

Molecular Weight Distribution of Wheat Proteins

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ABSTRACT

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The molecular weight distribution (MWD) of wheat proteins is becoming recognized as the main determinant of physical dough properties. Studies of high polymers have shown that properties such as tensile strength are related to a fraction of polymer with molecular weight above a critical value and the MWD of this fraction. Elongation to break is treated as a kinetic process with energies of activation for breaking noncovalent bonds and for chain slippage through entanglements. These considerations are related to tensile properties of wheat flour doughs such as those measured by the extensigraph. The MWD of wheat proteins is determined by the relative amounts of monomeric and polymeric proteins and the MWD of the polymeric proteins. The latter, in

turn, depends on the ratio of high molecular weight glutenin subunits (HMW-GS) to low molecular weight glutenin subunits (LMW-GS), the specific HMW-GS that result from allelic variation, and the presence of modified gliadins that act as chain terminators. The role of these compositional variables in determining dough extensibility is discussed in terms of present knowledge. Determination of MWD of wheat proteins is hindered by the difficulty of their solubilization and the lack of methods for reliably measuring very high molecular weights. Among the promising techniques for achieving these measurements are multi-angle laser light scattering (MALLS) and field flow fractionation (FFF).

It has long been known that the properties of hydrated gluten or wheat flour dough depend on two main types of protein, the glutenins and gliadins. Hydrated gliadin exhibits plasticity, whereas hydrated glutenin has strong elastic properties. The commercially desirable viscoelastic properties required for good performance in processing wheat flour doughs result from the combined contributions of these two main types of proteins. The reason protein governs dough properties is that during dough development it forms a continuous network throughout the dough. The physical properties of a colloidal system are invariably dependent on the continuous phase.

In recent times, research has increased our knowledge of the complex mixture of wheat proteins. However, the view that two main groups of proteins control dough properties has not changed. Currently, it is customary to think in terms of monomeric and polymeric proteins, although gliadins and glutenins are known to be the main components of these two groups, respectively. The monomeric proteins consist of single chain polypeptides. Their molecular weights range from approximately 20,000 (monomeric albumins and globulins) up to $\approx 70,000$ (ω -gliadins). In contrast, the polymeric proteins are multiple chain polymers in which the individual polypeptides or subunits are linked by disulfide bonds. The individual subunits form two main groups, HMW-GS and LMW-GS. The apparent molecular weight based on SDS-PAGE of the former (A subunits) fall in the range of 80,000–120,000 and the latter into two groups, 40,000–55,000 (B subunits) and 30,000–40,000 (C subunits). As a result of this polymerizing capacity, the molecular weight of glutenin can vary over wide limits. At the lower limit, this can be roughly 100,000. The upper limits have been difficult to determine, but values in the order of millions with a wide distribution have been obtained. Weight average molecular weights of 2×10^6 were reported by Jones et al (1961). Huebner and Wall (1975) estimated by gel-permeation chromatography that the largest size glutenin fraction that eluted at the void volume had a molecular weight $>20 \times 10^6$. Estimates of 5×10^6 have been

made for the largest glutenins based on gel-permeation chromatography (Schofield et al 1983). All these measurements are roughly in agreement with a size distribution of glutenin calculated by Ewart (1987) based on standard theory of high polymers assuming linear molecules. The most probable weight fraction had a molecular weight of $\approx 1 \times 10^6$, and molecular weights extended to $>5 \times 10^6$. More recent measurements have also pointed to molecular weights in the millions (Stevenson and Preston 1996, Wahlund et al 1996).

The MWD of wheat proteins can be varied in two main ways (Fig. 1): the relative amounts of monomeric and polymeric proteins can change or the MWD of polymeric protein can vary from one sample of wheat (or one cultivar) to another. Both the monomeric-to-polymeric ratio and the MWD of polymeric protein are genetically controlled but can be modified by environmental conditions.

Although, intuitively, it was thought that the gliadin-to-glutenin ratio should explain differences in properties between wheat flours, much of the early work in cereal chemistry failed to establish this link. One reason is the difficulty of measuring this ratio because the methodology depended on solubility in which pure fractions of gliadin and glutenin were unattainable. Another factor that increased the difficulty was the failure to take into account differences in the properties of glutenin between samples. For example, in theory, one wheat flour could have a higher ratio of glutenin to gliadin than another but, if its glutenin were of lower strength, the expected relationship would not be seen. Differences in strength of glutenins from different wheat samples are related to differences in MWD.

With the application of size-exclusion HPLC to wheat proteins pioneered by Bietz (1984) and developed by many workers (e.g., Dachkevitch and Autran 1989), it has become possible to measure the relative quantities of monomeric and polymeric proteins with accuracy. For example, the largest glutenin molecules can be broken down and solubilized by using sonication (Singh and MacRitchie 1989). Because sonication (like other mechanical degradation processes) is a selective process in which the largest molecules are split near the centers (Bueche 1960), the degradation products fall within the size range of glutenins. Nevertheless, measurement of the true MWD of glutenin remains a difficult problem for two reasons. The first reason is that no procedure has been developed for solubilizing the total wheat protein without modifying it in some way. The most difficult protein to solubilize is the largest

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size molecular glutenin. Two main factors that appear to contribute are the relative dearth of ionizable groups and the large size of the molecules. Other things being equal, a larger molecule will be more insoluble than a smaller one, simply because the number of arrangements with solvent molecules (entropy of mixing) is lower. The second reason is that there is a lack of reliable techniques for measuring molecular weights of very large molecules. In general, most methods for molecular weight determinations lose their sensitivity as the molecular weight increases. This article will be devoted to discussing what is known about MWD of wheat proteins and some of the newer approaches to tackling this difficult problem.

MOLECULAR WEIGHT DISTRIBUTION OF MACROMOLECULES

The gene products in wheat proteins are monomeric proteins and polymeric protein subunits. The polymeric protein subunits form large proteins by a posttranslational process that is not well understood but apparently occurs relatively slowly during grain development (Gupta et al 1996). As a result, a polydispersed protein is formed (i.e., a protein with a mixture of chemically similar compounds of various molecular weights). As well as confirming a polydispersed nature, experimental evidence favors a predominantly linear character with little, if any, branching (Ewart 1990), although other models have been proposed (Bernardin and Kasarda 1973, Graveland et al 1985, Ng et al 1992). The possibility of branching was considered by Bietz and Huebner (1980) and is inherent in the model of Graveland et al (1985). Based on the model of Ewart (1990), polymeric proteins could behave similarly to synthetic linear polymers. Consequently, much of the knowledge that has been built up in studies of synthetic polymers might be applied to polymeric proteins.

