

Wheat glutenin subunits and dough elasticity: findings of the EUROWHEAT project

Peter R. Shewry,^{*,†}

Yves Popineau,[‡]

Domenico Lafiandra[§] and

Peter Belton[¶]

^{*}IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton BS41 9AF, UK (tel: + 1275-392181; fax: + 1275-394299; e-mail: peter.shewry@bbsrc.ac.uk)

[‡]Institut National de la Recherche Agronomique, Centre de Recherches de Nantes, Laboratoire de Biochimie et de Technologie des Protéines, B.P. 71627, Rue de la Géraudière, Nantes 44316, Cedex 03, France

[§]Dipartimento di Agrobiologia ed Agrochimica, Via San Camillo de Lellis, Viterbo 01100, Lazio, Italy

[¶]Institute of Food Research, Norwich Laboratory, Norwich Research Park, Colney Lane, Norwich NR4 7UA, UK

Detailed studies of wheat glutenin subunits have provided novel details of their molecular structures and interactions which allow the development of a model to explain their role in determining the visco-elastic properties of gluten and dough. The construction and analysis of near-isogenic and transgenic lines expressing novel subunit combinations or increased amounts of specific subunits allows differences in gluten properties to be related to the structures and

properties of individual subunits, with potential benefits for the production of cultivars with improved properties for food processing or novel end users © 2001 Elsevier Science Ltd. All rights reserved.

Wheat is currently the most important crop in the world, with total annual yields of almost 600 million tonnes. It is adapted to a wide range of environments, being grown in all continents except Antarctica, ranging from upland sites in the tropics to the great plains of North America and steppes of Russia and the Ukraine. Much of the success of wheat stems from its processing properties and in particular its ability to form cohesive doughs which can be baked into bread or formed into pasta and noodles. These properties derive largely from the gluten proteins, which form a continuous visco-elastic network within the dough. Consequently, the gluten proteins have become the most widely and intensively studied group of plant proteins, with work dating back as far as 1745 when Beccari at the University of Bologna first reported the isolation of gluten.

Despite these studies the structures of the gluten proteins and the molecular basis for their role in processing are still incompletely understood. However, it has been clear for many years that both genotype and environment are important in determining grain quality, with wheats grown in the hot dry summers of North America generally having a higher level of gluten and dough elasticity (strength) and consequently, higher bread-making quality than wheat grown in the cool damp summers of Western Europe.

The last 30 years have seen a massive increase in the proportion of 'home-grown' wheat used for breadmaking in the European Union (EU), which has been made possible by improvements in intrinsic quality by plant breeding as well as more optimised agronomy and processing conditions. The EU has been the driving force for these changes, providing economic incentives to growers and processors and supporting community-wide research programmes to underpin the increased utilization.

The EUROWHEAT Project^{||} combined work from ten laboratories in six member states, with a budget

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[†]Corresponding author.

amounting to about two million Euro over three years. The aim was to provide a detailed understanding of the molecular basis for gluten visco-elasticity, focusing on one group of gluten proteins called the high molecular weight (HMW) subunits of glutenin. The most exciting results from this project are presented here in the wider context of gluten structure and functionality.

The HMW subunits and breadmaking quality

The HMW subunits are a group of proteins which are present in the grain solely as components of high molecular mass glutenin polymers. Interest has focused on them since the late 1970s, when Payne and co-workers at the Plant Breeding Institute, Cambridge, reported that the presence or absence of specific HMW subunit proteins was strongly correlated with differences in breadmaking quality between cultivars of European wheat, and in the progeny of crosses between these (reviewed in [1]). All cultivars of hexaploid bread wheat have six HMW subunit genes, with two genes (encoding one high M_r x-type subunit and one low M_r y-type subunit) being present on each of chromosomes 1A, 1B and 1D. However, the silencing of specific genes leads to variation in the number of subunits from three to five while allelic variation in the subunits encoded by active genes results in proteins with different mobility on electrophoresis (Fig. 1). Payne and co-workers showed that good breadmaking performance was particularly highly correlated with the presence of a 1Ax subunit protein and with specific allelic forms of subunits encoded by chromosome 1D. Further studies allowed all the individual subunits or pairs of subunits to be assigned 'quality scores' ranging from 1 to 5. Therefore, it should, in principle, be possible to predict the breadmaking performance of a cultivar based on its HMW subunit quality score. However, this is not always possible, reflecting the fact that HMW subunits are not the sole determinants of breadmaking performance. Never-

theless, the HMW subunits have been reported to account for between about 45 and 70% of the variation in breadmaking performance within European wheats [2–4], despite representing only 12% of the total flour proteins (corresponding to 1–1.7% of the flour dry weight) [5].

