THE ROYAL SOCIETY

*Published online* 25 February 2002

# The structure and properties of gluten: an elastic protein from wheat grain

**Peter R. Shewry[1*], Nigel G. Halford[1], Peter S. Belton[2] and Arthur S. Tatham[1]**

[1]*Institute of Arable Crops Research, Long Ashton Research Station, Department of Agricultural Science, University of Bristol, Long Ashton, Bristol BS41 9AF, UK*

[2]*School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, UK*

The wheat gluten proteins correspond to the major storage proteins that are deposited in the starchy endosperm cells of the developing grain. These form a continuous proteinaceous matrix in the cells of the mature dry grain and are brought together to form a continuous viscoelastic network when flour is mixed with water to form dough. These viscoelastic properties underpin the utilization of wheat to give bread and other processed foods. One group of gluten proteins, the HMM subunits of glutenin, is particularly important in conferring high levels of elasticity (i.e. dough strength). These proteins are present in HMM polymers that are stabilized by disulphide bonds and are considered to form the 'elastic backbone' of gluten. However, the glutamine-rich repetitive sequences that comprise the central parts of the HMM subunits also form extensive arrays of interchain hydrogen bonds that may contribute to the elastic properties via a 'loop and train' mechanism. Genetic engineering can be used to manipulate the amount and composition of the HMM subunits, leading to either increased dough strength or to more drastic changes in gluten structure and properties.

**Keywords:** wheat; gluten; protein elasticity; HMM subunits; transgenic plants

## 1. INTRODUCTION

Wheat is one of the three most important crops in the world, together with maize and rice. Approximately 600 million tonnes are harvested annually with cultivation extending over a vast geographical area, from Scandinavia to Argentina, including higher elevations in the tropics. Although the ability to give high yields under a range of conditions has contributed to the success of wheat, the most important factor has been the unique properties of wheat dough that allow it to be processed into a range of foodstuffs, notably bread, other baked products and pastas. These properties are usually described as viscoelasticity, with the balance between the extensibility and elasticity determining the end use quality. For example, highly elastic ('strong') doughs are required for breadmaking but more extensible doughs are required for making cakes and biscuits.

The grain proteins determine the viscoelastic properties of dough, in particular, the storage proteins that form a network in the dough called gluten (Schofield 1994). Consequently, the gluten proteins have been widely studied over a period in excess of 250 yr, in order to determine their structures and properties and to provide a basis for manipulating and improving end use quality (see Shewry *et al.* 1995).

## 2. THE ORIGIN OF THE WHEAT GLUTEN NETWORK

Gluten can be readily prepared by gently washing dough under a stream of running water. This removes the bulk of the soluble and particulate matter to leave a proteinaceous mass that retains its cohesiveness on stretching (figure 1*a*). Gluten comprises some 75% protein on a dry weight basis, with most of the remainder being starch and lipids. Furthermore, the vast majority of the proteins are of a single type called prolamins.

Prolamins are a group of proteins that were initially defined based on their solubility in alcohol–water mixtures (Osborne 1924), typically 60–70% (v/v) ethanol. This definition has since been extended to include related proteins, which are not soluble in alcohol–water mixtures in the native state, owing to their presence in polymers stabilized by interchain disulphide bonds. In wheat, these groups of monomeric and polymeric prolamins are known as gliadins and glutenins, respectively, and together form gluten (Shewry *et al.* 1986).

Wheat prolamins are the major storage proteins present in the starchy endosperm cells of the grain, where they are synthesized and deposited via the secretory system. Thus, the individual polypeptides are synthesized on ribosomes on the RER and pass via the usual translocation machinery into the lumen, with the loss of an N-terminal signal peptide. Once within the lumen it is probable that protein folding and disulphide bond formation occur with no further post-translational modifications taking place (i.e. no glycosylation or proteolysis as may occur with other types of seed storage protein).

The subsequent fate of the proteins may also vary with the protein type and with the age and stage of development of the tissue. Some of the proteins appear to be transported via the Golgi apparatus into the vacuole, where they form protein deposits (see Shewry 1999). However, others appear to accumulate directly within the

*Author for correspondence (peter.shewry@bbsrc.ac.uk).

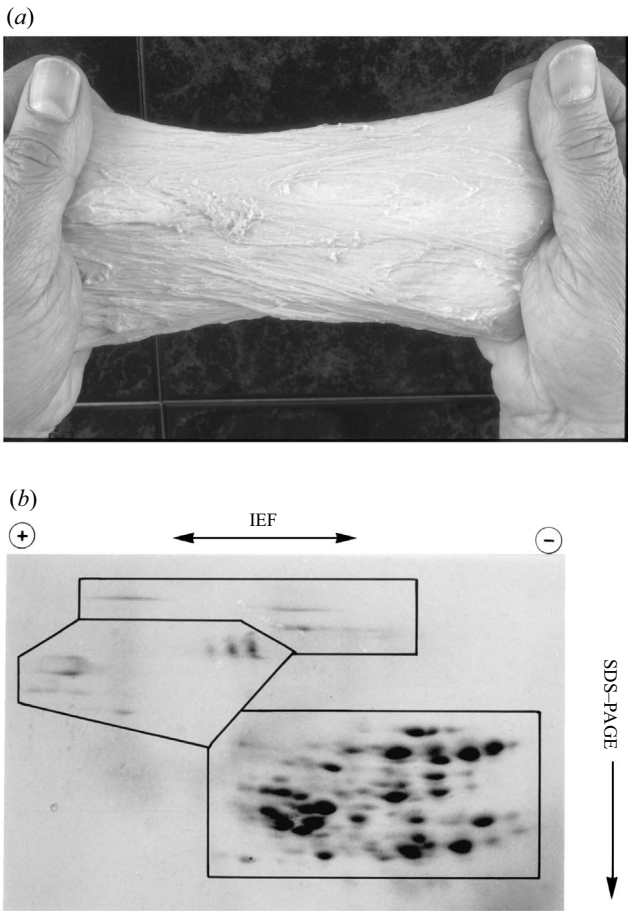


Figure 1. (*a*) A sheet of gluten stretched to demonstrate its cohesive properties. (*b*) Two-dimensional analysis (isoelectric focusing followed by SDS–PAGE) of wheat gluten proteins shows multiple components.

