited and reaches completion only later in grain development. Furthermore, the polymerisation is affected by growth conditions with larger polymers being formed under hot dry conditions. This raises the question of whether these later stages of polymerisation are random or specific and, if specific, how this specificity is determined. The emphasis on disulphide bond formation may also underestimate the contribution of H bonds to this polymerisation. The loss of water can be expected to increase H bonds which, if present in long regular arrays, can lead to the insolubility of polymers, even in chaotropic solvents (cf. cellulose). The importance of hydrogen bonding in stabilising gluten and their contribution to the elastic mechanism have been discussed by Belton (1999, 2005, 2007) and Shewry et al. (2003a). In this respect it is interesting that the molecular masses of polymers determined by AFFFF are lower when the mild chaotropic agent alcohol:water is used as a solvent (as discussed above).

8. Conclusions

We have focused on the structure of glutenin polymers and their assembly in developing grain, including the relationship between subunit composition and polymer structure and the role of disulphide and hydrogen bonds in stabilising individual polymers and "aggregates" of polymers and monomers, highlighting deficiencies in our knowledge of these processes. We have not considered the effects of dough mixing on polymer structure in detail but this is clearly an important topic if we are to fully understand the role of gluten polymers in determining processing quality.

Much of the work discussed was carried out in the last century, with some dating back 50 years, although important studies on grain development have been reported during the last decade. However, current interest in the topic is low which is unfortunate because the ability to make precise changes to protein structure and expression provide opportunities to exploit more detailed knowledge in designing improved types of wheat for future requirements. In the meantime, it is important that wheat scientists should appreciate that the current "dogma" on the structure and function of glutenin polymers is far from proven.

Declaration of competing interest

The authors have no conflicts of interest.

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References

Arfvidsson, C., Wahlund, K.-G., Eliasson, A.-C., 2004. Direct molecular weight determination in the evaluation of dissolution methods for unreduced glutenin. J. Cereal. Sci. 39, 1–8. https://doi.org/10.1016/S0733-5210(03)00038-9.

Ausennac, T., Rhazi, L., Branlard, G., 2020. Molecular weight distribution of polymeric proteins in wheat grains: the rheologically active polymers. Foods 9, 1675. https://www.mdpi.com/2304-8158/9/12/1905.

Belton, P.S., 1999. On the elasticity of wheat gluten. J. Cereal. Sci. 29, 103–107. https://doi.org/10.1006/jcrs.1998.0227.

Belton, P.S., 2005. New approaches to study the molecular basis of the mechanical properties of gluten. J. Cereal. Sci. 41, 203–211. https://doi.org/10.1016/j.jcs.2004.06.003.

Belton, P.S., 2007. Letter to the editor. J. Cereal. Sci. 46, 97–98. https://doi.org/10.1016/j.jcs.2007.04.001.

Bietz, J.A., Wall, J.S., 1980. Identity of high molecular weight gliadin and ethanol-soluble glutenin subunits of wheat: relation to gluten structure. Cereal Chem. 57, 415–420.

Branlard, G., Faye, A., Rhazi, L., Tahir, V., Aussenac, T., 2020. Genetic and Environmental factors associated to glutenin polymer characteristics of wheat. Foods 9, 683. https://doi.org/10.3390/foods9050683.

Carceller, J.-L., Aussenac, T., 1999. Accumulation and changes in molecular size distribution of polymeric proteins in developing grains of hexaploid wheats: role of the desiccation phase. Aust. J. Plant Physiol. 26, 301–331. https://doi.org/10.1071/PP99010.

Carceller, J.-L., Aussenac, T., 2001. Size characterisation of glutenin polymers by HPSEC-MALLS. J. Cereal. Sci. 33, 131–142. https://doi.org/10.1006/jcrs.2000.0356.

Dachkevitch, T., Autran, J.-C., 1989. Prediction of baking quality of bread wheats in breeding programs by size- exclusion high-performance liquid chromatography. Cereal Chem. 66, 448–456.

Danno, G., Xu, S.R., Natake, M., 1990. Molecular size distribution of glutenin from wheat flour and the polymerisation of proteins during purification. Agric. Biol. Chem. 54, 985–992. https://doi.org/10.1271/bbb1961.54.985.

Don, C., Lichtendonk, W., Plijter, J.J., Hamer, R.J., 2003. Glutenin macropolymer: a gel formed by glutenin particles. J. Cereal. Sci. 37, 1–7. https://doi.org/10.1006/jcrs.2002.0481.