HMW Subunit Structure

As implied by their name, the HMW subunits have the highest molecular masses of all the gluten proteins, ranging from about 67,500 to 73,500 (about 630–830 amino acids) for the commonly occurring allelic forms. Nevertheless, the full amino acid sequences of a number of subunits have been determined, including x-type and y-type proteins encoded by all three genomes, by sequencing of genomic DNA [7]. The individual proteins are very similar in sequence, the most striking feature being the presence of repeated sequence motifs which account for all except about 100 and 40 residues at the protein N- and C-termini, respectively. These motifs are rich in the amino acids proline, glutamine and glycine. Evidence from spectroscopy and scanning probe microscopy indicates that these motifs may form an unusual spiral supersecondary structure based on β -reverse turns in equilibrium with poly-L-proline II structure [8,9], although other workers have suggested that γ -turns may be present [10]. There is also evidence that the repetitive domain has an extended rod-shaped conformation. In contrast the non-repetitive N- and C-terminal domains appear to be more similar to globular proteins in structure, containing α -helix and aperiodic structure. These domains also contain most, if not all, of the cysteine residues which provide sites for the formation of inter-chain disulphide bonds.

The structural model shown in Fig. 2 is based on data from spectroscopic, hydrodynamic, predictive and scanning probe microscopy studies, as detailed in Shewry *et al.* [7]. Studies carried out on HMW subunit

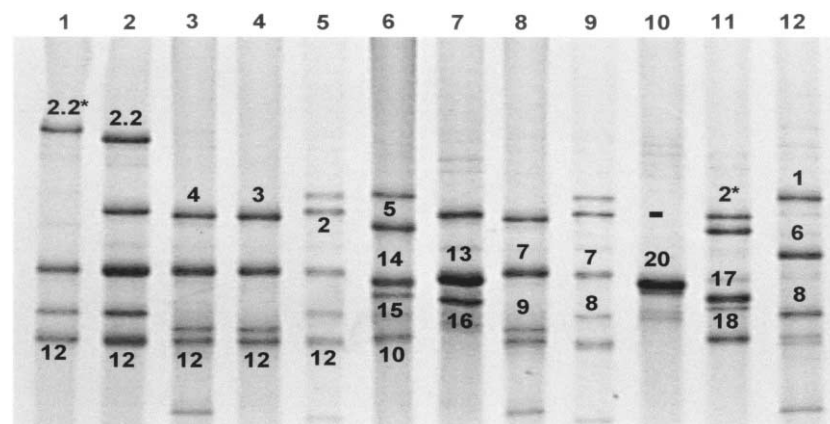


Fig. 1. SDS-PAGE of major allelic forms of HMW subunits present in different wheat cultivars and lines. Alleles at the *Glu-D1* locus are shown in lanes 1–6, at the *Glu-B1* from 6 to 12, at the *Glu-A1* from 11 to 12. The different alleles are numbered according to Payne and Lawrence [6].

films and on single molecules stretched by atomic force microscopy indicate that the β -spiral structure exhibits intrinsic elasticity but the precise contribution of this mechanism to the elastomeric properties of the polymer is not known.

Subunit interactions and gluten visco-elasticity

The high molecular weight subunits are present only in polymers, and in particular in polymers of high molecular mass (sometimes called ‘glutenin macropolymer’; see [12]). The importance of such polymers in determining gluten visco-elasticity has been appreciated for many years, although their size, complexity and low solubility have limited detailed studies. Similarly, the

importance of disulphide bonds in stabilizing these polymers was demonstrated by early studies in which the addition of disulphide reducing agents was shown to weaken doughs and result in increased glutenin solubility. Direct mapping of disulphide bonds has also demonstrated the presence of inter-chain bonds between HMW subunits and between HMW and LMW subunits, with one intra-chain bond within the N-terminal domain of an x-type subunit [13–15]. Disulphide bonds are, therefore, widely considered to be essential for glutenin visco-elasticity.

Figure 3 summarises a widely-held view of gluten structure in which the HMW subunits form an ‘elastic backbone’ consisting largely of head-to-tail polymers