lumen of the ER to form a second population of protein bodies. Galili (1997) has proposed that vesicles subsequently engulf the latter, with the contents being 'internalized' into vacuoles but to date this has not been confirmed by other workers. What is known is that during the later stages of grain maturation the starchy endosperm cells become disrupted and die and the protein bodies fuse to form a continuous matrix, which surrounds the starch granules and engulfs other organelles and membranes.

Galili (1997) has also suggested that the gliadins are preferentially transported to the vacuole via the Golgi apparatus, which is consistent with the lack of a classical ER retention sequence. We have also shown that a γ-gliadin is rapidly degraded in leaves and seeds of transgenic tobacco (presumably in the vacuole) unless a C-terminal ER retention sequence (the tetrapeptide His–Asp–Glu–Leu or Lys–Asp–Glu–Leu) is added (Napier *et al.* 1997). However, the glutenins also lack an obvious ER retention sequence but, nevertheless, Galili (1997) has proposed that they are preferentially retained in the ER. In this case, their rapid assembly into high $M_r$ polymers, which precipitate and accumulate directly within the ER lumen, could determine retention. It is also possible to envisage how the relative rates of trafficking via the ER and Golgi routes could vary with the level of protein synthesis and age of the tissue.

As a result of the formation of a protein matrix, individual cells of wheat flour contain networks of gluten proteins, which are brought together during dough mixing. The precise changes that occur in the dough during mixing are still not completely understood, but an increase in dough stiffness occurs that is generally considered to result from 'optimization' of protein–protein interactions within the gluten network. In molecular terms, this 'optimization' may include some exchange of disulphide bonds as mixing in air, oxygen and nitrogen result in different effects on the sulphydryl and disulphide contents of dough (Tsen & Bushuk 1963; Mecham & Knapp 1966).

Of course, the natural fate of the wheat grain is not to provide flour for humankind but to germinate to produce a new plant. The biological role of the gluten protein is, therefore, to provide a store of carbon, nitrogen and sulphur to support seed germination and seedling growth. The gluten proteins have no other known biological role and their viscoelastic properties appear to be a purely fortuitous consequence of their sequences and interactions.

## 3. THE HMM GLUTENIN SUBUNITS

Wheat gluten is a highly complex mixture of proteins with at least 50 individual components being separated by two-dimensional isoelectric focusing/SDS–PAGE of reduced total fractions (figure 1*b*). Furthermore, there is great variation in the component proteins present in different genotypes. This high level of polymorphism initially limited attempts to isolate and characterize individual components, but details of the structures and sequences of all of the major gluten protein types are now known (see Shewry *et al.* 1999). However, much of the work over the past 20 years has focused on one group of proteins, which are the subject of the remainder of this article. These are the HMM subunits of wheat glutenin (also called the HMW subunits).

Bread wheat is a hexaploid species with three genomes (called A, B and D) derived from related wild grass species. Single loci encoding HMM subunits are present on the long arms of the group 1 chromosomes (1A, 1B, 1D), each locus comprising two genes encoding subunits that differ in their properties and are called x-type and y-type subunits (Payne 1987). Although bread wheats could theoretically contain six HMM subunits (1Ax, 1Ay, 1Bx, 1By, 1Dx, 1Dy), the silencing of specific genes results in the presence of only three (1Bx, 1Dx, 1Dy) to five (1Ax, 1Bx, 1By, 1Dx, 1Dy) subunits (Payne *et al.* 1987).

The HMM subunits have been reported to account, on average, for about 12% of the total grain protein, corresponding to 1–1.7% of the flour dry weight (Seilmeier *et al.* 1991; Halford *et al.* 1992; Nicolas 1997). However, variation in the amount of HMM subunits (associated with the differences in gene silencing discussed above) and in the properties of expressed subunits have been reported to account for between 45 and 70% of the variation in breadmaking performance within European wheats (Branlard & Dardevet 1985; Payne *et al.* 1987, 1988). These correlative studies are supported by the development and analysis of near-isogenic lines that differ only in their HMM subunit composition. Analyses of such lines have confirmed that the subunits are largely responsible for determining dough viscoelasticity and that specific