Egorov, T.A., Odintsova, T.I., Shewry, P.R., Tatham, A.S., 1998. Characterisation of high M_r wheat glutenin polymers by agarose gel electrophoresis and dynamic light scattering. FEBS (Fed. Eur. Biochem. Soc.) Lett. 434, 215–217. https://doi.org/10.1016/S0014-5793(98)00983-1.

Ewart, J.E.D., 1977. Re-examination of the linear glutenin hypothesis. J. Sci. Food Agric. 28, 191–199. https://doi.org/10.1002/JSFA.2740280214.

Ferreira, M.S.L., Samson, M.-F., Bonicel, J., Morel, M.-H., 2012. Relationship between endosperm cells redox homeostasis and glutenin polymers assembly in developing durum wheat grain. Plant Physiol. Biochem. 61, 36–45. https://doi.org/10.1016/j.plaphy.2012.08.015.

Ferreira, M.S.L., Mangavel, C., Rogniaux, H., Bonicel, J., Samson, M.-F., Morel, M.-H., 2014. A MALDI-TOF based study of the in vivo assembly of glutenin polymers of durum wheat. Food Res. Int. 63, 89–99. https://doi.org/10.1016/j.foodres.2013.12.025.

Field, J.M., Shewry, P.R., Miflin, B.J., 1983. Solubilization and characterization of wheat gluten proteins; correlations between the amount of aggregated proteins and baking quality. J. Sci. Food Agric. 34, 370–377. https://doi.org/10.1002/jsfa.2740340409.

Graveland, A., Bosveld, P., Lichtendonk, W.L., Moonen, H.E., Scheepstra, A., 1982. Extraction and fractionation of wheat flour proteins. J. Sci. Food Agric. 33, 1117–1128. https://doi.org/10.1002/jsfa.2740331109.

Graveland, A., Bosveld, P., Lichtendonk, W.L., Moonen, H.E., Marseille, J.P., Scheepstra, A., 1985. A model for the molecular structure of the glutenins from wheat flour. J. Cereal. Sci. 3, 1–16. https://doi.org/10.1016/S0733-5210(85)80029-1.

Gupta, R.B., Masci, S., Lafiandra, D., Bariana, H.S., MacRitchie, F., 1996. Accumulation of protein subunits and their polymers in developing grains of hexaploid wheats. J. Exp. Bot. 47, 1377–1385. https://doi.org/10.1093/jxb/47.9.1377.

Hamauzu, Z., Khan, K., Bushuk, W., 1979. Studies of glutenin XIV. Gel filtration and sodium dodecyl sulphate electrophoresis of glutenin solubilised in sodium stearate. Cereal Chem. 56, 513–516.

Haward, S.J., Shewry, P.R., Marsh, J., Miles, M.J., McMaster, T.J., 2011. Force spectroscopy of an elastic peptide: effect of $D_{(2)}O$ and temperature on persistence length. Microscope Res. Tech. 74, 170–176. https://doi.org/10.1002/jemt.20888.

Huebner, F.R., Wall, J.S., 1976. Fractionation and quantitative differences of glutenin from wheat varieties varying in baking quality. Cereal Chem. 53, 258–269.

Inamine, E.S., Noble, E.G., Mecham, D.K., 1967. Solubilization and fractionation of wheat flour proteins insoluble in dilute acetic acid. Cereal Chem. 44, 143–151.

Jones, R.W., Babcock, G.E., Taylor, N.W., Senti, F.R., 1961. Molecular weights of wheat gluten fractions. Arch. Biochem. Biophys. 94, 483–488. https://doi.org/10.1016/0003-9861(61)90076-5.

Koga, S., Bocker, U., Wieser, H., Koehler, P., Uhlen, A.K., Molderstad, A., 2017. Polymerisation of gluten proteins in developing wheat grain as affected by desiccation. J. Cereal. Sci. 73, 122–129. https://doi.org/10.1016/j.jcs.2016.12.003.

Koga, S., Aamot, H.U., Uhlen, A.K., Seehusen, T., Veiseth-Kent, E., Hofgaard, I.S., Moldestad, A., Bocker, Uk, 2020. Environmental factors associated with glutenin polymer assembly during grain maturation. J. Cereal. Sci. 91, 108065 https://doi.org/10.1016/j.jcs.2019.102865.

Lafiandra, D., Shewry, R.R., 2022. Wheat glutenin polymers. 2 . The role of glutenin subunits in polymer formation and dough properties. J. Cereal. Sci. (in press).

