THE HIGH MOLECULAR WEIGHT SUBUNITS OF WHEAT GLUTENIN AND THEIR ROLE IN DETERMINING WHEAT PROCESSING PROPERTIES

PETER R. SHEWRY, NIGEL G. HALFORD AND ARTHUR S. TATHAM

IACR-Long Ashton Research Station
Department of Agricultural Sciences, University of Bristol
Long Ashton, Bristol BS41 9AF, UK

YVES POPINEAU

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

DOMENICO LAFIANDRA

Dipartimento di Agrobiologia ed Agrochimica
Università degli Studi della Tuscia
Via San Camillo de Lellis, Viterbo 01100, Italy

PETER S. BELTON

School of Chemical Sciences
University of East Anglia, Norwich NR4 7TJ, UK

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I. INTRODUCTION

The mature wheat grain contains about 9–15% protein, approximately half of which is storage proteins deposited in the starchy endosperm cells. These cells are separated from the outer layers (including aleurone) and embryo during milling to give white flour. When the white flour is mixed with water and kneaded to form dough the storage proteins form a continuous network, called gluten, which confers unique mechanical properties that underpin the utilization of wheat in many food systems. These properties are a combination of elasticity and viscosity, and the precise balance between these properties (dough strength) is important in determining the end use. In particular, relatively strong (i.e. highly elastic) doughs are required to make pan breads and weaker doughs to make flat breads, noodles, cakes and biscuits.

Wheat gluten can be readily prepared by washing dough with water, giving a cohesive mass comprising about 80% protein, 10% starch, 5%

lipid and 5% other components (minerals, fibre, etc.) on a dry weight basis. This has facilitated studies of the composition and biophysical properties of gluten but it should not be forgotten that these properties are also likely to be affected by interactions with other components in dough.

Because of their importance in wheat processing, the structures and properties of the wheat gluten proteins have been studied for many years, starting with the first description of the isolation of gluten by Beccari in 1745 (Beccari, 1745). Subsequent studies showed that gluten proteins could be separated into two fractions, which were either soluble or insoluble in alcohol (Taddei, 1819), and this division, with some modifications, has remained in use to the present day, with the gluten proteins that are readily soluble in alcohol–water mixtures (e.g. 60–70% ethanol) being called gliadins and those that are insoluble being called glutenins. These fractions are also important as they have functional significance, with the glutenins being mainly responsible for gluten and dough viscosty and elasticity and the gliadins for plasticity and extensibility (as discussed below). However, we now know that the two fractions contain proteins that are structurally related, with the differences in solubility resulting from their presence as monomers that interact by noncovalent forces (gliadins), or as high molecular mass polymers stabilized by inter-chain disulphide bonds. When present as reduced subunits the glutenin proteins are also soluble in alcohol–water mixtures and can therefore be defined together with the gliadins as prolamins. The ratio of gliadin to glutenin proteins in dough and gluten is generally about 1 : 1, although this ratio may vary with genotype and growth conditions with resulting effects on dough strength (Doekes and Wennekes, 1982; Graybosch *et al.*, 1995; Vereijken *et al.*, 2000; Johansson *et al.*, 2001).

A. OVERVIEW OF WHEAT GLUTEN PROTEINS

The gliadins and glutenins are not single homogeneous proteins but complex mixtures that can be separated using various electrophoretic and other procedures. In fact, the true extent of the complexity has never been conclusively established as it is difficult to determine the correspondence of components separated by different procedures or whether apparently single components actually contain two or more closely related proteins. However, it is generally accepted that at least 50 different gluten proteins are present in hexaploid bread wheat, and that only partial separation can be achieved by any single procedure, even by two-dimensional systems such as that shown in Figure 1. A high level of complexity is supported by the analysis of gluten protein genes, although the existence of pseudogenes (i.e. nonexpressed genes) makes it difficult to establish precise gene copy numbers (see, for example, Anderson and Greene, 1997).

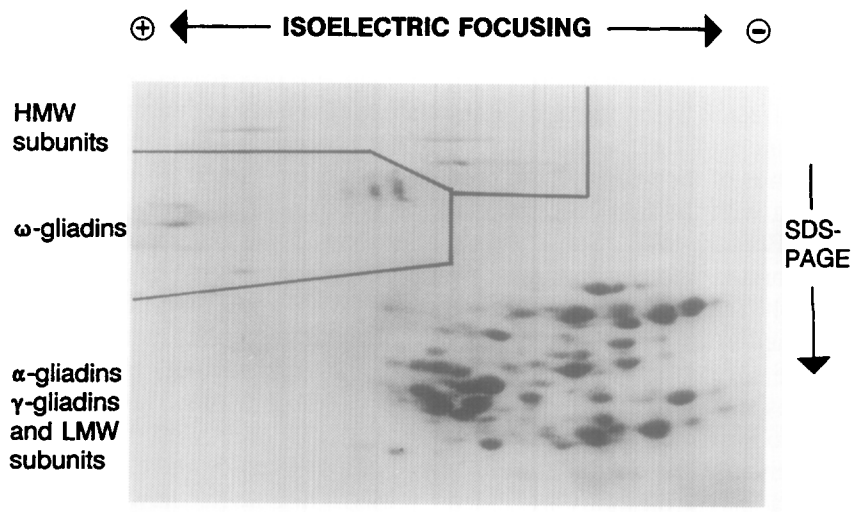


FIG. 1. The polymorphism of wheat gluten proteins (c.v. Chinese Spring) demonstrated by two-dimensional electrophoresis (isoelectric focusing followed by SDS-PAGE). The groups of HMW prolamins (HMW subunits), S-poor prolamins (ω-gliadins) and S-rich prolamins (α-gliadins, γ-gliadins and LMW subunits) are indicated. Taken from Shewry *et al.* (1987) with permission.

The gliadin proteins are traditionally separated into four groups, called (α, β- γ- and ω-gliadins, based on their mobilities when separated by electrophoresis at low pH (ω-gliadins being slowest) (Figure 2). We now know that α- and β-gliadins are closely related in sequence and structure and it is usual to refer to both as α-type gliadins, as opposed to the γ-type gliadins which are more distantly related. Furthermore, the α-type and γ-type gliadins are often classified together as sulphur-rich (S-rich) prolamins, while the ω-gliadins form a separate S-poor group.

The glutenin polymers can be separated on the basis of their size, as discussed below. However, in order to separate their component subunits it is necessary to first reduce the inter-chain disulphide bonds. Once this is done the subunits can be separated by electrophoresis in the presence of the detergent sodium dodecylsulphate (SDS) into two broad groups of subunits called high molecular weight (HMW) and low molecular weight (LMW) subunits, as shown in Figure 2.

The low molecular weight subunits can be further classified into three groups: the D-type subunits, which appear to be related to ω-gliadins; the C-type, which comprise components related to α-type and γ-type gliadins, and the B-type, which form a discrete group of S-rich prolamins. The

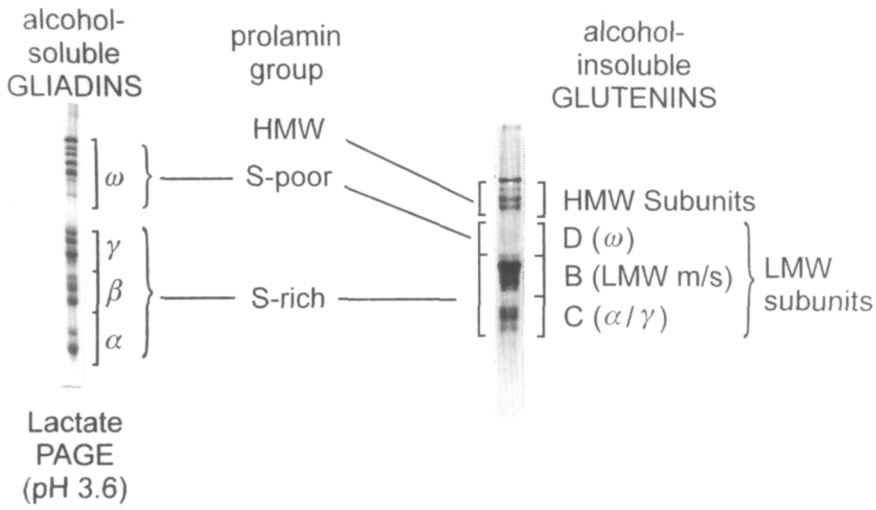


FIG. 2. The groups of gliadins and glutenin subunits separated by lactate-PAGE and SDS-PAGE, respectively. Taken from Shewry *et al.* (1999), with permission.

HMW subunits are not closely related to any other gluten proteins and form a distinct group called the HMW prolamins. This classification is summarized in Figure 3.

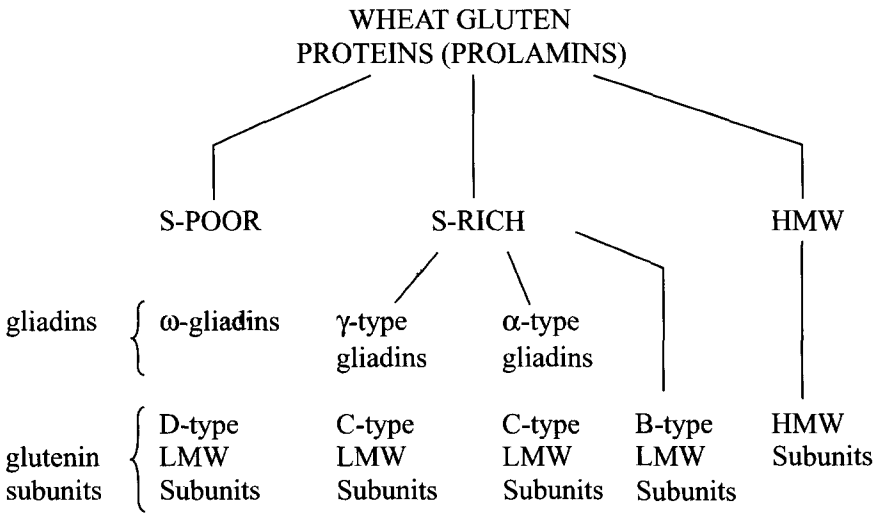


FIG. 3. The classification and nomenclature of wheat gluten proteins.

Although the HMW subunits were only identified and defined as a group about 20 years ago, they have since become the most widely and intensively studied group of gluten proteins. This is because of work started in the late 1970s which demonstrated correlations between HMW subunit composition and grain quality. However, before discussing these correlations in detail it is first necessary to discuss the polymorphism and genetic control of the HMW subunits.

II. THE HMW SUBUNITS OF GLUTENIN

A. GENETICS AND POLYMORPHISM OF HMW SUBUNITS

The use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for the analysis of the subunits resulting from the reduction of glutenin polymers allowed the determination of their genetic control. Wheat genetic stocks, such as the nulli-tetrasomic and ditelocentric lines analysed by Bietz *et al.* (1975), gave the first clear evidence for the chromosomal location of glutenin subunit genes in the bread wheat cv. Chinese Spring. Subsequently, the introduction of the discontinous SDS-PAGE system of Laemmli (1970) and two-dimensional electrophoretic separations have given more detailed information on the genetics of HMW glutenin subunits and the extent of their polymorphism in different bread wheat cultivars (Lawrence and Shepherd, 1980; Holt *et al.*, 1981; Payne and Lawrence, 1983). In particular, it is now firmly established that genes controlling the synthesis of HMW subunits are located on the long arms of the homoeologous group 1 chromosomes of hexaploid bread wheat at loci designated *Glu-A1*, *Glu-B1* and *Glu-D1*, with each *Glu-1* locus containing two tightly linked genes encoding subunits of high and low M_r, which are termed x- and y-type, respectively. These gene loci have also been mapped but contrasting results have been obtained (Payne *et al.*, 1981b; Singh and Shepherd, 1988). DNA sequencing of the genes corresponding to HMW glutenin subunits has revealed the structural characteristics of the corresponding subunits, including the presence of a large repetitive central domain that may provide a basis for major and rapid structural changes in the *Glu-1* genes by duplication and/or deletion of large segments as a result of unequal crossing over (Shewry *et al.*, 1989). These processes, together with the accumulation of small insertions, deletions or point mutations, have resulted in the existence of large numbers of allelic forms of subunits encoded by each locus, as detected by current electrophoretic techniques (Figure 4). Based on electrophoretic analyses of about 300 wheat varieties, Payne and

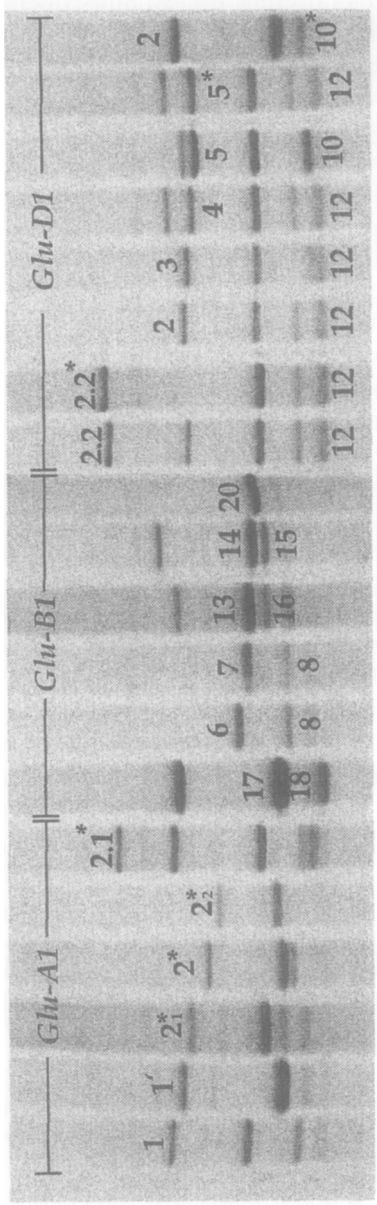


FIG. 4. SDS-PAGE of HMW subunits from a range of genotypes of wheat showing allelic variation in the mobilities of proteins encoded by the *Glu-A1*, *Glu-B1* and *Glu-D1* loci. The numbering is according to Payne and Lawrence (1983), with subsequent modifications by other workers.

Lawrence (1983) identified three alleles at the *Glu-A1* locus, eleven at the *Glu-B1* locus and five at the *Glu-D1* locus, and proposed a numbering system to designate the different alleles. Subsequent analyses of cultivars from different countries and of wheat collections stored in gene banks has resulted in a continued increase in the number of alleles detected at each of the three loci (Figure 4).

The detection of genes at the *Glu-D1* and *Glu-A1* loci encoding rare subunits with unusual high molecular mass (see for example lanes 6, 13 and 14 of Figure 4) and their characterization by polymerase chain reaction (PCR) have provided further evidence for the role of the sequence encoding the repetitive central domain in the molecular evolution of glutenin subunit genes (D'Ovidio *et al.*, 1994; Tahir *et al.*, 1996). Despite the fact that bread wheat possesses six HMW subunit genes, the number of expressed subunits ranges from three to five because of gene silencing processes that have occurred during its evolutionary history. In particular, the y-type gene present at the *Glu-A1* locus is always silent in cultivated wheat, while the x-type gene at the same locus and the y-type gene at the *Glu-B1* locus are expressed only in some cultivars. In contrast, the y-type subunit encoded by the *Glu-A1* locus is expressed in cultivated and wild diploid wheats (*Triticum monococcum* ssp. *monococcum* and ssp. *boeoticum*, *T. urartu*), in the wild tetraploid wheat *T. turgidum* ssp. *dicoccoides* (Waines and Payne 1987; Levy *et al.*, 1988) and in cultivated and wild forms of tetraploid wheats with the genomic formula AAGG (Margiotta *et al.*, 1998).

Studies of variation in HMW glutenin subunits present in old cultivars or landraces have also resulted in the identification of unusual allelic variants or mutant types characterized by the absence of subunits that are normally present in current bread wheat cultivars. For example, the absence of both HMW glutenin subunits encoded by chromosome 1D was reported by Bietz *et al.* (1975) in seeds of the landrace Nap Hal, while lines lacking either x- or y-type subunits encoded by the *Glu-D1* or *Glu-B1* loci have also been identified (Payne *et al.*, 1984; Lafiandra *et al.*, 1988). This type of material is being used for the development of genetic stocks suitable for elucidating the composition–functionality relationships, as discussed in a later section.

B. CORRELATIONS BETWEEN HMW SUBUNIT COMPOSITION AND GRAIN PROCESSING QUALITY

The first direct correlation between HMW subunit composition and grain processing quality was reported by Payne *et al.* (1979), who showed a correlation between the presence of HMW subunit 1Ax and quality

(measured using the indirect SDS sedimentation test) in the progeny of a cross between wheat cultivars of good (Maris Wigeon) and poor (Maris Ranger) breadmaking quality. Further studies showed a similar correlation with the subunit pair 1Dx5 + 1Dy10 (Payne *et al.*, 1981a). Although Payne and coworkers used an indirect test for breadmaking quality, an association of subunits 1Dx5 + 1Dy10 and 1Ax2* with loaf volume was subsequently reported by Moonen *et al.* (1982, 1983), while Burnouf and Bouriquet (1980) demonstrated that subunits 1Dx5 and 1Bx7 were present in cultivars with good breadmaking quality and high gluten strength. The association of specific subunits or pairs of subunits with good or poor breadmaking quality (i.e. high or low gluten strength) has since been confirmed by many studies carried out using a wide range of germplasm and by workers in many countries (as reviewed by Payne, 1987 and Shewry *et al.*, 1989, 1992), with general agreement on the following:

1. Subunits encoded by all three genomes (A, B, D) may be associated with quality.
2. The subunit pair 1Dx5 + 1Dy10 encoded by chromosome 1D is associated with the highest quality, whereas the allelic pairs 1Dx2 + 1Dy12, 1Dx3 + 1Dy12 and 1Dx5 + 1Dy12 are all associated with poor quality.
3. The presence of an x-type subunit encoded by chromosome 1A (1Ax1 or 1Ax2*) is superior to the null (i.e. silent) allele.
4. The subunit pair 1Bx17 + 1By18 is generally superior to other alleles encoded by chromosome 1B.

Furthermore, the combination of information from a wide range of studies has allowed "quality scores" to be assigned to individual subunits or subunit pairs (Payne *et al.*, 1987b; Branlard *et al.*, 1992), as shown in Table I.

TABLE I

QUALITY SCORES ASSIGNED TO INDIVIDUAL HMW SUBUNITS OR SUBUNIT PAIRS

	Locus		
Score	*Glu*-A1	*Glu*-B1	*Glu*-D1
4	–	–	5 + 10
3	1	17 + 18	–
3	2*	7 + 8	–
2	–	7 + 9	2 + 12
2	–	–	3 + 12
1	null	7	4 + 12
1	–	6 + 8	–

Taken from Payne *et al.* (1987b) with permission.

Quantitative analyses have shown that the HMW subunits account for up to about 12% of the total grain proteins, corresponding to about 1–1.7% of the flour dry weight (Seilmeier *et al.*, 1991; Halford *et al.*, 1992; Nicolas, 1997). Nevertheless, they account for between about 45 and 70% of the variation in breadmaking performance within European wheats (Branlard and Dardevet, 1985, Payne *et al.*, 1987b, 1988a). Consequently, the quality scores assigned to the HMW subunits can be exploited to select for breadmaking performance in breeding programmes.

III. THE SEQUENCES AND STRUCTURES OF HMW SUBUNITS

Although early studies resulted in the direct determination of the *N*-terminal amino acid sequences of a number of HMW subunits purified from wheat grain (Field *et al.*, 1982; Shewry *et al.*, 1984), the determination of full sequences resulted only from the isolation and sequencing of the corresponding genes. As a result we now know the sequences of nine HMW subunit proteins from bread wheat, including forms encoded by all three genomes (see Table II), and of homeologues from related cultivated (*T. timopheevi*) and wild (*T. tauschii*, *Aegilops cylindrica*) species (Mackie *et al.*, 1996; Wan *et al.*, 2002).

A. AMINO ACID SEQUENCES

The subunits which occur most widely in bread wheat comprise between 627 (1Dy10) and 827 (1Dx5) amino acid residues, with M_r ranging from 67 476 to 88 128 (Table II). Furthermore, their amino acid sequences can be divided into three distinct parts, or domains, with the central domains consisting of highly repeated blocks of amino acids and ranging in length from 481 to 696 residues (Figure 5).

The availability of complete amino acid sequences of HMW subunit proteins from cultivated wheat and related wild species of grasses allows a detailed comparison of their repeat motifs to be made. Because there are no apparent differences between the sequences of proteins from modern bread wheat, ancient cultivated wheats (*T. timopheevi*) and wild related species (*A. cylindrica*, *T. tauschii*), data from these species are combined in Table III and in the following discussion.

A major difference between x-type and y-type subunits is that the former contain tripeptide, hexapeptide and nonapeptide motifs (Table III), whereas the y-type subunits contain only hexapeptides and nonapeptides (Table III). The tripeptides in x-type subunits always occur in tandem with hexapeptides, forming essentially a nine-residue repeat motif (see Figure 5).

TABLE II

SUMMARY OF THE TOTAL NUMBER OF RESIDUES (RES), NUMBER OF CYSTEINE RESIDUES (CYS) AND NUMBER OF TRI-, HEXA- AND NONAPEPTIDE REPEAT UNITS (TRI, HEXA, NONA, RESPECTIVELY) IN EIGHT HMW SUBUNITS OF GLUTENIN FROM BREAD WHEAT AND THEIR THREE STRUCTURAL DOMAINS

			N-terminal domain		Repetitive domain					*C*-terminal domain		Whole protein	
Subunit	Cultivar	M_r	Res	Cys	Res	Cys	Tri	Hexa	Nona[a]	Res	Cys	Res	Cys
1Ax1	Hope	87680	86	3	681	0	15	65	23	42	1	809	4
1Ax2*	Cheyenne	86309	86	3	666	0	16	67	23	42	1	794	4
1Bx7	Cheyenne	82865	81	3	647	0	4	66	25	42	1	770	4
1Bx17	L88-69	80750	81	3	611	0	4	64	23	42	1	734	4
1By9	Cheyenne	73518	104	5	538	1	0	56	22	42	1	684	7
1Dx2	Yamhill	87000	88	3	687	0	20	73	21	42	1	817	4
1Dx5	Cheyenne	88137	89	3	696	1	23	73	21	42	1	827	5
1Dy10	Cheyenne	67495	104	5	481	1	0	47	21	42	1	627	7
1Dy12	Chinese Spring	68696	104	5	493	1	0	49	21	42	1	639	7

[a]Includes two long (11/12 residue) degenerate repeats.

Based on sequences reported in Halford *et al.* (1987, 1992); Anderson and Greene (1989); Anderson *et al.* (1989); Thompson *et al.* (1985); Sugiyama *et al.* (1985); Reddy and Appels (1993).