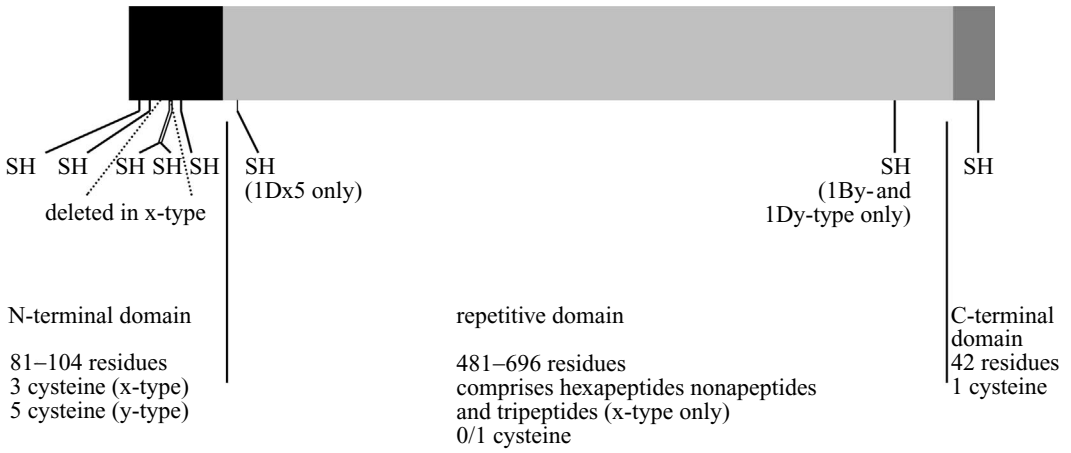


Figure 2. Schematic summary of the sequences of x-type and y-type HMM subunits.

allelic subunit pairs are associated with either high or low dough strength (Popineau *et al.* 1994).

A number of genes encoding HMM subunits have been isolated from bread wheat (see Shewry *et al.* 1992; Reddy & Appels 1993) and from related wheat species and wild relatives (Mackie *et al.* 1996; Wan *et al.* 2001). These show that the HMM subunits have conserved amino-acid sequences, comprising three distinct parts or domains (figure 2). The central domains of the proteins consist of repeated peptides, based on two or three short peptide motifs. They vary in length from about 420 to 700 residues and account for between 74 and 84% of the whole protein. These domains are flanked by short non-repetitive domains, which vary in length from 81 to 104 residues at the N-terminus but always comprise 42 residues at the C-terminus.

## 4. SEQUENCES OF THE REPETITIVE DOMAINS

The x-type and y-type subunits have essentially similar repeat structures, comprising mainly nonapeptide and hexapeptide motifs. Also, whereas tandem blocks of hexapeptides may be present, the nonapeptides are always interspersed with hexapeptides. Consequently, it is convenient to consider them as forming a 15 amino-acid motif. The x-type subunits also differ from the y-type in having additional tripeptide motifs, which also only occur in tandem with hexapeptides, forming a second nonapeptide motif. Figure 3 shows the sequences of the repetitive domains of typical x-type and y-type subunits (1Dx5 and 1Dy10, respectively) arranged to show their repeated block structure. The repeat motifs are rich in glutamine, proline and glycine, which together account for over 70% of the total amino-acid residues. No major differences are apparent between the homeoallelic proteins of bread wheat (A, B, D) or related genomes present in other wheat species or wild relatives (A, C and D), so combined data for five x-type and seven y-type subunits are presented in table 1.

Comparison of the patterns of amino-acid substitutions shows that some positions of the motifs appear to be more highly conserved than others. In particular, glutamine tends to be more highly conserved at specific positions than other consensus amino acids: at positions 3, 5 and 6 of the hexapeptide, 8 and 9 of the nonapeptide and 2 and 3 of the tripeptide. This may relate to the role of glutamine residues in stabilizing the structures and interactions of the subunits. Similarly, serine is conserved at position 6 of the nonapeptides. In contrast, positions 1 and 4 of the hexapeptide are poorly conserved, as is position 7 of the nonapeptide.

Some differences are also observed between the x-type and y-type subunits. Thus, replacement of Pro with Ser at position 1 of the hexapeptides is more common in x-type subunits, as is replacement of Gln with Pro at position 6. However, the latter only occurs in hexapeptides within a 15 residue (6 + 9) motif, rather than in the tandemly-arranged hexapeptides. Similarly, replacement of Tyr with His at position 2 and Thr with Ala at position 5 of the nonapeptides are more common in y-type subunits and these two substitutions usually occur together, giving the two consensus motifs GYYPTSLQQ and GHYPASLQQ compared with GYYPTSPQQ for x-type subunits.

There is no evidence that amino-acid substitution leads to replacement with similar amino-acid residues (i.e. conservative substitutions). Instead, analysis of codons indicates that most replacements are due to single nucleotide changes, with substitutions resulting from double nucleotide changes occurring more rarely. For example, proline (CCA) occurs at position 1 in 55% of x-type hexapeptides, with single nucleotide changes leading to the occurrence of leucine (CTA, 12%) and serine (TCA, 30%) and two nucleotide changes to isoleucine (ATA, 3%).

The failure to detect any appreciable differences between the consensus motifs and degree of conservation of the repetitive sequences present in the HMM subunits of cultivated and wild species (Wan *et al.* 2001) indicates that selection by plant breeders for dough strength, which has been carried out systematically for the last century and perhaps unconsciously over the 10 000 year life of bread wheat, has had little or no impact on the sequences (and hence structure) of the subunits. However, it is possible that the differences in degree of conservation within the motifs and the precise amino-acid residues that are present as substitutions at different positions within the motifs may relate to their role in determining the structure adopted by the domain.