Larroque, O.R., Gianibelli, M.C., Batey, I.L., MacRitchie, F., 1997. Electrophoretic characterisation of fractions collected from gluten protein extracts subjected to size-exclusion high-performance liquid chromatography. Electrophoresis 18, 1064–1067. https://doi.org/10.1002/elps.1150180706.

Lemelin, E., Aussenac, T., Violleau, F., Salvo, L., Lein, V., 2005. Impact of cultivar and environment on size characteristics of wheat proteins using asymmetric flow field-flow fractionation and multi-angle laser light scattering. Cereal Chem. 82, 28–33. https://doi.org/10.1094/CC-82-0028.

Lindsay, M.P., Skerritt, J.H., 1999. The glutenin macropolymer of wheat flour doughs: structure:function perspectives. Trends Food Sci. Technol. 10, 247–253. https://doi.org/10.1016/S0924-2244(00)00004-2.

Lutz, E., Wieser, H., Koehler, P., 2012. Identification of disulphide bonds in wheat gluten proteins by means of mass spectroscopy/electron transfer dissociation. J. Agric. Food Chem. 60, 3708–3716. https://doi.org/10.1021/jf204973u.

MacRitchie, F., 2007. Letter to the editor. J. Cereal. Sci. 46, 96–97. https://doi.org/10.1016/j.jcs.2007.02.002.

Masci, S., Egorov, T.A., Ronchi, C., Kuzmicky, D.D., Kasarda, D.D., Lafiandra, D., 1999. Evidence for the presence of only one cysteine residue in the D-type low molecular weight subunits of wheat glutenin. J. Cereal. Sci. 29, 17–25. https://doi.org/10.1006/jcrs.1998.0224.

Masci, S., Rovelli, L., Kasarda, D.D., Vensel, W.H., Lafiandra, D., 2002. Characterisation and chromosomal localisation of C-type low-molecular-weight glutenin subunits in the bread wheat cultivar Chinese Spring. Ther. Appl. Sci. 104, 422–428. https://doi.org/10.1007/s001220100761.

Morel, M.-H., Dehlon, P., Autran, J.C., Leygue, J.P., Bar-L'Helgouac'h, C., 2002. Effects of temperature, sonication time, and power settings on size distribution and extractability of total wheat flour proteins as determined by size-exclusion high-performance liquid chromatography. Cereal Chem. 77, 685–691. https://doi.org/10.1094/CCHEM.2000.77.5.685.

Morel, M.-H., Pincemaille, J., Chaveau, E., Louhichi, A., Voilleau, F., Menut, P., Ramos, L., Banc, A., 2020. Insight into gluten structure in a mild chaotropic solvent by asymmetrical flow field-flow fractionation (AsF1FFF). Food Hydrocolloids 103, 105676. https://doi.org/10.1016/j.foodhyd.2020.105676.

Mueller, E., Wieser, H., Koehler, P., 2016. Preparation and chemical characterisation of glutenin macropolymer (GMP) gel. J. Cereal. Sci. 70, 79–84. https://doi.org/10.1016/j.jcs.2016.05.021.

Naeem, H.A., MacRitchie, F., 2005. Polymerisation of glutenin during grain development in near-isogenic wheat lines differing at *Glu-D1* and *Glu-B1* in greenhouse and field. J. Cereal. Sci. 41, 7–12. https://doi.org/10.1016/j.jcs.2004.04.009.

Naeem, H.A., Paulon, D., Irmak, S., MacRitchie, F., 2012. Developmental and environmental effects on the assembly of glutenin polymers and the impact on grain quality of wheat. J. Cereal. Sci. 59, 51–57. https://doi.org/10.1016/j.jcs.2011.10.014.

Osipova, S.V., Permyakova, M.D., Permyakov, A.V., 2012. Role of Non-prolamin proteins and low molecular weight redox agents in protein folding and polymerization in wheat grains and influence on baking quality parameters. J. Agric. Food Chem. 60, 12065–12073. https://doi.org/10.1021/jf303513m.

Payne, P.I., 1987. Genetics of wheat storage proteins and the effect of allelic variation on breadmaking quality. Annu. Rev. Plant Physiol. 38, 141–153. https://doi.org/10.1146/annurev.pp.38.060187.001041.

Perutz, M.F., Johnson, T., Suzuki, M., Finch, J.Y., 1994. Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. Proc. Nat. Acad. U. S. A 91, 5355–5358. https://doi.org/10.1073/pnas.91.12.5355.

Pitkanen, L., Sontag-Strohm, T., Kaneva, P., 2014. Enhanced separation and characterisation of gluten polymers by asymmetrical flow field-flow fractionation coupled with multiple detectors. J. Cereal. Sci. 59, 126–131. https://doi.org/10.1016/j.jcs.2013.11.009.