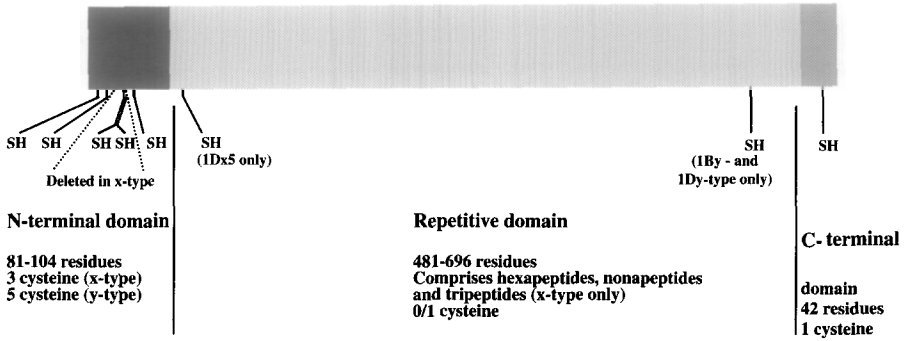


FIG. 5. Schematic summary of the sequences of x-type and y-type HMW subunits.

Similarly, in both types of subunit the hexapeptides occur either in tandem arrays or interspersed with nonapeptides, the latter forming a 15-residue repeat (Figure 6). Unlike the hexapeptides, the tripeptides and nonapeptides never occur in tandem arrays.

The hexapeptides have the same consensus motif in x-type and y-type subunits (Pro.Gly.Gln.Gly.Gln.Gln.), but subtle differences are apparent in the frequencies of substitutions at different positions. Notably, the replacement of Pro with Ser at position 1 is much more common in x-type subunits, as is the replacement of Gln with Pro at position 6. However, the latter only occurs in hexapeptides within a 15-residue (i.e. 6 + 9) motif, not in tandemly arranged hexapeptides. Most, but not all, of the 15 amino acid repeats in x-type subunits have Pro in this position.

Greater differences are present between the nonapeptides in the x-type and y-type subunits. Replacement of Tyr with His at position 2 and of Thr with Ala at position 5 are both common in y-type subunits but rare or absent in x-type. These two substitutions also usually occur together, resulting in two consensus sequences for y-type nonapeptides: Gly.Tyr.Tyr.Pro.Thr.Ser.Leu.Gln.Gln. and Gly.His.Tyr.Pro.Ala.Ser.Leu.Gln.Gln. The consensus motif of the x-type subunits also differs in having Pro in place of Leu at position 7.

The data in Table III also show clear differences in the frequency of amino acid substitutions at different position in the motifs. In particular, Gln is highly conserved wherever it occurs (positions 3, 5 and 6 of the nonapeptide and 2 and 3 of the tripeptide), with the exception of the substitution with Pro at position 6 of hexapeptides present in 15-residue repeats of x-type subunits, as discussed above. These conserved glutamine residues can, therefore, be regarded as forming a glutamine backbone to the repetitive domains.

TABLE III

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 (*Ae. CYLINDRICA*). (B) THE 339 HEXAPEPTIDE AND 123 NONAPEPTIDE REPEAT MOTIFS OF y-TYPE HMW SUBUNITS 1Ay (NOT EXPRESSED), 1By9 AND 1Dy10 (*T. AESTIVUM*); 1Ay (*T. TIMOPHEEVI*); 1Cy AND 1Dy (*Ae. CYLINDRICA*); 1Dy (*T. TAUSCHII*)

	Hexapeptides (%)						Tripeptides (%)		
A	1	2	3	4	5	6	1	2	3
	Pro 62	Gly 84	Gln 99	Gly 75	Gln 94	Gln 80	Gly 89	Gln 99	Gln 99
	Ser 26	Ala 7	Other 1	Trp 9	Leu 3	Pro 15	Asp 5	Arg 1	Arg 1
	Leu 10	Glu 4		Leu 7	Other 3	Ser 2	Ala 2		
	Ile 1	Arg 3		Glu 4		Arg 1	Arg 2		
	Other 2	Thr 2		Arg 2		Leu 1	His 1		
				Ala 1		Other 2			
				Other 1					

	Nonapeptides (%)								
	1	2	3	4	5	6	7	8	9
	Gly 84	Tyr 98	Tyr 97	Pro 90	Thr 96	Ser 100	Pro 70	Gln 88	Gln 94
	Arg 6	His 2	Asp 2	Leu 8	Ile 4		Ser 13	Leu 8	Leu 4
	Glu 3		Phe 1	Ser 2			Leu 11	Trp 3	Glu 2
	Trp 3						Ala 2	Arg 1	
	Val 2						Glu 2		
	Ala 1						--- 2		
	Lys 1						Arg 1		

	Hexapeptides (%)					
B	1	2	3	4	5	6
	Pro 65	Gly 92	Gln 96	Gly 76	Gln 94	Gln 94
	Ser 12	Glu 6	Lys 4	Glu 7	His 2	Glu 2
	Leu 10	Lys 2		Trp 7	Lys 2	His 2
	Ile 7	Other 1		Arg 4	Other 1	--- 2
	Thr 4			Ala 2		
	Gln 1			Val 2		
	Other 1			Lys 1		
				Other 2		

	Nonapeptides (%)								
	1	2	3	4	5	6	7	8	9
	Gly 96	Tyr 54	Tyr 85	Pro 91	Thr 60	Ser 97	Leu 54	Gln 97	Gln 90
	Trp 2	His 41	Cys 4	Leu 5	Ala 37	Tyr 2	Pro 21	His 3	His 7
	Arg 1	Gln 5	Asp 2	Arg 2	Ser 2	Phe 1	Gln 19		Glu 2
	Tyr 1		His 2	Ser 1	Ile 1		Val 4		*Stop* 1
			Ile 2	Thr 1			Gly 2		
			Phe 2				Ser 2		
			Arg 1				Ala 1		
			Asn 1						
			Glu 1						

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.

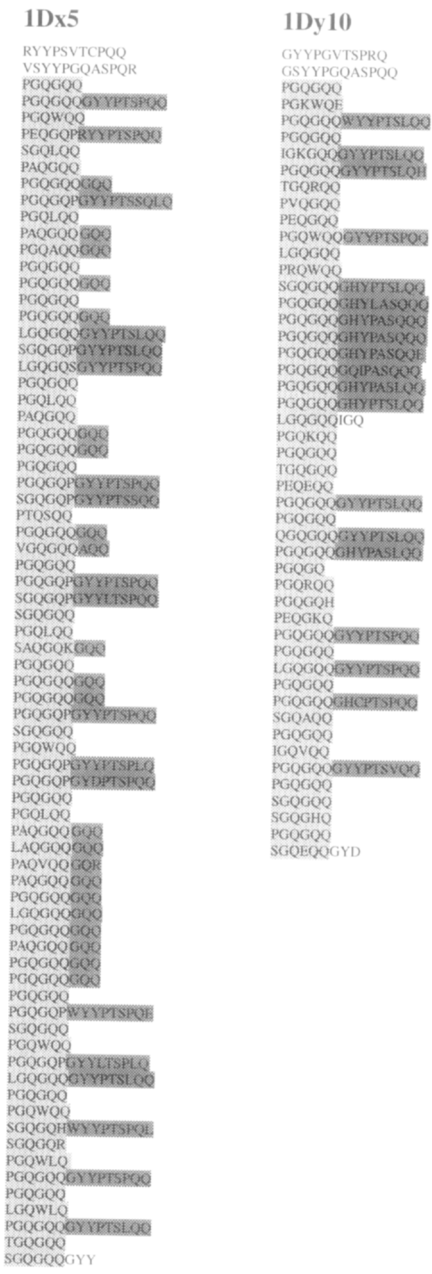


FIG. 6. Amino acid sequences of the repetitive domains of typical x-type (1Dx5) and y-type (1Dy10) HMW subunits arranged to show their repeat unit structures.

The x-type subunits are also highly conserved at positions 2, 3, 5, 6 and 9 of the nonapeptide, with the y-type subunits being more variable at these positions. Similarly, Ser is always present at position 6 of the x-type nonapeptides and is highly conserved (97%) in the y-type motifs.

However, comparison of the properties of the amino acids that occur at specific positions provides no evidence for conservative substitutions (i.e. replacement with an amino acid with similar properties). Instead, the most common replacements can be accounted for by single base mutations in the DNA codons, with substitutions requiring two mutations occurring more rarely. For example, 55% of the x-type hexapaptides have Pro (codon CCA) at position 1, with 12% containing Leu (CTA), 30% Ser (TCA) but only 3% Ile (ATA). Nevertheless it is possible that at least some of the differences in the degree of conservation within motifs relate to the role of individual residues in determining protein structure.

A number of studies have been carried out to determine the structures of the HMW subunits and their domains. However, it must be borne in mind that most of these have been carried out on proteins or peptides in the solution state rather than as hydrated solid, which is the "natural" state in protein bodies in the developing grain and in gluten and dough.

B. SIZE AND SHAPE OF HMW SUBUNITS

Data from a range of studies indicate that the HMW subunits have an extended rod-like structure, both in solution and the hydrated solid state (Table IV). Field *et al.* (1987) used intrinsic viscosity measurements to calculate the dimensions of subunit 1Bx20 purified from pasta wheat, showing overall dimensions ranging from 49×1.8 nm in 50% (v/v) aqueous propan-1-ol to 62×1.5 nm in trifluroethanol. Small angle X-ray scattering (SAXS) has shown similar lengths but different diameters, of 56.7×8.0 and 78.6×6.3 nm for undefined subunits in 50% (v/v) aqueous propan-1-ol and 0.1 M acetic acid, respectively (Matsushima *et al.*, 1992), and of 69×6.4 nm for subunit 1Bx20 in 50% (v/v) aqueous propan-1-ol (Thomson *et al.*, 1999).

Scanning tunnelling microscopy (STM) of the hydrated solid protein showed aligned rods of diameter about 1.8 nm (Miles *et al.*, 1991). It can therefore be suggested that the diameters obtained by SAXS represent side-to-side aggregates rather than individual proteins. Atomic force microscopy (AFM) of subunit 1Dx5 deposited onto mica or graphite substrate has indeed shown such filaments, with a diameter of about 20 nm (Figure 7). An M_r 58 000 peptide derived from the central repetitive domain of subunit 1Dx5 also formed filamentous structures with diameters about 20 nm, with connection points every 45–50 nm (Humphris *et al.*, 2000).

TABLE IV

COMPARISON OF THE MOLECULAR DIMENSIONS DETERMINED AND CALCULATED FOR HMW SUBUNIT MOLECULES

Method	Subunit	State	Dimensions (nm)		Reference
			Length	Diameter	
Viscometry	1Bx20	50% propan-1-ol	49	1.8	1
Viscometry	1Bx20	0.05 M acetic acid/ 0.01 M glycine	50	1.75	1
Viscometry	1Bx20	Trifluoroethanol	62	1.5	1
SAXS		0.01 M acetic acid	79	6.3	2
SAXS		50% propan-1-ol	57	8.0	2
SAXS	1Bx20	50% propan-1-ol	69	6.4	3
STM	1Bx20	hydrated solid	–	1.8	4
Modelling					
Model 1	650[a] residues	modelled in solvent sheath	53.5	1.7	5
Model 2			37.7	2.0	5
Model 3			54.5	2.1	5

[a]Modelled as repetitive domain comprising 650 residues of a 9 + 6 + 9 residue motif. References: 1, Field *et al.* (1987); 2, Matsushima *et al.*, 1992; 3, Thomson *et al.* (1999); 4, Miles *et al.* (1991); 5, Parchment *et al.* (2001).

C. STRUCTURES OF HMW SUBUNIT TERMINAL DOMAINS

The *N*- and *C*-terminal domains of the HMW subunits have proved to be difficult to study in isolation from the central repetitive domain. Early structure predictions indicated that these domains were essentially globular in structure, being rich in α-helix (Tatham *et al.*, 1984, 1985). More recently, van Dijk *et al.* (1998) have predicted that residues 1–33 of subunit 1Dx5 (and the corresponding regions of subunits 1Ax1, 1Ax2* and 1Bx7) form α-helices while residues 40–48, 52–59, 64–67 and 75–84 form β-sheet.

This is supported by more detailed prediction and modelling studies reported by Köhler *et al.* (1997). They proposed that residues 5–32 of subunit 1Dx5 form a continuous α-helix, as shown in Figure 8. In contrast, several short sections of α-helix are predicted for subunit 1Bx7 (residues 6–13, 16–20, 24–26), with an inverse β-turn between residues 14 and 16 allowing the formation of an intra-chain disulphide bond between the cysteine residues at positions 10 and 17 (Figure 8) (see below for a discussion of disulphide bond formation).

It has not so far proved possible to isolate a peptide corresponding to the *N*-terminal domain of an authentic HMW subunit, but van Dijk *et al.* (1998) have reported the expression in *Escherichia coli* and

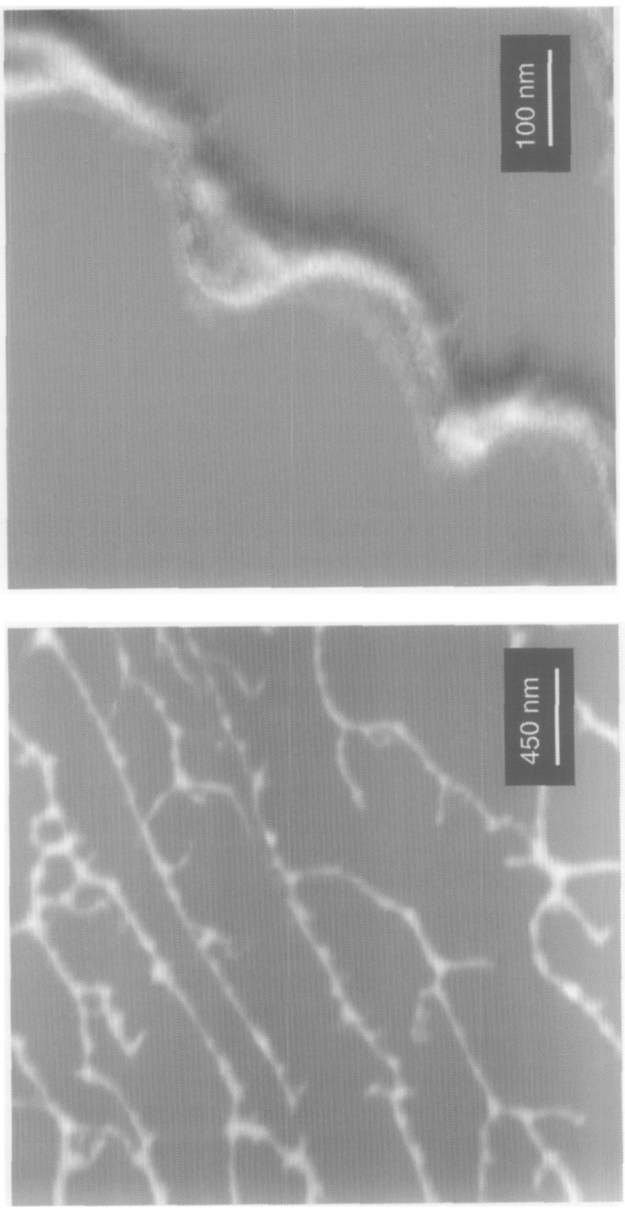


FIG. 7. Atomic force microscopy of reduced and alkylated subunit 1Dx5 deposited from 0.05 M acetic acid on a highly orientated pyrolytic graphite (HOPG) substrate shows network formation. Taken from Humphris *et al.* (2000), with permission.

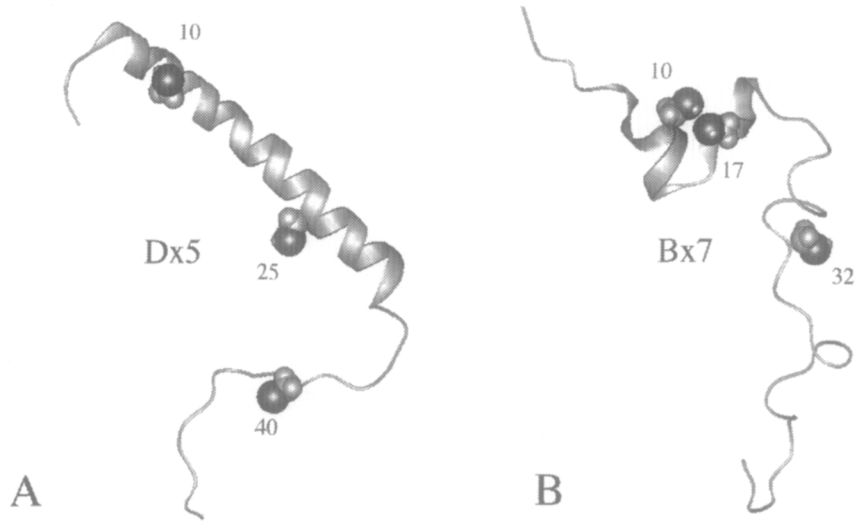


FIG. 8. Molecular models of the *N*-terminal domains of subunits 1Dx5 and 1Bx7, showing the backbone only in cartoon form. Sulphur atoms (10, 25 and 40 in 1Dx5; 10, 17 and 32 in 1Bx7) are shown in grey. Taken from Köhler *et al.* (1997), with permission.

characterization of a peptide corresponding to the *N*-terminal domain of subunit 1Dx5. The peptide was insoluble in water except in the presence of SDS and circular dichroism spectroscopy (in 0.1% SDS) indicated the presence of 26% α-helix and 33% β-sheet at pH 8.17 and 35% α-helix and 36% β-sheet at pH 3.59. The authors concluded that the insolubility of the *N*-terminal domain was responsible for the solubility properties of the whole subunits. However, the expressed peptide differed from the native *N*-terminal domain in the presence of a 21-residue signal sequence and it is possible that this hydrophobic sequence may have affected the structure and properties of the peptide, including the solubility.

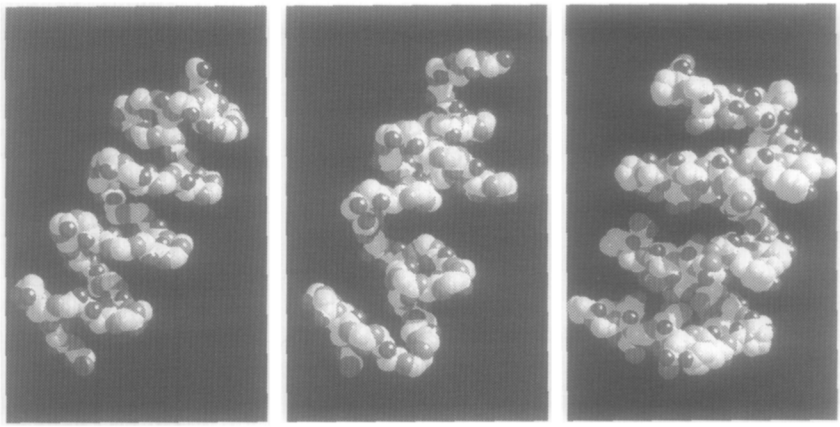
Tatham *et al.* (1984) also predicted that the short (42-residue) *C*-terminal domains of the HMW subunits were α-helical in structure. Bekkers *et al.* (1996) subsequently synthesized a peptide corresponding to the *C*-terminal domain of subunit 1Dx5 and showed that it was readily soluble in aqueous buffers over a wide pH range and adopted a random coil structure when dissolved in water. Nuclear magnetic resonance (NMR) spectroscopy in the "structure-inducing" solvent 40% (v/v) aqueous trifluoroethanol allowed a low-resolution structure to be determined which was "molten globule" - like, with α-helical regions formed by residues 5–20 and 26–32. It is not possible to conclude whether this structure is related to that adopted in the hydrated solid state (i.e. as in gluten) and it is also possible that the

structure of this domain, and of the *N*-terminal and central domains, is affected by interactions with other domains of the subunit or with other gluten proteins.

D. STRUCTURE OF HMW SUBUNIT REPETITIVE DOMAINS

The repetitive domains form the largest part of the HMW subunits, ranging from 481 to 696 residues. Initial studies using secondary structure prediction (Tatham *et al.*, 1984, 1985), circular dichroism (CD) and infrared (IR) spectroscopies of the whole protein (Tatham *et al.*, 1985) and synthetic peptides based on the repeat motifs (Tatham *et al.*, 1990) indicated β-turns as the dominant structural feature. β-Turns were predicted over residues QPGQ, QQGY, YPTS and SPQQ, and it was proposed that regular β-turn predictions led to the formation of a spiral structure, similar to the β-spiral described for a synthetic polypentapeptide based on the repeat motif present in the connective tissue protein elastin (Tatham *et al.*, 1985). Van Dijk *et al.* (1996a) used 2D-NMR, CD and IR of synthetic cyclic peptides to confirm the presence of β-turns: a type II β-turn at QPGQ, type I β-turns at YPTS and SPQQ and a type I or II β-turn at QQGY. They also reported *cis/trans* proline isomerism, with 50% of proline residues in the *cis* conformation in YPTS, and the other proline residues being more than 90% *trans*-conformation. Conversion of the *cis* form in YPTS to the *trans*-conformation destabilized the type I turn, but increased the stability of the β-turns at SPQQ and QQGY. Van Dijk *et al.* (1996b) also studied the solution structures of HMW subunits 1Bx6 and 1Bx7 and of an M_r 16 802 heterologously expressed peptide from the central repetitive domain of subunit 1Dx5. Using CD and IR, they concluded that the structure was compatible with β-turns stabilized by hydrogen bonds both within and between turns. More recently, variable temperature CD studies of an M_r 58 000 peptide derived from the central repetitive domain of subunit 1Dx5 indicated the presence of β-turn structures in equilibrium with a poly-L-proline II-like structure (an extended hydrated structure), with supporting evidence for this coming from IR studies (Gilbert *et al.*, 2000).