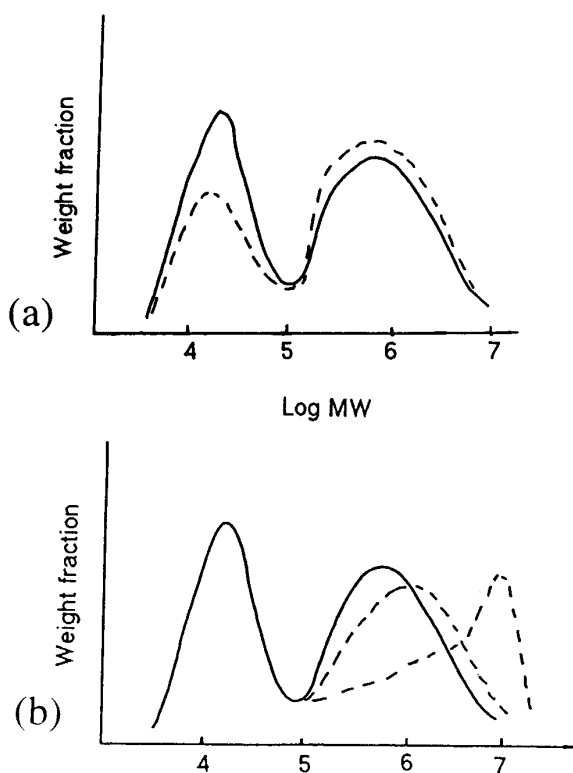


Fig. 1. Schematic representation of the two main ways in which the molecular weight distribution (MWD) of flour protein can be altered: (a) by changes in the relative proportions of monomeric and polymeric proteins; (b) by change in the size distribution of polymeric proteins. Dashed lines show alternate MWD (MacRitchie and Lafiandra 1997).

The dependence of physical properties of high polymers on molecular weight has been known for a long time. At low molecular weight, providing its glass transition temperature (T_g) is below ambient temperature, a polymer may exist as a viscous liquid. At higher molecular weight, it may become an elastomer with low strength and high elongation to break (extensibility) in tensile stress tests. Above a molecular weight of $\approx 10^5$, polymers show the phenomenon of molecular entanglements and have true rubbery behavior with greatly increased strength and elongation to break. In view of their polydispersed nature, attempts have been made to characterize polymers by an average molecular weight. Lansing and Kraemer (1935) were the first to draw a distinction between different kinds of average molecular weights expected to be obtained from different physical measurements. The simplest is the number-average molecular weight, which is the weight of the whole divided by the number of molecules in the sample. This is the average that is obtained from measurements of colligative properties such as osmotic pressure or freezing point depression. On the other hand, the weight-average molecular weight gives an extra weighting to large molecules. This is the average obtained by the measurement of any property whose intensity is proportional not only to the amount of polymer present but to the mass of the particle. Such properties are those measured by light scattering or ultracentrifugation. Another average, the z-average, which gives a still higher weighting to the large molecules of the size range, is also used.

$$\text{Number average, } \bar{M}_n = \frac{\sum_{i=1}^{\infty} N_i M_i}{\sum_{i=1}^{\infty} N_i} \quad (1)$$

$$\text{Weight average, } \bar{M}_w = \frac{\sum_{i=1}^{\infty} N_i M_i^2}{\sum_{i=1}^{\infty} N_i M_i} \quad (2)$$

$$\text{Z average, } \bar{M}_z = \frac{\sum_{i=1}^{\infty} N_i M_i^3}{\sum_{i=1}^{\infty} N_i M_i^2} \quad (3)$$

where N_i , M_i are the number of molecules and molecular weight of the i th component. From measurements of viscosity, a viscosity average molecular weight can be calculated, and its value lies between the number average and weight average, the exact relationship depending on the character of the individual polymer as well as the solvent (Schaeffgen and Flory 1948). These molecular weight averages are illustrated in Fig. 2. For a monodispersed polymer, the number and weight-average molecular weights coincide. As polydispersity increases, differences between them increase.

It has become well established that the largest effects on the physical properties of polymers are due to the molecular weight and the MWD. Specifications for wheat flours are, to a large extent, based on physical dough properties such as mixing characteristics (mixograph, farinograph) and uniaxial (extensigraph) and biaxial (alveograph) extension. Therefore, we will summarize some recent approaches to describing tensile properties of polymers in terms of molecular weight and MWD.

An equation of Flory (1945) has been used to predict the tensile strength (σ) of polymers:

$$\sigma = \sigma_0(1 - M_T/M_n) \quad (4)$$

where σ_0 = the limiting tensile strength at high molecular weight; M_T = a critical or threshold molecular weight; and M_n = the number-average molecular weight of the polymer

Bersted and Anderson (1990) found that this equation described well the tensile strength of monodispersed but not polydispersed polymers. Therefore, they developed a modified version of the

Flory equation (Eq. 4) that appeared to be more applicable to poly-dispersed polymers. Their basic premise was that only those molecules that formed effective entanglements contribute to strength:

$$\sigma = \sigma_0(1 - M_T/M_n^*)\phi \quad (5)$$

where σ_0 = the limiting tensile strength as in Equation 4; M_T = the threshold molecular weight for effective entanglements; ϕ = the fraction with molecular weight $>M_T$; M_n^* = the number-average molecular weight of this fraction (i.e., the fraction with molecular weight $>M_T$).

The problem of elongation to break (or draw ratio) has been tackled by Termonia and Smith (1987). Two kinetic processes were considered relevant. The first was the breaking of secondary valence bonds between polymer strands. Once these chain strands between entanglement nodes are fully stretched, further movement of chains relative to one another can only occur by chain slippage through entanglements. The two kinetic processes, breaking of non-covalent bonds and slippage through entanglements were treated by Eyring activation rate theory, each with its appropriate activation energy. The magnitude of the elongation to break (extensibility) is largely determined by the rate of chain slippage through entanglements relative to the rate of elongation of the sample (MacRitchie and Lafiandra 1997).

The above considerations are relevant to the physical properties of doughs that determine the suitability of wheat flours for different end uses. The theories of tensile strength and elongation to break in tensile measurements of polymers can be directly applied to understanding of how protein composition governs variability in dough strength and extensibility. Dough mixing characteristics can also be related to polymer behavior. The shear and tensile stresses in a dough mixer develop the gluten protein into a continuous network to impart viscoelastic properties to the dough. At a molecular level, it may be deduced that these forces stretch the large glutenin molecules into more extended conformations. With only monomeric proteins (gliadins) present in admixture with starch, there is no development stage and no dough-like properties with accompanying elasticity. The larger the size of the glutenin, the more energy is required to unravel the molecules to develop the dough. Consequently, the larger the molecules, the greater the restoring forces and, thus, the elasticity.