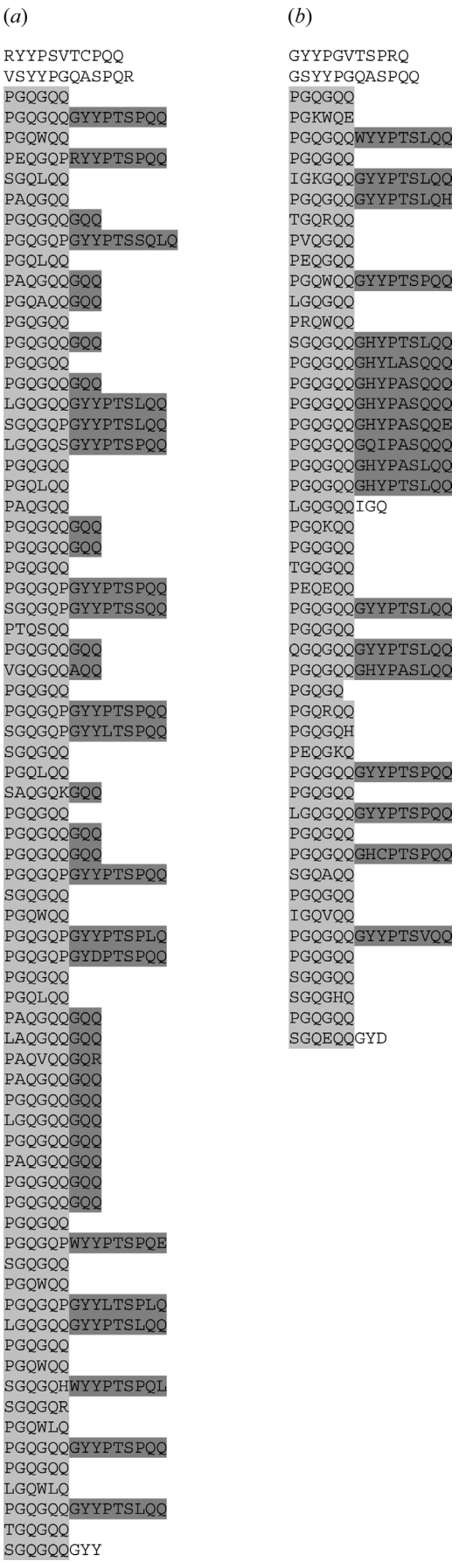


Figure 3. Amino-acid sequences of the repetitive domains of typical x-type (1Dx5,(*a*)) and y-type (1Dy10,(*b*)) HMM subunits arranged to show their repeat unit structures.

Cysteine residues occur only rarely in the repetitive sequences, with single cysteine residues present towards the C-terminal end of the repetitive domains (at position −73) of 1By and 1Dy subunits only. In addition, subunit 1Dx5 differs from all other subunits whose sequences are known in that a single additional cysteine is present at position 8 relative to the N-terminal end of the domain.

## 5. STRUCTURE OF THE HMM SUBUNIT REPETITIVE DOMAIN

Although several workers have attempted to determine the structure adopted by the HMM subunit repeats by X-ray crystallography of whole subunits or repetitive peptides, the crystals produced have failed to give clear diffraction patterns. Similarly, analysis of synthetic peptides based on the repetitive sequence motifs has not yet led to the determination of three-dimensional structures. Consequently, our current view of HMM subunit structure comes from a range of indirect studies.

Early hydrodynamic studies of subunit 1Bx20 purified from pasta wheat indicated that it had an extended rod-shaped conformation in solution, the dimensions ranging from *ca.* $500 \times 17.5$ to $620 \times 15$ Å depending on the solvent (Field *et al.* 1987). Detailed spectroscopic studies of whole subunits, of recombinant repetitive peptides and of linear and circular synthetic peptides (Tatham *et al.* 1985; Field *et al.* 1987; van Dijk *et al.* 1997*a*,*b*; Gilbert *et al.* 2000) have also been reported. The results are consistent with the repetitive sequences forming β-reverse turns which may be in equilibrium with poly-L-proline II structure, the latter predominating at low temperature (Gilbert *et al.* 2000). It has also been proposed that the β-turns are organized to give a regular spiral structure (termed a β-spiral) similar to that demonstrated for a synthetic polypentapeptide based on a repeat motif of elastin (Urry 1988). Molecular modelling can be used to generate such spiral structures (figure 4) whose dimensions (diameter, pitch and length) are consistent with those determined by viscometric analysis and revealed by STM of purified proteins in the hydrated solid state (Miles *et al.* 1991). However, Kasarda *et al.* (1994) have proposed that an alternative type of spiral structure is formed, based on γ-turns rather than β-turns.

## 6. SEQUENCES AND STRUCTURES OF THE NON-REPETITIVE DOMAINS

The N-terminal domains vary in length, being 81–89 residues in the x-type subunits and 104 residues in the y-type. This difference results from a deletion in the x-type subunits compared with the y-type, which involves the loss of two cysteine residues. Consequently, the N-termini of the x-type subunits usually contain three cysteine residues and those of the y-type subunits five. Structure prediction and molecular modelling studies indicate that this domain is 'globular' with one or more α-helices (Tatham *et al.* 1984, 1985; Van Dijk *et al.* 1998; Köhler *et al.* 1997).