Matsushima *et al.* (1990) attempted to model the nonapeptide repeat motif of an x-type subunit (GYYPTSPQQ), but concluded that as the repeat contained two proline residues, there were many degrees of freedom and hence many models could be constructed. They modelled structures with two or three β-turns per repeat and calculated diameters of 1.4–1.6 nm, but reported no detailed structures. Kasarda and coworkers have developed models based on hexapeptide and nonapeptide repeats and a hexa-nonapeptide (15-mer) repeat, fitted to the hexapeptide template (Kasarda, 1994; Kasarda *et al.*, 1994). Using energy minimization and molecular



(A) (B) (C)

FIG. 9. Space filling models of backbone structures of hexapeptide + nonapeptide + hexapeptide repeat motifs (PGQGQQ GYYPTSPQQ PGQGQQ): (A) with type II β-turns at QPGQ, YPTS, SPQQ and QGQQ; (B) with type II β-turns at QGYY and QPGQ and type-I/III β-turns at SPQQ and YPTS; (C) with distance-based β-turns at QPGQ, YPTS and SPQQ, β-sheet at GQGQQGY. Taken from Parchment *et al.* (2001) with permission.

dynamics they reported that when type II β-turns were used a distorted spiral with a flat ribbon shape resulted, but that this was unstable and other turn types did not improve the stability. When inverse γ-turns (a three-residue turn) were modelled a more stable spiral structure resulted, with a diameter of about 2.4 nm and a pitch of about 1 nm. The stability of the spiral arose from hydrogen bonding of the glutamine side chain amide groups to the backbone amide groups and to other glutamine side chains. However, the model did not include interactions with water, which might break up some or all of the hydrogen bonding that was proposed (Kasarda *et al.*, 1994).

Parchment *et al.* (2001) used structure prediction and molecular dynamics to generate three alternative spiral structures based largely on β-turns with diameters of about 2 nm. The β-turns were placed over residues identified by prediction and spectroscopic studies and spiral structures were generated in the presence of a water shell (Figure 9). The simplest model placed a type II β-turn at QPGQ and turns were forced at YPTS, SPQQ and QGQQ in the repeat motif, resulting in a spiral with a diameter of 1.7 nm and a pitch of 1.3 nm (Figure 9). In the second model type II β-turns were located at QPGQ and QGYY and type I/III β-turns at SPQQ and YPTS, resulting in a spiral structure with a diameter of about 2 nm and

pitch of 1.6 nm (Figure 9). The third model did not use standard β-turn types, but the positions of β-turns were based on distance criteria. These were located at QPGQ, YPTS and SPQQ, with the sequence GQGQQGY forming a β-sheet structure. The resulting spiral was flattened, with a cross-section of about 1.7 × 2.5 nm (Figure 9). Calculated dimensions for the models are shown in Table IV.

Arkin *et al.* (2000) used multicanonical simulation to model the structures of five common tetrapeptide sequences (QPGQ, QSGQ, YPTS, SPQQ and QPGY) in the central repetitive domains. They found that QOGQ and QPGY had the highest probabilities for β-turns, with lower probabilities for the others. They also considered the probabilities of γ-turn formation and found probabilities in all five tetrapeptides. They concluded that these may make a contribution to the overall structures of the central repetitive domains. Arkin *et al.* (2001) also modelled two hexapeptide repeats, PGQGQQ and SGQGQQ; these agreed with about 40% of the total occurrence of β-turns predicted by Tatham *et al.* (1985), but also concluded γ-turns may contribute to the proposed spiral structure.

The studies discussed above were either carried out on the HMW subunits in dilute solution, or were modelled *in vacuo* or hydrated in a water shell. However, the environment of the HMW subunits in dough and gluten is as a hydrated solid protein mass. Belton *et al.* (1995) used Fourier transform infrared (FT-IR) and NMR spectroscopy to study the hydration behaviour of subunits and found that the proportions of β-turns and β-sheet varied in relation to the water content. Thus, the content of intermolecular β-sheet structure increased when the protein was hydrated from the dry solid. Gilbert *et al.* (2000) also expressed an M_r 58 000 peptide from the central repetitive domain of subunit 1Dx5 in *E. coli* and studied its behaviour during hydration using FT-IR. They reported that β-turn rich structures formed in the dry and hydrated solid states, with significant contents of intermolecular β-sheet structure. In comparison with intact subunits, the M_r 58 000 peptide showed a lower propensity to form β-sheet structure, indicating that the *N*- and *C*-terminal domains may play a role in assembling the molecules to allow β-sheet formation to occur in the central repetitive domains. NMR studies indicate that the central repetitive domain is flexible, with increasing hydration resulting in increased flexibility (Belton *et al.*, 1995; Alberti *et al.*, 2001). However, two populations of glutamine residues were identified: one in a mobile environment that was tentatively identified with β-turn conformations, and a second population that was more hindered, possibly by hydrogen bonding, in protein segments containing glycine residues and possibly adopting a β-sheet conformation (Alberti *et al.*, 2001).

E. CROSS-LINK FORMATION BETWEEN HMW SUBUNITS

The HMW subunits are only present in oligomers or polymers that are stabilized by inter-chain disulphide bonds. However, little is known about their organization within these polymers.

Partial reduction of gluten with reducing agents followed by stabilization of the free cysteine sulphydryl groups with cystamine diHCl has been shown to result in the release of HMW subunit dimers (Lawrence and Payne, 1983; Werner *et al.*, 1992). These include a preponderance of x–y dimers, and in particular dimers involving 1Dy with 1Ax, 1Bx or 1Dx subunits. These studies indicate that x–y HMW subunit dimers may act as "building blocks" in glutenin polymers.

Evidence for head-to-tail bonds between HMW subunits was reported by Tao *et al.* (1992). They prepared a glutenin-enriched fraction from flour and digested this with the endoproteinase LysC. Fractionation of the digest led to the isolation of two peptides containing intact disulphide bonds, both of which consisted of the *C*-terminal part of subunit 1Bx17 linked to the *N*-terminal domain of subunit 1Dy10. However, the precise cysteine residue involved in subunit 1Dy10 could not be identified.

The isolation of x–y dimers and the identification of head-to-tail disulphide bonds are both consistent with the suggestion by Graveland *et al.* (1985) that alternating x-type and y-type subunits form the "backbone" structure of the glutenin polymers.

Further disulphide bonds involving HMW subunits have been reported by Köhler and coworkers (Köhler *et al.*, 1991, 1993, 1994; Keck *et al.*, 1995). These include one intra-chain bond within the *N*-terminal domain of subunit 1Bx7 (involving Cys 10 and Cys 17) (Figure 8) and one cross-link between a cysteine residue within the repetitive domain of subunit 1By9 (Cys 564) or 1Dy10 (Cys 507) and a low molecular weight subunit of glutenin. This could lead to the presence of LMW subunits as "branches" on the HMW subunit backbone, as proposed by Graveland *et al.* (1985).

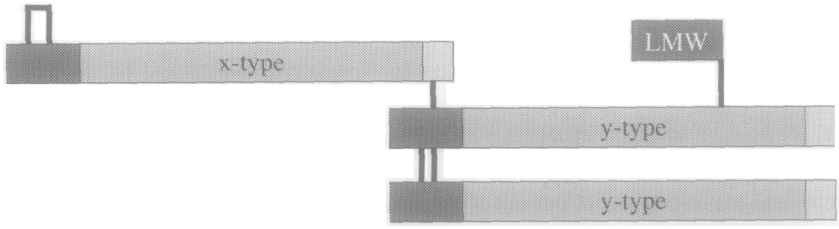


FIG. 10. Schematic summary of disulphide bonds identified involving HMW subunits. Based on details in Tao *et al.* (1992), Köhler *et al.* (1991, 1993, 1994) and Keck *et al.* (1995).

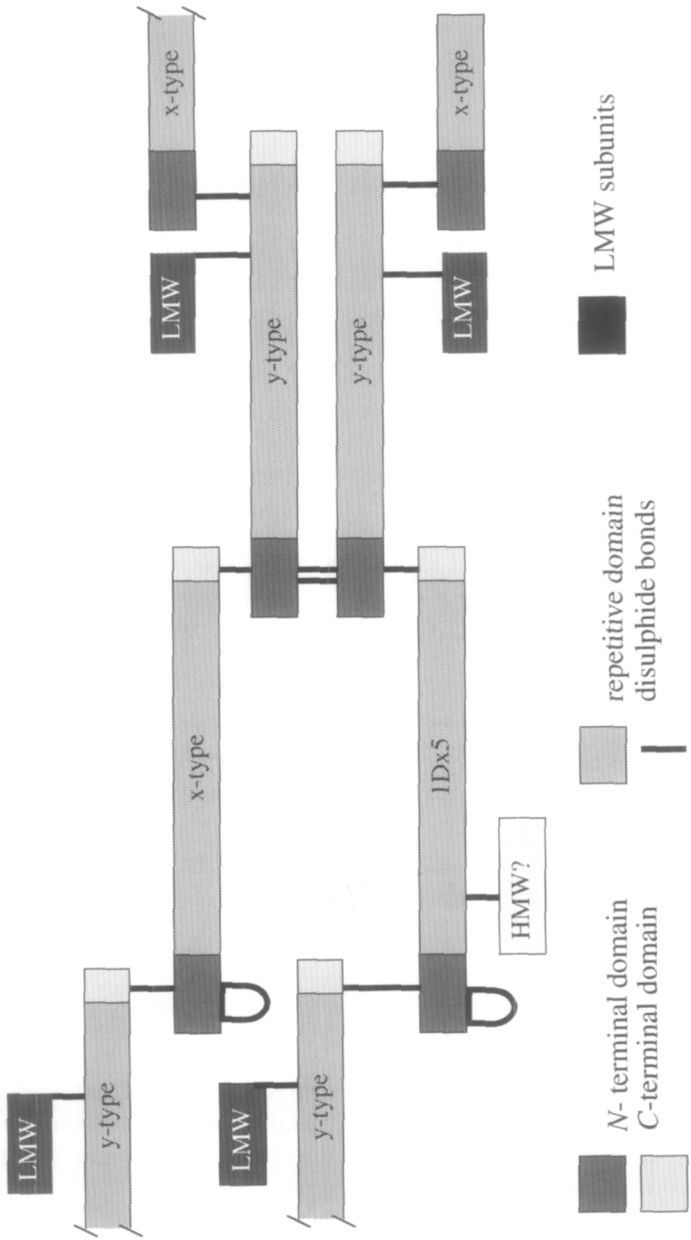


FIG. 11. Hypothetical structure for wheat glutenin polymers, based on mapped disulphide bonds (Figure 10). Not all cysteine residues are included. The additional cysteine residue present in the repetitive domain of subunit 1Dx5 is proposed to link to another HMW subunit, based on the effect on gluten rheology of transformation with additional gene copies.

In addition, two disulphide bonds were identified between the adjacent cysteine residues (Cys 44, Cys 45) in the *N*-terminal domains of two y-type subunits (1By9 and/or 1By10), linking the two subunits in parallel. Since hetero- or homodimers comprising two y-type subunits were not detected by Werner *et al.* (1992) or Lawrence and Payne (1983), it can be concluded that such cross-links between y-type subunits are either readily reduced or occur at low frequency.

Based on these studies, our current knowledge of the disulphide bonds formed by HMW subunits is summarized in Figure 10. This information can be used to propose a wider structural model for wheat glutenin polymers, based on x–y subunit dimers with branches to LMW subunits via y-type subunits (Figure 11). A more detailed discussion of the disulphide bond structure of gluten proteins is provided by Shewry and Tatham (1997).

Although disulphide bonds are classically considered to be the only type of covalent cross-links in wheat glutenin polymers, Tilley *et al.* (2001) have recently reported that a novel type of cross-link may occur. These are tyrosine cross-links formed specifically between the TyrTyr sequences present in the HMW subunit repeat motifs. Analysis of synthetic peptides (Tyr.Tyr.; Tyr.Tyr.Pro.Thr.Ser. and Gln.Gln.Gly.Tyr.Tyr.Pro.Thr.Ser.) demonstrated that the formation of such cross-links was enhanced by $KBrO_3$ which acts as an "improver" in breadmaking. The authors suggest that tyrosine bonds formed during mixing and baking contribute to the structure of the gluten network.

IV. EXPERIMENTAL EVIDENCE FOR THE ROLE OF HMW SUBUNITS IN DOUGH MIXING AND GLUTEN VISCOELASTICITY

Experimental evidence for the role of the HMW subunits in determining the viscoelastic properties of doughs comes from a range of studies in which gluten fractions, individual proteins and peptides have been studied in reconstituted systems.

A. GLUTEN FRACTIONATION AND RECONSTITUTION

A valuable approach to elucidate the role played by the different protein components in gluten and dough functionality is to fractionate and reconstitute gluten or flour and to observe the effects of adding or omitting components on the technological or rheological properties.

The earliest work used this approach to investigate the role of gliadin- and glutenin-rich fractions. In this way Finney and coworkers (for a synthesis, see Finney, 1985) showed that glutenin was involved in the mixing requirement

and that glutenins extracted from good or poor quality wheats had different properties. Further fractionation of glutenin into acid-soluble and acid-insoluble fractions, corresponding broadly to components with different polymer sizes and/or aggregation behaviour, showed a positive relationship between the acid-insoluble (or high molecular mass) glutenin aggregates and dough mixing strength (Orth and Bushuk, 1972; Huebner and Wall, 1976; Hamada *et al.*, 1982). More recently, it has been shown that increasing the glutenin to gliadin ratio (while maintaining a constant total protein content) increased the mixing time and the overmixing tolerance of dough. Extensibility was reduced by the addition of glutenin whereas resistance to extension was increased (Uthayakumaran *et al.*, 1999), corresponding to higher rupture viscosity and a lower rupture strain when dough was submitted to uniaxial elongation. In contrast, gliadin contributed to increased extensibility of dough by lowering the viscosity and enhancing the rupture strain (Uthayakumaran *et al.*, 2000a).

Rheological studies have also been carried out on isolated gliadin and glutenin fractions separated by the Osborne procedure. Gliadins were described as an elasto-viscous liquid whereas glutenins behaved like a viscoelastic solid (Khatkar *et al.*, 1995). The addition of protein fractions to gluten and gluten reconstitution with varied ratios of gliadins to glutenins showed that the storage (related to elasticity) and the loss (related to viscosity) moduli (G' and G'', respectively) of gluten were positively related to its content of glutenin and that increasing the glutenin content increased gluten elasticity (Khatkar *et al.*, 1995; Janssen *et al.*, 1996),

However, the results of this approach could be invalidated if the extraction procedure modified protein functionality (MacRitchie, 1985; Skerritt *et al.*, 1996) and it was considered that the extraction of gliadin with alcohol/water in the classical Osborne procedure could irreversibly change the aggregation of the glutenin fraction (Hoseney *et al.*, 1969). MacRitchie (1987) therefore proposed a new method for the sequential extraction of gluten proteins in dilute hydrochloric acid, a procedure which was considered to preserve their original functionality. The ten fractions obtained in this way contain mixtures of gliadin and glutenin in varying proportions, with the size distribution of the glutenin polymers also varying between the fractions (Lundh and MacRitchie, 1989). The effects of these fractions on the mixing properties and the storage modulus (G') of the dough, when added to a control flour, were related to their contents of glutenin polymers and to their aggregation state (MacRitchie, 1987; Eliasson and Lundh, 1989). The same fractionation procedure was also applied to gluten extracted in the laboratory. The fractions were analysed by size-exclusion high-performance liquid chromatography (SE-HPLC) under dissociating conditions (Cornec *et al.*, 1994), showing that the most readily extracted material

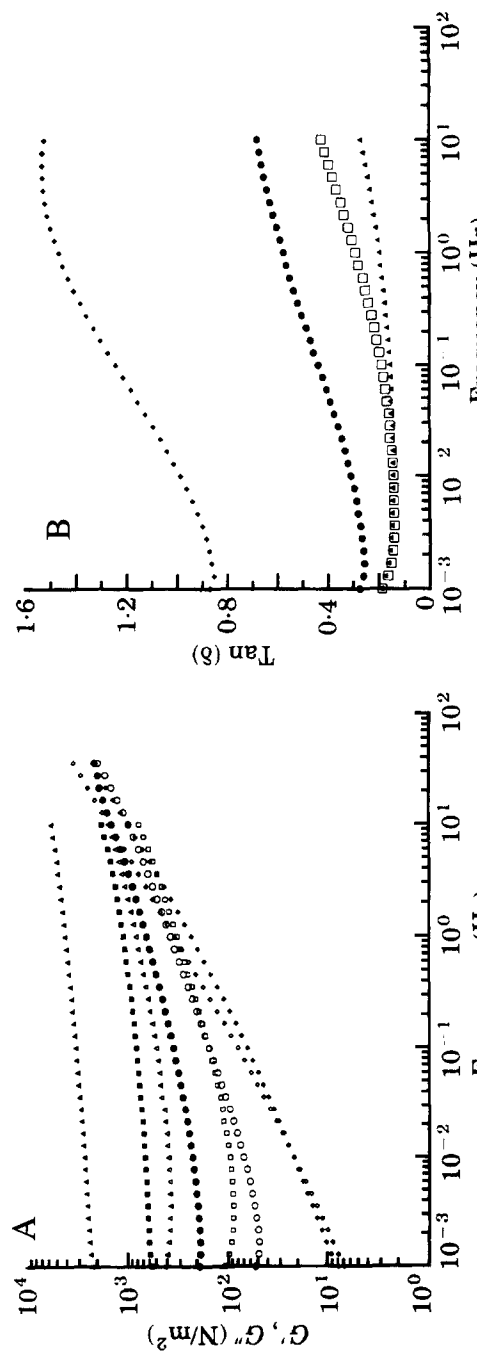


FIG. 12. Viscoelastic properties of gluten subfractions differing in their content and size distribution of glutenin polymers. The content of glutenin and the proportion of the largest glutenin polymers increase from fractions F3 to F8. Dynamic rheological assays in shear were performed at 20°C on gluten fractions fully hydrated with water using a Carri-Med CSL 100 constant stress rheometer (cone-plate geometry: cone angle, 4°; diameter, 2 cm; amplitude of strain at all frequencies 3%).

(A) Storage (G') and loss (G'') moduli: closed symbols, G'; open symbols, G''; diamonds, F3; circles F4; squares, F6; triangles, F8.

(B) Tangent of the loss angle. Symbols: diamonds, F3; circles F4; squares, F6; triangles, F8. Taken from Cornec *et al.* (1994), with permission.

contained mostly gliadin monomers. With decreasing extractability, the fractions became enriched in glutenin polymers of medium and large size with the HMW subunits being concentrated in the largest, most insoluble polymers. The mechanical spectra of the fully hydrated fractions were also recorded in a dynamic assay in shear (Cornec *et al.*, 1994). The viscoelastic properties of pure gliadin were too low to be measured with the Carrimed SL 100 stress rheometer but an increasing content of glutenin polymers was associated with increases in both the storage (G') and loss (G'') moduli but with a decrease in the G''/G' ratio (tangent δ), indicating that the elasticity of the material increased (Figure 12). For all the fractions except those containing the lowest amount of glutenin, G' was higher than G'' over the whole range of frequencies (with a cross-over of G' and G'' being observed for gliadin-rich fractions, as shown for F3 in Figure 12). The frequency range corresponding to the elastic plateau was also shifted towards higher values when the content of glutenin polymers increased. This behaviour is typical of a transient network structure and the connectivity of the network, measured by the height of the elastic plateau (G_N^0), increased when the extractability of the fractions decreased. Thus, G_N^0 was between about 3000 and 30 000 N m^{-2} for the glutenin-rich fractions and was strongly positively correlated with the content of large glutenin polymers (measured as the excluded peak in SE-HPLC), but not with that of medium-size glutenin polymers. Therefore, it was assumed that the density of transient cross-links in hydrated gluten was determined primarily by the proportion of the largest glutenin polymers. In this network, monomeric gliadins act as plasticizing elements (Cornec *et al.*, 1994). Using a different fractionation procedure, Tsiami *et al.* (1997a, b) confirmed that the major variable affecting the rheological properties of gluten proteins, and particularly of glutenins, is the size of the concatenations.

B. INCORPORATION OF PROTEIN FRACTIONS INTO DOUGH

In other studies, different types of isolated gliadins or glutenin subunits have been incorporated into gluten and dough to discriminate between the effects of individual proteins and to relate functionality to particular structural features. The addition of isolated gliadins confirmed that monomeric prolamins have a weakening effect on dough (Fido *et al.*, 1997), by increasing the extensibility and decreasing resistance. However, the extent of the effect depended on the gliadin type, and conflicting results were reported by different authors (Fido *et al.*, 1997; Uthayakamuran *et al.*, 2001), with both molecular mass and hydrophobicity being reported to determine gliadin functionality. Such experiments are relatively easy to perform with gliadins because they are present in the flour as monomeric proteins. A

simple mixing after addition of the protein into a control flour is sufficient to observe the changes in dough properties induced by the added components. In the case of the glutenin subunits a more complicated procedure must be used, because the subunits must be incorporated by covalent disulphide bonds into glutenin polymers in order to express their functionality in the same manner as in the native gluten. Special procedures to reduce and reoxidize flour proteins were therefore developed and applied in solution (Schropp *et al.*, 1995; Veraverbeke *et al.*, 2000a, b) or in dough (Békés *et al.*, 1994a; Uthayakamuran *et al.*, 2000b). When reoxidation of HMW subunits was performed in solution, only partial polymerization was obtained, with the extent depending on protein concentration and type and on the concentration of oxidizing agent (Schropp *et al.*, 1995; Veraverbeke *et al.*, 2000a). About 20–40% of the initial subunits remained as monomers, with intramolecular instead of intermolecular bonds being formed. Some conflicting results were also reported concerning the respective oxidizing efficiencies of $KBrO_3$, KIO_3 and H_2O_2 (Schropp *et al.*, 1995, Veraverbeke *et al.*, 2000a, b). Although no differences in the degree or pattern of polymerization were observed when a mixture of subunits 1Bx5, 1Bx7, 1By9 and 1Dy10 was compared with a mixture of subunits 1Dx2, 1Bx6, 1By8 and 1Dy12 (Schropp *et al.*, 1995), y-type subunits were found to be less highly polymerized than x-type subunits (Veraverbeke *et al.*, 2000b) and subunit 1Dy10 was found to form the lowest proportion of polymers (Antes and Wieser, 2001b). Antes and Wieser (2001b) also noted that subunit 1Dx5, which contains an additional cysteine residue, yielded a higher proportion of polymers than subunit 1Bx7, whereas the opposite was noted by Veraverbeke *et al.* (2000a). It is also notable that LMW glutenin subunits showed a higher propensity to polymerize *in vitro* than HMW subunits and that the polymers formed by HMW and LMW subunits did not differ in their size distribution (Veraverbeke *et al.*, 2000b). These results are not consistent with those obtained with native gluten, where the largest glutenin polymers contain a higher proportion of HMW subunits (Payne and Corfield, 1979; MacRitchie, 1989; Lundh and MacRitchie, 1989), nor with results obtained with lines depleted in HMW subunits, where the absence of HMW subunits increased the extractability and decreased the average size of the glutenin polymers (Popineau *et al.*, 1994; Gupta *et al.*, 1995; Lefebvre *et al.*, 2000). These conflicting results indicate that it is difficult to synthesize glutenin polymers *in vitro* from isolated subunits and that it may be unwise to draw conclusion about glutenin functionality in native gluten from such experiments.