PROTEIN COMPOSITION AND MWD FUNCTIONALITY RELATIONSHIPS

The task of relating wheat protein MWD to dough properties has proved elusive because of the difficulty in totally solubilizing the protein to characterize it. The use of sonication combined with size-exclusion HPLC (SE-HPLC) has enabled fairly accurate determinations of the proportions of the three major protein classes in flour samples (Singh et al 1990a). Therefore, of the two ways in which MWD can be varied, as depicted in Fig. 1, the variation in the polymeric-to-monomeric ratio can be determined. It enabled this MWD variable to be correlated with various dough property parameters (Gupta et al 1992). An example is shown in Fig. 3, where extensigraph maximum resistance (R_{max}) is plotted against the percentage of polymeric protein in the total protein (PPP) for a set of flour samples from 15 cultivars grown at six nitrogen fertilizer levels. The moderately high correlation ($r = 0.665^{***}$) explains $\approx 40\%$ of the variation. Similar behavior was found for mixograph dough development time (MDDT), another measure of dough strength closely related to R_{max} . A clue to the origin of the unexplained variation may be gleaned from the clustering of points corresponding to the different cultivars. For example, points for the cultivar Halberd fall above the line of best fit, whereas those for the cultivar Israel M68 fall well below this line. Densitometry of the SDS-PAGE patterns for polymeric protein from each of the two cultivars under reducing conditions (i.e., causing breaking of the interchain S-S bonds) gave average

values for the HMW/LMW ratio of 0.34 for Halberd and 0.18 for Israel M68. On this basis, it is suggested that the MWD of the polymeric protein from Halberd is shifted to higher molecular weight than that for Israel M68. Confirmation of the positive relationship between the HMW/LMW ratio and the MWD has been obtained by varying this ratio and obtaining relative estimations of the MWD from the proportion of polymeric protein unextractable by an SDS solution (UPP) (Gupta et al 1993) (Fig. 4). The range of the HMW/LMW ratio was obtained by using flour samples from a single wheat cultivar (Olympic) grown under varying sulfur fertilizer levels. The basis for this variation is that, as sulfur fertilizer becomes limiting, the proportions of relatively S-poor proteins increase to the detriment of the relatively S-rich proteins. HMW-GS are S-poor relative to LMW-GS. It is concluded that the unexplained variation in R_{max} deduced from the correlation coefficient for the linear regression of Fig. 3 may be related to the MWD of the polymeric protein.

UPP as a Relative Measure of MWD

The solubility of a polymer decreases with increasing molecular size. This relationship has been used to obtain a relative measure of the MWD of the polymeric protein based on the proportion that

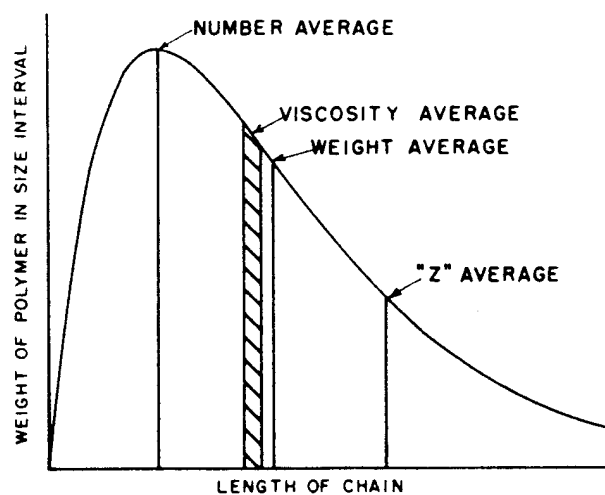


Fig. 2. Qualitative relationship of molecular weight averages (McGrew 1958).

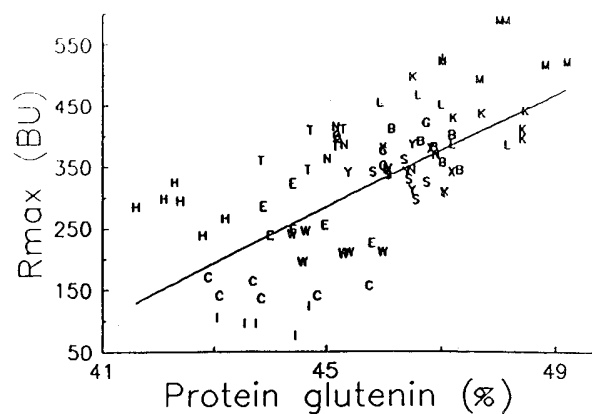


Fig. 3. Plot of extensigraph maximum resistance (R_{max}) vs. polymeric protein in the total protein (PPP) for 84 flour samples comprising 15 cultivars grown at six nitrogen levels (six samples omitted because of insufficient quantity): C, Chile 1B; N, Condor; K, Cook; E, Egret; B, Gabo; G, Gamenya; H, Halberd; I, Israel M68; M, Mexico 8156; L, Olympic; S, Osprey; X, Oxley; T, Timgalen; Y, Wyuna; W, WW15. Line of best fit is shown. Correlation for linear regression was 0.665^{***} (Gupta et al 1992).

is unextractable in SDS solution (Gupta et al 1993). The procedure is based on an initial extraction with SDS solution to extract practically all the monomeric and a portion of the polymeric protein. The remaining protein (UPP) is then solubilized by sonicating a suspension of the residue in SDS solution. A quantitative measure of the percentage of UPP is then obtained by running the extractable and unextractable protein samples on SE-HPLC. Simplifications to the procedure have subsequently been made by Sapirstein and Johnson (1996) and Bean et al (1998).

Application to Composition-Functionality Relationships

Although dough strength as measured by R_{max} has correlated with the percentage of polymeric protein (PPP) in surveys of a number of field trials, there have been some sets of flour samples where this relationship has either not been found or has been tenuous. However, in these cases R_{max} correlates well with UPP. This protein fraction has been termed the glutenin macropolymer in studies by Hamer and coworkers (Weegels et al 1996) where it was related to quality parameters. A schematic interpretation of the result is depicted in Fig. 5. The high correlation with UPP and not with PPP suggests that not all the polymeric protein, but only a fraction of the highest molecular weight, contributes to dough strength (R_{max}). This result has been further investigated in a study of 158 flour samples from 31 wheat lines grown at five locations (with one extra line grown at three of the locations). In this work,

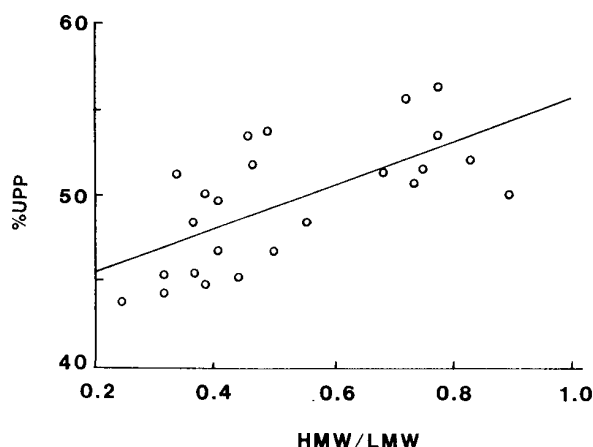


Fig. 4. Unextractable polymeric protein (UPP) vs. ratio of high molecular weight glutenin subunits to low molecular weight glutenin subunits (HMW/LMW-GS) for Olympic flour samples grown at different sulfur and nitrogen fertilizer levels (MacRitchie and Gupta 1993).