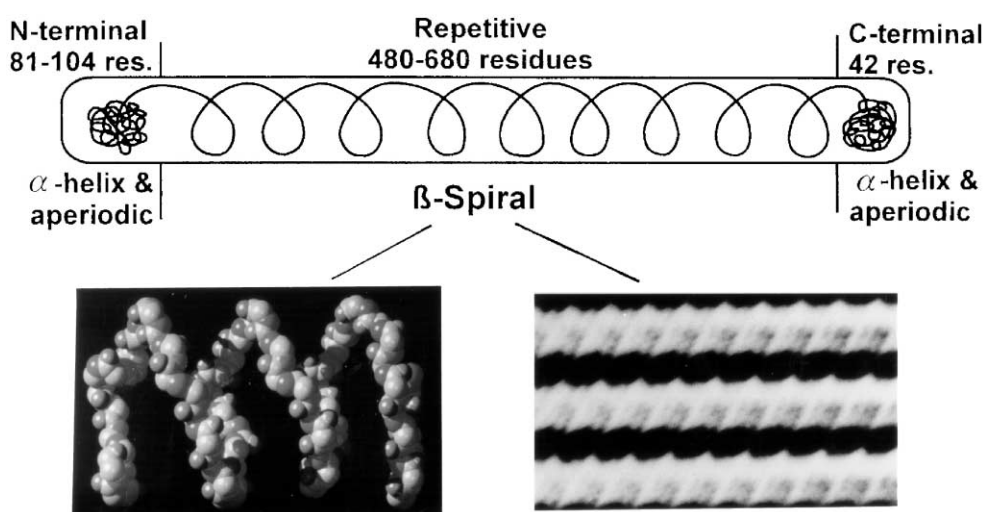


Fig. 2. A structural model for the HMW subunits of glutenin, based on predictive, spectroscopic and hydrodynamic studies [7]. Scanning probe microscopy (right) of a purified subunit reveals aligned rods with a diameter of about 18 Å showing spiral striations of pitch about 15 Å [11]. Computer modelling (left) can be used to develop a model of the peptide backbone as a loose spiral structure based on β -reverse turns (unpublished results of O Parchment, A.S. Tatham, P.R. Shewry and D. Osguthorpe).

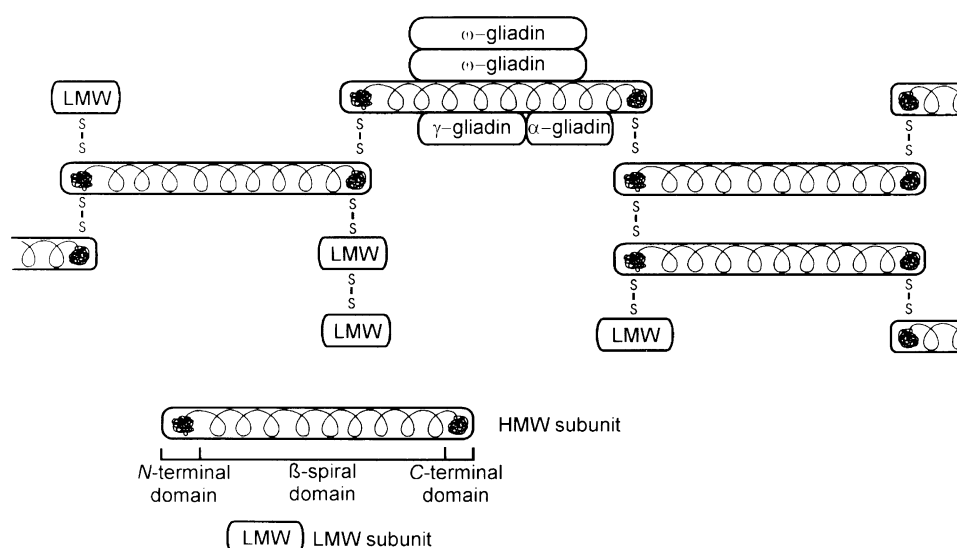


Fig. 3. A structural model for wheat gluten in which the HMW subunits provide a disulphide-bonded backbone which interacts with other gluten proteins by disulphide bonds (LMW subunits) and non-covalent interactions (gliadins).

with inter-chain disulphide bonds. This backbone forms a basis for LMW subunit 'branches' (linked by disulphide bonds). Gliadins may also interact with the glutenin polymers by non-covalent forces, although these interactions are traditionally considered to contribute to gluten viscosity rather than elasticity.

However, work carried out in the EUROWHEAT programme suggests that non-covalent interactions (i.e. hydrogen bonds) between glutenin subunits and polymers may also contribute to elasticity. Evidence for this comes from nuclear magnetic resonance (NMR) and Fourier-transform infra-red (FT-IR) of high molecular weight subunits and model proteins and peptides [16–19] and may be summarised as follows:

- The proteins are disordered and have little structure when dry
- On hydration the proteins have greater mobility and form β -sheet structures
- On further hydration there is a further increase in mobility accompanied by the formation of turn-like structures at the expense of the sheet-like structures.

It is important to note that these results were obtained with protein in the dry or hydrated solid state which reflects the situation in dough whereas results obtained on samples in solution do not reflect the role of protein-protein interactions and are therefore of limited validity in understanding dough behaviour.