Nevertheless, *in vitro* polymerized glutenins have been incorporated into gluten and dough to test their effect on the rheological properties. The addition of glutenin polymers composed solely of HMW subunits clearly

increased the resistance of gluten and dough to extension, provided some free thiol groups were available to enable the added polymers to be linked to endogenous proteins (Schropp and Wieser, 1996; Antes and Wieser, 2001a). If no thiol groups were available, extensibility was decreased. Incorporation of polymers comprising LMW glutenin subunits also resulted in decreased extensibility, while the incorporation of mixed polymers decreased both the resistance and extensibility (Antes and Wieser, 2001a).

In other experiments, isolated glutenin subunits were directly incorporated into the dough using a partial reduction/reoxidation procedure, initially developed by Békés *et al.* (1994b). Provided the conditions were carefully controlled, reduction by dithiothreitol (DTT) could be completely reversed by addition of KIO_3, as judged by the mixing properties. However, the optimal conditions of reduction/reoxidation for mixing studies were recently shown to be unsuitable for extension and baking testing and specific procedures were therefore developed for each test (Uthayakumaran *et al.*, 2000b). Extension tests were also shown to be more discriminating than mixing tests for determining the rheological properties of the treated doughs with the shape of the resistance vs. extensibiliy curve being especially sensitive to redox conditions. Even under the conditions determined as "optimal", the overall shape of the curve after reduction and reoxidation was different from the original one, indicating that the structure of the protein network and the rheological behaviour of the dough were not restored to an identical condition, even if maximal resistance and extension were similar. Incorporation of HMW subunits always had a positive effect on strength, whereas the effects of incorporation of LMW subunits depended on the type of subunit added (Sissons *et al.*, 1998). Increasing the HMW : LMW subunit ratio improved all parameters in the mixing test, with increased resistance and decreased extensibility of the dough. These changes were correlated with an increase in the amount of "unextractable polymeric proteins" (i.e. the largest glutenin polymers) (Uthayakumaran, 2000b).

C. INCORPORATION OF PURIFIED HMW SUBUNITS INTO DOUGH

Different HMW glutenin subunits have been incorporated into control doughs using reduction/reoxidation procedures. Incorporation of subunit 1Bx20 from the durum wheat cultivar Bidi 17 increased dough strength (i.e. increased mixing time and peak resistance and, decreased the breakdown in Mixograph tests). The amount of the largest glutenin polymers was also increased and the 1Bx20 subunit was recovered mainly from this polymer fraction (Békés *et al.*, 1994b). On the other hand, the simple addition of the subunit (with no reduction and reoxidation to form inter-

chain disulphide bonds) resulted in a decrease in dough strength. This demonstrated that increasing the proportion of HMW glutenin subunit in glutenin polymers was associated with increases in their size and/or aggregation state, and significantly enhanced the technological properties of the flour. Using the same method, the effects of partially purified 1Dx and 1Dy subunits expressed in *E. coli* were investigated. Incorporation of the subunits increased dough strength, but the effect of 1Dx subunits was greater than that of 1Dy subunits. When incorporated in pairs, the most efficient associations were 1Dx + 1Dy, and subunit 1Dx5 combined with 1Dy10 resulted in a greater enhancement of strength than subunit 1Dx2 with 1Dy10 (Békés and Gras, 1994). The subunit pair 1Dx5 + 1Dy10 was also superior to the 1Dx2 + 1Dy12 pair. These results were confirmed by mixing, extension and baking tests on dough in which single subunits or

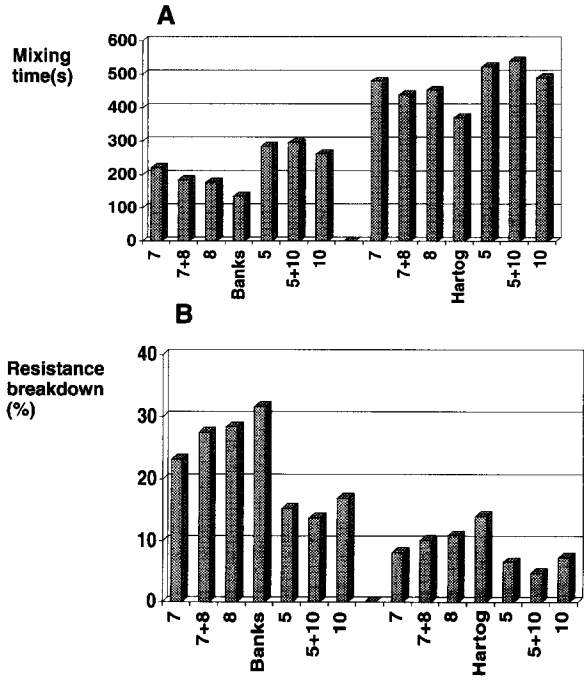


FIG. 13. Effect of incorporation of HMW glutenin subunits 1Bx7, 1By8, 1Dx5 and 1Dy10 on the mixing properties of two base flours, Banks and Hartog. The purified HMW glutenin subunits labeled on the abscissa were incorporated into the base flours using the reduction–oxidation procedure of Békés and Gras (1994). (A) Mixing time; (B) resistance of dough to breakdown. Taken from Uthayakumaran *et al.* (2000c), with permission.

pairs of subunits, extracted from flours or expressed in *E. coli*, were incorporated (Uthayakumaran *et al.*, 2000c) (Figure 13). These experiments showed that chromosome 1B-encoded subunits were less able to increase dough strength than chromosome 1D-encoded subunits. When single subunits were compared, subunit 1Dx5 had a greater effect than subunit 1Bx7 while subunit 1Dy10 had a greater effect than subunit 1By8. Synergy was also noted for the chromosome 1D-encoded subunits in that the subunit pairs 1Dx5 + 1Dy10 or 1Dx2 + 1Dy12 had greater positive effects than each single component incorporated at the same total concentration (Békés and Gras, 1994; Uthayakumaran *et al.*, 2000c). This was not the case for the chromosome 1B-encoded subunits 1Bx7 and 1By8. The greatest effect of subunits 1Dx5 + 1Dy10 was also demonstrated using baking tests (Uthayakumaran *et al.*, 2000c).

The results obtained with the subunit pairs are consistent with the presence in gluten of dimers composed of x- and y- subunits (see above) and also provide experimental support for the quality scores of glutenin subunits based on correlations between the HMW composition of genotypes and their technological properties (Payne *et al.*, 1987b; Branlard *et al.*, 1992).

D. INCORPORATION OF HMW SUBUNIT PEPTIDES INTO DOUGH

In order to determine whether the HMW subunit repetitive domain was alone able to affect the mixing properties of dough, Buonocore *et al.* (1998) expressed a series of repetitive peptides in *E. coli* and then incorporated them into dough using the 2 g Mixograph. The peptides had M_r of about 58 000 and consisted of residues 103 to 643 of subunit 1Dx5 (comprising most of the repetitive domain), with the addition of short linking sequences at the *N*- and *C*-termini. In addition, a series of mutants were constructed with 0, 1 and 2 cysteine residues as substitutions close to the *N*- and/or *C*-termini.

When incorporated into dough, the peptide with two additional cysteine residues at the *N*- and *C*-termini (called 2 + 2) resulted in substantial increases in the mixing time and peak resistance (Figure 14), demonstrating that the nonrepetitive *N*- and *C*-terminal domains of the HMW subunits are not essential for the formation of the viscoelastic glutenin polymers.

V. MANIPULATING HMW SUBUNIT COMPOSITION

Although it is possible to obtain information on the functional properties of individual HMW subunits using *in vitro* approaches, it is important to

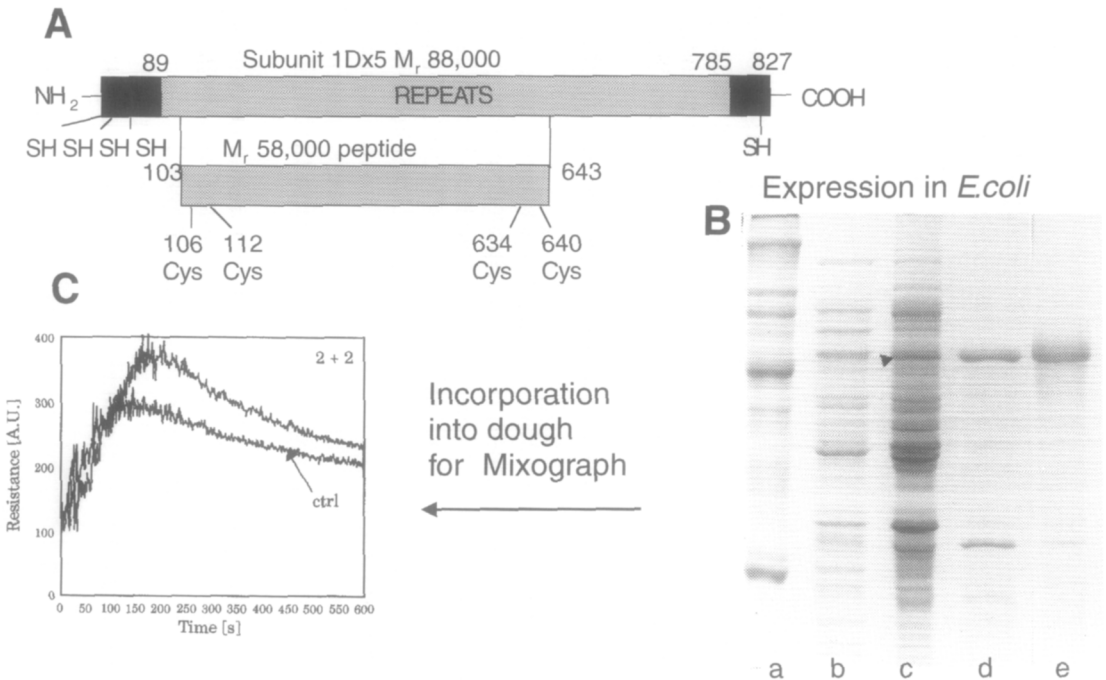


FIG. 14. The effect of an M_r 58 000 repetitive peptide from subunit 1Dx5 on the mixing properties of dough.

A subclone encoding residues 103 to 643 from subunit 1Dx5 mutated to encode cysteine residues at positions 106, 112, 634 and 640 (A) was expressed in *E. coli*, purified (B) and incorporated into dough using the 2g Mixograph (C).

Tracks in part (B) are a, M_r marker proteins; b, total proteins from uninduced *E. coli* cells; c, total proteins from *E. coli* cells after induction to express the M_r 58 000 peptide (arrowed); d, the proteins extracted from induced cells of *E. coli* with 70% (v/v) ethanol and e, the purified M_r 58 000 peptide. Taken from Buonocore *et al.* (1998) with permission.

also determine their properties when incorporated into glutenin polymers *in vivo*. In order to do this it is advantageous to compare the properties of individual subunits when expressed in the same genetic background. This can be achieved by classical crossing to produce near isogenic lines, or by transformation to insert additional genes. These approaches can be regarded as complementary, with the transgenic approach being used to generate variation beyond that which is available naturally, for example, to add additional copies of expressed genes or to express single genes or novel combinations of single genes which are usually inherited as tightly linked allelic pairs.

A. NEAR ISOGENIC LINES

The demonstration by Payne and coworkers (see review by Payne, 1987) that specific HMW subunit alleles are associated with good and poor breadmaking performance has led to their selection in plant breeding programmes. Although work was initially carried out on defined crosses (Payne *et al.*, 1981a), the correlations demonstrated have since been confirmed and extended using collections of genotypes and different genetic stocks such as biotypes, null lines and near isogenic lines. Of these approaches, the development of near isogenic lines (NIL) is of greatest value for studies of structure–functionality relationships as they contain different combinations of HMW subunits transferred into a common genetic background by repeated back-crossing. A number of such series of lines are now available, in addition to near isogenic pairs such as the recently reported transfer of a novel 1Bx subunit into the breadwheat cultivar Fiorello (Margiotta *et al.*, 2000).

1. The Sicco NIL

Payne *et al.* (1987a) have described the production of lines in the cultivar Sicco differing in their number (two to five) and composition of HMW subunits. These contained the following subunit combinations:

	Glu-A1	*Glu-B1*	*Glu-D1*
Sicco	1	7 + 9	5 + 10
2 + 12	1	7 + 9	2 + 12
1Ax null	null	7 + 9	5 + 10
1D null	1	7 + 9	null
1Ax/1D null	null	7 + 9	null

Subsequently, Rogers *et al.* (1991) used the same Sicco background to

produce three additional NIL differing in the absence of x-type or y-type subunits. They had the following compositions:

	Glu-A1	*Glu-B1*	*Glu-D1*
Sicco	1	7 + 9	5 + 10
1Dx null	1	7 + 9	null + 36
1Dy null	1	7 + 9	2 + null
1Bx null	1	null + 8	5 + 10

2. *The Gabo NIL*

Lawrence *et al.* (1988) combined null mutations at all three *Glu-1* loci to develop NIL in the cultivar Gabo with subunit numbers ranging from zero to five, as listed below.

Glu-A1	*Glu-B1*	*Glu-D1*
1	17 + 18	5 + 10
null	17 + 18	5 + 10
1	null	5 + 10
1	17 + 18	null
null	17 + 18	null
null	null	5 + 10
1	null	null
null	null	null

3. *The Galahad NIL*

Payne and Seekings (1996) described a series of NIL containing only single 1B subunits (either 1Bx6, 1Bx7 or 1By8) in the cultivar Galahad. These four lines have been used for detailed studies of the functional properties of HMW subunits as discussed below. In addition, two more recently produced series of NIL will undoubtedly also prove to be valuable in this respect.

4. *The Pegaso NIL*

Margiotta *et al.* (2000) have repored the development of lines in the bread wheat cultivar Pegaso (Figure 15). They include lines expressing novel x-type and y-type subunits with higher and lower mobilities on SDS-PAGE than the more widely occurring alleles, which may reflect the presence of differences in the length of their repetitive domains. Furthermore, a *Glu-A1* locus expressing genes for both x-type and y-type subunits was

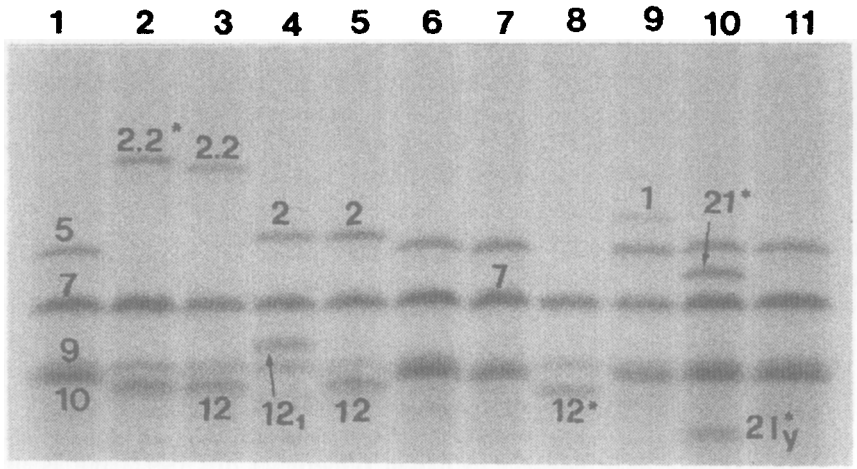


FIG. 15. SDS-PAGE of HMW subunits from near isogenic lines of the bread wheat cultivar Pegaso, showing the expression of novel 1Ax (21*), 1Ay (21*y), 1Dx (2.2, 2.2*) and 1Dy (12*, 12_1) subunits.

introduced from the wild tetraploid wheat *T. dicoccoides*, allowing a NIL expressing six subunits to be produced.

5. Single subunit lines

Lafiandra *et al.* (2000) have reported the production of a series of lines expressing single x-type (1Ax1, 1Dx2, 1Bx7) or y-type (1By8, 1Dy10, 1Dy12) subunits in the background of Gabo, by combining spontaneous mutations identified in collections of old wheat varieties. Crossing of these lines will allow the properties of novel combinations of x-type and y-type subunits (e.g. 1Dx2 + 1Dy10) to be determined.

B. TRANSGENIC LINES

The development of methods for the transformation of wheat lagged behind those for most other major crops (including rice and maize), with the first fertile transformed plants being reported barely a decade ago (Vasil *et al.*, 1992). Nevertheless, wheat transformation has now been established in a number of laboratories world-wide, resulting in renewed interest in quality targets.

The most widely used wheat transformation system is based on direct gene transfer, the DNA being coated onto the surface of microscopic gold

particles and literally shot into the cells using high-pressure helium gas. It is necessary to use a recipient tissue that can be regenerated into a whole plant and it is usual to use immature embryos for bread wheat and either immature embryos or immature inflorescence explants for pasta wheat (see Barcelo *et al.*, 2001). The exogenous DNA appears to integrate in a random fashion into the genome of cells of the recipient tissue, but only some cells are transformed. This could result after regeneration in a chimaeric plant containing a mixture of transformed and non-transformed cells. Consequently it is usual to also use a "selectible marker" gene which will usually co-integrate with the gene of interest and allow the selective regeneration of transformed cells. The most widely used selectible marker genes confer resistance to toxic herbicides or antibiotics, which can therefore be used to kill non-transformed cells. An alternative gene delivery system, based on using the bacterium *Agrobacterium tumefaciens* as a vector, has been applied to wheat (Cheng *et al.*, 1997) but is not widely used.

The delayed development of wheat transformation systems meant that genes for HMW subunits and information on their relationship to grain processing quality were already available when reliable systems become available, with the result that the HMW subunits were the earliest target selected for the improvement of wheat by transformation.

The first success was reported by Blechl and Anderson (1996), who constructed a chimaeric gene encoding a hybrid subunit comprising residues 1 to 124 of the mature 1Dy10 subunit fused to residues 130 to

TABLE V

PUBLISHED REPORTS OF THE EXPRESSION OF HMW SUBUNIT GENES IN TRANSGENIC WHEAT

Species	Line	Subunit	Reference
Bread wheat	Bobwhite	1Dy10/1Dx5 hybrid	Blechl and Anderson (1996)
Bread wheat	Bobwhite	1Ax1	Altpeter *et al.* (1996)
Bread wheat	L88-6	1Ax1	Barro *et al.* (1997)
	L88-31	1Dx5	
Bread wheat	Bobwhite	1Dx5+1Dy10	Anderson and Blechl (2000)
Bread wheat	Canon	1Ax1	Pastori *et al.* (2000)
	Cadenza	1Ax1	
Bread wheat	Pro INTA	1Ax1	Alvarez *et al.* (2000)
	Federal	1Dx5	
Bread wheat	L88-6	1Dx5 mutants	He *et al.* (2000)
	L88-31	1Dx5 mutants	
Pasta wheat	L35	1Ax1	He *et al.* (1999)
	Ofanto	1Dx5	
Tritordeum	Three lines	1Ax1	Rooke *et al.* (1999a)
		1Dx5	

848 of subunit 1Dx5. This essentially combined the *N*-terminal domain of subunit 1Dy10 with the repetitive and *C*-terminal domains of 1Dx5. The novel subunit was readily resolved from the endogenous subunits present in the recipient cultivar (Bobwhite) by SDS-PAGE and analysis of seeds showed accumulation at levels comparable to those of the native proteins. However, subsequent studies (Shimoni *et al.*, 1997) showed that the novel subunit formed circular monomeric structures stabilized by head-to-tail disulphide bonds rather than becoming incorporated into glutenin polymers.

Since then HMW subunit genes have been successfully expressed in a number of lines of bread and pasta wheat, including commercial cultivars as well as model lines (Table V). They include two mutant forms of subunit 1Dx5 in which the length of the repetitive domain has been increased or decreased to determine the effects on the gluten properties. Also, HMW subunit genes have been used to transform cultivars of durum wheat and lines of tritordeum, the latter being a novel cereal produced by

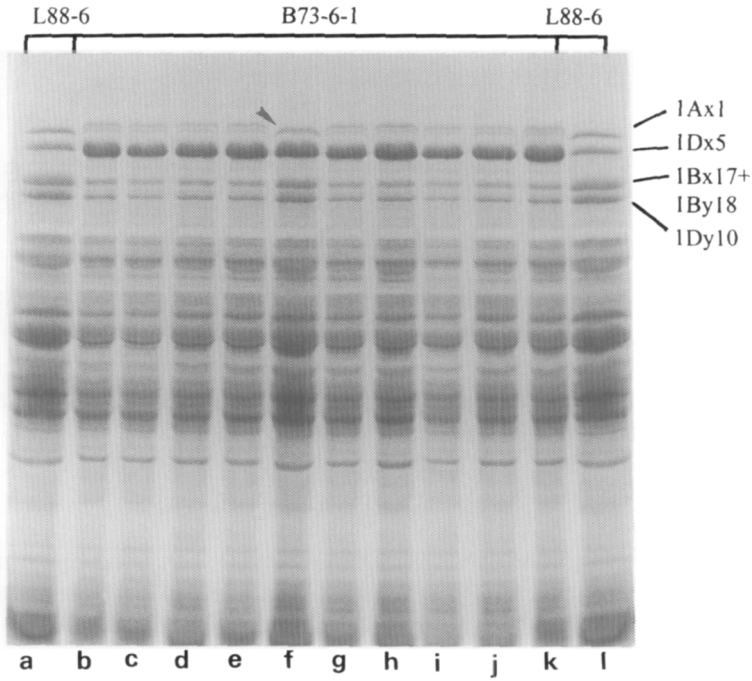


FIG. 16. SDS-PAGE of ten single seeds of the transgenic line B73-6-1 showing expression of the 1Dx5 transgene in the L88-6 background. The minor band indicated by an arrow is observed to segregate between the progeny. Taken from Rooke *et al.* (1999b), with permission.

combining the genomes of pasta wheat and the wild barley species *Hordeum chilense* (Martin *et al.*, 1999). In all cases expression levels up to or exceeding those of the endogenous genes have been reported, with expression being stable over a number of generations. However, both Blechl *et al.* (1998) and Alvarez *et al.* (2000) reported that some lines exhibited silencing or reduced expression of endogenous subunits, presumably via a co-suppression mechanism (see Barcelo *et al.*, 2001).

Of particular interest are some lines in which the levels of expression of the transgenes greatly exceed those of the endogenous genes. An example of this is shown in Figure 16 (Rooke *et al.*, 1999b). The line B73-6-1 was produced by transforming the line L88-6 (expressing subunits 1Ax1, 1Dx5, 1Bx17, 1By18, 1Dy10) with about 15 copies of the 1Dx5 transgene, resulting in a four-fold increase in the proportion of subunit 1Dx5 (from about 2.7 to 10.7% of the total seed proteins) and a 1.6-fold increase in total HMW subunits (from about 12.7 to 20.5% of the total). B73-6-1 and several other transgenic lines have now been grown in replicate field trials at two UK sites (Long Ashton Research Station near Bristol and Rothamsted near London) over four seasons (1998–2001) (Fido et al., 2000; Popineau *et al.*, 2001). The effects of the transgenes on the functional properties are discussed in a later section.