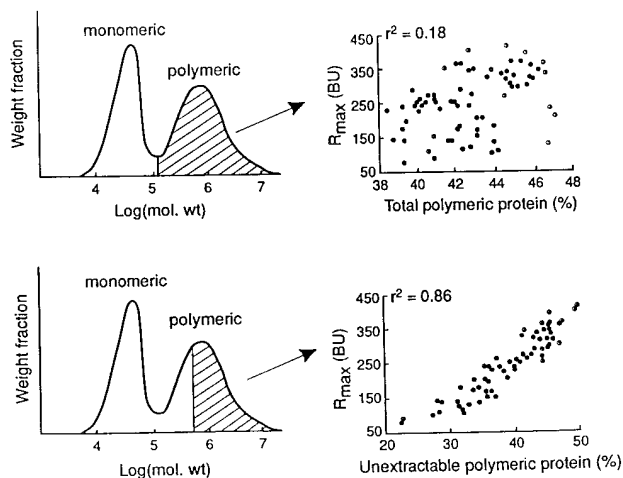


Fig. 5. Schematic representation of the total and unextractable polymeric protein and plots of extensigraph maximum resistance (R_{max}) vs. those for 74 recombinant inbred lines (MacRitchie and Lafiandra 1997).

the area under the SE-HPLC trace was evaluated at elution time intervals of 0.4 min as shown in Fig. 6, and the area up to each elution time was correlated with the R_{max} and extensibility (Ext) values for all lines. The elution times at which maximum correlation coefficients (r) were found for R_{max} and Ext at each of the locations and for the whole sample set are summarized in Table I. For both R_{max} and Ext, r increased as elution time increased; reached a maximum value different for each parameter; and subsequently declined. In agreement with Fig. 5, a maximum value for r occurred for R_{max} at an elution time of 13.2 min for the whole sample set (i.e., the fraction of polymeric protein eluting from zero to that time gave the highest correlation). The cut-off point at 13.2 min corresponded to a molecular weight of $\approx 250,000$ based on a calibration of the SE-HPLC column using standard proteins. This can only be considered to be a rough estimation because it assumes a similar conformation for glutenin molecules to standard globular proteins. Nevertheless, the basic conclusion that only polymeric proteins above a certain molecular size contribute to dough strength appears to be well established. This result is in agreement with the theory of Bersted and Anderson (1990). For the sample set considered, the fraction contributing to strength based on the elution time for highest r value represented $\approx 60\%$ of the total polymeric protein. Table I shows that the maximum values of r for R_{max} are quite low, thus explaining only a relatively small part of the variation in R_{max} . In the theory of Bersted and Anderson (1990), two main variables determine tensile strength: the fraction of polymer above the threshold molecular weight and the number-average molecular weight of this fraction. The latter parameter was not amenable to measurement in this work, as most

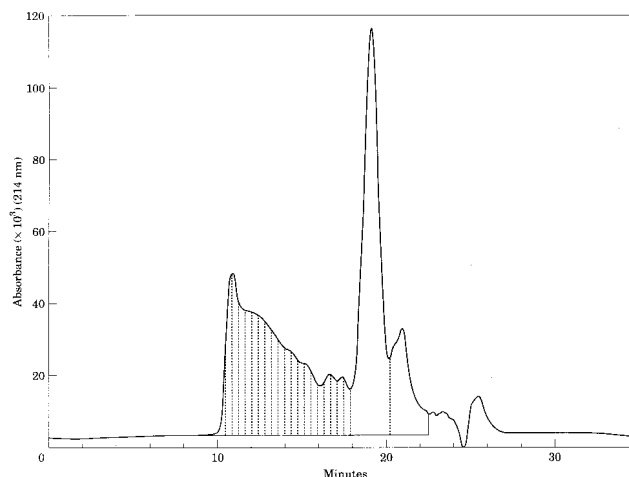


Fig. 6. Size-exclusion HPLC protein profile of one flour sample from the 158 sample set illustrating division of chromatogram at elution intervals of 0.4 min (Bangur et al 1997).

TABLE I
Elution Times for Highest Correlations of Extensigraph Maximum Resistance (R_{max}) Against Cumulative Percentage of Polymeric Protein (PPP) and Extensibility (Ext) vs. Cumulative Flour Polymeric Protein (FPP) at Five Sites and for Combined Data at All Sites^a

Site	Elution Times (maximum correlation coefficients)	
	R_{max} vs. PPP (min)	Ext vs. FPP (min)
Dooen ($n = 31$)	14.4 (0.510***) ^b	16.0 (0.662***)
Narrabri ($n = 32$)	13.2 (0.430**)	16.4 (0.791***)
Turretfield ($n = 32$)	13.6 (0.696***)	16.8 (0.742***)
Wagga ($n = 32$)	12.8 (0.643***)	16.4 (0.744***)
Wongan Hills ($n = 31$)	13.2 (0.446**)	16.8 (0.649***)
All sites ($n = 158$)	13.2 (0.489***)	16.8 (0.859***)

^a Set of 31 wheat cultivars grown at all sites and an extra one at three locations.

^b ***, ** = Differences at 0.01 and 0.05 levels of significance (Bangur et al 1997).

of the protein in this range elutes at the void volume and therefore gives no indication of MWD. It appears reasonable to believe that there would be appreciable differences of the MWD in this region, and that this could account for another large part of the variation in R_{max} .

Dough Extensibility

The two extensigraph parameters, R_{max} and Ext, have different dependence on protein composition (Table I). Unlike R_{max} , Ext appears to relate to the total proportion of flour polymeric protein (FPP) in agreement with previous results (Gupta et al 1992). For more information on the factors crucial to extensibility, two pairs of wheat lines were chosen for a more detailed study (Larroque et al 1999). The individual lines from each pair differed appreciably in extensibility, this difference being maintained over five different locations. Table II summarizes the extensigraph data, averaged over the five sites, for each sample from the pair of lines DD118 and RAC704, together with values of flour protein content, PPP, and UPP determined by SE-HPLC. In Table III, corresponding data for each line of the other pair (VF304 and RAC746) are tabulated. The DD118-RAC704 pair of lines is interesting because their HMW-GS composition is identical and their LMW-GS composition is similar. As a result, the average values of R_{max} over the five locations are similar. The glutenin subunit composition of the RAC746-VF304 pair are different. RAC746 has HMW-GS 5+10 (*Glu-D1*) and 7+9 (*Glu-B1*), known to contribute strength, whereas VF304 has HMW-GS 2+12 (*Glu-D1*) and 20x+20y (*Glu-B1*), linked to lack of strength. This is reflected in the higher values of R_{max} for RAC746 than VF304. For each of the four lines, there is high correlation between Ext and FPP, consistent with previous observations. For the DD118-RAC704 pair, most of the difference in Ext appears to be explainable by a difference in FPP between the two lines. In contrast, FPP is closely similar for the lines RAC746 and VF304. However, UPP is significantly different between these two lines, which is not the case for the DD118 and RAC704. The presence of certain HMW-GS that are associated with dough strength (e.g., 5+10) have increased UPP and by deduction shift the MWD of the glutenin to higher molecular weights (Gupta et al 1994). These have also contributed to decreased extensibility in some cases (Gupta and MacRitchie 1994). The results suggest that larger sized glutenins in the flour from RAC746 are contributing to its lower extensibility. In the theory of Termonia and Smith (1987), the rate of slippage of chain strands through entanglement nodes becomes lower for larger molecules because of the stronger network that is formed. This would then contribute to lower extensibility.