In order to explain this behaviour a model, which is now termed the 'loop and train model' has been developed [20]. This model is illustrated in Fig. 4. In the low hydration state there are many protein-protein interactions through interchain hydrogen bonding of glutamine residues. As the levels of hydration increase, plasticisation of the system allows the formation of hydrogen bonded structures between the chains (intermediate hydration). Further increases in hydration lead to the formation of hydrogen bonds between water and glutamine, resulting in the formation of regions in

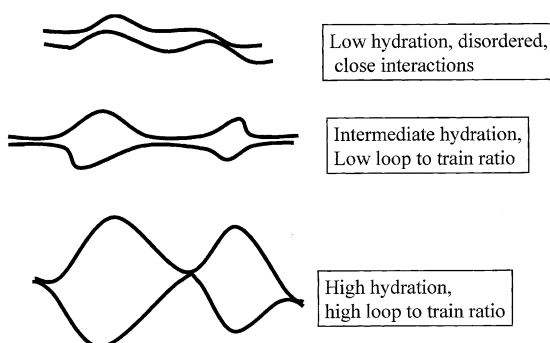


Fig. 4. Model for the effect of hydration on the loop to train ratio of HMW subunits.

which interchain interactions are broken (high hydration). These regions are mobile and associated with the β -turn structure revealed by the FTIR spectra. It may be expected that the mobile turn regions would become dominant as the water content increased further, and that the protein would dissolve. However, this does not happen because the numbers of glutamine residues are high and the statistical likelihood of all the interchain bonds breaking simultaneously will, therefore, be very low. It is also possible that such hydrogen bonded structures form between other gluten proteins, notably the LMW subunits of glutenin.

The glutenin subunits form a disulphide-bonded network in doughs. Extension of the dough will, therefore, result in strain in the network, with the loops becoming stretched and the train regions becoming 'unzipped' at low extension, and extended stiff chains formed as further extension occurs (Fig. 5). It may be assumed that the relaxation rate of the polymers is low in a concentrated polymer matrix such as exists in dough, implying that the stiff extended polymers will not relax on the time scale of the mixing rate. The formation of the extended chains is therefore, a mechanism by which elastic energy is stored in the dough and can account for the increased resistance to extension of dough during the mixing process. The formation of hydrogen bonds between glutamine residues also explains the effects on dough rheology that have been observed on esterification of glutamine residues and the addition of D_2O , rather than H_2O , with the former decreasing and the latter increasing the resistance to extension.

Water clearly plays a critical role in the ratio of loops to trains. Since it would be expected that the deformation of loops would require less energy than the unzipping of trains, increasing the water will result in a more easily deformable system. Thus, the mechanism of the plasticisation of dough by water can be understood.

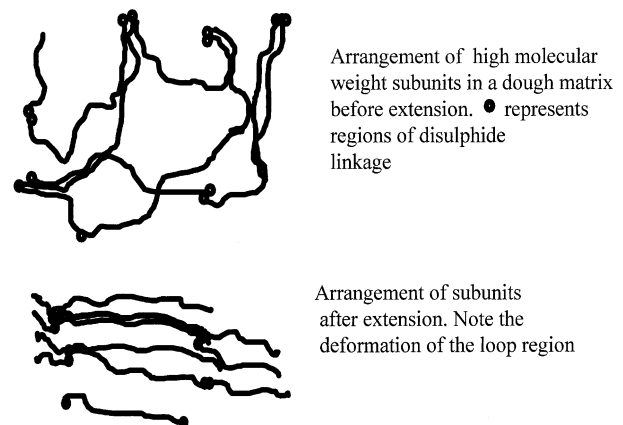


Fig. 5. Model for the arrangement of HMW subunits in a dough matrix before (top) and after (bottom) extension.

However, the role of disulphide bonds is clearly also critical in that they hold the network together. Disulphide interchange during mixing could operate in two different ways, neither of which is exclusive. Breaking and remaking disulphide bonds under extension could result in the formation of a network which is aligned along the direction of extension. This in turn would result in an apparent increase in extensibility along the direction of extension. Alternatively, the continued input of energy into the dough could result in the breakdown of the network, with the reformation of disulphide bonds being a repair mechanism that would restore the network, deferring the loss of elastic energy and maintaining resistance to extension.

The loop and train model is, therefore, able to explain a number of features which are observed in model systems and real doughs:

- The spectroscopic properties of high molecular weight subunits and related systems
- Esterification of glutamine residues decreases the coherence and resistance to extension of doughs. [21,22]
- Deuteration increases dough strength [23]
- Dough is able to store elastic energy [24]
- Water plasticizes dough [24].

Manipulation of HMW subunit composition

Plant breeders routinely exploit variation in HMW subunit composition, selecting for optimal combinations of subunits for different end uses: highly viscoelastic doughs for breadmaking but more extensible doughs for cakes and biscuits.