VI. EXPERIMENTAL EVIDENCE FOR DIFFERENTIAL EFFECTS OF INDIVIDUAL HMW SUBUNITS ON MIXING AND RHEOLOGICAL PROPERTIES

In vivo evidence for differential effects of individual HMW subunits comes from comparative analyses of the functional properties of lines with different compositions of HMW subunits. These may be collections of cultivars, which led to the concept of "quality scores" discussed above, or more defined near isogenic and transgenic lines.

A. VARIATION BETWEEN CULTIVARS

The close association of subunits 1Dx5 + 1Dy10 with good breadmaking quality when compared with subunits 1Dx2 + 1Dy12 has been largely confirmed by analysis of a range of cultivars from around the world (Branlard and Dardevet, 1985; Cressey *et al.*, 1987; Campbell *et al.*, 1987; Payne *et al.*, 1988a; Ng and Bushuk, 1988; Lukow *et al.*, 1989; Mosleth and Uhlen, 1991; Dong *et al.*, 1991; Gupta *et al.*, 1991a; Dong *et al.*, 1992; Manley *et al.*, 1992). Kolster *et al.* (1991) have reported that the alleles present at the *Glu-D1* locus also modify the effects of alleles at the *Glu-*

A1 and *Glu-B1* loci on breadmaking quality. Thus, in the absence of subunits 1Dx5 + 1Dy10 correlations may be observed between *Glu-A1* and *Glu-B1* alleles and dough or gluten properties, as demonstrated by analysis of a collection of cultivars from southern Japan which contained subunits 1Dx2 + 1Dy12 or subunits 1Dx2.2 + 1Dy12 but not subunits 1Dx5 + 1Dy10 (Nagamine *et al.*, 2000).

Furthermore, genetic variation in the LMW subunit composition must also be taken into account when predicting technological properties based on glutenin subunit composition. A study including 101 genotypes (48 from Australia and 53 from around the world) showed that allelic variation in both LMW and HMW glutenin subunit composition explained the rheological properties of dough as determined by extensimetry (Gupta *et al.*, 1991a). In the world set of genotypes, most of the dough resistance was explained by the HMW subunits, with a marked difference between subunits 1Dx5 + 1Dy10 and 1Dx2 + 1Dy12. However, this was not observed in Australian lines, where the LMW subunit composition was more strongly correlated with resistance than the HMW subunit composition. This association was attributed to the presence of particular combinations of HMW and LMW subunit (*Glu-1* and *Glu-3*) alleles in these genotypes, with high-quality *Glu-1* alleles often being associated with low quality *Glu-3* alleles.

Analysis of the dough properties of biotypes with identical gliadin compositions but different HMW subunit alleles indicated that those with the *Glu-D1* subunits 1Dx5 + 1Dy10 had higher dough resistances than those with the subunits 1Dx3 + 1Dy12 and that these lines also contained a greater proportion of unextractable glutenin polymers (Gupta and MacRitchie, 1994). The effects of alleles at the *Glu-A1* and *Glu-B1* loci was much smaller (Lawrence *et al.*, 1987). The dough properties and HMW glutenin subunit composition of the F3 population of the cross Nuri 70 (1Ax1, 1Bx17 + 1By18, 1Dx5 + 1Dy10) × UL 72 (1Ax null, 1Bx7 + 1By8, 1Dx2 + 1Dy12) were determined by Lagudah *et al.* (1988). No significant correlations were observed between dough properties and gliadin composition, but the resistance of the dough was strongly dependent on the HMW subunits alleles, especially those encoded at the *Glu-D1* locus. In particular, the progeny containing subunits 1Dx5 + 1Dy10 showed higher dough resistance and lower resistance breakdown than those with subunits 1Dx2 + 1Dy12. On the other hand, no differences were observed between the *Glu-B1*-encoded subunit pairs 1Bx7 + 1By8 and 1Bx17 + 1By18. However, no relationship was found between HMW subunit composition and dough extensibility. Similar studies with other parental lines confirmed these results. Allelic variation at the *Glu-D1* locus (i.e. subunits 1Dx5 + 1Dy10 vs 1Dx2 + 1Dy12)

explained most of the variation in the SDS sedimentation test values of random lines from the Cheyenne (1Ax2*, 1Bx7 + 1By9, 1Dx5 + 1Dy10) × MG 27116 (1Ax null, 1Bx7 + 1By8, 1Dx2 + 1Dy12) cross, whereas the *Glu-B1* alleles had a much smaller effect (Benedettelli *et al.*, 1992). Analysis of recombinant inbred lines also showed that subunits 1Dx5 + 1Dy10 were associated with superior qualitative traits compared with subunits 1Dx2 + 1Dy12 (Rousset *et al.*, 1992).

The loci encoding the HMW and LMW glutenin subunits have therefore been ranked as follows according to their contribution to the dough quality (essentially resistance) (Gupta et al., 1994): *Glu-D1* > *Glu-B1* > *Glu-B3* > *Glu-A3* > *Glu-D3* = *Glu-A1*. However, additive and epistatic effects between the loci were also noted, and the differences in quality could only be assessed by taking into account the alleles present at the *Glu-1* loci (encoding HMW subunits) and the *Glu-3* loci (encoding LMW subunits) (Rousset *et al.*, 1992; Gupta *et al.*, 1994). It was also noted that factors other than the quantity of the HMW subunits were responsible for the effects of alleles at the *Glu-D1* and *Glu-B1* loci on the development time and the resistance of the doughs, in particular the size distribution of the polymeric proteins (Singh *et al.*, 1990a, b; Gupta and MacRitchie, 1994), which was largely determined by allelic variation in the HMW (and LMW) glutenin subunits. Thus, lines with subunits 1Dx5 + 1Dy10 contained a higher proportion of unextractable polymers and showed a longer dough development time than other lines. Similarly, the presence of high molecular mass glutenin polymers and of unextractable polymeric glutenin proteins had previously been shown to be strongly correlated with dough strength (Dachkevitch and Autran, 1989; Singh *et al.*, 1990b; Gupta *et al.*, 1992, 1993). This indicates that the individual HMW subunits do not have the same propensity to polymerize, which could be the basis for the differences in dough properties associated with allelic variation in their composition. This is substantiated by preliminary studies of the *in vitro* polymerization of isolated subunits (Candler *et al.*, 1996). Subunits encoded by the *Glu-1* locus oxidized more slowly than subunits encoded by the *Glu-D1* locus, with the x-type subunits oxidizing faster than the y-type for each locus. A synergistic effect was observed when x- and y-type subunits were mixed, accelerating the oxidation and leading to a higher proportion of large polymers.

Glutenin macropolymer (GMP) has been defined as the wheat protein fraction unextractable in 1.5% (w/v) SDS (Graveland *et al.*, 1980). It contains principally glutenin polymers and its composition is strongly affected by dough mixing and resting (Hamer and Lichtendonk, 1987). The highest contents of GMP were found in flours, but mixing resulted in a significant decrease in the GMP content of dough and dough resting in

an increase. These changes in GMP content (i.e. in the extractability of glutenin polymers) were interpreted as arising from de-polymerization and re-polymerization of glutenin (Hamer and Lichtendonk, 1987). The contents of GMP in flours and doughs of 14 cultivars were also compared and related to the mechanical properties and breadmaking performance of doughs (Weegels *et al.*, 1997a). The de-polymerization/re-polymerization during dough mixing and resting were observed in all cultivars. The GMP contents also influenced dough rheology, with the contents after a 45 min rest explaining about 90% of the dough resistance to extension. Globally, GMP contents were better related to quality parameters than the classical Osborne protein fractions (i.e. albumins, globulins, gliadins and glutenins).

GMP is composed of about 70% LMW and 30% HMW glutenin subunits (Weegels *et al.*, 1995). However, each type of subunit was not affected to the same extent by de-polymerization and re-polymerization (Weegels *et al.*, 1997b). After mixing, GMP contained a lower proportion of HMW subunits than flour GMP and a selective de-polymerization of x-type HMW subunits was also observed during mixing (Skerritt *et al.*, 1999). However, subunit 1Dx5 was found to be particularly resistant to de-polymerization (Aussenac et al., 2001). HMW subunits were also more prone to become extractable through de-polymerization, but resting of the dough restored their initial concentrations in GMP. In this respect, the HMW subunits can be considered to be more reactive than the LMW glutenin subunits; this could be due either to their structures and conformations or to the conformations of the glutenin polymers. Furthermore, y-type subunits were re-incorporated in GMP more rapidly than x-type subunits during dough resting. As a consequence, GMP became more enriched in y-type subunits after glutenin re-polymerization (Weegels *et al.*, 1997b). These data on the dynamics of glutenin polymers during dough processing showed that they undergo complex rearrangements in their composition and properties in dough.

B. NEAR ISOGENIC LINES

Direct experimental evidence for a functional relationship between dough or gluten properties and HMW subunits comes from analyses of the series of NIL.

The first important result from analysis of the Sicco isogenics was to show that the absolute amount of HMW subunit protein had a significant effect on the dough properties, with the absence of one or three subunits having significant effects on the SDS sedimentation values and breadmaking performance. The absence of subunit 1Ax had a small but significant negative effect on the quality parameters, whereas the absence

of subunits 1Dx5 + 1Dy10 had a major negative effect, as observed previously with two sister lines of wheat exhibiting contrasting bread-making properties (Payne et al., 1988b). The absence of the 1Ax and 1Dx + 1Dy subunits had an even more pronounced negative effect.

Furthermore, substitution of subunits 1Dx5 + 1Dy10 by subunits 1Dx2 + 1Dy12 gave less elastic dough (Payne *et al.*, 1987a). This demonstrated that a qualitative factor, probably related to the specific structures of the individual subunits, was also involved in the determination of the dough elasticity. Further studies of gluten extracted from these Sicco NIL showed that absence of HMW subunits encoded by *Glu-A1* and *Glu-D1* decreased the total content of HMW subunits by about 50%. This increased the extractability of the glutenin, with the amounts of large glutenin polymers, as determined by sequential extraction and SE-HPLC, being reduced. The rheological properties of the hydrated gluten were also affected, with considerable change in both the storage and loss moduli (Popineau *et al.*, 1994b) (Figure 17). The height of the elastic plateau dropped to less than 1/30 the value for the standard line and the G''/G'

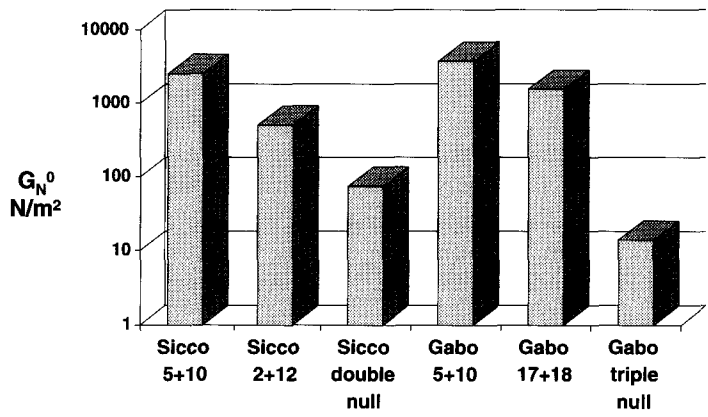


FIG. 17. The height of the elastic plateaux (G_N^0) of glutens extracted from near-isogenic lines of Sicco and Gabo wheats differing in their composition of HMW glutenin subunits encoded at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci. Dynamic rheological assays in shear were performed at 20°C on glutens fully hydrated with water using a Carri-Med CSL 100 constant stress rheometer (cone-plate geometry: cone angle, 4°; diameter, 2 cm, amplitude of strain at all frequencies, 3%).

Sicco 5+10 (1Ax1, 1Bx7 + 1By9, 1Dx5 + 1Dy10); Sicco 2 + 12 (1Ax1, 1Bx7 + 1By9, 1Dx2 + 1Dy12); Sicco double null: (1A null, 1Bx7+1By9, null); Gabo 5+10 (1A null, 1B null, 1Dx5 + 1Dy10); Gabo 17 + 18 (1A null, 1Bx17 + 1By18, 1D null); Gabo triple null (1A null, 1B null, 1D null).

increased four-fold, representing a dramatic collapse of gluten elasticity. The substitution of the subunit pair 1Dx5 + 1Dy10, normally present in the Sicco genotype, by the 1Dx2 + 1Dy12 pair did not modify the total amounts of HMW subunits or the concentrations of individual components. Nevertheless, the gluten in the 1Dx2 + 1Dy12 line contained a lower proportion of large glutenin polymers and the height of the elastic plateau was decreased five-fold. The relationship between the contents of the largest glutenin polymers in gluten fractions and the height of the viscoelastic plateau, previously shown in fractionation experiments (Cornec *et al.*, 1994), was also valid with the Sicco NIL, showing that the HMW subunit composition determines gluten viscoelasticity by modifying the polymer size distribution and the aggregative properties of glutenin (Popineau *et al.*, 1994b).

Analysis of the Gabo NIL (Lawrence *et al.*, 1988) confirmed these data. The mixing time of the dough (determined by Mixograph analysis) was decreased when HMW subunits were absent, the shortest time being recorded for the triple null line lacking all of the HMW subunits. In this study, a quantitative effect of the HMW subunits was observed and no differences were found between the contributions of subunits 1Ax1, 1Dx5, 1Bx17, 1By18 and 1Dy10 to dough functionality. Complementary experiments on the same lines indicated, however, that the presence of subunits 1Dx5 + 1Dy10 resulted in a longer dough mixing time and higher maximum resistance than subunits 1Bx17 + 1By18 (Gupta *et al.*, 1995). The viscoelastic properties of glutens extracted from four of these NIL (the control, 1A/1B null, 1A/1D null and triple null lines) were compared by dynamic assay in shear (Lefebvre *et al.*, 2000). Similar behaviour to that of the Sicco NIL was observed (Figure 17). When HMW glutenin subunits were absent, the rheological behaviour of the gluten was drastically modified: the height of the viscoelatic plateau decreased to a value equal to only 1/250th of the plateau in the standard line, and the position of the plateau was shifted to lower frequencies. Glutenin polymers accounted for 35% of total proteins in this triple null line but they were composed only of LMW subunits and contained only 5% of unextractable glutenin. When only HMW subunits encoded by the *Glu-B1* locus (1Bx17 + 1By18) were present, the height of the plateau was lowered to about one half the value in the standard line. Furthermore, gluten from the 1Dx5 + 1Dy10 NIL exhibited the same plateau value as the standard line (Lefebvre *et al.*, 2000). The size distribution of the glutenin also depended on the number and the type of HMW subunits that were absent. Thus, the absence of all of the subunits resulted in a very large reduction in the proportion of unextractable glutenin polymers (i.e. those requiring sonication to be extracted) in the flour (Gupta *et al.*, 1995),

and their quasi-absence from the gluten, whereas the presence of the subunits 1Dx5 + 1Dy10 resulted in a higher amount of unextractable polymers than in the line with subunits 1Bx17 + 1By18. This was in agreement with the rheological properties of the glutens (Hargreaves *et al.*, 1996, Lefebvre *et al.*, 2000). From these assays, it was concluded that HMW glutenin subunits are practically indispensable for the formation of large aggregative polymers and that these cannot be formed by LMW subunits alone. It can therefore be concluded that HMW subunits constitute the basis for gluten viscoelasticity, because polymers below a critical size limit cannot efficiently entangle and so cannot contribute to the gluten strength properties (Gupta and MacRitchie, 1994; Bangur *et al.*, 1997). Furthermore, a minimum amount of large glutenin polymers is necessary in order to confer viscoelastic behaviour to gluten fractions (Cornec *et al.*, 1994). In addition, the different alleles have different "viscoelastic potential", which is higher for subunits 1Dx5 + 1Dy10 than for subunits 1Dx2 + 1Dx12 or 1Bx17 + 1By18 and is related to their ability to form aggregates or polymers. These differences must arise from structural features of the individual subunits such as the presence of an additional cysteine residue in the sequence of subunit 1Dx5 or to the regularity of the conformation of the repetitive domain (Anderson *et al.*, 1989; Flavell *et al.*, 1989; Goldsborough *et al.*, 1989; Shewry *et al.*, 1992). This is discussed in more detail in a later section.

In the studies reported above the effect of allelic variation was analysed for pairs of subunits encoded by the *Glu-B1* and *Glu-D1* loci. The contributions of individual x-type and y-type subunits was studied by Rogers *et al.* (1991) using the Sicco null lines. The two *Glu-D1* subunits showed a much greater positive effect on gluten strength and dough quality than the y-type *Glu-1B* subunit, confirming the ranking of the loci reported previously (Gupta *et al.*, 1994) (Table I). Although the 1Dx subunit appeared to be superior to the 1Dy subunit, this observation was not conclusive because allelic variation was superimposed on the deletion of subunits (Rogers *et al.*, 1991). Further experiments on the Galahad NIL (Galahad 6, Galahad 7 and Galahad 8, containing only the 1B-encoded HMW subunits 6, 7 and 8, respectively) demonstrated that gluten containing only a single y-type subunit was less extensible and more sensitive to heat treatment (Payne and Seekings, 1996). This was attributed to the additional cysteine residues present in y-type subunits, which could allow more extensive cross-linking of glutenin polymers.

C. TRANSGENIC LINES

The creation of transgenic lines of wheat differing in their HMW subunit

composition makes it possible to determine the effects of individual subunits on the rheological and technological properties of wheat gluten and thus to interpret functionality in terms of protein structure. Transgenes encoding subunits 1Ax1 (containing two cysteines available for intermolecular disulphide bonds) and subunit 1Dx5 (with three cysteines available for intermolecular disulphide bonds) were therefore expressed in bread wheat (Barro *et al.*, 1997, Rooke *et al.*, 1999b), pasta (durum) wheat (He *et al.*, 1999) and tritordeum (Rooke *et al.*, 1999a).

The introduction of one (1Ax1) or two (1Ax1 + 1Dx5) transgenic HMW glutenin subunits in a Gabo NIL line (L88-31) containing only the *Glu-B1*-encoded subunits 17 and 18 resulted in progressive increases in the mixing time of dough (Barro *et al.*, 1997), demonstrating that the subunits encoded by the transgenes were incorporated into the gluten structure in the same way as normal glutenin subunits and contributed to dough strength. In contrast, expression of subunit 1Ax1 in the cultivar Bobwhite showed only small effects on mixing time and loaf volume (Vasil *et al.*, 2001), although this could be related to the fact that this cultivar contains the 1BL/1RS chromosome translocation, which results in "sticky dough" and poor mixing and baking performance. Similarly, although Anderson and Blechl (2000) reported that the expression of subunits 1Dx5 and 1Dy10 (presumably in the cultivar Bobwhite) resulted in greatly increased mixing time (from about 4 to 17 minutes), the peak resistance was greatly reduced.

The 1Ax1 and 1Dx5 transgenes were also expressed in durum wheat lines that lack the D genome associated with high gluten viscoelasticity and were also silent for the *Glu-1A* locus. The absence of subunits 1Ax1 and 1Dx5 from the donor durum wheat lines therefore made it easy to evaluate the technological effects of transgene expression. Both transgenic subunits increased the strength and stability of the dough, with the presence of subunit 1Dx5 resulting in an overstrong dough that could not be properly mixed under normal conditions. However, blending of the transgenic 1Dx5 flour with a weak flour resulted in fortification when added at medium doses (He *et al.*, 1999). The same subunits were also expressed in tritordeum, a fertile amphiploid between wild barley and durum wheat, which is also generally unsuitable for breadmaking. These lines also lacked subunits 1Ax1 and 1Dx5. The expression of subunit 1Ax1 resulted only in a small increase in the dough strength, whereas the expression of the subunit 1Dx5 improved both the mixing time and the resistance breakdown (Rooke *et al.*, 1999a), but this difference could have resulted partially from the different expression levels of the two subunits.

More detailed studies have been carried out on the effects of the 1Ax1 and 1Dx5 transgenes on Gabo NIL expressing HMW subunits 1Ax1,

1Bx17 + 1By18, 1Dx5 + 1Dy10 (the high-quality line, L88-6) and only subunits 1Bx17 + 1By18 (the low-quality line, L88-31). Over-expression of subunit 1Dx5 in the transgenic line B73-6-1 doubled the proportion of the HMW subunits in the total proteins, reaching 20%, and increased the concentration of subunit 1Dx5 by four-fold (Rooke *et al.*, 1999b), but the gliadin/glutenin ratio was only slightly altered. Dough from this transgenic line did not develop properly under the condition of hydration and mixing speed (88 rpm) used in the 2g Mixograph test, whereas the control line L88-6 behaved normally. A high-speed mixing was necessary to form a continuous and cohesive dough. Blending experiments with a weak flour also indicated that the B73-6-1 line was overstrong (Rooke *et al.*, 1999b).

Further experiments were carried out on the L88-6 and L88-31 lines and the transgenic lines B73-6-1 (1Dx5 in L88-6), B102-1-2 (1Ax1 in L88-31) and B72-8-11b (1Dx5 in L88-31) grown in the field in 1998, including protein fractionation and determination of the size distribution and rheological properties of extracted gluten (Popineau *et al.*, 2001). Subunits 1Ax1 and 1Dx5 accounted for about 50 and 70% of the HMW subunits in the transformed lines, respectively, compared with 0% (1Ax1 in L88-31) and 26% (1Dx5 in L88-6) in the control lines. Overexpression of subunits

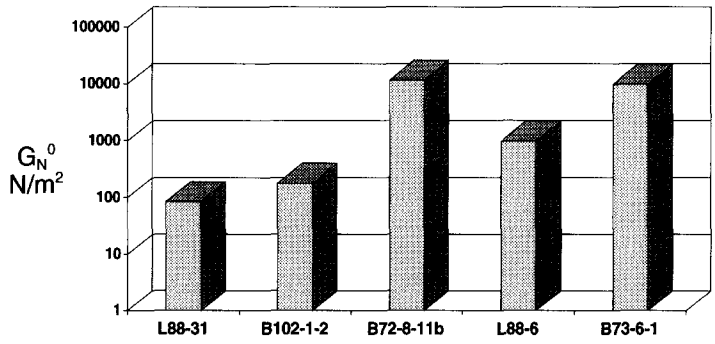


FIG. 18. The height of the viscoelastic plateaux (G_N^0) of glutens extracted from control and transgenic lines of wheat. Dynamic rheological assays in shear were performed at 20°C on glutens fully hydrated with water using a Carri-Med CSL 100 constant stress rheometer (cone-plate geometry: cone angle, 4°; diameter, 2 cm; amplitude of strain at all frequencies, 3%).