In summary, two factors emerge that appear to govern Ext, the FPP and the MWD of this protein. FPP depends on FP, which is largely environmentally determined, and PPP, which is largely genetically controlled. This is clear when there is a wide range of PPP depending on the genotype (Fig. 3). For example, Mexico 8156 is characterized by a high value for PPP, whereas lines such as Halberd and Israel M68 have low values. On the other hand, the MWD of the polymeric protein is mainly genetically determined, if we are comparing cultivars grown under the same conditions. If, however, we compare a cultivar grown at different locations or in different seasons, MWD can be affected by environment.

EFFECTS OF SUBUNIT COMPOSITION ON MWD

HMW-GS/LMW-GS Ratio

The origins of the differences in PPP (or the related parameter, polymeric-to-monomeric protein ratio) in different genotypes has been discussed by Singh et al (1990b) in terms of the numbers of proteins and subunits in the different protein classes and their degree of expression. This provides one of the main ways of varying the MWD as illustrated in Fig. 1. In this article, we are mainly concerned with the other variation in the MWD, namely

changes in the size distribution of the polymeric and, in particular, the glutenin proteins. There are several possible ways of effecting this variation. We have already seen from Figs. 3 and 4 that the effect of increasing the HMW-GS/LMW-GS ratio is to shift the MWD to higher molecular weight. This conclusion is consistent with analyses of subfractions of polymeric protein which showed that this ratio decreased as the molecular weight decreased (Larroque et al 1997). In addition, this work indicated that the ratio of B/C LMW-GS decreased as the molecular weight attained lower values. Evidence that the B/C LMW-GS ratio related positively to MWD and dough strength has also been reported by Gupta and MacRitchie (1994). Further confirmation of the dependence of the MWD (and dough strength based either on R_{max} and MDDT or dynamic rheological measurements of the gluten) on the HMW-GS/LMW-GS ratio has been obtained by studying special genetic lines in which either the HMW or LMW subunits have been progressively deleted (Gupta et al 1991, Gupta et al 1995). Loss of all *Glu-1* subunits (HMW-GS), on an equal weight basis, reduced the amounts of large polymers to a much greater extent than loss of the *Glu-3* subunits (LMW-GS).

Allelic Variation in HMW-GS

Important work by Payne and coworkers (1987) established that dough strength and baking performance of wheat cultivars were related to allelic variation in HMW-GS. As a result of correlations of different alleles with dough properties, a system of quality scores was assigned to HMW-GS. The nomenclature for HMW-GS was based on electrophoretic mobility, where subunit 1 had the lowest mobility. As more HMW subunits have been discovered, it has been necessary to modify the nomenclature so that there are now subunits with lower mobility than that designated as subunit 1, and numbers such as 2.1* have been introduced. The quality scores assigned to the HMW-GS range from 0 (null allele) to 4. The HMW-GS pair 5+10 coded by *Glu-D1* has been assigned a score of 4 as this pair has been associated with the greatest dough strength. Its *Glu-D1* counterpart, the pair 2+12, on the other hand, has been assigned a score of 2, reflecting its association with dough weakness. Based on similar correlations at the *Glu-B1* locus, the pair 17+18 is given a score of 3, whereas the subunits 20x+20y, also coded at *Glu-B1*, is given a score of 1. In subsets of certain wheat lines, the expected effects of allelic variation at HMW-GS loci have not always been observed. One reason may be the swamping effects of other variation in the genetic background. This variability in background was eliminated in a

TABLE II
Extensigraph-Protein Composition Data for Two Wheat Lines^a

Line	R_{max} (BU)	Ext (cm)	FPP (%)	UPP (%)
RAC704	452	18.1	5.3	50.2
DD118	454	23.5	5.9	51.3
LSD ^b	104	1.65	0.35	1.9

^a Averages for five sites (Larroque et al 1999). R_{max} = maximum extensigraph resistance; Ext = extensigraph extensibility; FPP = flour polymeric protein; UPP = unextractable polymeric protein; Correlations: $r = 0.84^{***}$ (Ext-FPP) and $r = 0.24$ (Ext-UPP).

^b Least significant difference ($P < 0.05$).

TABLE III
Extensigraph-Protein Composition Data for Two Wheat Lines^a

Line	R_{max} (BU)	Ext (cm)	FPP (%)	UPP (%)
RAC746	398	17.4	4.5	51.2
VF304	274	22.4	4.6	45.4
LSD ^b	99	3.4	0.2	2.2

^a Averages for five sites (Larroque et al 1999). R_{max} = maximum extensigraph resistance; Ext = extensigraph extensibility; FPP = flour polymeric protein; UPP = unextractable polymeric protein; Correlations: $r = 0.78^{***}$ (Ext-FPP) and $r = -0.82^{***}$ (Ext-UPP).

^b Least significant difference ($P < 0.05$).

study by Gupta and MacRitchie (1994) by comparing three pairs of near-isogenic lines differing only in allelic expression at the *Glu-D1* locus, one line of each pair with subunits 5+10 and the other subunits 2+12.

The association of HMW-GS with dough strength is related to their effects on MWD of the glutenin proteins estimated from the UPP. Figure 7 shows some results for three pairs of near-isogenic lines that differ at the *Glu-D1* locus, one line of each pair with subunits 5+10 and the other with subunits 2+12. The differences in dough strength (measured by MDDT) between the lines of each pair cannot be explained by differences in flour protein content or PPP but correlate well with UPP (Gupta and MacRitchie 1994). Similar conclusions were reached in a study of 74 randomly selected recombinant inbred lines. In this case, lines with the *Glu-D1d* allele (5+10 subunits) had significantly higher R_{max} and UPP than lines with the *Glu-D1a* allele (2+12 subunits). Furthermore, lines with the *Glu-B1i* allele (17+18 subunits) had superior R_{max} as well as UPP to lines with the *Glu-B1e* allele (20x+20y subunits) (Gupta and MacRitchie 1994). The origin of the allelic effects on MWD have not been resolved.

Although the presence of allelic HMW-GS pairs (e.g., 5+10 vs. 2+12) exhibits differences in the molecular weight of glutenins, in many cases, the amounts of the subunits are not different. It has seemed logical to assume that intrinsic differences in the subunits (and therefore in their polymerization behavior) are responsible for the effects. Some differences in HMW-GS have been found. For example, subunit 5 has one additional cysteine residue and this may be related to the higher molecular weight (Greene et al 1988). On the other hand, HMW-GS are remarkably similar in structure. Recently, a preliminary study of the changing protein composition in developing grain of two near-isogenic lines differing only at the *Glu-D1* locus (5+10 vs. 2+12) suggests an alternative explanation for the allelic effects (Gupta et al 1996). The line with subunits 5+10 (Lance C) showed a more rapid accumulation of HMW-GS and total glutenin than the line with 2+12 subunits (Lance A)