Popineau, Y., Cornec, M., Lefebvre, J., Marchylo, B., 1994. Influence of high *Mr* glutenin subunits on glutenin polymers and rheological properties of glutens and gluten sub-fractions on near-isogenic lines of wheat Sicco. J. Cereal. Sci. 19, 231–241. https://doi.org/10.1006/jcrs.1994.1030.

Rao, U.J.S., Nigam, S.N., 1988. Chromatography of glutenin on Sepharose CL-4B in dissociating solvents: molecular weight composition of covalently bonded glutenin. Cereal Chem. 65, 373–374.

Rhazi, L., Cazalis, R., Ausennac, T., 2003. Sulfhydryl-disulphide changes in storage proteins of developing wheat grain: influence on the SDS-unextractable polymer. J. Cereal. Sci. 38, 3–13. https://doi.org/10.1016/S0733-5210(03)00019-5.

Schmid, M., Wieser, H., Koehler, P., 2017. Disulphide structure of high-molecular-weight (HMW-) gliadins as affected by terminators. J. Cereal. Sci. 78, 66–74. https://doi.org/10.1016/j.jcs.2016.12.007.

Shewry, P.R., Tatham, A.S., 1997. Disulphide bonds in wheat gluten proteins. J. Cereal. Sci. 25, 207–227. https://doi.org/10.1006/jcrs.1996.0100.

Shewry, P.R., Miflin, B.J., Lew, E.J.-L., Kasarda, D.D., 1983. The preparation and characterization of an aggregated gliadin fraction from wheat. J. Exp. Bot. 34, 1403–1410. https://doi.org/10.1093/jxb/34.11.1403.

Shewry, P.R., Gilbert, S.M., Savage, A.W.J., Tatham, A.S., Wan, Y.-F., Belton, P.S., Wellner, N., D'Ovidio, R., Bekes, F., Halford, N.G., 2003a. Sequence and properties

of HMW subunit 1Bx20 from pasta wheat (*Triticum aestivum*) which is associated with poor end use properties. Ther. Appl. Sci. 106, 744–750. https://doi.org/10.1007/s00122-002-1135-6.

Shewry, P.R., Halford, N.G., Tatham, A.S., Popineau, Y., Lafiandra, D., Belton, P.S., 2003b. The high molecular weight subunits of wheat glutenin and their role in determining wheat processing properties. Adv. Food Nutr. Res. 45, 221–302. https://doi.org/10.1016/s1043-4526(03)45006-7.

Shewry, P.R., D'Ovidio, R., Lafiandra, D., Jenkins, J.A., Mills, E.N.C., Bekes, F., 2009a. Wheat grain proteins. In: Khan, K., Shewry, P.R. (Eds.), Wheat: Chemistry and Technology, fourth ed. AACC, St Paul Mn, pp. 223–298.

Shewry, P.R., Underwood, C., Wan, Y., Lovegrove, A., Bhandari, D., Toole, G., Mills, E.N. C., Denyer, K., Mitchell, R.A.C., 2009b. Storage product synthesis and accumulation in developing grains of wheat. J. Cereal. Sci. 50, 106–112. https://doi.org/10.1016/j.jcs.2009.03.009.

Singh, N.K., Donovan, G.R., MacRitchie, F., 1990. Use of sonication and size-exclusion high-performance liquid chromatography in the study of wheat proteins II. Relative quantity of glutenin as a measure of breadmaking quality. Cereal Chem. 67, 161–170.

Stevenson, S.G., Preston, K.R., 1996. Flow field-flow Fractionation of wheat proteins. J. Cereal. Sci. 23, 121–131. https://doi.org/10.1006/jcrs.1996.0012.

Stevenson, S.G., You, s., Izydorczyk, M.S., Preston, K.R., 2003. Characterization of polymeric wheat proteins by flow field-flow fractionation/MALLS. J. Liq. Chromatogr. Relat. Technol. 26, 2771–2781. https://doi.org/10.1081/JLC-120025044.

Sutton, K.H., 1996. Analysis of wheat glutenin protein aggregate size using photon correlation spectroscopy. In: Wrigley, C.W. (Ed.), Gluten '96. RACI, North Melbourne, pp. 317–320.

Tahir, M., Pavoni, A., Tucci, G.F., Turchetta, T., Lafiandra, D., 1996. Detection and characterisation of of a glutenin subunit with unusual high *Mr* at the *Glu-A1 l*ocus in wheat. Ther. Appl. Sci. 92, 654–659. https://doi.org/10.1007/BF00226085.