L88-31: control line (1A null, 1Bx17 + 1By18, 1D null); B102-1-2: transformed line expressing subunit 1Ax1 transgene in the L88-31 background. B72-8-11b: transformed line expressing subunit 1Dx5 transgene in the L88-31 background. L88-6: control line (1Ax1, 1Bx17 + 1By18, 1Dx5 + 1Dy10); B73-6-1: transformed line expressing the subunit 1Dx5 transgene in the L88-6 background.

1Ax1 and 1Dx5 in the transgenic lines B102-1-2 and B72-8-11b doubled the proportion of the HMW subunits in the total proteins, when compared which their respective control lines. Clear differences were observed, however, between the effects of subunits 1Ax1 and 1Dx5 on gluten physicochemical properties, emphasizing that differences in subunit structure can influence their contribution to gluten structure and rheology.

Sequential extraction and SE-HPLC showed that the expression of the subunit 1Ax1 transgene increased glutenin aggregation, but did not appear to result in increased cross-linking by disulphide bonds. Thus, only the average size of glutenin polymers may have been increased. Gluten viscoelasticity was only moderately altered by the expression of the subunit 1Ax1 transgene (with slightly higher storage and loss moduli) (Figure 18), which mainly increased the dough resistance to elongation during mixing (Figure 19). In contrast, overexpression of subunit 1Dx5 generated very large and insoluble aggregates, probably through covalent cross-linking of polymers. As a result, the glutenin was only completely extracted after reduction of disulphide bonds. The connectivity and viscoelastic moduli of the gluten network were also greatly increased (Figure 18). This effect can be attributed primarily to the presence of an additional cysteine residue available for intermolecular cross-linking in subunit 1Dx5 as compared with subunit 1Ax1 (see Shewry *et al.*, 1992). The very high gluten strength also resulted in abnormal dough mixing behaviour, irrespective of whether the genetic background was L88-6 or L88-31 (Figure 19). It can be postulated that an excess of subunit 1Dx5 modified the glutenin (gluten) structure and hindered the formation of a homogeneous protein network. Furthermore, subunit 1Dx5 is always expressed as a pair with subunit 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 (Lawrence and Payne, 1983; Gao *et al.*, 1992; Tao *et al.*, 1992; Werner *et al.*, 1992; Shani *et al.*, 1994). Over-expression of subunit 1Dx5 in the absence of additional subunit 1Dy10 (or another y-type subunit) could therefore have resulted in extensive restructuring of the glutenin polymers, with important consequences for gluten strength and for the mixing and baking properties of dough.

VII. THE MOLECULAR BASIS FOR CORRELATIONS BETWEEN HMW SUBUNITS AND QUALITY

It is clear from the preceding sections that we now know a great deal about the amino acid sequences of individual HMW subunits, which has led to

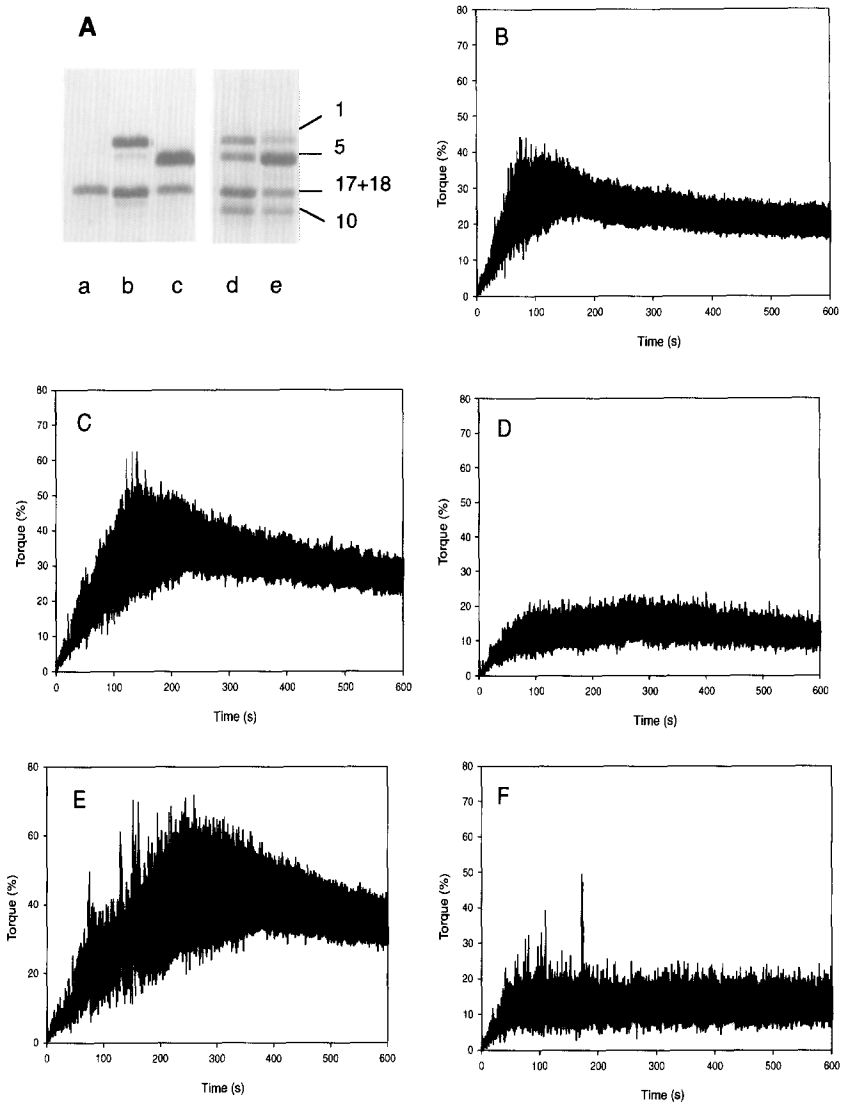


FIG. 19. Analysis of the mixing properties of transgenic wheats expressing additional HMW subunits using the 2g Mixograph. (A) SDS-PAGE of the HMW subunits from a, L88-31: control line (1A null, 1Bx17 + 1By18, 1D null); b, B72-8-11b: transformed line expressing 1Dx5 subunit transgene in the L88-31 background; c, B102-1-2: transformed line expressing 1Ax1 subunit transgene in the L88-31 background; d, L88-6: control line (1Ax1, 1Bx17 + 1By18, 1Dx5 + 1Dy10); e, B73-6-1: transformed line expressing 1Dx5 subunit transgene in the L88-6 background. (B–F) are mixographs of (B) L88-31; (C) B102-1-2; (D) B72-8-11B; (E) L88-6; (F) B73-6-1. The resistance is given as torque % and the mixing time in seconds. Taken from Popineau *et al.* (2001), with permission.

the development of generalized models for their structures, interactions and role in gluten structure and properties. However, it has proved more difficult to identify the precise molecular basis for the differential effects of individual subunits on processing properties that have allowed "quality scores" to be assigned to them (Table I). We will now consider our current knowledge of the molecular basis for these effects.

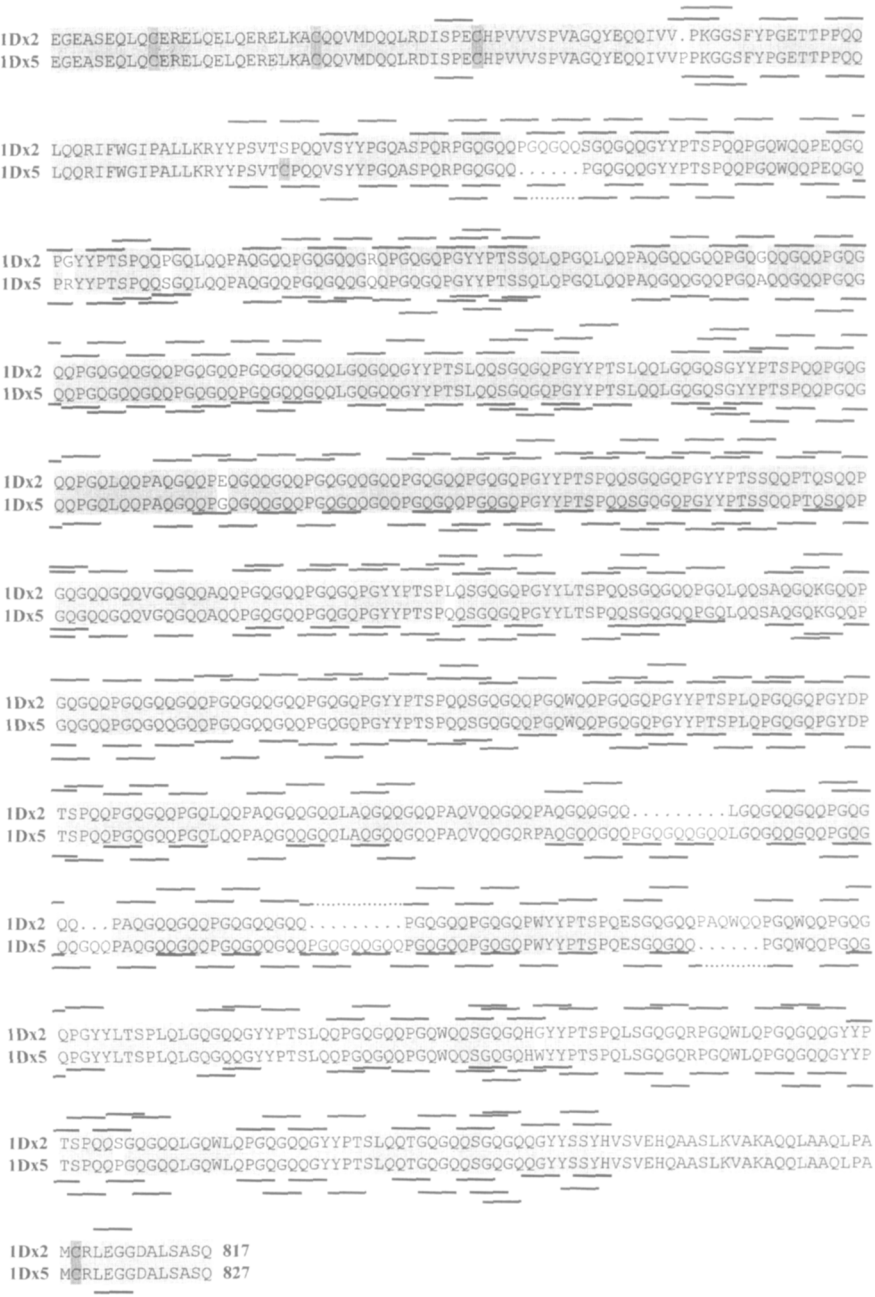
A. PROTEIN AMOUNT

There is good evidence from several studies that the presence of a 1Ax subunit (1Ax1 or 1Ax2*) is associated with an increase in the total amount of HMW subunit protein present in the grain, by about 1.5 to 2.0% of the total grain proteins when compared with the null allele (Seilmeier *et al.*, 1991; Halford *et al.*, 1992). It is probable that this quantitative difference accounts for the quality score of 3 assigned to subunits 1Ax1 and 1Ax2*, as broad correlations between the total amount of HMW subunit protein and good processing properties have been reported by other workers (Payne *et al.*, 1988a; Lawrence *et al.*, 1988; Gupta *et al.*, 1991b; Békés *et al.*, 1994b; Popineau *et al.*, 2001).

B. PROTEIN SEQUENCES

The availability of complete amino acid sequences for allelic pairs of 1Dx and 1Dy subunits associated with good (1Dx5 + 1Dy10) and poor (1Dx2 + 1Dy12) quality allows comparisons to be made to identify features which may relate to their different properties (Figure 20). Comparisons between the sequences of subunits 1Dy10 and 1Dy12 show that they have identical *N*- and *C*-terminal domains, but their repetitive domains differ by 12 single amino acid substitution and by the deletion of two hexapeptides and two adjacent residues in subunit 1Dy10 and of two adjacent residues in subunit 1Dy12. Although these differences are minor, they may nevertheless affect the structure and stability of the protein domain. Thus, Flavell *et al.* (1989) noted that the differences in sequence resulted in a higher proportion of consensus repeat motifs in subunit 1Dy10, which should result in a more regular pattern of β-turns, while Hickman (1995) predicted the presence of 130 β-turns present mainly in the repetitive domain of subunit 1Dy10 but only 125 β-turns in subunit 1Dy12 (Figure 20). This difference in regularity could affect the stability and intrinsic elasticity of the subunits and their ability to form "trains" in gluten.

Similar minor differences between the amino acid sequences of the repetitive domains of subunits 1Dx2 and 1Dx5 are also observed with 13 single amino acid substitutions and the deletion of two hexapeptides and


1Dx2 EGEASEQLQCERELQELQERELKACQQVMDQQLRDISPECHPVVVSPVAGQYEQQIVV.PKGGSFYPGETTPPQQ
1Dx5 EGEASEQLQCERELQELQERELKACQQVMDQQLRDISPECHPVVVSPVAGQYEQQIVVPPKGGSFYPGETTPPQQ
1Dx2 LQQRIFWGIPALLKRYYPSVTSPQQVSYYPGQASPQRPGQGQQPGQGQQSGQGQQGYYPTSPQQPGQWQQPEQGQ
1Dx5 LQQRIFWGIPALLKRYYPSVTCPQQVSYYPGQASPQRPGQGQQ.....PGQGQQGYYPTSPQQPGQWQQPEQGQ
1Dx2 PGYYPTSPQQPGQLQQPAQGQQPGQGQQGRQPGQGQPGYYPTSSQLQPGQLQQPAQGQQGQQPGQGQQGQPGQG
1Dx5 PRYYPTSPQQSGQLQQPAQGQQPGQGQQGQQPGQGQPGYYPTSSQLQPGQLQQPAQGQQGQQPGQAQQGQQPGQG
1Dx2 QQPGQGQQGQQPGQGQQPGQGQQGQQLGQGQQGYYPTSLQQSGQGQPGYYPTSLQQLGQGQSGYYPTSPQQPGQG
1Dx5 QQPGQGQQGQQPGQGQQPGQGQQGQQLGQGQQGYYPTSLQQSGQGQPGYYPTSLQQLGQGQSGYYPTSPQQPGQG
1Dx2 QQPGQLQQPAQGQQPEQGQQGQQPGQGQQGQQPGQGQQPGQGQPGYYPTSPQQSGQGQPGYYPTSSQQPTQSQQP
1Dx5 QQPGQLQQPAQGQQPGQGQQGQQPGQGQQGQQPGQGQQPGQGQPGYYPTSPQQSGQGQPGYYPTSSQQPTQSQQP
1Dx2 GQGQQGQQVGQGQQAQQPGQGQQPGQGQPGYYPTSPLQSGQGQPGYYLTSPQQSGQGQQPGQLQQSAQGQKGQQP
1Dx5 GQGQQGQQVGQGQQAQQPGQGQQPGQGQPGYYPTSPQQSGQGQPGYYLTSPQQSGQGQQPGQLQQSAQGQKGQQP
1Dx2 GQGQQPGQGQQGQQPGQGQQGQQPGQGQPGYYPTSPQQSGQGQQPGQWQQPGQGQPGYYPTSPLQPGQGQPGYDP
1Dx5 GQGQQPGQGQQGQQPGQGQQGQQPGQGQPGYYPTSPQQSGQGQQPGQWQQPGQGQPGYYPTSPLQPGQGQPGYDP
1Dx2 TSPQQPGQGQQPGQLQQPAQGQQGQQLAQGQQGQQPAQVQQGQQPAQGQQGQQ.....LGQGQQGQQPGQG
1Dx5 TSPQQPGQGQQPGQLQQPAQGQQGQQLAQGQQGQQPAQVQQGQRPAQGQQGQQPGQGQQGQQLGQGQQGQQPGQG
1Dx2 QQ...PAQGQQGQQPGQGQQGQQ.....PGQGQQPGQGQPWYYPTSPQESGQGQQPAQWQQPGQWQQPGQG
1Dx5 QQGQQPAQGQQGQQPGQGQQGQQPGQGQQGQQPGQGQQPGQGQPWYYPTSPQESGQGQQ.....PGQWQQPGQG
1Dx2 QPGYYLTSPLQLGQGQQGYYPTSLQQPGQGQQPGQWQQSGQGQHGYYPTSPQLSGQGQRPGQWLQPGQGQQGYYP
1Dx5 QPGYYLTSPLQLGQGQQGYYPTSLQQPGQGQQPGQWQQSGQGQHWYYPTSPQLSGQGQRPGQWLQPGQGQQGYYP
1Dx2 TSPQQSGQGQQLGQWLQPGQGQQGYYPTSLQQTGQGQQSGQGQQGYYSSYHVSVEHQAASLKVAKAQQLAAQLPA
1Dx5 TSPQQPGQGQQLGQWLQPGQGQQGYYPTSLQQTGQGQQSGQGQQGYYSSYHVSVEHQAASLKVAKAQQLAAQLPA
1Dx2 MCRLEGGDALSASQ 817
1Dx5 MCRLEGGDALSASQ 827

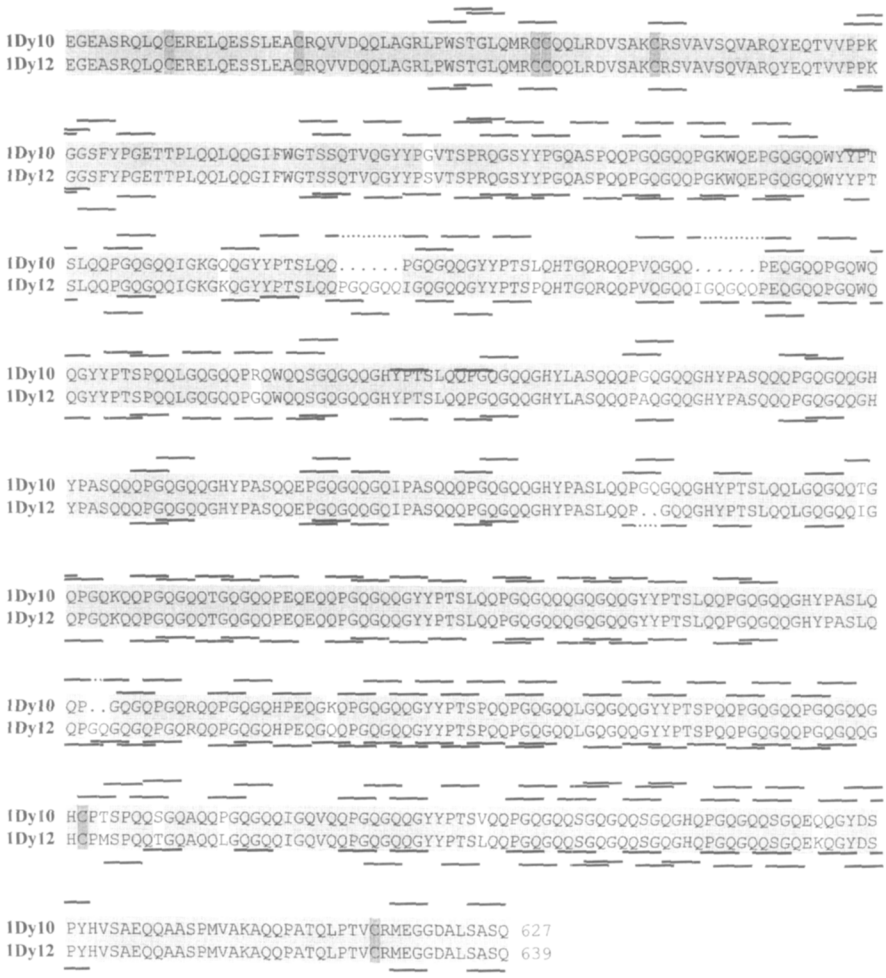


FIG. 20. Alignment of the amino acid sequences of subunits 1Dx2, 1Dx5, 1Dy10 and 1Dy12. The lines indicate β-turns predicted by the method of Chou and Fasman (1978), using a significance level of 1×10^{-4}.

insertion of three nonapeptides and two hexapeptides in subunit 1Dx5. In addition, they differ in one single amino acid deletion in the *N*-terminal domain of subunit 1Dx2. These differences are predicted to have little effect on the *β*-turn structure, with 211 and 212 turns predicted to be present mainly in the repetitive domains of subunits 1Dx2 and 1Dx5, respectively (Figure 20).

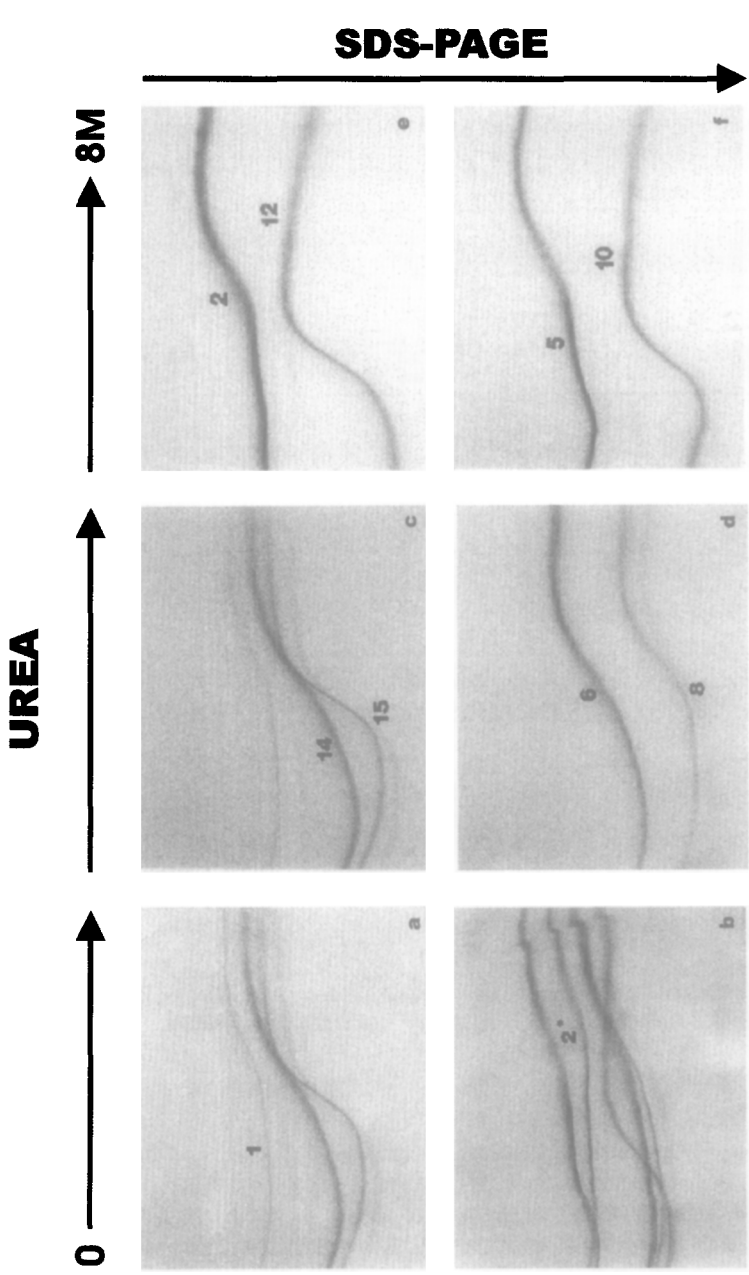


FIG. 21. Transverse urea gradient gel electrophoresis of a range of x-type (1Ax1, 1Ax2*, 1Dx2, 1Bx6, 1Bx14) and y-type (1By8, 1By15, 1Dy10, 1Dy12) HMW subunits. Taken from Lafiandra *et al.* (1999), with permission.