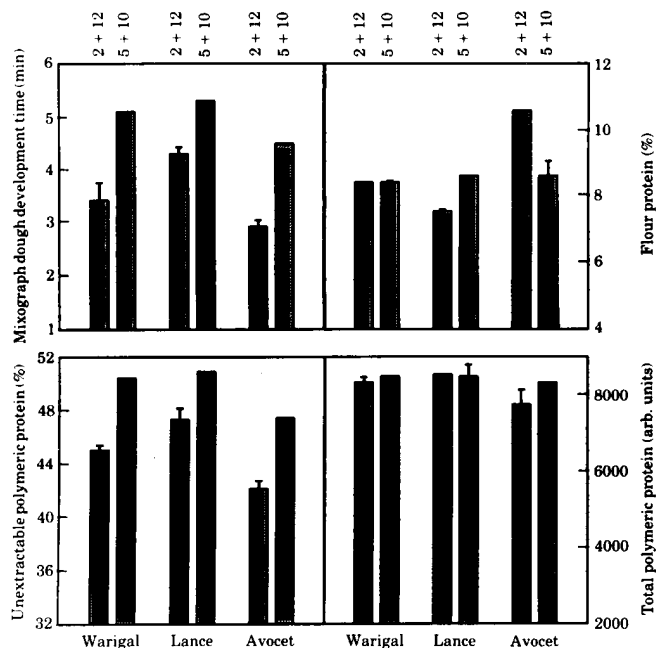


Fig. 7. Mean maximum dough development time (MDDT), proportion of protein in flour (% FP), absolute quantity of total polymeric protein in the flour (1% flour protein and 1 mg of flour), and proportion of unextractable polymeric protein in the total protein (% UPP) for pairs of near-isogenic lines from three Australian wheat cultivars differing only in high molecular weight glutenin subunits (HMW-GS) 5+10 and 2+12 at the *Glu-D1* locus. Standard error of the difference for each comparison is shown on the genotype with the lower value for these parameters (Gupta and MacRitchie 1994).

during a period 22–34 days after anthesis (DAA), although the amounts of glutenin were similar at maturity. The effect of this difference in the times of accumulation was that, in the later stages of development up to maturity, Lance C produced glutenin with the MWD shifted to higher values than Lance A, as deduced from measurements of the UPP (Fig. 8). These results suggest that some regulatory mechanism may be responsible for the allelic effect rather than structural differences between the subunits, although this possibility needs to be more fully tested.

Chain Propagators and Terminators

For a glutenin subunit to participate in a growing polymer, it needs to have at least two cysteine residues. Structural studies of HMW-GS and LMW-GS have shown that this requirement is usually fulfilled so that they can act as chain propagators (Kasarda 1989, Shewry et al 1989). However, certain subunits that are incorporated into the polymeric protein are modified gliadins with an odd number of cysteine residues, unlike the normal gliadins that have either an even number or none (e.g., ω -gliadins) (Lew et al 1992, Masci et al 1993, Gianibelli et al 1996, Masci et al 1999). These proteins, therefore, behave as glutenin subunits but are expected to act as chain terminators and effect shifts in the MWD to lower values.

NEW APPROACHES TO MWD MEASUREMENT

Many of the methods for determining molecular weight are unsuitable for the size distribution of the largest glutenin molecules as they have upper molecular weight limits beyond which their

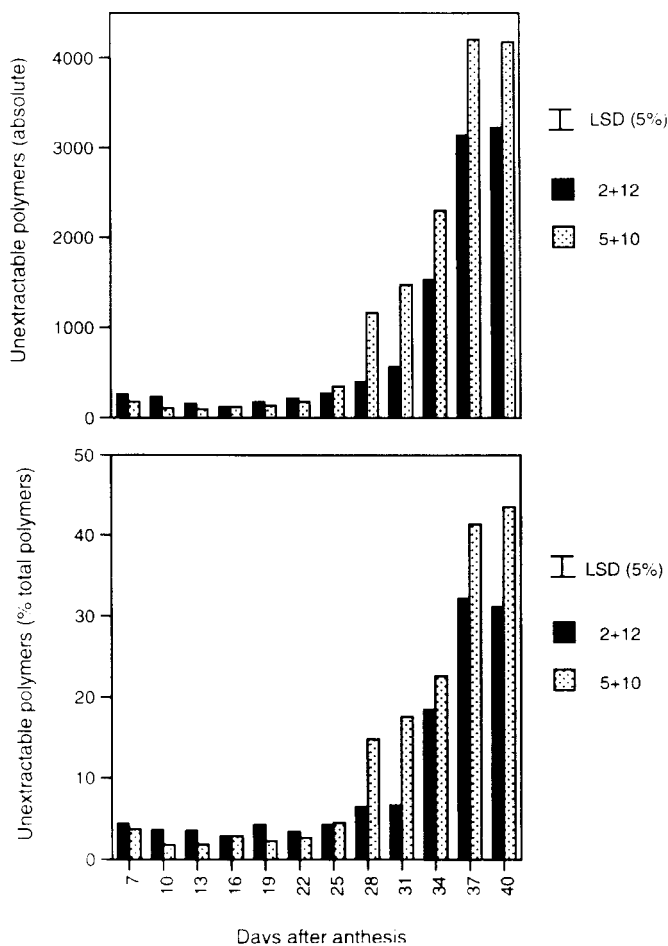


Fig. 8. Amounts of unextractable polymeric protein (UPP) for the near-isogenic lines Lance A (HMW-GS 2+12) and Lance C (HMW-GS 5+10) measured at different stages of grain development. Least significant difference (LSD) at the 5% probability level (Gupta et al 1996).

sensitivity is lost. Methods not limited in this way include FFF, MALLS, and multistacking SDS-PAGE.

FFF is a relatively new technique (Giddings 1976) that separates macromolecules, particles, or colloids over a broad range of molecular size. Size-separation is based on the intrinsic diffusivity, which is inversely related to the effective diameter, of a macromolecule in solution. Separation occurs in a thin (10–250 μm) ribbon-like channel (≈ 30 cm long) that replaces the column in SE-HPLC. Like SE-HPLC, solvent travels longitudinally down the channel. The two techniques diverge with the application of a field perpendicular to the channel flow in FFF. The field may be a solvent flow, a centrifugal field, a thermal field, or an electric field (Giddings 1993). FFF utilizes a cross flow of solvent for the field, this being achieved with upper and lower (symmetric flow) or only lower (asymmetric) porous sintered glass channel walls. The lower channel in each case is overlaid by an ultrafiltration type membrane with a molecular mass cut-off to allow only passage of solvent molecules. A cross-flow rate is used where the sample is carried close to the membrane of the lower channel wall at static channel flow and the sample is allowed to reach equilibrium. The lower molecular weight components with the greater diffusivities migrate against the cross flow to a distance further away from the membrane than the higher molecular weight components. Differential separation of this size distribution within the channel is achieved by the parabolic channel flow front resulting from friction within the very thin channel. As a result, the lower molecular mass components residing further away from the channel wall elute in a higher velocity flow lamina, and before the higher molecular mass components in the lower velocity lamina. FFF has been used in measurements of wheat flour proteins by Preston and Stevenson (1996) using symmetrical flow and of wheat protein fractions by Wahlund et al (1996) using asymmetrical flow.