However, limited variation is present in the HMW subunit composition of European wheats and research-

ers have therefore, taken two approaches to introduce additional variation. These are the use of exotic wheats and the introduction of additional genes by genetic engineering. Both approaches were used in the EURO-WHEAT programme, in order to determine the effects of additional, novel and mutant subunits on grain functional properties, determined in common genetic backgrounds.

Analysis of a wide range of wheat lines in gene banks led to the discovery of a number of novel HMW subunit alleles. These have all been crossed into the Italian variety Pegaso, resulting in a near-isogenic series of lines (NILs) which have identical compositions of gluten proteins except for variation in HMW subunit composition (Fig. 6). They include lines expressing between three and six subunits and containing subunits with longer repetitive domains such as 1D \times 2.2, 1D \times 2.2* and 1Dy12₁. Preliminary studies of the lines containing six subunits indicate beneficial effects of increased subunit numbers on dough properties.

A further near isogenic series was available prior to the start of the project, provided by Lawrence and co-workers at CSIRO, Canberra, Australia [25]. These lines were produced by crossing naturally occurring null mutants in the Australian cultivars Gabo and Olympic, giving lines silent for the HMW subunit genes on chromosomes 1A, 1B and 1D. Combination of these null mutants resulted in a series of lines with variation in subunit number from nil (triple null line) to five (subunits 1A \times 1, 1B \times 17+1By18, 1D \times 5+1Dy10).

This series of NILs allowed the expression of genes at the individual loci to be related to functional properties. Furthermore, the series also provided an ideal background for transformation with additional HMW subunit genes [26]. A 1A/1D null line expressing only the

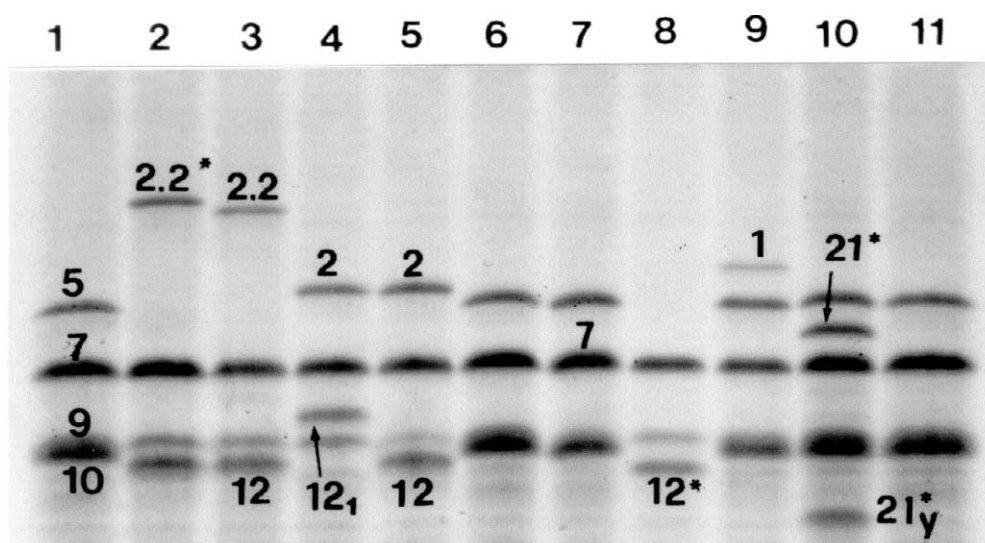


Fig. 6. SDS-PAGE of HMW subunits present in the bread wheat cultivar Pegaso (lanes 1, 6 and 11) and derived near isogenic lines. Lanes 2, 3 and 4 show lines containing subunits with larger repetitive domains.

HMW subunits encoded by chromosome 1B (1B \times 17+1By18) was selected for transformation with active genes from chromosome 1A (1A \times 1) and 1D (1D \times 5), to determine their effects in a poor quality background. Additional copies of the subunit 1D \times 5 gene were transferred to the line expressing genes on chromosomes 1A, 1B and 1D to determine the effects of increasing the number of active genes.

The transformation was carried out using a standard particle bombardment system, the DNA being coated onto gold particles and shot into the cells of cultured immature embryos using helium gas at high pressure. The embryos were then regenerated to give whole plants.