However, one of the single amino acid substitutions could have an impact on the interactions of the subunits in gluten. This is the substitution of a cysteine residue for serine at position 97 (at the *N*-terminal end of the repetitive domain) of subunit 1Dx5. This could result in the formation of more highly cross-linked, and hence more elastic, polymer. Evidence for this comes from the expression of the 1Dx5 transgene in developing grain, as discussed above.

C. STABILITY OF ALLELIC SUBUNITS

It is possible that the differences in the degree of conservation of the repeat motifs present in allelic subunits lead to differences in protein conformation and/or conformational stability that affect their functional properties. A simple way to determine conformational stability is by gel electrophoresis in a transverse gradient of 0–8 M urea, as described by Goldenberg and Creighton (1984). Data obtained with this technique can provide information on the thermodynamics and the kinetics of the unfolding process and demonstrate changes in stability following introduction of a given mutation in the protein (Goldenberg, 1992). In general, proteins that unfold in a single, cooperative, two-state transition exhibit an abrupt decrease in mobility, with a single inflection point at the midpoint of the transition. Studies on the unfolding behaviour of different allelic variants of HMW subunits have been carried out (Lafiandra *et al.*, 1999), demonstrating different behaviour for x-type and y-type subunits (Figure 21). The x-type subunits generally show a broad unfolding pattern with no clear transition as the urea concentration increases, suggesting the existence of several conformational intermediates. In contrast, most y-type subunits show an abrupt decrease in mobility on unfolding, with a single inflection point at the midpoint of the transition indicative of a protein unfolding in a single, cooperative, two-state transition. The free energy values associated with the unfolding process were calculated for the y-type subunits, which showed a two-state transition. Different values were calculated for the allelic subunits 1Dy10 and 1Dy12, with the former showing greater stability. This is consistent with the differences in conservation of the repetitive domains of these two subunits, as discussed above, and may provide a partial explanation for association of the subunit pair 1Dx5 + 1Dy10 with good quality when compared with subunits 1Dx2 + 1Dy12.

D. SUBUNIT INTERACTIONS

These studies indicate, therefore, that both the structure and stability of subunit 1Dy10 and the cross-linking behaviour of subunit 1Dx5 may

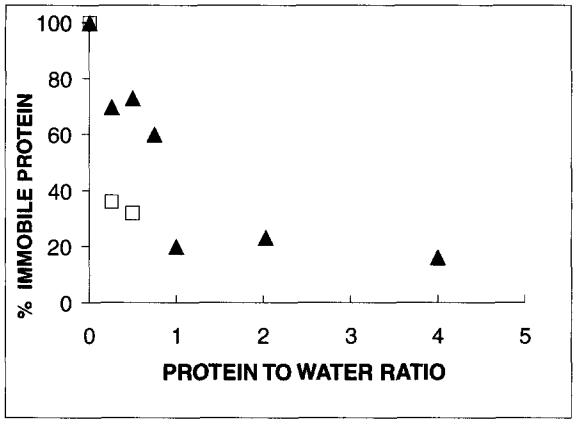


FIG. 22. Relationship between the proportion of mobile protein and the water content of HMW subunits. Triangles: data from Belton *et al.* (1994) calculated assuming that all populations of relaxation times less than or equal to 125 μs contributed to the immobile signal. Open squares: data from Gil *et al.* (2001) using the average figures for the subunits 1Dx5 + 1Dy10.

contribute to the greater dough strength associated with subunits 1Dx5 + 1Dy10 compared with 1Dx2 + 1Dy12. However, it is also possible that specific interactions occur between the individual subunits of the allelic pairs (i.e. 1Dx5 with 1Dy10, 1Dx2 with 1Dy12), resulting in synergistic effects on quality.

One of the well-documented effects of adding water to high molecular weight subunits is the change in mobility observed by NMR. These observations have contributed to the development of the loop and train hypothesis discussed below (see p. 281). It seems logical, therefore, that any interactions between different subunits that affect the rheology should be reflected in changes in mobility. Gil *et al.* (2001) have compared the ^{13}C CPMAS (cross-polarization magic angle spinning) spectra of subunits 1Dx5, 1Dx2, 1Dy10 and 1Dy12 separately and in combination. The CPMAS spectra are only sensitive to the immobile parts of the proteins. Therefore, the experiment directly measures only those parts of the protein that are not moving rapidly and therefore correspond to regions in which the hydrogen-bonded interprotein interactions are present (these are referred to as chains in the following section). Similar measurements of the amount of immobile material can also be made using proton NMR relaxation time measurements. (Belton *et al.*, 1994). Figure 22 shows relaxation time data obtained for a mixture of high molecular weight subunits, together with average values for subunits 1Dx5 and 1Dy10 calculated using CPMAS data. While the scatter in both sets of data is

TABLE VI
VARIATION IN THE AMOUNT OF IMMOBILE PROTEIN IN HMW SUBUNIT MIXTURES

	Percentage of immobile protein			
Water : protein ratio	1Dx5 + 1Dy10 Predicted value	1Dx5 + 1Dy10 measured value	1Dx2 + 1Dy12 predicted value	1Dx2 + 1Dy12 measured value
Dry	100	100	100	100
0.6	36	50	38	95
1.8	32	40	52	29

Data recalculated from Gil *et al.* (2001).

high, the same trends are evident. The scatter arises, in part, because of the intrinsic difficulty of measurement, but also because the two methods of measurement are not exactly the same.

When different subunits are compared using the CPMAS method differences in behaviour are observed. Table VI compares the amounts of immobile protein measured for mixtures of subunits 1Dx5 + 1Dy10 and 1Dx2 + Dy12 with the predicted values arrived at by simply averaging the values of the two components in the mixture. It is difficult to estimate the errors in the data, but the variation between the predicted and measured values for subunits 1Dx5 + 1Dy10 is probably not significant. For the mixture of subunits 1Dx2 + 1Dy12 the differences are much larger and suggest that the sensitivity to water content has been greatly changed by interactions of the two subunits. In particular, at intermediate water contents the interactions between the two subunits appear to result in a higher population of trains (see p. 281). If this were the case it would seem to run counter to the observation that enhanced dough strength arises from interactions of the subunits 1Dx5 + 1Dy10 rather than subunits 1Dx2 + 1Dy12. This is an area where further work is needed to compare the molecular level information obtained from spectroscopy with the macroscopic rheological information.

VIII. THEORETICAL BASIS FOR PROPERTIES OF THE HMW SUBUNITS AND THEIR ROLE IN GLUTEN VISCOELASTICITY AND DOUGH MIXING

Much of the above discussion has focused on providing a biochemical and genetic explanation for the role of the HMW subunits in determining breadmaking quality. However, recent work allows us to develop more sophisticated biophysical models to explain the fundamental properties of

HMW subunits and the molecular basis for their role in viscoelasticity and mixing.

A. WHY ARE HMW SUBUNITS INSOLUBLE?

It is often stated that gluten proteins are insoluble in water or that particular prolamins are insoluble or soluble in specific solvents. Unfortunately, the terminology is often used with respect to some pragmatic measure such as the ability to dissolve the protein to a sufficient extent to carry out some biochemical analysis. However, in thermodynamic terms the solubility of a component in a liquid at equilibrium can be defined by the equilibrium condition:

$$\mu_S = \mu_{0L} + RT \ln a_L \tag{1}$$

where μ is the chemical potential of the component in the solid (S) or the liquid (L). The subscript 0 refers to the standard state of the component, while a is the activity of the component. Thus, for a system at equilibrium with a pure solid $\mu_S = \mu_{0S}$, and

$$RT \ln a_L = \mu_{0S} - \mu_{0L} \tag{2}$$

with the term on the right-hand side being a constant. In practice it is very difficult to achieve this condition for proteins since the standard state is a crystalline solid. In general, therefore, the equation must be modified to take account of the fact that the solid is not in its standard state. Assigning an activity to the noncrystalline protein may achieve this, if pseudo-equilibrium between solid and liquid is assumed. Thus:

$$\mu_{0S} + RT \ln a_S = \mu_{0L} + RT \ln a_L \tag{3}$$

This equation reflects the importance of the activities in both the phases. The term a_S is a measure of the deviation of the solid phase from its standard state, which for proteins will be a function of sample history. The solubility as measured, therefore, will depend on the state of the solid sample and should be measured in a steady state for defined and reproducible solids and solvents. Unfortunately, pragmatic biochemical observations are often treated as if they were thermodynamic measurements. Nevertheless, it is obvious that addition of excess water to gluten or native high molecular weight subunits does not result in the formation of a clear solution as is the case with soluble proteins such as lysozyme or bovine serum albumin, and it is therefore legitimate to infer that the

factors favouring the formation of a solution are insufficient in the case of gluten.

In order for a solution to form there must be net decrease in the free energy of the protein/solvent system on mixing. Conceptually one can imagine that two steps have to take place: the first is the formation of a liquid from the solid phase, and the second is the mixing of the liquid solute and the solvent. The total free energy change (ΔG_{sol}) is the free energy of the formation of the liquid solute (ΔG_{fus}) plus the free energy of formation of the mixture (ΔG_{mix})

$$\Delta G_{sol} = \Delta G_{fus} + \Delta G_{mix} \quad (4)$$

In order for solution to occur there must be net decrease of the free energy, i.e. ΔG_{sol} must be negative, ΔG_{fus} will be positive and thus for solution to occur ΔG_{mix} must be negative and greater in magnitude than ΔG_{fus}. The magnitude of ΔG_{fus} will depend on the strength of the intermolecular interactions in the solid; if these are strong then solution will not be favoured. The importance of considering both terms seems to have been neglected by some authors. For example, Singh and MacRitchie (2001) only consider the Flory free energy of mixing a liquid polymer with a liquid solvent (Flory, 1953) and ignore the role of ΔG_{fus}. If this logic were applied to the solubility of inorganic salts one would expect compounds such as barium sulphate or lithium fluoride to be readily soluble, as the free energies of hydration of the component ions are quite favourable. However, in both of these cases the very high energy of formation of the crystalline state results in lack of solubility.

Free energy contains both enthalpic and entropic contributions, and some authors have suggested that gluten proteins (including HMW subunits) are insoluble because the entropy of solution is unfavourable. Assumptions about the importance of entropic terms can arise because of the neglect of the ΔG_{fus} term (Singh and MacRitchie, 2001), or because of the assumption that hydrophobic interactions in gluten make it insoluble. If a substance is hydrophobic, its interactions with water cause an increase in the hydrogen bonding of the water. As this is a more ordered state, the entropy of the system decreases on solution. Because this is not compensated by any enthalpic interaction, solution is not favoured. This view of gluten protein solubility is also based on the assertion that gluten proteins are hydrophobic. However, consideration of the amino acid composition does not provide support for this. In fact, gluten proteins are very rich in hydrophilic amino acids. The hydrophilic/hydrophobic balance can also be calculated using published data for individual amino acids and typical amino acid composition of whole gluten and HMW subunits (see

TABLE VII

FREE ENERGIES OF HYDRATION FOR AMINO ACIDS IN GLIADINS, GLUTENINS, WHOLE GLUTEN AND AN AVERAGE OF THE AMINO ACID CONTENTS OF 314 PROTEINS

Amino acid	Free energy of hydration (kcal/mol)	Mol% amino acid in gliadins	Mol% amino acid in glutenins	Mol% amino acids in 1Dx5	Mol% amino acids in 1Dy10	Average amino content of 314 proteins (mol%)	Free energy of hydration for an average protein (kcal/mol)	Free energy of hydration for gliadins (kcal/mol)	Free energy of hydration for glutenins (kcal/mol)	Free energy of hydration for 1Dx5 (kcal/mol)	Free energy of hydration for 1Dy10 (kcal/mol)
asp	–3.11	2.8	3.7	0.48	0.64	9.8	–30.478	–8.708	–11.507	–1.4928	–1.9904
thr	–1.69	2.4	3.5	2.9	3.83	6.1	–10.309	–4.056	–5.915	–4.901	–6.4727
ser	–2.36	6.1	7	5.68	6.7	7	–16.52	–14.396	–16.52	–13.4048	–15.812
gln	–3.15	34.5	28.9	37.97	35.57	9.9	–31.185	–108.675	–91.035	–119.606	–112.046
pro	0.23	16.2	11.9	13.8	11	5.2	1.196	3.726	2.737	3.174	2.53
gly	–0.23	3.1	7.5	20.07	18.02	8.4	–1.932	–0.713	–1.725	–4.6161	–4.1446
ala	–0.06	3.3	4.4	3.02	3.67	8.6	–0.516	–0.198	–0.264	–0.1812	–0.2202
cys	–0.27	3.3	2.6	0.61	1.12	2.9	–0.783	–0.891	–0.702	–0.1647	–0.3024
val	0.04	4.8	4.8	1.69	2.55	6.6	0.264	0.192	0.192	0.0676	0.102
met	–0.1	1.3	1.4	0.24	0.48	1.7	–0.17	–0.13	–0.14	–0.024	–0.048
ile	0.07	4.4	3.7	0.48	0.64	4.5	0.315	0.308	0.259	0.0336	0.0448
leu	0.07	6.9	6.5	4.36	3.83	7.4	0.518	0.483	0.455	0.3052	0.2681
tyr	–2.82	1.8	2.5	5.56	5.42	3.4	–9.588	–5.076	–7.05	–15.6792	–15.2844
phe	–0.28	4.3	3.6	0.29	0.32	3.6	–1.008	–1.204	–1.008	–0.0812	–0.0896
lys	–3.77	0.6	2	0.73	1.12	6.6	–24.882	–2.262	–7.54	–2.7521	–4.2224
his	–2.18	1.9	2	0.48	2.07	2	–4.36	–4.142	–4.36	–1.0464	–4.5126
arg	–6.85	2	3	1.21	2.07	4.9	–33.565	–13.7	–20.55	–8.2885	–14.1795
trp	–0.88	0.4	1.3	1.09	0.96	1.3	–1.144	–0.352	–1.144	–0.9592	–0.8448
						Totals	**–164.147**	**–159.794**	**–165.817**	**–169.616**	**–177.224**

The data for this table are taken from Janssen (1992) (amino acid contents) and Oobtake and Tatsuo (1993) (hydration free energies)
1 cal = 4.18 J.

Table VII). The result is consistent with the calculations of Belton (1995) and shows that the free energies of hydration for HMW subunits are very close to the average value calculated from a sample of 314 proteins of -164 kcal mol^{-1} (-686 kJ mol^{-1}). From this point of view, gluten and high molecular weight subunits are typical hydrophilic proteins. This is entirely consistent with the swelling behaviour of gluten in water (Grant *et al.*, 1999). Furthermore, comparison of the behaviour of the truly hydrophobic protein elastin with HMW subunits (Belton *et al.*, 1994) shows that the latter absorb water on heating whilst elastin ejects it, clearly demonstrating the hydrophilic nature of HMW subunits.

These results do not imply that hydrophobic interactions do not exist, but they do imply that the reason for lack of solubility cannot solely be hydrophobicity. Singh and MacRitchie (2001) have argued that the lack of charged groups may also contribute to the lack of solubility of gluten proteins. This is undoubtedly the case, but the authors fail in their analysis to take account of the importance of polar groups in contributing to polymer solubility. Polar groups such as OH groups can account for the solubility of many polysaccharides; gluten is rich in glutamine and thus polar NH_2 groups, which would be expected to interact favourably with water.

It is clear, therefore, that the insolubility of HMW subunit proteins needs some explanation. We consider that the answer lies mainly in the ΔG_{fus} term, not in the ΔG_{mix} term. The polypeptide polyglutamine is not soluble in water, but exists as an insoluble material with a β-sheet conformation. Similarly, Perutz (1996) has identified the formation of "polar zippers" formed between stretches of glutamine residues in pathological forms of human proteins. Recent modelling work (Belton *et al.*, 2000) has suggested that the formation of intermolecular β-sheet involving glutamine residues present in HMW subunits may also occur. The hydrogen bonding pattern will also have the effect of placing the hydrophobic side chains within the interior of the protein. The role and importance of β-sheet formation will be discussed in more detail later, but for the present we note that the reason for the insolubility of the HMW subunits and other gluten proteins in water is probably the formation of strong inter- and intra-protein interactions rather than hydrophobic interactions with water.

The question still remains as to why it is possible to dissolve HMW subunits in alcohol–water mixtures, and in this case the answer may lie in the balance of hydrophobic and hydrophilic forces. The addition of alcohol will favour the interaction of the hydrophobic groups with the solvent; in addition alcohols are polar and so can interact with the amide groups of the glutamine residues. This interaction will clearly be less favourable than the interaction with water but, combined with favourable

interactions with hydrophobic side chains, may be enough to tip the balance in favour of solubility.

It is important to recognize that disulphide bonds interlink much of the protein present in doughs. This creates very large polymeric units (Lindsay and Skerrit, 1999) that appear to contain both HMW and LMW subunits, with the LMW subunits acting to both extend and terminate the chains of polymers. However, the extractability of glutenins from dough has been shown to be a strong function of mixing (Weegels *et al.*, 1996) and it is assumed that extractability is related to polymer size. Clearly, if the polymer network is very extended, then the behaviour of the dough may be better understood as akin to a swollen polymer gel. However, it is important to note that the presence of the polymer does not alone account for the insolubility of the proteins in water since reduction of the disulphide bonds does not result in water solubility.

B. INTERACTIONS BETWEEN HMW SUBUNITS

As discussed above, consideration of the factors affecting subunit solubility suggest that hydrogen bonding may play a role. Early NMR relaxation time studies of C hordein, the barley homologue of the wheat ω-gliadins, indicated that significant changes in the dynamics of the protein occurred as water was added, even though the protein did not dissolve (Belton and Gil, 1993; Belton *et al.*, 1994). Similar results have since been observed with HMW subunits (Belton *et al.*, 1995) and ω-gliadins (Belton *et al.*, 1998). The C hordeins and ω-gliadins both resemble HMW subunits in containing repeat motifs that are rich in proline and glutamine residues. The similar behaviour of these proteins may therefore be attributed to competition with water for hydrogen bonding with glutamine. Increasing the water content results in a decrease in glutamine–glutamine hydrogen bonds and an increase in glutamine–water hydrogen bonds, leading to an increase in the mobility of the polymer chains.

A number of authors have used FT-IR spectroscopy to determine the structure of the proteins in gluten and related systems (Pezolet *et al.*, 1992; Popineau *et al.*, 1994b; Belton *et al.*, 1995; Wellner *et al.*, 1996; Grant *et al.*, 1999; Gilbert *et al.*, 2000). The broad conclusion from these studies is that gluten and the individual proteins contain β-sheet, β-turn and some α-helix, the latter mainly being associated with nonrepetitive sequences in the *C*- and *N*-termini of the HMW subunits (see above). Detailed studies of the behaviour of HMW subunits and ω-gliadins show that the proteins also undergo changes in conformation with water content. When dry, the proteins are largely disordered, but as water is added there are initially increases in the amounts of β-sheet and then increases in β-turns. More

recent work (Feeney *et al.*, 2002) has shown that similar behaviour is observed with model peptides based on the repeat motifs of the HMW subunits.

The behaviour of the HMW subunits on hydration can be explained in terms of the formation and breaking of hydrogen bonds: at low water levels there are many intra- and inter-chain interactions. However, because there is very little molecular motion, the proteins remain in the disordered state that they adopt during sample preparation (typically freeze-drying). As more water is added, some molecular rearrangements to the lowest energy state will occur, which for glutamine-rich materials will be the β-sheet conformation. As yet more water is added, the water and the side chain amide residues will compete for hydrogen bond formation, decreasing the population of inter-chain β-sheet conformers and increasing the population of extended hydrated β-turn conformers. The transition from β-sheet to β-turn may be correlated with the changes in mobility observed by NMR; the β-sheet regions are of low mobility and the hydrated extended β-turns of high mobility.

C. HIGH MOLECULAR WEIGHT SUBUNITS AND DOUGH VISCOELASTICITY

This interpretation of the spectroscopic results has led to a model for the interactions of HMW subunits in doughs that offers an explanation for dough viscoelasticity. However, before considering this it is important to recognize that a theory of dough rheology is not a theory of breadmaking quality. Although appropriate dough mechanical properties are necessary for breadmaking, they are not sufficient. Bubble stability, oven spring, and the stabilization of structure by starch gelation depend on other factors. Theories of dough rheology can, therefore, only be expected to explain those observations that relate to the mechanical properties of dough.

Before considering theoretical models in more detail, it is important to clarify the thermodynamic basis of elasticity. It is often assumed that the elastic component of gluten viscoelasticity is purely entropic. This can arise because of a misunderstanding of the basic thermodynamics involved. For example, Ewart (1989) assumed that the second law of thermodynamics ensures that all spontaneous changes are entropy-driven. Although it is true that the entropy must increase in a spontaneous change for whole closed systems, this may occur because of enthalpic effects causing a rise in temperature. It is certainly not true for any individual component of that system, and if it were crystallization could not occur. The second law does not, therefore, imply that elasticity must be the result of entropic forces; the total free energy change is critical. In addition to these *a priori* reasons

for not assuming that elasticity is driven by entropy in gluten, recent studies by Tatham *et al.* (2001), in which the temperature coefficient of the elasticity of high molecular weight subunits was measured, have shown empirically that elasticity is not entropic.

Some of the key observations on the mechanical behaviour of dough are as follows:

1. Dough is viscoelastic.
2. The mechanical behaviour of dough is strongly dependent on water content.
3. The mechanical behaviour of dough is strongly dependent on the nature and concentration of HMW subunits present.
4. Mixing in D_2O rather than H_2O results in stronger dough.
5. Esterification of glutamine residues reduces the coherence and the resistance to extension of doughs.
6. The presence of oxidants and reducing agents affects dough rheology.
7. The resistance to extension of doughs reaches a maximum on mixing.