The intrinsic diffusion coefficient of a macromolecule is dependent on its hydrodynamic diameter. In FFF, the shape as well as the molecular mass influence the effective diameter of a molecule. For example, the hydrodynamic diameter (d) of random coil polymers is related to molecular mass M by the expression:

$$d = AM^b \quad (6)$$

where A and b are constants that depend on the polymer-solvent system and on the shape of the macromolecule. Only when these constants are known can retention times be converted directly into molecular mass. Wahlund et al (1996) utilized Equation 6 to estimate the upper and lower limits for the molecular mass of glutenin fractions. The lower limit was defined as $d = 0.0542M^{0.498}$ for a flexible random coil polymer, and the upper limit was defined as $d = 0.159M^{1/3}$ corresponding to a spherical shape. Values for the upper limit were in the range of 440,000 to 11 million. This, of course, assumes a spherical shape which does not seem likely because the solvent contained SDS which would tend to impart an electrical charge on the glutenin molecules and cause extension. On the other hand, the solutions had been sonicated so that the very largest glutenins would have been reduced in size. This effect of sonication time in narrowing the size distribution has been shown by Southan et al (1998).

An automated FFF system with frit inlet and frit outlet has been developed by Stevenson et al (1999) for measurements of wheat proteins. These modifications have resulted in improved resolution and better reproducibility.

The MALLS technique has been applied to HMW-GS (Lookhart 1997), but there is great potential for application of light scattering to glutenin polymers (Egorov et al 1998). In theory, MALLS is capable of measuring MWD which is what is needed for wheat proteins. Because MALLS enables measurements of molecular shape, it seems that it could be used in tandem with FFF to give accurate measurements of MWD of wheat polymeric proteins. Multistacking SDS-PAGE has been used to measure size distribution of gluten proteins (Khan and Huckle 1992, Bekes et al

1996). Successive layers containing progressively increasing polyacrylamide concentrations to separate proteins of different size ranges are used, followed by densitometry of the stained gels.

APPLICATION TO PREDICTION AND MANIPULATION OF FUNCTIONALITY

It is becoming apparent that the MWD of the protein is one of, if not the most important, parameter in explaining functional properties of flours. Of course, it is not a static property. It obviously changes during grain development, but it also may change during storage, during dough mixing, and during subsequent steps in processing. However, the manner in which it changes postharvest is determined by the initial MWD of the flour protein from the mature grain, and this is controlled by both genetic and environmental factors. The challenge is to determine the optimum MWD for a given end-use requirement and then to use this knowledge to try to adjust it in the processing or, more indirectly, to achieve it in breeding. In processing, oxidizing and reducing agents are commonly used to alter the MWD although their action and precise effects may not always be completely understood.

In this article, we discuss some of the approaches that can be adopted in breeding to predict the MWD of the protein and to put into practice strategies to fit the final MWD to that most suitable for processing of given products. In this way, the cereal chemist can provide an input for wheat breeders to include in their programs in addition to their strategies for optimizing yield, disease resistance, and other agronomic characteristics.

Genetic Factors

As already mentioned, one way to alter the MWD is to change the polymeric-to-monomeric protein ratio. This ratio appears to depend to some extent on the numbers of proteins or subunits (and therefore on the number of genes coding for them) in each class (Singh et al 1990b). The loss of genes for one class of protein or subunit does not seem to produce any appreciable compensation for the loss. This can be inferred from data in which HMW-GS or LMW-GS genes are not expressed (Lawrence et al 1988, Gupta et al 1991). Since these deletions have not affected grain protein level, the loss of certain proteins or subunits presumably is compensated by an increase in expression of all other proteins and subunits. Of course, a sample of flour protein may have >100 individual proteins, so that removal of a few will effect only a slight increase in the remaining ones if the total increase is shared more or less equally. These considerations lead to the view that relatively large changes in the polymeric-to-monomeric ratio can be produced by deletion of one locus (e.g., for the HMW-GS and LMW-GS). This, in turn, can produce large changes in flour functionality. Examples are the wheat-rye translocation lines. The 1B/1R lines in which the short arm of chromosome 1B of wheat has been replaced by the short arm of chromosome 1 of rye, although introducing valuable genes for resistance to pathogens, have weak and sticky dough properties. This effect is associated with an appreciable reduction in the quantity of LMW-GS as a result of the loss of the *Glu-B3* locus. Because, in general, a greater number of LMW-GS are controlled by this locus than the other *Glu-3* loci, and because LMW-GS are normally in excess of HMW-GS by a factor of two or three, there is a resultant dramatic decrease in the proportion of glutenin (thus a decrease in the polymeric-to-monomeric protein ratio). There are also indications that the shift in the ratio is accentuated because the gliadins that are removed with the *Glu-B3* locus are overcompensated by secalins introduced by the rye chromosome short arm (Dhaliwal and MacRitchie 1990). Some suggestions for remedying the dough problem in conventional breeding programs have been made based on increasing the strength of the glutenin either by 1) introducing an extra HMW-GS at *Glu-A1* (in hexaploid wheats, only one HMW-GS is expressed at this locus) or 2) bolstering the

gluten strength by introducing HMW-GS that are associated with strength (e.g., 5+10 at *Glu-D1* and 17+18 at *Glu-B1*). In terms of the MWD, it has to be realized that not only has the polymeric-to-monomeric protein ratio been decreased by elimination of the *Glu-B3* locus but the HMW/LMW ratio has also been increased. From our previous discussion, this would be expected to shift the MWD to higher values, thus tending to compensate partially for the decrease in strength. Some preliminary results support this effect. The 1B/1R translocation cultivar Grebe had a higher extensigraph R_{max} (and lower Ext) than its normal parent line but showed greater dough stickiness (Bariana, Gupta, Ellison and MacRitchie, unpublished results). This suggests that dough stickiness is strongly related to the polymeric-to-monomeric protein ratio, whereas R_{max} , as we have seen, relates to the proportion of glutenin with molecular weight above a critical value. The change in the HMW-GS/LMW-GS ratio thus may shift the MWD of Grebe protein to higher molecular weight, causing the observed increase in R_{max} . To correct the problem of 1B/1R lines, the change in the balance of the proteins and its effect need to be taken into account. Greater strength as measured by R_{max} can lead to undesirable effects on dough properties such as reduced extensibility and increased dough requirements without necessarily correcting the problem of dough stickiness. One approach to increasing the polymeric-to-monomeric protein ratio without affecting the MWD of the glutenin is to make use of lines that are null at the *Gli-2* loci (loci on the short arms of group 6 chromosomes coding for α - and β -gliadins). The introduction of null loci into the 1B/1R lines would thus eliminate a substantial amount of gliadins and be expected to shift the polymeric-to-monomeric ratio to higher values without having a large effect on the MWD of the glutenin. Manipulation of proteins on the group 1 chromosome short arms is more problematic because of the linkage of genes coding for gliadins and those coding for LMW-GS. However, selection of parent lines with relatively low

amounts of LMW-GS coded at *Glu-B3* and high amounts of LMW-GS coded at *Glu-A3* and *Glu-D3* would help to minimize the effects of removal of the *Glu-B3* locus. Of course, approaches other than conventional breeding are being pursued which show promise in overcoming the functionality defects caused by the rye translocation (Shepherd et al 1991). Our discussion is meant to serve as an example of how the MWD of the protein needs to be taken into account when considering strategies to counteract any detrimental effects on functionality caused by introduction of alien genes.