The C-terminal domains of all of the subunits comprise 42 residues with single cysteine residues at position −13 with respect to the C-terminus. Structure prediction indicates that this domain may be α-helical (Tatham *et al.*

Table 1. Frequency of occurrence of different amino acid residues in each position of: (*a*) The 347 hexapeptide, 103 nonapeptide and 81 tripeptide repeat motifs of x-type HMW subunits 1Ax1, 1Bx7 and 1Dx5 (*T. aestivum*); 1Ax (*T. timopheevi*); 1Dx (*A. cylindrica*). (*b*) The 339 hexapeptide and 123 nonapeptide repeat motifs of y-type HMW subunits 1Ay (not expressed), 1By 9 and 1Dy10 (*T. aestivum*); 1Ay (*T. timopheevi*); 1Cy and 1Dy (*A. cylindrica*); 1Dy (*T. tauschii*). (Percentages may not add up to 100 because of rounding. Residues present at less than 1% are either included as 'other' if together they add up to 1%, or are not shown.)

| | hexapeptides (%) | | | | | | tripeptides (%) | | | nonapeptides (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (*a*) | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| | Pro 62 | Gly 84 | Gln 99 | Gly 75 | Gln 94 | Gln 80 | Gly 89 | Gln 99 | Gln 99 | Gly 84 | Tyr 98 | Tyr 97 | Pro 90 | Thr 96 | Ser 100 | Pro 70 | Gln 88 | Gln 94 |
| | Ser 26 | Ala 7 | Other 1 | Trp 9 | Leu 3 | Pro 15 | Asp 5 | Arg 1 | Arg 1 | Arg 6 | His 2 | Asp 2 | Leu 8 | Ile 4 | | Ser 13 | Leu 8 | Leu 4 |
| | Leu 10 | Glu 4 | | Leu 7 | Other 3 | Ser 2 | Ala 2 | | | Glu 3 | | Phe 1 | Ser 2 | | | Leu 11 | Trp 3 | Glu 2 |
| | Ile 1 | Arg 3 | | Glu 4 | | Arg 1 | Arg 2 | | | Trp 3 | | | | | | Ala 2 | Arg 1 | |
| | Other 2 | Thr 2 | | Arg 2 | | Leu 1 | His 1 | | | Val 2 | | | | | | Glu 2 | | |
| | | | | Ala 1 | | Other 2 | | | | Ala 1 | | | | | | — 2 | | |
| | | | | Other 1 | | | | | | Lys 1 | | | | | | Arg 1 | | |
| (*b*) | 1 | 2 | 3 | 4 | 5 | 6 | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| | Pro 65 | Gly 92 | Gln 96 | Gly 76 | Gln 94 | Gln 94 | | | | Gly 96 | Tyr 54 | Tyr 85 | Pro 91 | Thr 60 | Ser 97 | leu 54 | Gln 97 | Gln 90 |
| | Ser 12 | Glu 6 | Lys 4 | Glu 7 | His 2 | Glu 2 | | | | Trp 2 | His 41 | Cys 4 | Leu 5 | Ala 37 | Tyr 2 | Pro 21 | His 3 | His 7 |
| | Leu 10 | Lys 2 | | Trp 7 | Lys 2 | His 2 | | | | Arg 1 | Gln 5 | Asp 2 | Arg 2 | Ser 2 | Phe 1 | Gln 19 | | Glu 2 |
| | Ile 7 | Other 1 | | Arg 4 | Other 1 | — 2 | | | | Tyr 1 | | His 2 | Ser 1 | Ile 1 | | Val 4 | | Stop 1 |
| | Thr 4 | | | Ala 2 | | | | | | | | Ile 2 | Thr 1 | | | Gly 2 | | |
| | Gln 1 | | | Val 2 | | | | | | | | Phe 2 | | | | Ser 2 | | |
| | Other 1 | | | Lys 1 | | | | | | | | Arg 1 | | | | Ala 1 | | |
| | | | | Other 2 | | | | | | | | Asn 1 | | | | | | |
| | | | | | | | | | | | | Glu 1 | | | | | | |

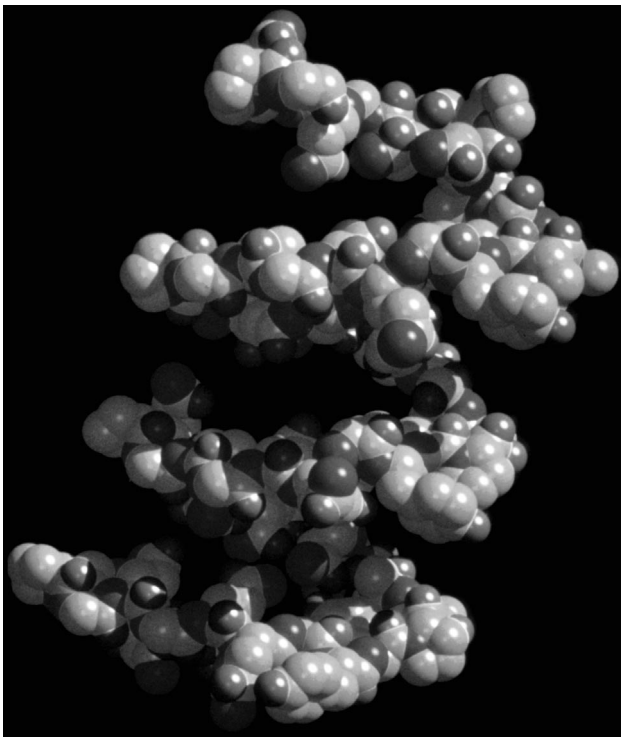

Figure 4. Molecular model developed for a β-spiral structure based on the amino-acid sequence of a repetitive domain of a HMM subunit. The backbone structure only is shown (D. J. Osguthorpe, O. Parchment, P. R. Shewry & A. S. Tatham, unpublished results).