A reasonable starting assumption is that dough consists of a protein matrix in which are embedded starch granules. This matrix contains a cross-linked polymer network with some noncovalently bonded readily extractable proteins. The readily extractable proteins are unlikely to contribute to the elasticity of the dough since elasticity requires an interconnected network, albeit not necessarily a covalently bonded one. Elasticity therefore resides in the network. The network comprises both HMW and LMW subunits, which may interact with each other through covalent disulphide bonds and non-covalent interactions. Although the exact organization and role of the low molecular weight subunits in the polymer is not clear (Lindsay and Skerrit, 1999; Shewry *et al.*, 2001), it is possible that they can act both as chain terminators and extenders. Our current knowledge of the disulphide bond structure of glutenin indicates that most disulphide bonds between subunits are end-to-end, although some branches also occur (see above). Located between these cross-links are the repetitive domains that interact with the repetitive domains of other proteins by hydrogen bonds. Interactions between HMW subunits will therefore tend to increase the cohesiveness of the dough, while interactions between HMW and LMW may increase or decrease cohesiveness, depending on the precise interactions. If the interaction results in the masking of the "interactive surface" of a HMW subunit, then the cohesiveness may be diminished. If, however, the external surface of the LMW subunit can hydrogen bond, then cohesiveness may be unaffected or increased. Interactions between HMW and LMW subunits could, therefore, affect dough rheology by both hydrogen bonding and disulphide interactions. This is illustrated in Figure 23.

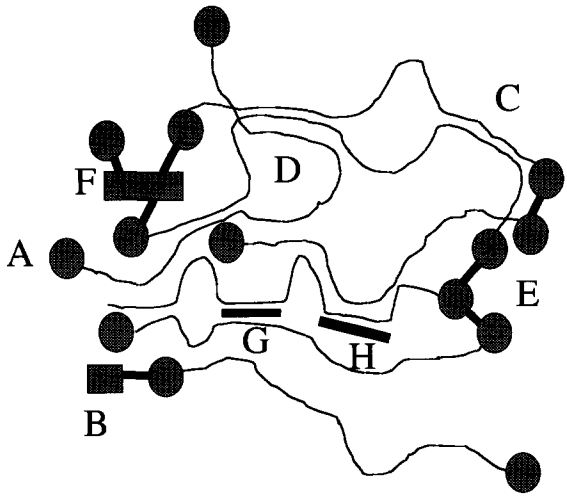


FIG. 23. Schematic representation of the interactions in a glutenin macropolymer. (A) *C*- and *N*-termini of HMW subunits containing cysteine residues; (B) HMW *C*- and *N*-termini blocked by chain-terminating LMW subunit; (C) train region of HMW subunit; (D) loop region of HMW subunits; (E) disulphide cross-linked *C*- and *N*-termini; (F) LMW subunit acting as chain extender; (G) LMW subunits acting as a hydrogen bonding cross-linking unit; (H) LMW subunit preventing the formation of inter-chain hydrogen bonds.

Irrespective of the exact role of LMW subunits, the starting point for the theory of dough rheology is that elasticity originates mainly from interaction between the HMW subunits, although these may be mediated by the LMW subunits. The hydrogen bonded interactions between repeat regions (which we term chains) of these would be expected to be mediated by water. Thus, in the dry state strong interactions would be expected. Addition of water would result in competition between water and amino acid residues to form hydrogen bonds, breaking some of the inter-chain interactions. However, the density of glutamine residues and the proximity of the chains would result in many inter-chain interactions, and it is unlikely that all would be broken simultaneously. Two types of interaction will therefore be present in equilibrium in the hydrated system: hydrated regions, which, as above, are identified with mobile, extended β-turn structures, and regions of subunit interactions identified with rigid β-sheet structures. These two regions have been termed "loops and trains" (Belton, 1995, 1999a), in analogy with the interactions of polymers with surfaces (Dickinson, 1992). In the latter the polymer has many interactions with the surface, making it almost impossible to wash it from the surface once it has been absorbed. Even if the individual interactions

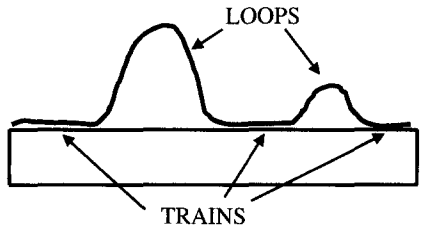


FIG. 24. Schematic depiction of loops and trains formed by a polymer at an interface.

are quite weak, the statistical probability of all the interactions being broken simultaneously is very low. Polymers at interfaces thus exist in train regions where there are a number of interactions between the polymer and surface and solvated loop region. This is illustrated in Figure 24. The "loop and train" hypothesis has been supported by recent work of Alberti *et al.* (2001), who showed, using proton magic angle spinning NMR, that hydrated HMW subunits contained two types of glutamine residues. It is probable that one of these is associated with loops and the other with trains.

As mechanical extension is applied to the gluten network, the first effect will be to extend the loops, as this will require relatively little energy. Further extension will strain the loops and pull the β-sheet train regions apart, while even further extension may result in breakage of the inter-chain disulphide bonds. When the extending force is relaxed the tendency will be to restore the balance between loops and trains, since this is the equilibrium situation. If the time scale for extension is faster than the intrinsic relaxation rate of the polymers, then the application of mechanical force will result in the build up of stored elastic energy of the system. This is illustrated diagrammatically in Figure 25, in which the polymeric network is simplified and shown as a group of cross-linked linear polymers and the LMW subunits omitted.

The model leads to an explanation of some of the points made above. It explains why dough is viscoelastic (point 1) as it provides a mechanism for both extension and elastic recovery. The effects of water (point 2) may be seen in the changes that hydration causes in the ratio of loops to trains. Thus, increasing the water content will result in an increase in the loop to train ratio, which will make extension easier. However, the loop to train ratio cannot increase without limit as it is constrained by the disulphide cross-links that will keep the proteins in close proximity, by the solubility of the proteins which is in turn limited by the interchain interactions, and by breadmaking practice that typically maintains the water content of

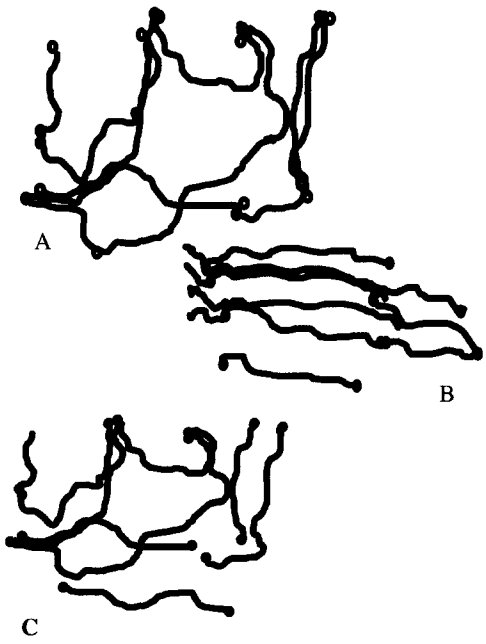


FIG. 25. Schematic depiction of changes in the polymer network in dough during extension. A two-dimensional network is shown for clarity; in reality the network is three-dimensional. (A) Network before extension; (B) the extended network; (C) the network after relaxation. Note that after relaxation some polymers have become detached from the network.

dough at about 65%. Assuming that the water is evenly distributed between all components, this would result in about six water molecules per amino acid residue. A similar calculation has been reported by Ewart (1989). This is a very high protein concentration but it is still not high enough for glass formation at room temperature. The system is, therefore, in a rubbery state (Noel *et al.* 1995; Kalichevsky *et al.*, 1992) and is liable to plasticization by water by the mechanism proposed. It should be noted that the term rubbery is used in this context to denote the opposite of glassy and does not imply that dough behaves according to rubber elasticity theory.

In the hypothesis outlined above the HMW subunits play a crucial role and are assumed to be responsible for most of the gluten viscoelasticity. This may seem surprising, as they only account for about 10% of the total grain protein. However, it is not unusual for the rheology of a food system to be governed by the behaviour of a minor component, and high

molecular mass polymers can radically change the rheology of systems even when concentrations are low. A good example of this phenomenon is agar, which at 1% (w/v) will transform liquid water to a solid self-supporting gel by formation of a continuous network. Thus, the crucial role of the HMW subunits, which account for only about 20% of the total glutenin subunits, is not surprising. Any change in the concentration of the HMW subunits or any changes that might make a difference to the ratio of loops to trains will therefore directly affect the mechanical properties of the system (point 3).

The role of the polymer interactions can also explain the effects of mixing in the presence of D_2O and of the esterification of glutamine residues. Deuterium would form stronger hydrogen bonds than hydrogen in the β-sheet regions, which would result in stronger interactions and stronger doughs. Conversely, the esterification of glutamine residues would reduce the likelihood of β-sheet formation and thus weaken doughs (points 4 and 5).

Oxidizing and reducing agents (point 6) would be expected to have effects on disulphide bonds, with oxidizing agents being assumed to increase the number of disulphide bonds and reducing agents to decrease them (Weegels *et al.*, 1996). Thus, the effects of oxidizing and reducing agents may be through their effects on the connectivity of the network. However, both oxidizing and reducing agents also increase the extractability during mixing. It is probable that reducing agents increase the extractability by reducing the number of disulphide bonds. In contrast, increasing cross-links by oxidation would increase dough stiffness and work input. This could result in more bond breakage, resulting in a fragmented structure and thus increased protein extractability (Weegels *et al.*, 1996). It should be noted that implicit in the interpretation of extractability measurements is the assumption that the controlling factor for extraction is the nature of the polymer network. However, as noted above, equilibrium considerations do not apply in such systems and the controlling factor may be the kinetics of the extraction process. This may depend on such factors as fissures and cracks in the dough improving solvent ingress and solute egress and the effective dough surface area. So far these factors do not appear to have been investigated.

D. EXPLANATION FOR THE ROLE OF THE HMW SUBUNITS IN DOUGH MIXING

In order to understand the maximum resistance observed in doughs during mixing (point 7), it is necessary to explore the process in a more quantitative manner using a simple kinetic model. Figure 25(A) shows a

diagrammatic representation of the network of polymers in dough. In this model the HMW subunit network is simplified to a network of polymers that are cross-linked at the ends and are elastic. The elasticity, as explained above, results from the tendency of the HMW subunit network to return to its equilibrium ratio of loops to trains after extension. After extension the rate of restoration of equilibrium is relatively slow compared with the extension rate in the mixer. Thus, if there are N_0 polymers in the network, on extension due to mixing, the network is stretched and there will be N_S stretched polymers formed. The effect of mechanical extension of the stretched network is to break N_B polymers away from the network with N_U polymers remaining unstretched. If the system is left for a suitably long time after mixing it relaxes to its original unstretched state, but N_B polymers remain broken from it.

Following the work of Gras *et al.* (2000), the best characterized mixing process is that of the Mixograph. They showed that the action of the Mixograph was purely extensional and that extension resulted in the storage of elastic energy in the dough. They reported the behaviour of three different wheat flours in the system. The flours showed the usual behaviour in reaching a maximum in resistance to extension, the height of which deceased with increased water content. Water content also affected the behaviour of the dough after resting, with the resistance to extension decreasing with water content and the degree of extension to break increasing. Longer mixing decreased the resistance to extension and decreased the degree of extension to break of doughs of constant water content, which were rested after different mixing times.

The effects of extension on the network shown in Figure 25 may for simplicity be modelled by first-order kinetics (these are, of course, not realistic for a mixing experiment, but the kinetics of the process are complex and currently not known). First-order models are, therefore, used to illustrate the principles involved and demonstrate that realistic models based on a network theory, combined with the loop and train model, can reproduce some of the features observed during mixing. In the following it is assumed that each revolution of the Mixograph results in the creation of stretched polymers and that the rate of relaxation of the polymers from their stretched state is slow compared with the rate of mixing. Thus, mixing stores mechanical energy in the dough. Further, it is assumed that the formation of the network and the uniform hydration of the dough are fast compared with the mixing process.

The latter assumption may not be true for all mixing processes and some of the observed increase in the resistance of the dough may be due to network build up. However, this would not explain the observation of maxima in dough resistance, merely a monotonic increase and levelling,

and for simplicity it is ignored here. Such an effect could be modelled, however, by simply adding a term for the rate of increase in the number of polymers in the network.

After R revolutions the system can be described by the following equations:

$$dN_S/dR = k_1N_U - k_2N_S \quad (5)$$

$$dN_U/dR = -k_1N_U \quad (6)$$

$$dN_B/dR = k_2N_S \quad (7)$$

and

$$N_U + N_S + N_B = N_0 \quad (8)$$

where k_1 is the rate constant for the rate of creation of stretched polymers and k_2 is the rate constant for the rate of breakage of the polymers.

In order to relate these equations to resistance to extension, additional assumptions about the elasticity of the polymer networks need to be made. Resistance, ρ, is related to the number of polymers by the simple linear equation

$$\rho = E_UN_U + E_SN_S + E_BN_B \quad (9)$$

where E is the resistance to extension per polymer unit.

The most important factor controlling the extensibility of the network will be the number of polymers it contains that are already extended, because as more polymers are extended the possibilities for further extension will decrease. Unextended polymers that are attached to the network will resist extension since they are coupled to it by trains and will have the natural resistance to extension of any polymer. Broken polymers will only contribute viscous drag to the system, thus $E_S > E_U > E_B$.

The variation of the numbers of the various types of polymers with the number of revolutions is shown in Figure 26. Clearly the number of stretched polymers and hence the resistance reaches a maximum. Figure 27 shows the effects of varying the rate constant for the formation of broken polymers. A large value of k_2 results in a sharp maximum whereas a low value results in a flattening of the curve. It may be expected that stiffer doughs will result in the input of more mechanical energy for dough extension and hence to more breakages in the polymer network.

The effects of water content may be understood in terms of the behaviour E_U and E_B: as water content increases it would be expected that the loop to train ratio would increase. This would result in easier extension

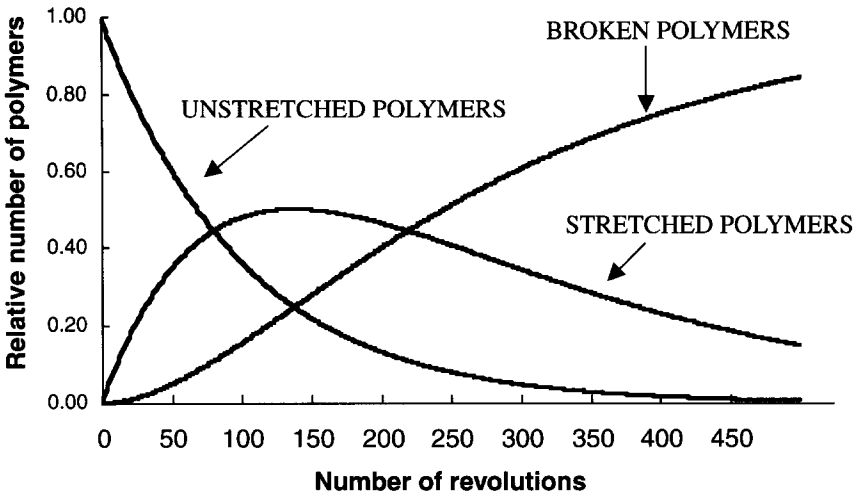


FIG. 26. The effects of the number of mixer revolutions on the populations of unstretched, stretched and broken polymers in a dough.

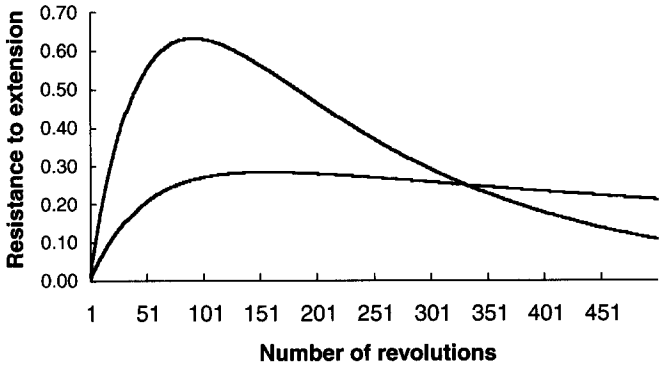


FIG. 27. The effect of changing the rate constant for polymer breakage (k_2) on the shape of the mixing curve. The flattened curve has a low value of k_2 and the curve with a sharper maximum has a higher value of k_2. Values of k_1 are kept constant and resistance values are normalized to be on a similar scale.

of the unstretched polymers and would therefore decrease the resistance to extension of the dough. This would in turn result in a lower maximum resistance, since the total resistance is that of the stretched polymers plus that of the other polymers. It would also result in a lower breakage rate of the polymers since less energy would be required to extend the dough. The

changes in resistance to extension after resting can also be understood. Energy input into the doughs will result in an increase in the number of broken polymers. After resting, the polymer network will relax to its equilibrium configuration but the number of polymers in the network will have decreased. This will result in decreased resistance to extension, as the number of polymers in the network available to be stretched will be lowered. In addition, there will be a reduced degree of extension to break since the network integrity will be reduced by the loss of polymers from it.

E. IS THE LOOP AND TRAIN HYPOTHESIS CONSISTENT WITH OTHER MODELS?

The combination of the loop and train model with a simple kinetic approach offers an explanation of the role of the HMW subunits in determining many of the phenomena observed in dough viscoelasticity and mixing. By recognizing the importance of network formation by covalent linkages and the role of hydrogen bonding in understanding of the elastic component of dough behaviour, a testable hypothesis is formulated that is consistent with both mechanical and spectroscopic behaviour. Some other theories of the mechanical behaviour of doughs have also been formulated. (For a thoughtful review of some earlier work see El-Dash, 1991.) Ewart (1989) has formulated a hypothesis that assumes that the "glutenin molecules" are arranged in chains of folded units; on stretching these units unfold and the elastic restoring force is said to be entropic. As pointed out above, this argument is based on a misunderstanding. The glutenin molecules are assumed to be oriented by the shearing process in dough and overlap between oriented molecules is suggested to augment noncovalent interactions between these molecules. This proposal is not dissimilar to the loop and train model in that it recognizes the role of noncovalent interactions and a role for the stretching of the molecules in elasticity. However, explicit mechanisms are lacking and, apart from the prediction of the effects of shear on orientation, little is offered in the way of testable predictions.

Singh and MacRitchie (2001) consider the applications of polymer science to the properties of gluten. Some of the problems of their approach have been discussed above. The core of their argument is that resistance to extension of the dough arises through chain entanglements. However, these are not the generalized "entanglements" of classical polymer theory in which interactions between polymers are represented by a constraining tube (Doi and Edwards, 1986; Belton, 1999b), but specific points at which it appears that the polymers form tangles analogous to those observed on the macroscopic scale in string. Slippage through these entanglements is

proposed to cause resistance to extension. The authors then consider the effects of molecular weight on slippage and the draw ratio of the material. The problem with this approach is that it is nonspecific; it does not explain why any polymer of suitable molecular weight does not behave like dough or why specific effects of D_2O and esterification are observed. The effects of water are discussed in terms of the glass transition temperature of polymer–water systems and it is suggested that zein proteins of maize do not show dough-like properties at room temperature because of glass transition effects. In fact, the glass transition temperatures of wheat gluten are below 20°C for gluten when it has greater than 15% water content (Kalichevsky *et al.*, 1992; Noel *et al.*, 1995) and similar figures are reported for zein (Madeka and Kokini, 1996). Arguments about a role for glass transitions in dough rheology seem therefore to be untenable.

El-Dash (1991) has proposed a mechanism of viscoelasticity that has some features in common with the loop and train model and with Ewart's model. He emphasizes that the relative rarity of cysteine residues in glutenin ensures that disulphide interchange is unlikely to be a cause of network formation and assumes that the disulphide-bonded network pre-exists. Noncovalent interactions are considered to be responsible for holding coiled, but not necessarily ordered, molecules together in chains and sheets; these are interconnected by the relatively rare disulphide bridges. Mixing enhances noncovalent intermolecular interactions by bringing more molecules into contact with each other while continued mechanical stress causes unfolding of the coiled molecules. The recovery of the coiled state is the mechanism of elasticity. Interestingly, he points out that any polymeric system in which covalent cross-links exist together with non-covalent intermolecular interactions might be made to demonstrate similar viscoelasticity to gluten.

All of the other models proposed therefore offer some insights, but none of them have been sufficiently developed to make testable predictions about the nature of dough viscoelasticity or to generate testable hypotheses.

IX. CONCLUSIONS AND FUTURE PROSPECTS

The initial demonstration of correlations between HMW subunit composition and breadmaking quality of wheat stimulated a wide range of studies (biochemical, biophysical and genetic) that demonstrated that these proteins play a crucial role in determining the viscoelastic properties of hydrated gluten and the mixing properties of dough.

Furthermore, the relationship between the allelic composition of the HMW subunits and dough mechanical properties has been shown to result

from both quantitative (i.e. subunit amount) and qualitative effects, the latter relating to differences in the structures and properties of individual subunits. In this respect the glutamine-rich repeated sequence domain may be particularly important in establishing inter-molecular interactions that contribute to both cohesiveness and elasticity.

Although the primary and secondary structures of the HMW subunits have been studied in some detail, we still know very little about their higher-order structures. However, it is clear that the functional state of gluten consists of proteins assembled into polymers or aggregates, stabilized by both covalent disulphide bonds and noncovalent forces (principally hydrogen bonds). These protein assemblies are essential for the expression of the mechanical properties; isolated HMW subunits are not viscoelastic. Despite the paucity of our knowledge of the structures of these assemblies, it is possible to propose a plausible physicochemical mechanism for the role of the HMW subunits in gluten viscoelasticity, based on studies of the structures and interactions of isolated subunits.

In addition to the genetic effects discussed above, it is clear that environmental factors also affect the structure and properties of the gluten polymers and hence grain processing quality. This results in extensive year-to-year and site-to-site variation, posing problems for the grain utilizing industries. Furthermore, there is also evidence for the existence of genotype × environment (G × E) interactions, meaning that cultivars may be differentially affected by environmental factors.

The elucidation of the detailed structures and properties of the glutenin polymers is clearly an important goal for wheat scientists. This will not only require the analysis of glutenin polymers isolated from dough, but also the determination of their mechanisms of synthesis and its regulation, their interactions with other dough components (notably lipids, starch and arabinoxylans) and dynamic aspects of their changes in structure during grain maturation and dough mixing. This is clearly a formidable challenge, but one that can be faced using the combination of modern genetic, molecular, biochemical and biophysical approaches discussed above.

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