Tatham, A.S., Hayes, L., Shewry, P.R., Urry, D.W., 2001. Wheat proteins exhibit a complex mechanism of protein elasticity. Biochem. Biophys. Acta. 1548, 187–193. https://doi.org/10.1016/S0167-4838(01)00232-1.

Tilley, K.A., Benjamin, R.E., Bagorogoza, K.E., Okot-Kotber, B.M., Prakask, O., Kwena, H., 2001. Tyrosine cross-links: molecular basis of gluten structure and function. J. Agric. Food Chem. 49, 2627–2632. https://doi.org/10.1021/jf010113h.

Tosi, P., 2012. Trafficking and deposition of prolamins in wheat. J. Cereal. Sci. 56, 81–90. https://doi.org/10.1016/j.jcs.2012.02.004.

Van Herpen, T.W.J.M., Cordewener, J.H.G., Klok, H.J., Freeman, J., America, A.H.P., Bosch, D., Smulders, M.J.M., Gilissen, L.J.W.J., Shewry, P.R., Hamer, R.J., 2008. The origin and early development of wheat glutenin particles. J. Cereal. Sci. 48, 870–877. https://doi.org/10.1016/j.jcs.2008.07.002.

van Vliet, T., Hamer, R.J., 2007. Letter to the editor. J. Cereal. Sci. 46, 98–99. https://doi.org/10.1016/j.jcs.2007.04.002.

Venzel, W.H., Tanaka, C.K., Altenbach, S.B., 2014. Protein composition of wheat gluten polymer fractions determined by quantitative two-dimensional gel electrophoresis and tandem mass spectrometry. Proteome Sci. 12, 8. https://doi.org/10.1186/1477-5956-12-8.

Wahlund, K.G., Gustavsson, M., MacRitchie, F., Nylander, T., Wannerberger, L., 1996. Size characterisation of wheat proteins, particularly glutenin, by asymmetrical flow field-flow fractionation. J. Cereal. Sci. 23, 113–119. https://doi.org/10.1006/jcrs.1996.0011.

Weegels, P.L., 1994. Depolymerisation and Re-polymerisation of Wheat Glutenin during Dough Processing and Effects of Low Mr Wheat Proteins. PhD thesis, University of London.

Weegels, P.L., Hamer, R.J., Schofield, J.D., 1995. RP-HPLC and capillary electrophoresis of subunits from glutenin isolated by SDS and Osborne fractionation. J. Cereal. Sci. 22, 211–224. https://doi.org/10.1006/jcrs.1995.0058.

Weegels, P.L., van de Pijpekamp, A.M., Graveland, A., Hamer, R.J., Schofield, J.D., 1996. Depolymerisation and re-polymerisation of wheat glutenin during dough processing. I. Relationships between glutenin macropolymer content and quality parameters. J. Cereal. Sci. 23, 103–111. https://doi.org/10.1006/jcrs.1996.0010, 1996.

Wellner, N., Mills, E.N.C., Brownsea, G., Wilson, R.H., Brown, N., Freemen, J., Halford, N.G., Shewry, P.R., Belton, P.S., 2005. Changes in protein secondary structure during gluten deformation by dynamic Fourier transform infrared spectroscopy. Biomacromolecules 6, 255–261. http://doi.org/10.1021/bm049584d.

Wellner, N., Marsh, J.T., Savage, A.W.J., et al., 2006. Comparison of repetitive sequences derived from high molecular weight subunits of wheat glutenin, an elastomeric protein. Biomacromolecules 7, 1096–1103. https://doi.org/10.1021/bm050893t.

Wieser, H., 2000. Comparative investigations of gluten proteins from different wheat species. 1. Qualitative and quantitative composition of gluten protein types. Eur. Food Res. Technol. 211, 262–268. https://doi.org/10.1007/s002170000165.

Wieser, H., 2007. Chemistry of gluten proteins. Food Microbiol. 24, 114–119. https://doi.org/10.1016/j.fm.2006.07.004.

Wrigley, C.W., 1996. Giant protein with flour power. Nature 381, 738–739. https://doi.org/10.1038/381738a0.

Zhang, X., Cai, J., Wollenweber, B., Liu, F., Dai, T., Cao, W., Jiang, D., 2013. Multiple heat and drought events affect grain yield and accumulations of high molecular weight glutenin subunits and glutenin macropolymers in wheat. J. Cereal. Sci. 57, 134–140. https://doi.org/10.1016/j.jcs.2012.10.010.