1984) and NMR spectroscopy of a synthetic peptide dissolved in 40% (v/v) aqueous trifluoroethanol (a 'structure-inducing' solvent) allowed a low-resolution structure containing two α-helices to be determined (Bekkers *et al.* 1996).

## 7. HMM SUBUNIT STRUCTURE AND GLUTEN ELASTICITY

The HMM subunits are only present in glutenin polymers, particularly in high $M_r$ polymers, the amounts of which are positively correlated with dough strength (Field *et al.* 1983). This provides support for the genetic evidence (see § 3) that the HMM subunits are the major determinants of dough and gluten elasticity.

Two features of HMM subunit structure may be relevant to their role in glutenin elastomers: the number and distribution of disulphide bonds and the properties and interactions of the repetitive domains.

Direct sequence analysis of disulphide-linked peptides released by enzymic digestion of glutenin or gluten fractions has revealed a number of inter- and intrachain disulphide bonds involving HM W subunits (Köhler *et al.* 1991, 1993, 1994; Tao *et al.* 1992; Keck *et al.* 1995). These are summarized diagrammatically in figure 5 and include one interchain disulphide bond within the N-terminal domain of an x-type subunit, two parallel disulphide bonds between the N-termini of y-type subunits, an interchain bond between a y-type subunit and a LMM glutenin subunit and a bond linking y-type and x-type subunits in a 'head-to-tail' fashion. The latter is consistent with the results obtained by partial reduction of glutenin, which leads to the release of dimers comprising x-type + y-type subunits (Lawrence & Payne 1983; Tao *et al.* 1992). Such dimers have therefore been proposed to form the 'building blocks' of glutenin (Graveland *et al.* 1985). However, our knowledge of the detailed disulphide structure of glutenin is not sufficiently complete to allow us to relate disulphide distribution to biomechanical properties.

Although it is now widely accepted that disulphide-linked glutenin chains provide an 'elastic backbone' to gluten, evidence from spectroscopic studies (using NMR and FTIR spectroscopy) of HMM subunits and of model peptides based on the repeat motifs suggests that non-covalent hydrogen bonding between glutenin subunits and polymers may also be important (Belton *et al.* 1994, 1995, 1998; Wellner *et al.* 1996; Gilbert *et al.* 2000). These studies have shown that the dry proteins are disordered with little regular structure, but that their mobility increases and β-sheet structures form on hydration. Further changes occur if hydration continues, with a further increase in protein mobility and the formation of turn-like structures at the expense of β-sheet.

These observations led to the development of a 'loop and train' model (Belton 1999), which is summarized in figure 6. This proposes that the low hydration state has many protein–protein interactions, via hydrogen bonding of glutamine residues in the β-spiral structures. As the hydration level increases the system is platicized, allowing the orientation of the β-turns in adjacent β-spirals to form structures that resemble an 'interchain' β-sheet. Further hydration leads to the breaking of some of the interchain hydrogen bonds in favour of hydrogen bonds between glutamine and water, which then leads to the formation of loop regions. However, it does not result in the complete replacement of interchain hydrogen bonds, and hence solution of the protein, as the number of glutamine residues is high and the statistical likelihood of all the interchain bonds breaking simultaneously is therefore low. The result is an equilibrium between hydrated 'loop' regions and hydrogen-bonded 'chain' regions, with the ratio between these being dependent on the hydration state.

The equilibrium between 'loops' and 'trains' may also contribute to the elasticity of glutenin, as an extension of the dough will result in stretching of the 'loops' and 'unzipping' of the 'trains'. The resulting formation of extended chains may be a mechanism by which elastic energy is stored in the dough, thus providing an explanation for the increased resistance to extension that occurs during dough mixing. The formation of interchain hydrogen bonds between glutamine residues may also account for the observations that the esterification of glutamine residues results in decreased resistance to extension, while mixing in the presence of deuterium oxide ($D_2O$) rather than water results in increased resistance (Beckwith *et al.* 1963; Mita & Matsumoto 1981; Bushuk 1998).

## 8. MANIPULATION OF HMM SUBUNIT COMPOSITION IN TRANSGENIC WHEAT

The major aim of determining the structures of the HMM subunits and their role in gluten and dough elasticity is to facilitate the improvement of the end use

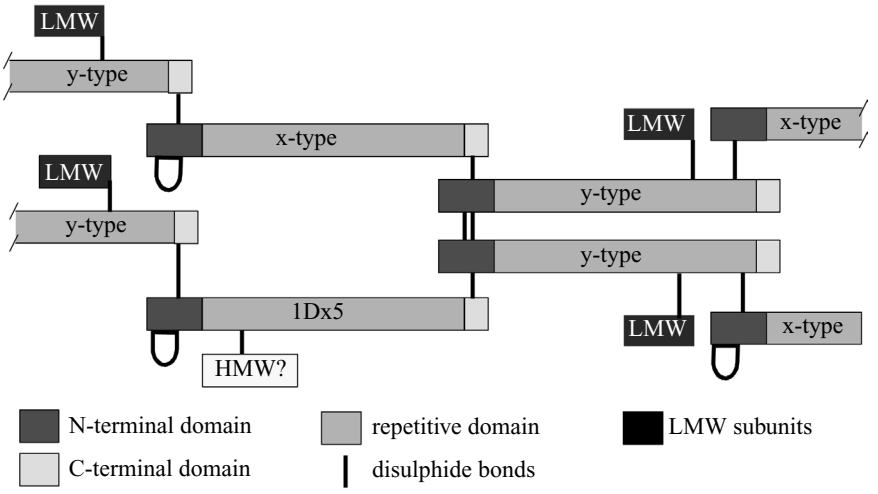


Figure 5. Schematic model of the structure of HMM subunit polymers, based on mapped disulphide bonds (Köhler *et al.* 1991, 1993, 1994; Tao *et al.* 1992; Keck *et al.* 1995)