Analysis of gluten structure and functional properties of near-isogenic and transgenic lines

Comparison of the glutenin composition and rheological properties of glutes extracted from the NILs provided information on the functional role of the HMW glutenin subunits. Loss of all the HMW subunits (in the triple null line) resulted in glutenin that contained almost no large aggregated glutenin polymers, demonstrating that these cannot be formed by LMW subunits alone. Gluten visco-elasticity was also considerably reduced when compared to the other lines. Thus, the HMW glutenin subunits are indispensable for the formation of large glutenin polymers and for the expression of gluten visco-elasticity. However, individual subunits differ in their 'visco-elastic potential' which is expressed through the properties of glutenin polymers and aggregates. This was shown by comparing the two double null lines expressing either subunits 1D \times 5+1Dy10 (1A/1B null) or 1B \times 17+1By18 (1A/1D null). Gluten from the 1A/1B null line contained more highly aggregated glutenin polymers and had higher visco-elasticity than that from the 1A/1D null line. Similar differences were found also between NILs expressing subunits 1D \times 5+1Dy10 or 1D \times 2+1Dy12 [27]. Furthermore, the rheological properties of the 1A/1B null line were similar to those of the control line expressing five subunits. This indicates that subunits 1D \times 5+1Dy10 have very high visco-elastic potential. These results support the quality scores for HMW glutenin subunit alleles based on relationships between glutenin composition and flour technological properties, subunits 1D \times 5+1Dy10 being given the highest quality score of 30 compared with a score of 18 for subunits 1B \times 17+1By18 [28].

The analysis of NILs does not allow us to determine the effects of individual subunits which are inherited as pairs encoded by closely linked genes but this can be achieved by the production of transgenic lines. We therefore compared the effects of expression of transgenes coding for subunit 1A \times 1 or for subunit 1D \times 5 on glutenin aggregation, gluten visco-elasticity and the

mixing properties of doughs. The 1A \times 1 and 1D \times 5 transgene subunits were both overexpressed, accounting for 50–70% of the HMW subunits in the transformed lines (Fig. 7A). However, clear differences were observed between their effects on the physicochemical properties of the gluten (Fig. 7B–F).

Expression of the subunit 1A \times 1 transgene increased glutenin aggregation, but did not appear to result in increased crosslinking by disulphide bonds. Thus, only the average size of glutenin polymers appeared to have been increased. Gluten visco-elasticity was also only moderately affected, with the main impact being increased resistance of the dough to elongation during mixing. In contrast, overexpression of subunit 1D \times 5 generated very large and insoluble aggregates, probably through increased covalent cross-linking. The connectivity and visco-elasticity of the gluten network were also considerably increased. These effects may be attributed to differences in the cysteine content of the subunits, with subunit 1D \times 5 containing an additional cysteine residue available to form inter-molecular cross-links [7]. The very high gluten strength of lines expressing the 1D \times 5 transgene was associated with abnormal dough mixing behaviour and it can be postulated that this resulted from modification of the glutenin structure to prevent the formation of a homogeneous network. However, subunit 1D \times 5 is always expressed as a pair with 1Dy10 and there is evidence that dimers between these two subunits, and between other x-type and y-type subunits, are present as 'building blocks' in the glutenin polymers [30,31]. Consequently, over-expression of subunit 1D \times 5 in the absence of additional subunit 1Dy10 (or another y-type subunit) could result in extensive restructuring of the glutenin polymers with important consequences for gluten strength and for the mixing and baking properties of dough.

The studies of near isogenic and transgenic lines have provided new information on the effects of variation in HMW subunit composition and amount on gluten structure and properties. They do not, however, provide information on the biochemical basis for these effects. In order to explore this a new series of transgenic lines is being produced. These include the transformation of the 1A/1D null line with mutant genes encoding forms of subunit 1D \times 5 in which the length of the repetitive domain has been increased by 22.5% or decreased by 17.2 or 36.6% [32]. It can be hypothesised that these changes will lead to effects on the ratio of loops to trains and hence on the visco-elasticity, but these analyses have not yet been carried out due to the need to select and multiply the transformed lines.

Good and poor quality subunits

Comparisons of cultivars expressing different numbers of subunits and analyses of near isogenic and transgenic lines as discussed above indicate that there is

a quantitative relationship between HMW subunit gene expression, the amount of subunit protein and gluten visco-elasticity, at least within certain limits. This may explain the association of chromosome 1A-encoded subunits with quality when compared with 1A null cultivars. However, it does not explain why allelic pairs of chromosome 1B and 1D-encoded subunits show clear differences in quality despite being expressed at approximately similar levels. The most striking example of this subunits is 1D \times 5 + 1Dy10 which are associated with higher quality than the allelic pairs 1D \times 2 + 1Dy12,

1D \times 3 + 1Dy12 and 1D \times 4 + 1Dy12. Because these pairs of subunits are encoded by tightly linked genes it has proved impossible to determine whether the higher quality results from the properties of subunit 1D \times 5 or subunit 1Dy10 or whether the specific combination of these subunits is required. Comparative analyses of purified subunits and comparison of gene sequences has shown that the allelic proteins have similar structures and properties, with two exceptions. Firstly, subunit 1D \times 5 contains one more cysteine residue than subunit 1D \times 2, located close to the N-terminal end of the