Dough strength (as measured by R_{max} and MDDT) is related to the proportion of polymeric protein above a critical molecular weight. If the aim is to increase strength, this can be achieved by 1) increasing the HMW-GS/LMW-GS ratio, 2) substituting strength-associated HMW-GS (e.g., 5+10) for weakness-associated HMW-GS (e.g., 2+12), and 3) minimizing potential chain terminators.

Extensibility relates to the proportion of total polymeric protein in the flour but this protein should not shift MWD to too high a molecular weight if high extensibility is sought. Thus, high extensibility requires a high ratio of polymeric-to-monomeric protein (at least up to a certain point) and not too high a molecular weight of the glutenins, achievable by a low HMW-GS/LMW-GS ratio, substitution of strength-associated GS, and the presence of potential chain terminators. Thus, some requirements for high strength and high extensibility are, to an extent, in contradiction. A compromise is needed to meet given specifications. Protein composition requirements for product quality such as breadmaking performance depends very much on the particular test used to assess baking performance (Gupta et al 1992). A suitable balance between dough strength and extensibility appears to be necessary.

Environmental Factors

The main proteins that determine functional properties of wheat flours are controlled by nine major loci (three *Glu-1*, three *Gli-1/Glu-3*, and three *Gli-2*), each locus exhibiting allelic variation. In theory, by knowing the relation between protein composition and different functional properties, it should be possible to design a genotype with the optimum allelic composition for a given end-use. However, even if this could be done, environmental conditions during growth of the plant can alter the protein composition in ways that may not have been anticipated. The general effects of certain environmental variables are understood to some extent. These include nitrogen and sulfur fertilizer levels and the effect of temperature during plant growth.

High nitrogen availability translates into high protein contents in the grain and flour. Increased protein content usually results in higher dough extensibility and breadmaking potential. However, changes in protein composition also occur. With increasing protein content, gliadin proteins tend to increase at a greater rate than other proteins (Gupta et al 1992). This can lead to decreases in the polymeric-to-monomeric protein ratio and may mean that dough strength as measured by a parameter such as R_{max} could decrease as the flour protein content is raised.

When sulfur fertilizer level is limiting, this can lead to a rearrangement of the relative quantities of different groups of proteins with dramatic effects on MWD and, as a result, on functional properties. Under these conditions, the relatively sulfur-poor proteins increase in amounts to the detriment of the sulfur-rich proteins (Wrigley et al 1984). The ω -gliadins are the most sulfur-poor of the proteins, while the α - and β -gliadins, albumins, and globulins are relatively sulfur-rich. Most importantly, the HMW-GS are relatively sulfur-poor compared with the LMW-GS. The result is that, when sulfur availability is limiting, the HMW/LMW-GS ratio increases dramatically (Fig. 9). The effect of the changing ratio on the UPP, and by deduction on the MWD, is shown in Fig. 4. Deficiency of sulfur availability shifts the MWD to higher molecular weight, causing increased dough

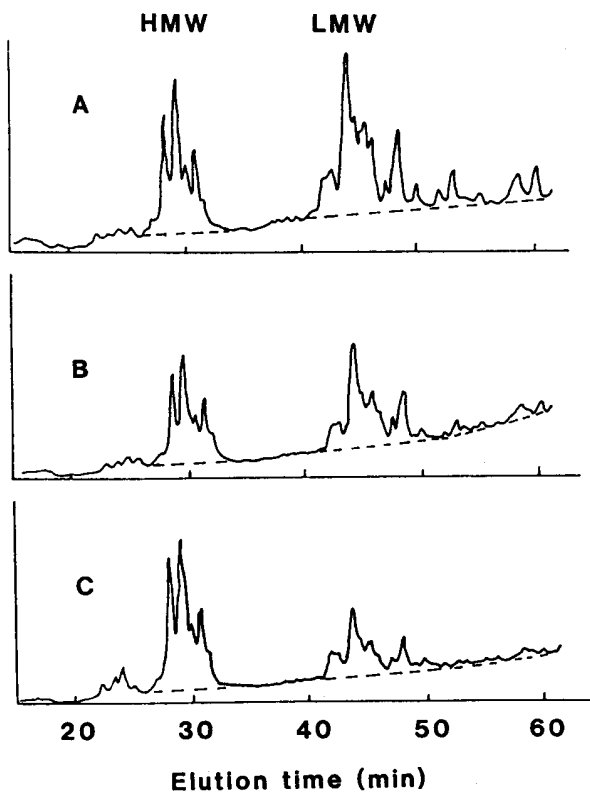


Fig. 9. Reversed-phase HPLC profiles of glutenins from three Olympic flour samples varying in sulfur (S) and protein (P) contents. **A**, S = 0.146%, P = 10.4%. **B**, S = 0.100%, P = 7.8%; **C**, S = 0.075%, P = 9.7% (MacRitchie and Gupta 1993).

strength and mixing requirements and reduced extensibility. There is also a nitrogen-sulfur interaction to consider. Sulfur deficiency is accentuated by higher nitrogen levels (Wooding et al 1994).

Considering the effects of temperature during grain filling, it has been reported that increases up to 30°C in daily mean temperature increased dough strength, but that temperatures >30°C produced weaker doughs (Randall and Moss 1990). A number of studies have investigated the molecular processes involved, including the presence of heat-shock proteins and the effects on the polymeric-to-monomeric protein ratio (Blumenthal et al 1993, Bernardin et al 1994, Stone and Nicolas 1994). Ciaffi et al (1994) reported that the weakening of dough properties as a result of heat stress was associated with a decrease in the UPP, indicating that the MWD had been shifted to lower values by the high temperatures.

CONCLUSIONS AND FUTURE DIRECTIONS

The effects of MWD of wheat proteins in determining properties such as dough mixing and rheological characteristics is well established. The MWD required to achieve a specific balance of these properties is likely to be an area of research in the future. Two main variables are available for manipulation, the polymeric-to-monomeric protein ratio and the MWD of polymeric protein. Some of the ways to vary these two parameters have been discussed in this article, based on their genetic control. MWD is also affected by environmental factors, and one of the challenges will be to produce wheat cultivars in which the MWD of the protein is relatively stable to these influences. This will require further studies of how factors such as high temperatures and other stresses influence the synthesis of the different protein classes and the polymerization of the polymeric protein subunits during grain development.

A stumbling block has been the difficulty of measuring MWD. No solvent has been found for solubilizing the total wheat protein without chemical alteration. This does not mean that it will not be possible to devise ways of estimating the MWD, but it will require ingenuity in designing experiments to accomplish it. One possible approach is to mathematically model degradation of the largest size glutenin by shear, such as in sonication. This would enable extrapolation of the MWD (which can be measured on the solubilized material) to the undegraded protein. The new methods for measuring molecular weight such as FFF and MALLS are in their early stages of application to wheat proteins and show great promise because these methods do not have an upper molecular weight limit.

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