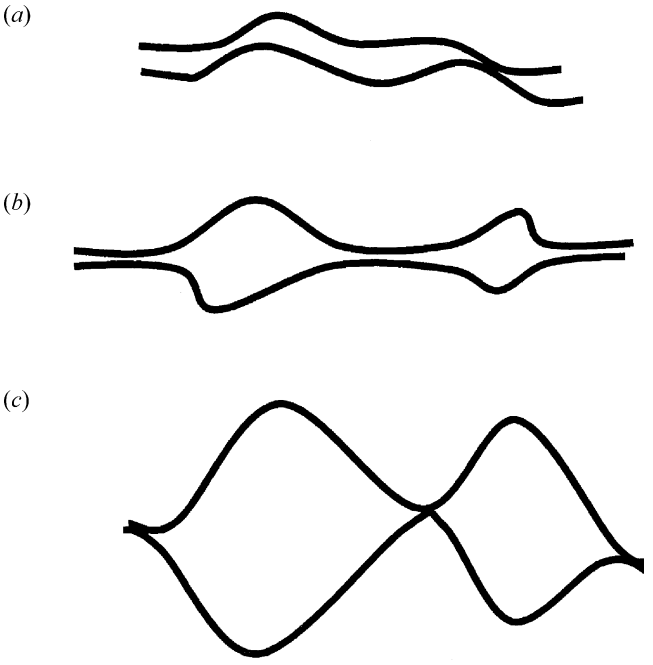


Figure 6. Model for the effect of hydration on the loop to train ratio of HMM subunits. (*a*) Low hydration, disordered, close interactions; (*b*) intermediate hydration, low loop to train ratio; (*c*) high hydration, high loop to train ratio.

properties of wheat. Substantial improvement in the processing performance of wheat has already been achieved by a combination of classical plant breeding and optimization of the agronomic and processing conditions. However, it is unlikely that these approaches will be sufficient in the long term and genetic engineering therefore provides an important additional approach. We are, therefore, using genetic engineering of wheat in order to further study the role of the HMM subunits in determining processing properties and to define strategies for the production of improved germplasm for incorporation into plant breeding programmes.

Most of our work, to date, has focused on transformation of two 'model' lines of wheat with two different HMM subunit genes. The model lines form part of a near isogenic series, which have been produced by crossing lines differing in their expression of HMM subunit genes. Thus, line L88-31 expresses only two HMM subunit genes (encoding subunits 1Bx17 and 1By18), while L88-6 also expresses genes encoding subunits 1Ax, 1Dx5 and 1Dy10 (Lawrence *et al.* 1988). The two genes used for transformation encode subunits 1Ax1 and 1Dx5, the latter always occurring as part of an 'allelic pair' with subunit 1Dy10. The three transgenic lines that have been studied in detail express the 1Ax1 subunit in L88-31 at a level of about 5.7% of the total protein (compared with 0% in the control line) and the 1Dx5 subunit in L88-31 and L88-6 at 8.7% (compared with 0%) and 17% (compared with 4.2%) of the total protein, respectively (Barro *et al.* 1997; Popineau *et al.* 2001).

The effects of the transgenes on dough strength were determined using a Mixograph. This measures the energy input during the mixing of dough and is routinely used for quality testing in a number of countries. When dough is mixed the resistance increases up to a certain level, after which it decreases. The increase in resistance may result from limited exchange of disulphide bonds (see § 2) and formation of the most stable patterns of hydrogen bonding (i.e. to form extensive 'train' regions). In contrast, the subsequent decrease in resistance is thought to result from disruption of these interactions by overmixing. Consequently, beneficial effects of the transgenes on dough strength and stability should be observed as increases in the PR (i.e. the maximum resistance that is observed) and the MT (i.e. the time taken to mix to PR) and a decrease in RBD (i.e. the rate of decrease in the resistance on overmixing beyond PR).

The results obtained with expression of the two transgenes in the L88-31 background are summarized in figure 7 (Popineau *et al.* 2001). The control line has low dough strength, which is consistent with the expression of only two endogenous HMM subunit genes, and the expression of the 1Ax1 transgene results in substantial increases in PR and MT. In contrast, expression of the 1Dx5 transgene in the same line was clearly detrimental to the mixing properties. An even more extreme effect was observed