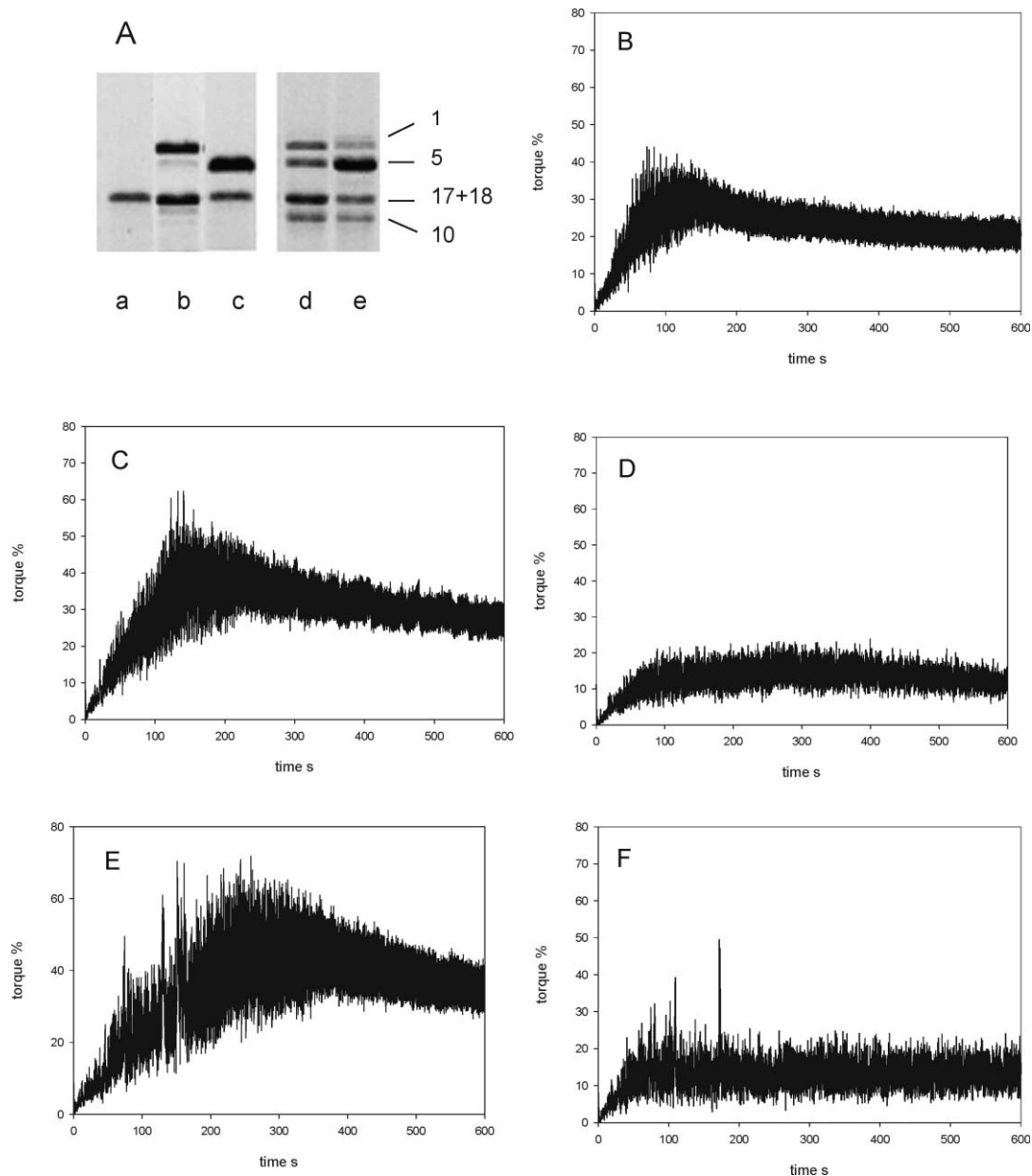


Fig. 7. Analysis of the mixing properties of transgenic wheats expressing additional HMW subunits using the 2g Mixograph. (A) SDS-PAGE of the HMW subunits from (a) control line L88-31 (subunits 1B \times 17 + 1By18); (b) L88-31 expressing the 1A \times 1 transgene; (c) L88-31 expressing the 1D \times 5 transgene; (d) control line L88-6 (subunits 1A \times 1, 1D \times 5 + 1Dy10, 1B \times 17 + 1By18); (e) L88-6 expressing the 1D \times 5 transgene. (B)–(F) are Mixographs of B, L88-31; (C) L88-31 expressing the 1A \times 1 transgene, D, L88-31 expressing the 1D \times 5 transgene; (E) L88-6; (F) L88-6 expressing the 1D \times 5 transgene. The resistance is given as torque % and the mixing time in seconds (s). Taken from Popineau *et al.* [29] with permission.

repetitive domain. This cysteine could be involved in the formation of inter-chain disulphide bonds resulting in a more highly elastic gluten, providing an explanation for the ‘overstrong’ characteristics resulting from the over-expression of subunit 1D \times 5 in the transgenic lines discussed above. Secondly, we have shown that subunit 1Dy10 shows significantly higher stability than 1Dy12 when unfolded on transverse urea gradient gels (with a free energy of 7.4 compared with 5.1 Kcal/mol). This may indicate that deformation on mixing would require a greater energy input, although it is difficult to make direct extrapolations between the results obtained by electrophoresis in the presence of a detergent with the behaviour in dough [33]. These comparisons nevertheless suggest that individual features of subunits 1D \times 5 and 1Dy10 may independently contribute to dough strength.

It is also possible that specific interactions also occur between individual subunits, particularly those inherited as allelic pairs. These are much more difficult to study but preliminary data from the EUROWHEAT project indicates that some combinations of subunits form more

stable interactions than others. This may be due to a higher degree of β -sheet formation resulting from subtle differences in repeat unit structure.

Conclusions

Figure 8 summarises our current knowledge of the various structural features of the HMW subunits which contribute to gluten visco-elasticity, based on a range of studies including important contributions from the EUROWHEAT project. This provides a sound basis for future work aimed at dissecting the mechanism of gluten visco-elasticity in more detail. It also provides targets for improvement of wheat quality, by identifying characteristics which can be selected in classical plant breeding or manipulated by genetic engineering. However, there is one topic which is clearly in need of further study. This is the specific interaction of individual subunits, particularly those inherited as allelic pairs. Understanding and optimising these interactions will be crucial for future attempts to fine-tune the structure and properties of gluten.

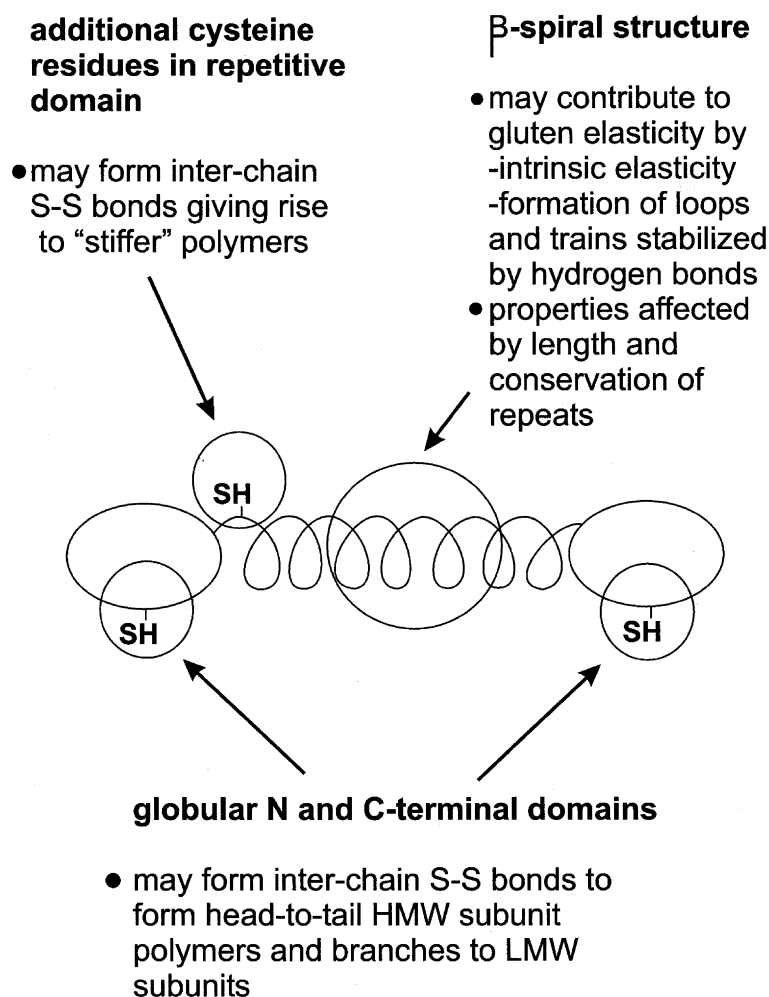


Fig. 8. Summary of our current knowledge of the structural features of the HMW subunits which may determine the elasticity of gluten and dough.

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