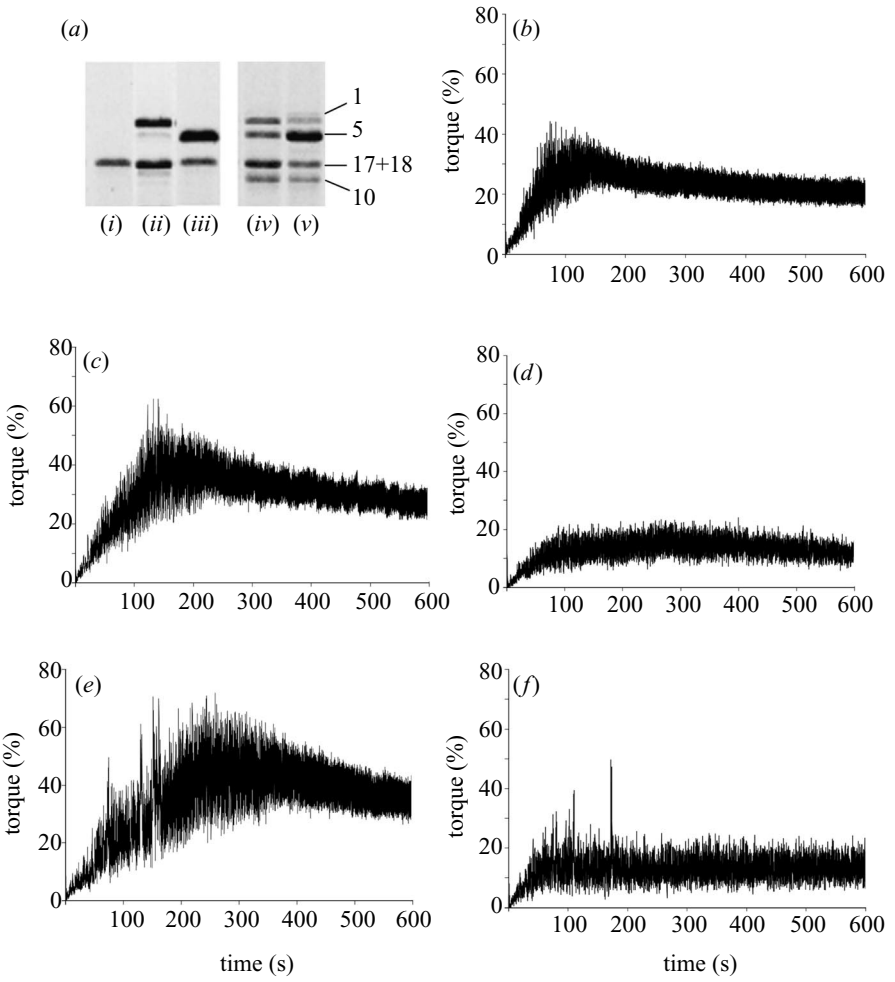


Figure 7. Analysis of the mixing properties of transgenic wheats expressing additional HMM subunits using the 2g Mixograph. (*a*) SDS–PAGE of the HMM subunits from (*i*), control line L88-31 (comigrating subunits 1Bx17 + 1By18); (*ii*), L88-31 expressing the 1Ax1 transgene; (*iii*), L88-31 expressing the 1Dx5 transgene; (*iv*), control line L88-6 (subunits 1Ax1, 1Dx5 + 1Dy10, 1Bx17 + 1By18); (*v*), L88-6 expressing the 1Dx5 transgene. (*b–f*) Mixographs of (*b*), L88-31; (*c*), L88-31 expressing the 1Ax1 transgene; (*d*), L88-31 expressing the 1Dx5 transgene; (*e*), L88-6; (*f*), L88-6 expressing the 1Dx5 transgene. The resistance is given as torque (%) and the MT in seconds (s). Taken from Popineau *et al.* (2001), with permission.

when the subunit 1Dx5 transgene was expressed in the L88-6 line which had much stronger mixing properties (figure 7*f*). In fact, both lines expressing the 1Dx5 transgene failed to absorb water and form a normal dough in the mixing bowl.

Rheological studies were also carried out on gluten fractions from the transgenic lines, showing that the expression of subunit 1Dx5 resulted in large increases in elasticity (measured as the storage and loss moduli, $G'$ and $G''$, and the viscoelastic plateau, $G_N^0$) while only a small increase was associated with expression of subunit 1Ax1. In fact, the effect of subunit 1Dx5 was similar to that previously observed when gluten was modified by treatment with transglutaminase to introduce interchain lysyl–glutamyl cross-links (Popineau *et al.* 2001).

The expression of the subunit 1Dx5 transgene was also associated with an increase in the amounts of glutenin subunits that were only extracted from flour by sonication with detergent (2% sodium dodecylsulphate) in the presence of reducing agent (1% dithiothreitol) – from 2–3% of the total flour proteins in the control lines to over 18% in the L88-31 transgenic line and almost 30% in the L88-6 transgenic line. These subunits can be assumed to be present in insoluble glutenin polymers. In contrast, expression of the 1Ax1 transgene was associated with a modest increase in the amount of subunits present in polymers that were extracted by sonication in the absence of reducing agent, but had no effect on the amount of subunits present in insoluble polymers.

These results suggest that the proteins encoded by the two transgenes had fundamentally different effects on the structure of the glutenin polymers in the two lines, with the 1Dx5 protein leading to the formation of highly cross-linked polymers that resulted in high gluten strength, unusual hydration behaviour and failure to form a homogeneous network during mixing. In contrast, the expression of subunit 1Ax1 resulted in similar effects on gluten composition and properties to those observed when comparing near-isogenic lines differing in HMM subunit composition.

As discussed in § 4, the 1Dx5 subunit protein differs from other characterized subunits in the presence of an additional cysteine residue within the repetitive domain and this may be responsible for the formation of highly

cross-linked polymers in the transgenic lines. However, it must also be borne in mind that, in non-transgenic wheat, subunit 1Dx5 is always found together with 1Dy10 and that dimers of these subunits are released by partial reduction of glutenin. Consequently, a precise molar balance of these two subunits may be required to give a 'normal' glutenin polymer structure.

These results demonstrate, therefore, that transformation of bread wheat with different HMW subunit genes may have fundamentally different effects on gluten structure and properties, which may relate to the expression levels, structures and interactions of the individual proteins.

I. A. C. R. and I. F. R. receive grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom.

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